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Analysis of Extracellular West Nile Virus Particles Produced by Cell Cultures from Genetically Resistant and Susceptible Mice Indicates Enhanced Amplification of Defective Interfering Particles by Resistant Cultures

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[^{3}H]uridine-labeled extracellular West Nile virus (WNV) particles produced by cell cultures obtained from genetically resistant C3H/RV and congenic susceptible C3H/HE mice were compared by sucrose density gradient centrifugation as well as by analysis of the particle RNA. Defective interfering (DI) WNV particles were observed among progeny produced during acute infections in both C3H/RV and C3H/HE cells. Although only a partial separation of standard and DI particles was achieved, the DI particles were found to be more dense than the standard virions. Particles containing several species of small RNAs consistently constituted a major proportion of the total population of virus progeny produced by C3H/RV cells, but a minor proportion of the population produced by C3H/HE cells. Decreasing the multiplicity of infection or extensive plaque purification of the WNV inoculum decreased the proportion of small RNAs found in the progeny virus. The ratio of DI particles to standard virus observed in progeny virus was determined by the cell type used to grow the virus. The ratio could be shifted by passaging virus from one cell type to the other. Homologous interference could be demonstrated with WNV produced by C3H/RV cells but not with virus produced by C3H/HE cells. Continued passage of WNV in C3H/HE cells resulted in a cycling of infectivity. However, passage in C3H/RV cells resulted in the complete loss of infectious virus. Four size classes of small viral RNA, with sedimentation coefficients of about 8, 15, 26, and 34S, were observed in the extracellular particles. A preliminary analysis of these RNAs by oligonucleotide fingerprinting indicated that the smaller RNAs were less complex than the 40S RNA and differed from each other. The data are consistent with the conclusion that WNV DI particles interfere more effectively with standard virus replication and are amplified more efficiently in C3H/RV cells than in congenic C3H/HE cells. The relevance of these findings to the further understanding of genetically controlled resistance to flaviviruses is discussed.

The flaviviruses constitute one genus of the togavirus family and are small (50 to 60 nm in diameter), round, enveloped viruses which contain a single-stranded 40S RNA genome of positive polarity (30). Relatively little is presently known about the details of flavivirus replication. The only viral mRNA detected on polysomes in flavivirus-infected cells is the 40S genome RNA (3, 20). However, the mechanism by which individual viral proteins are generated from this RNA is not known (30). Among the togaviruses, the detailed analysis of defective interfering (DI) particles (14) has so far been limited to members of the *Alphavirus* genus (24), primarily Sindbis virus and Semliki Forest virus.

In mice, a host gene has been identified that codes for resistance to flavivirus-induced encephalitis. Resistance has been demonstrated to be inherited as a Mendelian autosomal dominant allele (19, 21, 26). Mice that possess the resistance allele support the replication of flaviviruses, but virus titers in the brain tissues of these mice are lower (by 2 to 3 $\log_{10}$) and the spread of the infection is slower than in susceptible mice (7, 10, 12). Cultures of cells obtained from various tissues of resistant C3H/RV mice produce lower yields of several representative flaviviruses than do comparable cultures of cells from congenic susceptible C3H/HE mice (6, 27). The replication of all other types of viruses, including alpha togaviruses, is unaffected by the presence of the flavivirus resistance gene (6). Immunofluorescence studies have indicated that a similar percentage of cells in C3H/RV and

C3H/HE cultures is infected by a particular flavivirus inoculum (4, 6). In addition, interferon does not play a specific role in flavivirus genetic resistance, as it does in the expression of genetic resistance to myxoviruses (5).

A previous report of mine (6; some of my previous work was published under the name M. B. Darnell) indicated that serial high-multiplicity passage of the flavivirus West Nile virus (WNV) in embryo fibroblast cultures from resistant C3H/RV animals yielded virus with detectable homologous interfering activity, but interfering activity could not be detected in virus passaged in the same manner in cells from susceptible C3H/HE mice. Recently, Smith (23) obtained evidence that Banzi virus obtained from the brains of C3H/RV mice injected intraperitoneally but not intracerebrally showed demonstrable interfering activity. These results suggested, but did not conclusively prove, that flavivirus DI particles were at least produced by C3H/RV cells. A recent study in my laboratory showed that mouse cell cultures that were persistently infected with WNV stopped producing infectious virus during extended subculture but continued to produce nonplaquing virus (4). The majority of the virus particles obtained from these long-term, persistently infected cultures contained several species of RNA smaller than genome RNA and were capable of interfering with standard WNV.

I report here the detection of WNV DI particles in progeny obtained from acute infections of both C3H/HE and C3H/RV embryo fibroblast cultures. This represents the first physical chemical demonstration of the production of flavivirus DI particles during an acute infection. The DI particles consistently represented a significantly larger proportion of the progeny populations produced by the C3H/RV cells than of those produced by the C3H/HE cells under the same conditions.

MATERIALS AND METHODS

Cells. The congenic mouse strains C3H/HE and C3H/RV are maintained as inbred colonies. Embryo fibroblast cultures are routinely prepared from 14- to 18-day-old embryos and propagated in Eagle minimal essential medium supplemented with glutamine (0.29 mg/ml), gentamycin (50 μg/ml), and 10% (vol/vol) fetal calf serum. These cultures were used for experiments after the first or second subcultures.

BHK-21/WI2 cells isolated by Vaheri et al. (25) were used for plaque titration of virus, which was carried out as described previously (4).

Virus. WNV strain E101 (11) was obtained from Walter Brandt of the Walter Reed Army Institute of Research. This stock had been frozen in 1962 after eight passages in suckling mouse brain. After a single plaque purification step, a clarified 10% (wt/vol) brain homogenate pool was prepared in 3-day-old Swiss mice. This pool was designated WNV-9 and contained 2.5×10^8 PFU/ml.

Subsequently, WNV-9 was sequentially plaque purified six times. The resulting virus was used to infect BHK-21/WI2 cells at a multiplicity of infection (MOI) of 1, and culture fluid was harvested 30 h after infection. This virus pool was designated standard WNV and had a titer of 1.5×10^7 PFU/ml.

Pools of WNV were also prepared in cultures of C3H/HE or C3H/RV cells. T-150 flasks of each cell type were infected with WNV-9 at an MOI of 10, and progeny virus was pelleted from clarified culture fluid 48 h after infection. These pellets were resuspended in 10% minimal essential medium at one-tenth of the original volume of culture fluid. The infectivity of the C3H/HE–WNV-9 pool was 6×10^7 PFU/ml, whereas that of the C3H/RV–WNV-9 pool was 4.5×10^5 PFU/ml, as determined by plaque assay in BHK-21/WI2 cells.

A pool of Sindbis virus strain SAAR 339 was prepared in BHK-21 cells. Culture fluid was harvested 24 h after infection of cells with an MOI of 1. The titer of this pool was 1.1×10^9 PFU/ml.

Analysis of extracellular virions. Confluent monolayers of C3H/HE, C3H/RV, or BHK cells in T-150 flasks were infected with virus. Culture fluids were supplemented with 20 μCi of [^{3}H]uridine (28 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml at the times indicated. Harvested fluids were clarified by centrifugation, layered directly onto 15 to 45% sucrose gradients made in 0.01 M Tris-hydrochloride (pH 7.2)–0.1 M NaCl, and centrifuged in an SW27 rotor at 24,500 rpm for 16 h at 4°C. Fractions of 1.2 ml were collected by means of a density gradient fractionator and a recording UV analyzer (Isco 640 and VA-2; Instrumentation Specialties Co., Lincoln, Nebr.). Radioactivity incorporated into trichloroacetic acid-insoluble material was measured on glass fiber filters in a toluene-based scintillation cocktail.

Analysis of extracellular virion RNA. [^{3}H]uridine-labeled RNA was extracted from virions centrifuged on sucrose gradients as described above. Virus-containing gradient fractions were pooled, and vanadylribonucleoside complex (VRC) (Bethesda Research Laboratories, Inc., Rockville, Md.) and self-digested pronase (Sigma Chemical Co., St. Louis, Mo.) were added to final concentrations of 2 mM and 20 μg/ml, respectively. Samples were incubated at 37°C for 10 min, and then sodium dodecyl sulfate (SDS) was added to a final concentration of 1%. After a second incubation at 37°C, carrier yeast RNA (10 μg) was added, and the RNA was precipitated in ethanol at −20°C. The precipitated RNA was suspended in 10 mM Tris-hydrochloride (pH 7.2) containing 1% SDS and 2 mM VRC and layered onto linear 15 to 30% (wt/vol) SDS-sucrose gradients made in a solution composed of 0.05 M NaCl, 0.01 M Tris-hydrochloride (pH 7.2), 0.001 M EDTA, and 0.5% (wt/vol) SDS. Centrifugation was carried out in an SW27 rotor at 24,500 rpm for 16 h at 22°C. Fractions were collected and analyzed for acid-insoluble radioactivity.

Alternatively, RNA was extracted from virions pelleted from clarified culture fluids from T-75 flasks by centrifugation at $80,000 \times g$ for 2.5 h at 4°C in an SW27 rotor. The virus pellets were suspended in 10 mM Tris-hydrochloride (pH 7.2) containing 2 mM VRC and 20 μg of self-digested pronase per ml. After incubation at 37°C for 10 min, SDS was added to a final concentration of 1%. The samples were then incubated for 10

min at 37°C and centrifuged on 15 to 30% (wt/vol) SDS-sucrose gradients as described above.

Agarose gel electrophoresis. ^{32}P-labeled WNV RNAs from sucrose gradient fractions were precipitated with ethanol. RNA pellets were suspended in 10 μl of 50 mM Tris-acetate (pH 7.8)–20 mM Na acetate–2 mM EDTA–10 mM methyl mercury(II) hydroxide–10% glycerol–0.01% bromophenol blue and electrophoresed on 1.2% agarose gels (Bethesda Research Laboratories) made in 50 mM Tris-acetate (pH 7.8)–20 mM Na acetate–2 mM EDTA–4 mM methyl mercury(II) hydroxide. Electrophoresis was at 100 V for 4 h with buffer consisting of 50 mM Tris-acetate (pH 7.8), 20 mM Na acetate, and 2 mM EDTA. Gels were treated with 1% 2-mercaptoethanol for 1 h and then dried onto Gel-bond film (FMC Corp., Rockland, Md.) and exposed to Kodak AR-5 film at 4°C.

Oligonucleotide fingerprinting. Confluent cell cultures were infected with WNV-9 at an MOI of 10, and the cultures were refed with phosphate-free minimal essential medium supplemented with 5% dialyzed fetal calf serum. At 6 h after infection, 2 mCi of carrier-free $^{32}P_i$ (Amersham Corp., Arlington Heights, Ill.) was added per T-150 flask. Labeled virions were pelleted from clarified culture fluid harvested 48 h after infection. Virus pellets were suspended in 10 mM Tris-hydrochloride (pH 7.2) and incubated with VRC, pronase, and SDS as described above. The samples were phenol extracted three times, and then the viral RNA was ethanol precipitated in the presence of yeast carrier RNA. The RNA was sedimented on 15 to 30% SDS-sucrose gradients as described above, and fractions of 1.2 ml were collected. A 0.05-ml aliquot of each fraction was analyzed for acid-insoluble radioactivity, and peak fractions were pooled and precipitated with ethanol. Each of the RNAs was suspended in 1 ml of 10 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA and resedimented on 15 to 30% SDS-sucrose gradients. Peak fractions were identified as before, and the RNAs were precipitated with ethanol. The RNA pellets were washed twice with 70% ethanol and then once with 95% ethanol and suspended in water. The RNAs were lyophilized and then digested with RNase T_1 (Worthington Diagnostics, Freehold, N.J.) at 37°C for 30 min in 8 μl of 10 mM Tris-hydrochloride (pH 7.2)–1 mM EDTA. Urea (4 mg per sample) and 6 μl of gel loading buffer (0.1% bromophenol blue, 0.1% xylene cyanol FF, 5 M urea, and 1.4 M sucrose) were added, and the oligonucleotides were electrophoresed according to the method of de Wachter and Fiers (9). The first-dimension gel contained 10% acrylamide, 0.32% *N,N'*-methylene bisacrylamide, 6 M urea, and 0.025 M citric acid. Electrophoresis was carried out at 350 V until the dye front had moved 18 cm. The second-dimension gel consisted of 21.8% acrylamide, 0.7% bisacrylamide, and 0.04 M Tris-borate (pH 8). Electrophoresis was carried out at 30 mA per gel. Gels were exposed to Kodak AR-5 film at 4 or −70°C.

RESULTS

Density gradient analysis of WNV particles produced by C3H/HE or C3H/RV cells. The infectivity titer of WNV grown in C3H/RV cells has been consistently found to be lower than that of WNV grown in C3H/HE cells (6). Extracellular virus particles produced by both types of cells were compared physically as one means of determining the reason for the differential production of infectivity by the two types of cells. [^{3}H]uridine (20 μCi/ml) was added to the culture fluid at 6 h after infection of C3H/RV and C3H/HE cultures with WNV-9 (MOI = 10). Fluids were harvested at 32 h, clarified, layered directly onto 15 to 45% sucrose gradients and centrifuged as described above. Fractions of 1.2 ml were collected, and portions of each fraction were analyzed for acid-insoluble radioactivity (Fig. 1B and G) and infectivity (Fig. 1A and F). The titers of the virus progeny produced by the C3H/RV and C3H/HE cells were 7.5×10^5 and 7×10^7 PFU, respectively. A broad peak of radioactivity was observed between gradient fractions 15 and 25 with virus from both cell types (Fig. 1B and G). This region of the gradient also contained the majority of the infectivity (Fig. 1A and F). No [^{3}H]uridine-labeled material was detected in this region of the gradient in fluid obtained from uninfected cultures. The ratio of PFU to [^{3}H]uridine counts per minute was calculated for each gradient fraction (Fig. 1C and H). The virus particles with densities of approximately 1.17 g/cm^3 possessed more infectivity per amount of labeled RNA than did the particles with densities of 1.19 to 1.2 g/cm^3. Also, the infectivity-to-RNA ratio of the particles produced by C3H/HE cells was more than 10-fold higher than the ratio obtained for virus particles produced by C3H/RV cells.

The RNA present in particles obtained from the more dense and the less dense sides of the virus gradient peaks was analyzed separately. Gradient fractions were pooled as indicated in Fig. 1B and G. The virus particles were disrupted by the addition of SDS in the presence of 2 mM VRC, and viral RNA was precipitated with ethanol and sedimented on 15 to 30% SDS-sucrose gradients as described above. The majority of the RNA obtained from the C3H/HE virus particles in both pool 1 (Fig. 1D) and pool 2 (Fig. 1E) was found to be 40S RNA. However, a larger proportion of the total RNA from pool 2 consisted of RNAs with sedimentation coefficients of less than 40S. In contrast to what was observed with the virus produced in C3H/HE cells, more than 10-fold less 40S RNA was observed in the pooled fractions from gradients containing virus produced by C3H/RV cells. Several species of RNA with sedimentation coefficients of less than 40S were observed in both pool 3 and pool 4 (Fig. 1I and J). These small RNA species constituted the majority of the RNA found in both of these pools.

Effect of MOI and plaque purification of inoculum on virus progeny RNA produced by C3H/HE and C3H/RV cells. The results of the above-

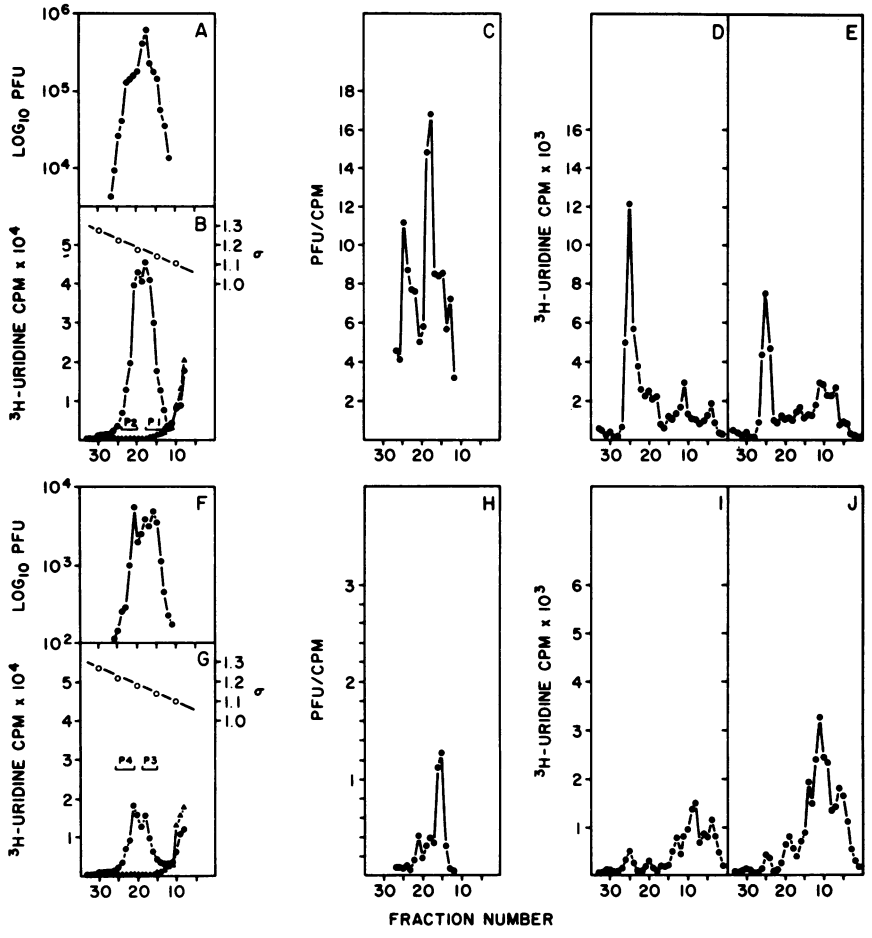

FIG. 1. Isopycnic density gradient centrifugation of WNV produced by C3H/HE and C3H/RV cells. Cultures were labeled with [^{3}H]uridine at 6 to 32 h after infection with WNV-9 at an MOI of 10. The infectivity of the infecting virus was determined by plaque assay in BHK cells. Harvested culture fluids were clarified and centrifuged directly on 15 to 45% isopycnic sucrose density gradients. The direction of sedimentation is from right to left. Virus produced by C3H/HE cells is shown in panels A–E, whereas that produced by C3H/RV cells is in panels F–J. Samples of each gradient fraction were analyzed for acid-insoluble radioactivity (B and G) and infectivity (A and F). The ratio of infectivity to uridine counts was calculated for each gradient fraction (C and H). Fractions were pooled as indicated, and the extracted RNA was sedimented on 15 to 30% SDS-sucrose gradients (panels D, E, I, and J for pools 1 through 4, respectively). Symbols: ●, virus-infected culture fluids; ▲, uninfected control culture fluid; ○, σ values. The direction of sedimentation is from right to left.

described experiments indicated that WNV DI particles present in the WNV inoculum interfered more effectively with standard WNV replication in C3H/RV cells than in C3H/HE cells. If this is indeed the case, then a reduction of the number of DI particles in the inoculum should result in an enhanced production of standard virus by C3H/RV cultures. Two methods were used which are known to reduce the possibility of cells being coinfected with DI particles and standard virus. First, progeny virus was compared after infections with WNV-9 used at multiplicities of 10, 1, or 0.1. Cultures of infected C3H/RV and C3H/HE cells were incubated in the presence of [^{3}H]uridine beginning at 4 h after infection. RNA in progeny virus harvested and pelleted at either 24 or 51 h after infection was extracted and sedimented on SDS-sucrose gradients as described above. The RNAs found in progeny produced in C3H/RV and C3H/HE cells infected with a multiplicity of 10 are shown in Fig. 2a through d. The patterns obtained after infections with a multiplicity of 1 are shown in panels e through h, and those obtained with a multiplicity of 0.1 are shown in panels i and j. The amount of 40S RNA in progeny virus produced by both C3H/HE and C3H/RV cultures was found to be increased after infection at

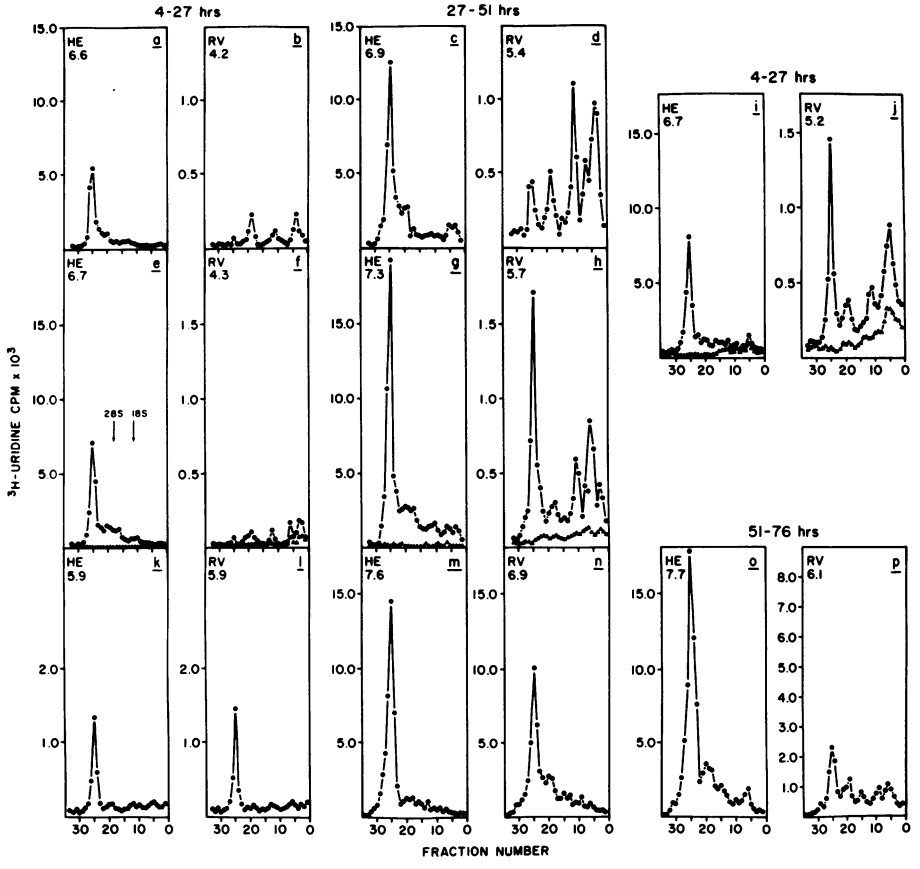

FIG. 2. Effect of multiplicity or plaque purification on progeny virus RNA populations. [^{3}H]uridine-labeled RNA in progeny virus harvested at 27, 51, or 76 h after infection was extracted and sedimented on SDS-sucrose gradients as described in the text. RNA was measured in progeny produced after infection with WNV-9 at MOIs of 10 (a–d), 1 (e–h), and 0.1 (i and j) and after infection with WNV-9 plaque purified six times at an MOI of 1 (k–p). The host cell is indicated in the upper left-hand corner of each panel, as is the total infectivity ($\log_{10}$) of each progeny virus sample. Symbols: ●, infected; ▲, uninfected. The direction of sedimentation is from right to left.

lower multiplicities. An increase in the infectivity of the progeny virus produced was also observed with decreasing multiplicity. Not enough extracellular virus 40S RNA was produced by C3H/RV cultures infected with a multiplicity of 10 or 1 to be detected by [^{3}H]uridine labeling during the first 27 h after infection (Fig. 2b and f). However, when a multiplicity of 0.1 was used, extracellular virions containing detectable levels of 40S RNA were produced during the first 27 h after infection (Fig. 2j).

The second method used to reduce the concentration of DI particles in the WNV inoculum was extensive sequential plaque purification of the WNV-9 stock. The resulting standard virus pool, prepared as described above, was then used to infect cultures of C3H/RV and C3H/HE cells at a multiplicity of 1. Between 4 and 27 h after infection with standard WNV, similar amounts of 40S RNA were observed in progeny virus produced by both C3H/RV and C3H/HE cell cultures, and no other species of labeled RNAs were detected (Fig. 2k and l). The infectivity of the virus produced by the two cell types during this period was identical. Between 27 and 51 h, the amount of 40S RNA in progeny virus produced by the C3H/RV cells was only about 75% of that produced by the C3H/HE cells (Fig. 2m and n), and low amounts of RNAs with sedimentation coefficients of less than 40S were detected in the progeny virus produced by the C3H/RV cells (Fig. 2n). The infectivity of the virus produced by the C3H/RV cells was approximately 10-fold less than that of virus produced by the C3H/HE cells during this period. The difference in the sedimentation patterns of RNA obtained from progeny virus produced by the C3H/HE and C3H/RV cultures between 51 and 76 h was even more striking (Fig. 2o and p). The virus produced during this period by both

C3H/RV and C3H/HE cells contained four size classes of small RNAs. These small RNAs represented a significantly larger proportion of the total viral RNA in the sample produced by the C3H/RV cells than in the sample from the C3H/HE cells. The infectivity of the virus produced by the C3H/RV cells during this period was 1.5 $\log_{10}$ lower than that of the virus produced by the C3H/HE cells.

RNA in WNV-9 progeny produced by BHK cells was also analyzed. The production of 40S RNA was found to be even more efficient in BHK cells than in the C3H/HE cells. This correlated well with the observation that BHK cells produce yields of infectious virus which are about 10-fold higher than those produced by comparable cultures of C3H/HE cells. When BHK cells were infected with the WNV-9 pool at a multiplicity of 10, several species of labeled small RNAs were observed in the progeny virus harvested 30 h after infection (data not shown). However, these small RNAs represented only a minor proportion of the RNA, and the RNA sedimentation pattern closely resembled that shown in Fig. 2g. When BHK cells were infected with the standard WNV pool, only a very sharp peak of 40S RNA was detected in virus harvested at 30 h after infection. Since BHK cells show extensive virus-induced cytopathology beginning about 33 h after a WNV infection, RNA in progeny virus from BHK cells was not analyzed past 30 h. These results indicated that the various species of small RNAs observed were WNV specific, since they were produced by both mouse and hamster cells and their level decreased as a result of decreased MOI or plaque purification of the inoculum. An assay developed by Barrett et al. (2) has demonstrated that inhibition of the synthesis of viral RNA is a sensitive and effective means of detecting the presence of alphavirus DI particles.

Modulation of progeny virus particle populations by the host cell. As a means of further investigating the effect exerted by the host cell on the population of progeny virus produced, C3H/RV and C3H/HE cultures were infected with WNV pools prepared in either CH3/RV or C3H/HE cells as described above, and the progeny virus RNAs were analyzed at various times after infection. The C3H/RV and C3H/HE WNV pools were first tested for their ability to interfere with the replication of a standard WNV pool prepared as described above. Confluent monolayers of C3H/RV and C3H/HE cells in six-well culture dishes were infected first with either C3H/RV-WNV or C3H/HE-WNV, and then after a 0.5-h incubation, standard WNV was added at an MOI of 0.1. Adsorption was then allowed to proceed for a further 0.5 h. Control cultures were infected with each pool of virus alone. The titers shown in Table 1 are averages obtained from titration of virus produced by duplicate wells and titrated in duplicate. Only the C3H/RV-WNV pool demonstrated significant homologous interference.

(i) C3H/HE-passaged virus. Replicate cultures of C3H/RV and C3H/HE cells were infected with the C3H/HE-WNV pool and incubated with [^{3}H]uridine beginning at 4 h after infection. The RNA associated with pelleted extracellular virus harvested at either 28 or 52 h after infection was extracted and sedimented on SDS-sucrose gradients. The majority of the RNA found to be associated with the extracellular particles produced by C3H/HE cells infected with C3H/HE-WNV was 40S during both time periods examined (Fig. 3a and c). However, low levels of several subgenomic RNAs were observed in virus produced between 4 and 28 h, and the amounts of these RNAs increased in virus produced between 28 and 52 h. The RNA associated with the virus particles produced between 4 and 28 h by C3H/RV cells infected with the C3H/HE-WNV pool was also primarily 40S (Fig. 3b), although the total amount of infectious virus and 40S RNA was about 10-fold less than that produced by the similarly infected C3H/HE cells. In contrast, the RNA associated with the virus particles produced by the C3H/RV culture between 28 and 52 h was not predominately 40S (Fig. 3d).

(ii) C3H/RV-passaged virus. Significantly different patterns of progeny virus RNA were observed with C3H/RV and C3H/HE cell cultures were infected with the C3H/RV-passaged WNV pool. Between 4 and 28 h, little [^{3}H]uridine-labeled RNA was detected in extracellular particles from either C3H/HE or C3H/RV cells (Fig. 3e and f). However, a very small peak of 40S RNA was detected in the sample from the C3H/HE cells that was not present in the sample from the C3H/RV cells. Between 28 and 52 h,

TABLE 1. Assay of interference between C3H/HE- or C3H/RV-passaged WNV and standard WNV

Virus[a]	WNV yield ($\log_{10}$ PFU/ml) at 48 h	
	C3H/RV cells	C3H/HE cells
Standard WNV	5.6	6.3
C3H/HE-WNV	5.2	5.9
C3H/HE-WNV + standard WNV	5.4	6.4
C3H/RV-WNV	3.5	4.6
C3H/RV-WNV + standard WNV	4.7	5.7

[a] MOIs were as follows: standard WNV, 0.1; C3H/HE-WNV, 4; C3H/RV-WNV, 0.06. These virus pools were prepared as described in the text.

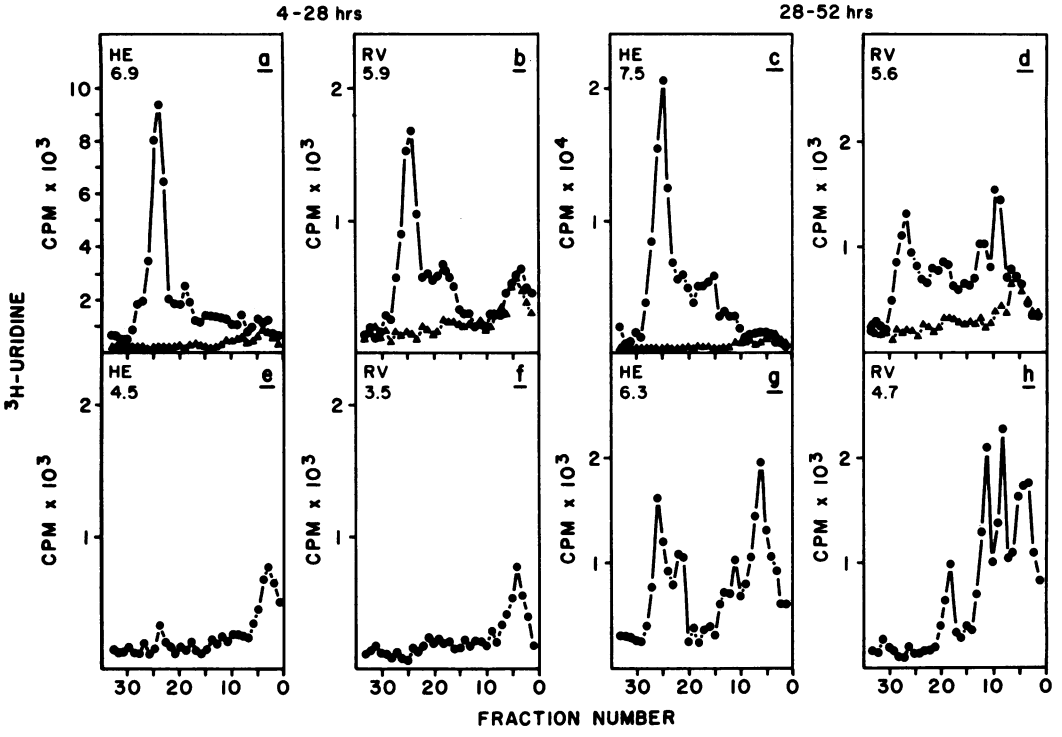

FIG. 3. Sedimentation profiles of RNA from extracellular progeny virus produced after infection with either C3H/RV-WNV or C3H/HE-WNV. Cultures were incubated with [^{3}H]uridine, and virus was harvested at 28 or 52 h after infection. The RNA was extracted and sedimented on 15 to 30% SDS-sucrose gradients. Panels: a–d, virus produced by cells infected with C3H/HE-WNV (MOI = 1); e–h, virus produced by cells infected with C3H/RV-WNV (MOI = 0.1). The host cell is indicated in the upper left-hand corner of each panel, as is the total infectivity ($\log_{10}$) of the harvested progeny virus. Symbols: ●, infected; ▲, uninfected control. The direction of sedimentation is from right to left.

the virus produced by C3H/HE cultures contained several species of small RNAs and 40S RNA. However, the 40S RNA was not the predominant species (Fig. 3g) as had been observed in the progeny virus from infections of C3H/HE cells with C3H/HE-passaged virus or stock brain pool virus. The progeny virus particles produced between 28 and 52 h by C3H/RV cultures infected with C3H/RV-WNV (Fig. 3h) contained essentially no labeled 40S RNA, and the several species of small RNAs represented the majority of the RNA observed. Although in the case of WNV-9, a decrease in the MOI to 0.1 resulted in a more efficient production of progeny containing 40S RNA (Fig. 2j), this was not the case with infections with the C3H/RV-WNV pool at an MOI of 0.1. These results indicate that the C3H/RV virus pool contained a higher ratio of defective to standard particles than did the WNV-9 pool.

As a further means of demonstrating the host cell effect on the populations of progeny virus produced, an undiluted passage series was carried out in each cell type. After each passage in C3H/RV cells, the ability of the C3H/HE cells to preferentially amplify the standard virus and so increase the infectivity in the progeny was tested. Conversely, after each passage in C3H/HE cells, the ability of C3H/RV cells to preferentially amplify DI particles and so decrease total infectivity was also tested. Confluent cultures of C3H/RV and C3H/HE embryo fibroblasts in T-75 flasks containing 10^7 cells were infected with WNV-9 at an MOI of 10. At 3 days after infection, one-half of the medium from a flask was used to infect a fresh culture of the same cell type, and the other half was used to infect a fresh culture of the other cell type. After adsorption for 2 h at room temperature, each new flask was refed with fresh medium. This procedure was repeated at 3-day intervals for six passages. Infectivity was assayed by using portions obtained at the time of each passage. The level of virus infectivity produced by the C3H/HE cultures remained relatively high, but infectivity levels cycled during passage. In contrast, infectious virus was not detectable in C3H/RV culture fluids after passage 2 (Table 2). At each

passage level, virus from the C3H/HE cell series (Table 2, series A) was found to produce a lower yield of virus when transferred to C3H/RV cells. Conversely, virus from the C3H/RV cell series (Table 2, series B) produced a higher yield of infectious virus when transferred to C3H/HE cells through passage 3. Thereafter, infectious virus was not detectable in fluids from either type of culture in the C3H/RV cell series.

Virus specificity of the particle-associated small RNAs. A DNA complementary to 40S WNV-9 genome RNA was prepared as described previously (4) and hybridized to WNV RNA from ethanol-precipitated pooled gradient fractions that had been spotted directly onto nitrocellulose filters. The cDNA was found to hybridize to RNA within each of the four size classes of small RNA (data not shown). This result was similar to that reported previously with the small RNAs obtained from WNV particles produced by persistently infected cell cultures (4).

TABLE 2. Serial undiluted passage of WNV in RV or HE cell cultures[a]

Series	Passage	72 hour virus yield (log_{10} PFU/ml) C3H/HE	C3H/RV
A	1	5.9	—
	2	4.5	1.3
	3	5.2	<1
	4	4.6	3.1
	5	4.5	<1
	6	4.1	2.3
		5.4	2.9
B	1	—	4.3
	2	4.1	1.8
	3	2.8	<1
	4	2.1	<1
	5	<1	<1
	6	<1	<1
		<1	<1

[a] In series A, C3H/HE cells were initially infected with WNV-9 at an MOI of 10. In series B, C3H/RV cells were initially infected with WNV-9 at an MOI of 10. After 3 days, culture medium was used to infect fresh cultures as indicated by the arrows.

The RNA obtained from extracellular WNV particles was also analyzed on agarose gels under denaturing conditions (Fig. 4). Virus RNA labeled during replication with $^{32}P_i$ was sedimented on SDS-sucrose gradients. The peaks were pooled, and the RNA was precipitated with ethanol. The initial gradient purification step effected a relatively good separation of the various small RNA species, since each species also migrated as a distinct size class under denaturing conditions (Fig. 4). However, some cross-contamination of species was observed, especially in lanes C and B. Each size class of partially purified small RNA was, therefore, subsequently sedimented through a second SDS-sucrose gradient before it was used for oligonucleotide fingerprint analysis.

A representative oligonucleotide fingerprint of the 40S genome RNA of WNV-9 is shown in Fig. 4A. In Fig. 4B through E representative fingerprints of the small WNV RNAs are shown. The 34S RNA fingerprint showed all the observable unique spots found in the 40S RNA (Fig. 4B). The pattern obtained for the 26S RNA had at least three new spots not found in the 40S RNA (Fig. 4C, arrows). The 15S (Fig. 4D) and 8S (Fig. 4E) RNAs were much simpler than the 26, 34, and 40S RNAs, as indicated by the decreased number of oligonucleotides observed. Each of the two smallest WNV RNAs displayed a distinct oligonucleotide pattern, indicating that they were not generated from the 40S RNA by a random breakdown process. The oligonucleotide fingerprint for a particular small RNA was the same when the RNA was isolated from virus produced by either C3H/RV, C3H/HE, or BHK cells (data not shown). Further analysis of individual T_1 oligonucleotides is needed before the precise relationship of the various small RNAs to each other or to the 40S RNA can be determined.

DISCUSSION

The data presented here represent the first physical demonstration of extracellular flavivirus DI particles among progeny virus produced during an acute infection. A density gradient analysis indicated that the majority of the WNV DI particles found were denser than the standard virions. This could be due to the packaging of more than one deleted RNA molecule per capsid or to a slight reduction in the overall size of the DI particles as compared with the standard virus. The several species of small RNAs observed are presumed to have been present in the WNV-9 stock virus. Cloning of this stock virus by six plaque purification steps

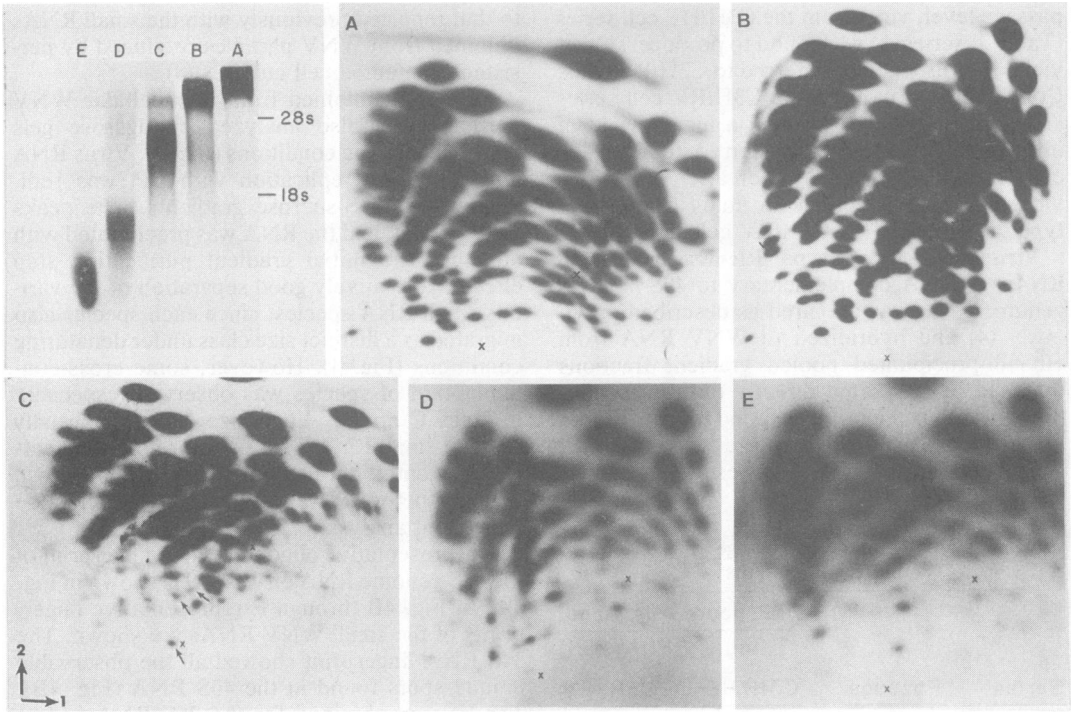

FIG. 4. Oligonucleotide fingerprints of WNV genome and small RNAs. ^{32}P-labeled RNA was extracted from extracellular virions produced by C3H/HE cells infected with WNV-9 at an MOI of 10 and sedimented on 15 to 30% SDS-sucrose gradients. Gradient fractions containing 40, 34, 26, 15, and 8S RNAs were pooled separately. A sample of each RNA was electrophoresed on a 1.2% agarose gel in the presence of CH_3HgOH (upper right-hand panel). Each RNA species was rebanded on a sucrose gradient before digestion with RNase T_1. Oligonucleotides were electrophoresed on a two-dimensional gel system according to the method of de Wachter and Fiers (9). Panels: A, 40S WNV genome RNA; B, 34S RNA; C, 26S RNA; D, 15S RNA; E, 8S RNA. Arrows indicate the positions of unique spots not found in the 40S RNA. The positions of dye markers are indicated by ×'s.

reduced the amount of DI particles present, but did not completely eliminate them.

It is not surprising that other investigators have not previously detected the presence of DI particles in progeny produced by acute infections with flaviviruses. Flaviviruses are routinely grown in the most permissive host cells available to facilitate good virus yields for subsequent experimental manipulation. Even if DI particles were present in the inoculum, the progeny virus produced under these conditions would primarily consist of virus-containing, full-size genome RNA, as observed in virus produced by C3H/HE and BHK cells. A further disadvantage of most of the highly permissive cell lines is that flavivirus infection induces a rapid cytopathic effect and so eliminates the possibility of multiple cycles of virus replication. It will be of interest to analyze other isolates of WNV. The WNV strain E101 stock was found to contain a number of DI particles, but stocks of other WNV isolates and other flaviviruses would be expected to differ significantly in the amounts and types of DI particles they contain. Preliminary data obtained with several strains of St. Louis encephalitis virus support this hypothesis (manuscript in preparation).

The host cell was found to influence significantly the composition of viral progeny populations. WNV replication in C3H/RV cells yielded progeny with a high proportion of DI particles, whereas progeny virus produced by congenic C3H/HE cells consisted primarily of standard virus. Four size classes of small RNAs with sedimentation coefficients of approximately 8, 15, 26, and 34S were consistently observed to be associated with extracellular particles. It is clear from oligonucleotide fingerprint analyses that the 26 and 34S RNAs are related to the 40S RNA, but further analysis is required to describe the relationship of the 15 and 8S RNAs to the 40S RNA. For a particular host cell-virus combination, the sedimentation pattern of the RNAs obtained from extracellular particles was reproducible from experiment to experiment. Similar peaks of labeled small RNAs have not been

observed in fluids from mock-infected cultures. Further, the small RNAs appear to be mostly of plus strand polarity, since they hybridized to a cDNA made to the 40S genome RNA. Wengler et al. (29) previously reported the identification of two species of small WNV-specific RNAs of positive polarity, but these RNAs were not tested for autointerfering activity. It is not yet known whether all four size classes of small RNA observed in the present experiments are capable of interfering with standard viral RNA synthesis. However, the two smallest species appeared in the greatest relative abundance in progeny virus from C3H/RV cells, which demonstrates detectable interfering activity. Although the 34S species did not show a detectable change in its fingerprint pattern from that of the 40S RNA, this RNA is not just a conformational variant of the 40S RNA since it migrated more rapidly than 40S RNA on gels under denaturing conditions. Studies of DI RNAs of alpha togaviruses have shown that they are generated from the genome RNA by a complex multistep process (15, 18). Whether the various WNV small RNAs have been generated by a simple deletion mechanism or by a more complex process is not yet known. According to the currently accepted theory, DI RNAs with rearrangements of their 3'-terminal sequences that confer enhanced affinity for the binding of polymerase would be expected to be most effective at interfering with the synthesis of helper virus RNA (17, 22).

It is tempting to speculate that the host proteins specified by the alleles of the murine flavivirus resistance gene may interact specifically at the level of the flavivirus RNA replication complex, affecting template-polymerase interactions. Data from both negative-strand RNA (17, 22) and positive-strand RNA (1, 8) virus systems indicate that host factors are involved in viral RNA replication. It has also been suggested that interactions between viral polymerase and host factors could explain the apparent role of the host cell in DI particle generation (13, 16, 17, 22). If a host factor does interact with the flavivirus polymerase and if the C3H/HE and C3H/RV cells contain different alleles for this factor, then an interaction between the viral polymerase and the host factor could influence the production of or extent of interference by flavivirus DI particles. Wengler and Wengler (28) have described a seven-base sequence which is separated from the 3' terminus of a WNV genome RNA by two bases and from the 3' terminus of the minus-strand RNA by seven bases. They have suggested that this seven-base sequence represents a recognition signal for the flavivirus polymerase.

The results presented here were obtained with tissue culture cells. If the same progeny virus selection mechanism occurs in C3H/RV animals, the rapid amplification of DI particles present in the infecting flavivirus inoculum could bring about the lowered yield of infectious virus and the reduced spread of infection observed in C3H/RV animals. If virus is given peripherally, such a process of DI particle amplification would be very effective in protecting C3H/RV animals from virus-induced encephalitis. However, if virus were injected intracerebrally, the concentration and type of DI particles present in the virus inoculum, as well as the virulence of the standard virus (a genetic property of the virus which determines the extent of central nervous system cell damage it causes), would be important in determining the survival of the C3H/RV animals. In support of this hypothesis, Smith (23) found that biological evidence of DI particles in virus produced in the brains of C3H/RV animals could only be detected after peripheral infections with Banzi virus.

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