Regeneration of DI Particles of Virulent and Attenuated Rabies Virus: Genome Characterization and Lack of Correlation with Virulence Phenotype

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(Accepted 3 June 1980)

SUMMARY

Two strains of fixed rabies virus were examined for their ability to regenerate defective interfering (DI) particles and for possible correlation of DI particle production with the expression of virulence. A plaque-purified stock of the attenuated ERA strain (ERA<sub>pp</sub>), which characteristically caused an auto-interfering death response in adult mice inoculated i.c., was serially passed at a high m.o.i. in BHK-21 cells. By the sixth passage, DI particles were regenerated that corresponded in sedimentation velocity and DI/RNA size to the smallest of three sizes of DI particles produced by the parental stock virus. The regeneration of ERA DI particles in vivo was not detected during 15 serial high or low m.o.i. passages of infected newborn mouse brain, though the passaged virus consistently elicited an auto-interfering-type death response when assayed in adult mice. The attenuated Flury HEP<sub>pp</sub> strain regenerated up to three unique size classes of DI particles during serial passage in BHK-21 or murine neuroblastoma C1300 clone NA cells compared with the one band of DI particles produced by the parental Flury HEP stock virus. The BHK-21 cell-adapted Flury HEP<sub>pp</sub> virus failed to kill adult mice when inoculated at high concentrations after two serial passages in NA cells. However, the virus became fully virulent and a single band of regenerated DI particles was visible. Additional bands of defective particles were visible following the third serial passage in NA cells. Single-stranded RNA with a mol. wt. of 0.62 x 10<sup>6</sup> was extracted from the first DI particle population to be regenerated. This corresponded in mol. wt. to the DI/ssRNA characteristic of the parental attenuated Flury HEP virus. However, in the parental type DI/RNA, partially dsRNA could be isolated in addition to ssRNA. Double-stranded RNA could not be detected in the regenerated DI particles derived from the virulent NA cell-propagated Flury HEP<sub>pp</sub> virus. These results suggest that the virulence phenotype of fixed rabies viruses does not depend on the presence or absence of DI particles.

INTRODUCTION

Defective interfering (DI) particles of rabies virus have been identified as a product of infection in vitro with the Flury low egg passage (LEP; Crick & Brown, 1974), Flury high egg passage (HEP; Holland & Villarreal, 1975; Kawai et al. 1975; Holland et al. 1976) and ERA strains (Wiktor et al. 1977). In a comparative study of the above strains and CVS and Pitman-Moore viruses, it was demonstrated that rabies viruses propagated under similar conditions are capable of producing DI particles with strain-specific physical characteristics.
(size) and biological properties (Clark et al. 1980). Holland & Villarreal (1975) also described defective particle production in vivo in newborn mice inoculated intracerebrally (i.c.) with clonally pure infectious B particles of Flury HEP virus. In each case the defective particles were defined characteristically as deletion mutants according to Huang & Baltimore (1970). Rabies DI particles were capable of interfering with the replication of homologous standard virions but not with heterologous virus (Crick & Brown, 1974; Holland & Villarreal, 1975; Wiktor et al. 1977; Clark et al. 1980).

The early observations of mortality caused by Flury HEP virus showed a distinct auto-interference response in hamsters and guinea-pigs (Koprowski, 1954; Koprowski et al. 1954). This interference phenomenon was later observed in vitro in Flury HEP virus-infected primary chick embryo fibroblasts (Yoshino et al. 1966).

Kawai et al. (1975) demonstrated that cells persistently infected with Flury HEP virus produced infectious virus particles that were identified as a major factor in establishing and maintaining a persistent rabies infection. This observation was confirmed with the ERA strain of rabies virus by Wiktor et al. (1977). Production of defective particles has also been associated with several chronically-infected cultures of mouse neuroblastoma and BHK-21 cells which failed to release rabies infectious particles (unpublished observations). On the basis of those findings in vitro, we have investigated the possible role of DI particles in the regulation of the expression of virulence in mice.

In recent papers, the biological phenomenon of the auto-interfering death response induced in i.c.-inoculated adult mice has been described (Wunner & Clark, 1978; Clark, 1979; Clark et al. 1980). Attenuated BHK-21 cell-adapted Flury HEP virus containing DI particles failed to kill mice inoculated with high doses but caused some deaths in mice inoculated with limiting dilutions. Flury HEP virus that was plaque-purified to remove detectable DI particles, however, induced a similar pattern of mortality, implying that the DI particles offered little or no protection. When plaque-purified Flury HEP virus was serially passaged in neuroblastoma cells, the virus regained virulence for adult mice (Clark, 1978). It was decided to use this system to monitor reversion of attenuated virus and DI particle production simultaneously.

DI particle regeneration in vitro and in vivo using the ERA strain that was cloned free of three distinct size classes of DI particles was also examined. Since the parental and cloned ERA stock virus killed mice according to an auto-interference-type dose response (Wunner & Clark, 1978; Clark et al. 1980), it was decided to try to demonstrate that this phenomenon was independent of DI particles produced by the virus. The results of these studies show that the presence of DI particles in rabies virus populations is not necessarily correlated with changes in virulence phenotype.

METHODS

Cells. Monolayer cultures of BHK-21 cells and mouse neuroblastoma C1300, clone NA (NA), cells were maintained as described elsewhere (Clark et al. 1980).

Viruses. The history of each strain of attenuated fixed rabies Flury HEP and ERA (WI) adapted to growth in BHK-21 cells has been described elsewhere (Clark et al. 1980). The size classes of DI particles produced by these strains have also been described (Wunner & Clark, 1978; Clark et al. 1980). HEP_{pp} and ERA_{pp} virus stocks were prepared from their respective parental stocks by serial cloning of isolated plaques in BHK-21/S13 cells three to five times to remove all traces of DI particles. The cloned isolates were then grown in BHK-21 cells to a titre of 10^7 to 10^8 plaque-forming units (p.f.u.) per ml. The viruses were titrated for p.f.u. in BHK-21/S13 cells suspended in agarose (Sedwick & Wiktor, 1967) and for lethality in 4-week-old ICR random-bred mice given 0.03 ml inoculum i.c.
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**Infection of cells and labelling virus RNA.** Virus inoculum was adsorbed to confluent monolayer cultures of cells at the m.o.i. indicated in the text for 0.5 h at 33°C. Cell cultures were maintained in Eagle’s minimal essential medium plus 0.2% bovine serum albumin (Sokol & Clark, 1973) at 33°C for 4 to 7 days post-infection (p.i.). When two harvests of individual cultures were analysed, 4-day-old culture fluid was collected and the infected cells were incubated further with fresh medium to day 7 when maximum c.p.e. was often observed. The RNA was labelled on day 2 with 10 μCi/ml ³H-uridine (New England Nuclear, Boston, Mass., U.S.A.) and harvested on day 4. Virus labelled on day 4 in the presence of fresh incubation medium was harvested on day 7. Infected adult and suckling mouse brains were homogenized individually in 10% suspension with 2% foetal calf serum in Stoker’s medium (MacPherson & Stoker, 1962) and centrifuged at 300 g for 20 min at 4°C. The clarified mouse brain homogenates were diluted 1:5 (per adult mouse) or 1:5 to 1:10 (per newborn mouse) for inoculation of BHK-21 cells.

**‘Herring-bone’ experiment.** The passage sequence to compare the induction of DI particles and acquisition of virulence of Flury HEP virus during passage in NA and BHK-21 cell cultures is shown in Fig. 1. In the pattern shown, HEpp virus was used to infect cultures in individual T-75 flasks at two different m.o.i. After incubation for 4 days, the undiluted culture fluid from each culture infected at the higher m.o.i. (3 p.f.u./cell) was used to pass progeny virus serially into fresh cultures. The culture fluid from the flasks infected at the lower m.o.i. (0.003 p.f.u./cell) was diluted 1:1000 before each serial passage. At each passage level, undiluted culture fluid inoculum was also used to infect triplicate cultures of BHK-21 cells in Blake bottles and virus was titrated in adult mice by i.c. inoculation. The RNA of the virus thus produced was labelled with ³H-uridine (10 to 20 μCi/ml) in one of the three infected cultures and later analysed.

**Virus purification and separation of DI particles.** Culture fluids from 4- and 7-day harvests were clarified separately and virus was concentrated and purified as described elsewhere (Clark et al. 1980). Visible bands of standard size virions (B band) and truncated...
defective (T and M band) particles in sucrose gradients were removed by side puncture. Virus gradients without visible bands were collected in 12-drop fractions from the bottom of the tube. Isolated virus bands were diluted in NTE buffer, virus was pelleted and re-suspended in 0.2 ml NTE buffer (Clark et al. 1980) before a second or third cycle of gradient purification or before extraction of virion RNA.

**Assay of radio-isotope incorporation.** ³H-uridine incorporation into RNA was measured in gradient fractions or in samples of extracted RNA by precipitating 10 to 20 μl of sample dried on Whatman No. 1 filter paper with 5% (w/v) trichloroacetic acid (TCA). The precipitates were washed seven times with a 5% TCA solution containing 5% (w/v) sodium pyrophosphate followed by two washes each with 95% ethanol and diethyl ether. The precipitated ³H-RNA was counted in a Packard Tri-carb liquid scintillation spectrometer.

**Analysis of DI particle RNA (DI/RNA).** Gradient-purified standard and defective virions in NTE buffer were digested with 100 μg/ml (final concentration) proteinase K (PK; Boehringer Mannheim) in PK buffer (10 mM-tris-HCl, pH 8, 10 mM-NaCl, 10 mM-EDTA, 0.5% SDS). After 2 h at 37°C, the digestion mixture was layered on to a 15 to 30% (w/v) sucrose density gradient containing 1% SDS in 10 mM-tris-HCl, pH 7.2, 0.1 M-NaCl and 10 mM-EDTA and centrifuged at 21,000 g for 16 h at 22°C in an SW 41 rotor. Gradient fractions were collected and ³H-virus RNA was assayed in individual fractions. The ³H-RNA in peak fractions was mixed with 0.3 M-NaOAc, pH 7.4, and precipitated by ethanol. RNA precipitates were washed in 70% (v/v) ethanol containing 2% (w/v) NaOAc and finally with 100% ethanol, and dried under nitrogen. The RNA was dissolved in E buffer and analysed by SDS-polyacrylamide gel electrophoresis as described by Bishop et al. (1967). Electrophoresis of RNA was conducted at 8 mA/gel for 4 h at room temperature. RNA samples were applied in 5% sucrose (RNase-free) and 0.2% SDS. Unlabelled ribosomal 28S RNA (1.7 × 10⁶) and 18S RNA (0.7 × 10⁶) mol. wt. markers were mixed with each sample of labelled RNA and were located in the gel after electrophoresis with a Joyce-Loebl Scan 400 u.v.-scanner. The 10 cm-long gels were then frozen, cut into 1 mm slices and counted in a liquid scintillation spectrometer after soaking overnight in a solution of nine parts Soluene-350 (Packard), one part water and 90 parts toluene-based scintillation fluid.

**RESULTS**

**Appearance of DI particles in HEPₚₚ virus infection coincides with increased HEPₚₚ virus virulence**

BHK cell-adapted HEPₚₚ virus that failed to kill adult mice when inoculated in high concentration but caused death in some mice inoculated at lower dosages (Fig. 2a) was used to infect NA (HEPₚₚ/NA) and BHK-21 (HEPₚₚ/BHK) cells at both high and low m.o.i. in the ‘herring-bone’ passage pattern described in Methods. Fig. 2 shows that HEPₚₚ/BHK virus, after one undiluted passage in NA cells, caused a non-typical dose response in which some mice were killed at a high concentration of virus. After a second passage, the virus became fully virulent, killing adult mice according to a normal dose response curve. Virus that was passaged at a low m.o.i. in NA cells was also fully virulent after the second serial passage although the virus did not cause deaths at high concentration after the first low m.o.i. passage. No change in virulence phenotype was detected for the HEPₚₚ virus after three passages in BHK-21 cells at either m.o.i. (data not shown). These results confirm our earlier observations of the enhancement of virus lethal efficiency with serial passage in NA cells (Clark, 1978).
Regeneration of rabies virus DI particles

Fig. 2. Effect of Flury HEPₚₚ virus passage in NA cells on virus lethal potential in adult mice. Groups of five mice were inoculated i.c. with serial 10-fold dilutions of virus that were (a) propagated in BHK-21 cells (4.0 × 10⁷ p.f.u./ml) or (b to e) recovered after one or two passages in NA cells of the 'herring-bone' experiment described in Fig. 1. Virus inoculum derived from NA cells infected at (b) high m.o.i. (9.0 × 10⁷ p.f.u./ml) and with (c) non-diluted culture fluid from (b) (HEPₚₚ/NAₐ, 2.9 × 10⁵ p.f.u./ml) were compared with virus obtained from NA cells infected at (d) low m.o.i. (8.0 × 10⁶ p.f.u./ml) and with (e) culture fluid from (d) diluted 10⁻³ (HEP/NAₐ, 5.0 × 10⁴ p.f.u./ml).

When HEPₚₚ/NA virus was purified from NA cells by velocity sedimentation in sucrose density gradients, a minimum of five visible bands was usually generated (data not shown). RNA analysed by SDS-polyacrylamide gel electrophoresis from each of these putative defective particle and standard virion bands (as described below) was heterogeneous in size, ranging from 9S RNA to full-length rabies genomic RNA (42S). The full range of RNA sizes was detected in each virus particle band although a larger proportion of the high mol. wt. RNAs was found in the bands that sedimented more rapidly. These results suggested that the defective and standard virions were either aggregated when isolated from NA cells or polyploid with respect to the genomic RNA molecules they contained.

Since the virulence acquired after HEPₚₚ virus passage in NA cells was unaltered by subsequent passage of HEPₚₚ/NA virus in BHK-21 cells (Clark, 1978) and particle aggregation after growth in BHK-21 cells was less apparent, it was advantageous to grow large quantities of HEPₚₚ/NA virus in BHK-21 cells for molecular and biochemical analysis. When HEPₚₚ/NA₁ and HEPₚₚ/BHK₁ (passage 1) viruses were grown in BHK-21
(a) (b) (c)

Fig. 3. Velocity sedimentation analysis of virus particles derived from serially passaged HEP<sub>pp</sub>
virus in NA and BHK-21 cells. BHK cell-adapted HEP<sub>pp</sub> virus was serially passaged from non-
diluted culture fluid in NA cells or in BHK-21 cells after initial infection at high m.o.i. (3 p.f.u./cell).
Progeny virus from the infected cell cultures at each passage level was grown in individual cultures
of BHK-21 cells. (a) Passage 1 standard (B band) virus particles derived from the initial infection
of NA and BHK-21 cells. (b) Passage 2 standard (B band) virions and defective (T band) particles
derived from NA and BHK-21 cells. (c) Passage 3 standard (B band) virions plus defective (T<sub>1</sub>,
T<sub>2</sub> and M band) particles derived from NA and BHK-21 cells. A faint particle band (designated
by slash bars) sedimented ahead of B band virions.

infectious particles in either cell type were produced from both high (Fig. 3a) and low (not shown)
multiplicity infections. The majority of <sup>3</sup>H-RNA isolated from the virion band in each
case sedimented in a sucrose gradient as 42S RNA; a shoulder representing no more
than 5% of total RNA was consistently observed trailing the 42S RNA (data not shown).
HEP<sub>pp</sub>/NA<sub>2</sub> and HEP<sub>pp</sub>/BHK<sub>2</sub> (passage 2) viruses from high multiplicity infections grown
in BHK-21 cells each produced a single defective particle T band plus a B band (Fig. 3b);
low multiplicity infections (serial inoculum dilution of 10⁻³) in either cell type produced
only a B band at the second passage level. Passage 3 virus from the high multiplicity
infections in NA and BHK-21 cells produced three defective particle bands after a single
passage each in BHK-21 cells (Fig. 3c). Two of the particle bands, T<sub>2</sub> and M, were
detected in addition to the pre-existing T band particles and tended to be less abundant.
A faint band, which contained aggregates of standard virions, sedimented ahead of the B
particle band. Often the T<sub>2</sub> and M bands from both cell types could be detected only in
the second of two harvests (day 7 p.i.), whereas only standard virions or standard virions
plus T band particles were detected in the first virus harvest (day 4 p.i.). No T band
particles were detected in the low multiplicity infections of either cell type by passage 3.

Analysis of HEP/BHK DI/RNA

DI/RNA from the parental BHK-21 cell-adapted Flury HEP virus was analysed first
by sedimentation in SDS-sucrose gradients. RNA of selected gradient fractions was then
subjected to SDS-polyacrylamide gel electrophoresis, since only gradient-fractionated
RNA (as compared with RNA precipitated directly from PK digests) migrated repro-
ducibly in an SDS-polyacrylamide gel. Fig. 4 shows the resolution by SDS-polyacrylamide
gel electrophoresis of <sup>3</sup>H-RNA from the T band particles of HEP/BHK virus. Three
size classes of DI/RNA between 16S and 28S RNA were initially detected by velocity
sedimentation in sucrose gradients; a predominant RNA peak at 16 to 18S, accompanied
by lesser peaks at approx. 20 to 22S and 24 to 28S. Pooled gradient fractions were analysed
on SDS-polyacrylamide gel (Fig. 4a to d). In the pool of 16 to 18S DI/RNA (Fig. 4a),
a major RNA component of apparent mol. wt. 0.62 × 10⁶ migrated faster than 18S
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Fig. 4. SDS-polyacrylamide gel electrophoresis of 3H-RNA from T band particles of parental HEP/BHK virus. BHK-21 cells were infected with BHK cell-adapted Flury HEP virus (0.1 p.f.u./cell), and RNA was labelled with 3H-uridine as described in Methods. Standard virus and DI particles were purified by velocity sedimentation in a sucrose density gradient. T band particles were isolated and 3H-RNA was extracted and centrifuged in an SDS-sucrose density gradient as described in Methods. Pooled gradient fractions of (a) 16 to 18S RNA, (b) 19 to 22S RNA, (c) 24 to 26S RNA, and (d) 28S RNA, were analysed on 2.4% SDS-polyacrylamide gels. Unlabelled ribosomal 28S (1.7 x 10^6 mol. wt.) and 18S RNA (0.7 x 10^6 mol. wt.) markers are indicated by arrows.

rRNA. The rest of the RNA migrated slower than 28S rRNA and appeared to be heterogeneous in size. The entire (16 to 18S) RNA fraction was sensitive to pancreatic ribonuclease (50 μg/ml, high salt, 37 °C, 30 min). The 19 to 22S DI/RNA (Fig. 4b) contained the 0.62 x 10^6 mol. wt. ssRNA in addition to a major RNA component with an apparent mol. wt. of 3 x 10^6. Each of the two remaining pooled RNA fractions (24 to 26S and 28S, Fig. 4c, d) contained the 3 x 10^6 mol. wt. RNA peak plus minor peaks of heterogeneous RNA. No 0.62 x 10^6 mol. wt. ssRNA was present in either of these fractions, suggesting that the single T band of HEP/BHK DI particles contained a heterogeneous population of RNA species. However, if the slow migrating RNA (3 x 10^6 mol. wt.) component in the gel were single-stranded, it would have an apparent mol. wt. too large for the size of corresponding DI particles. This RNA was found to be 30 to 40% resistant to pancreatic RNase (50 μg/ml, high salt, 37 °C, 30 min) and soluble in 2 M-LiCl at 4 °C.
Fig. 5. SDS-polyacrylamide gel electrophoresis of $^3$H-RNA from DI particle and standard virion bands of HEP$_{pp}$ virus after three serial passages in NA cells. HEP$_{pp}$/NA$_3$ virus was grown in BHK-21 cells and RNA was labelled with $^3$H-uridine as described in Methods. $^3$H-RNA was extracted from (a) T$_1$ (16 to 26S), (b) T$_2$ (16 to 26S), (c) M (28 to 30S) and (d) B (40 to 42S) band particles after separation by velocity sedimentation in a sucrose density gradient (see Fig. 3). $^3$H-RNA was centrifuged in an SDS-sucrose gradient and peak fractions from each gradient were pooled and analysed on 2.4% SDS-polyacrylamide gels. Unlabelled ribosomal 28S and 18S RNA markers are indicated by arrows.

Analysis of HEP$_{pp}$/NA DI/RNA

$^3$H-RNA was extracted from DI particles at each passage of HEP$_{pp}$ virus in NA and BHK-21 cells (Fig. 3) and analysed by sucrose gradient centrifugation and SDS-polyacrylamide gel electrophoresis. The DI/RNA from T$_1$, T$_2$ and M band particles, as well as RNA from standard B band virions of HEP$_{pp}$/NA$_3$ virus is shown in Fig. 5. The T$_1$...
Regeneration of rabies virus DI particles

Table 1. Regeneration of DI particles by serial passage of ERA<sub>pp</sub> rabies virus in BHK-21 cells

<table>
<thead>
<tr>
<th>Passage no.</th>
<th>Sucrose gradient-banded particles</th>
<th>Particle RNA size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B virions</td>
<td>42S</td>
</tr>
<tr>
<td>2</td>
<td>n.d.*</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>B virions</td>
<td>42S</td>
</tr>
<tr>
<td>4</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>B virions</td>
<td>42S</td>
</tr>
<tr>
<td>6</td>
<td>B virions + T particles</td>
<td>42S + 20S</td>
</tr>
<tr>
<td>7</td>
<td>B virions + T particles</td>
<td>42S + 20S</td>
</tr>
<tr>
<td>8</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>B virions + T particles</td>
<td>42S + 20S</td>
</tr>
<tr>
<td>10-14</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>B virions + T particles</td>
<td>42S + 20S</td>
</tr>
</tbody>
</table>

* n.d. = not determined.

DI/RNA migrated in SDS-polyacrylamide gel electrophoresis as a single major peak. The mol. wt. was estimated to be 0.62 × 10<sup>6</sup>. This RNA was completely RNase-sensitive and sedimented in an SDS-sucrose density gradient as a broad (16 to 26S) peak. It was identical in size to the single species of DI/RNA from HEP<sub>pp</sub>/NA<sub>2</sub> virus (data not shown) and the ssDI/RNA from parental HEP/BHK virus (Fig. 4). This DI/RNA also corresponded closely to the DI/RNA (0.65 × 10<sup>6</sup> mol. wt.) that was extracted from T band particles of HEP<sub>pp</sub>/BHK<sub>2</sub> virus and T<sub>1</sub> band particles of HEP<sub>pp</sub>/BHK<sub>3</sub> virus (data not shown). Very little larger (heterogeneous) RNA and no smaller size RNA were detected in the DI particles derived from HEP<sub>pp</sub> virus in BHK-21 cells.

The T<sub>2</sub> DI/RNA extracted from HEP<sub>pp</sub>/NA<sub>2</sub> virus (Fig. 5b) also sedimented in a sucrose density gradient as a broad (16 to 26S) peak and contained ssRNA corresponding to that of the slower sedimenting (T<sub>1</sub>) DI particles. In addition, a broad peak of RNA was revealed in SDS-polyacrylamide gel electrophoresis that migrated more slowly than the 18S rRNA marker and corresponded to the heterogeneous RNA previously associated with the T band DI/RNA of the parental HEP/BHK virus (Fig. 4c). M band DI/RNA (28 to 30S) migrated in a broad peak in SDS-polyacrylamide gel electrophoresis (Fig. 5c) more slowly than the 28S rRNA marker, but did not have the double-strand characteristics of the slow migrating DI/RNA from the parental HEP/BHK DI particles. It appeared to represent a discrete size of subgenomic RNA, distinguishable by SDS-polyacrylamide gel electrophoresis from the 42S genome size RNA that was extracted from B band particles (Fig. 5d).

Regeneration of DI particles in vitro and in vivo

Our parental stock of ERA strain rabies virus produced DI particles of three unique sizes of subgenomic RNA (Wunner & Clark, 1978). The regeneration of these DI particles from ERA<sub>pp</sub> virus serially passaged in BHK-21 cells and in newborn mouse brain was monitored as described above. Initially, BHK-21 cells were infected with the virus at 135 p.f.u./cell. By the third serial passage of undiluted culture fluid, the harvested virus produced all three sizes of ERA DI particles, corresponding in both sedimentation velocity and size of RNA to the T<sub>1</sub>, T<sub>2</sub> and M band particles of the parental ERA stock virus. The rapid reappearance of all known ERA DI particles precluded any observation of a possible sequential reappearance. The experiment was, therefore, repeated with ERA<sub>pp</sub> virus at an initial m.o.i. of 0.1/cell and subsequent passage of non-diluted culture fluids. The results obtained after fifteen serial passages are presented in Table 1. The single DI particle size that appeared by the sixth passage contained a single peak of 20S RNA in sucrose. This DI/RNA migrated in SDS-polyacrylamide gel as a single species with a mol. wt. of 0.9 × 10<sup>6</sup> (Fig. 6).
The regenerated DI/RNA was compared with DI/RNA from the parental virus stock. Fig. 7(a) shows the recombined T₁ and T₂ band DI/RNAs from individual DI particle preparations (Fig. 7b, c). The presence of minor DI/RNA components in the individual preparations is presumably due to incomplete separation of adjacent DI particles in T₁, T₂ and M bands. Fig. 7(d) shows the migration of a mixture of DI/RNAs from parental ERA T₂ and regenerated (passage 7) DI particles. The DI/RNA from regenerated particles migrated closely with the ERA T₁ band DI/RNA. DI particle production was stabilized in serially passaged virus-infected BHK-21 cells through passage 15 with no evidence of regeneration of either T₂ or M band DI particles.

To examine DI particle regeneration in mouse brain, ERA<sub>pp</sub> virus was injected i.c. into newborn mice and serially passaged by i.c. inoculation of brain homogenate at 4-day intervals. Brain homogenates were also inoculated on to BHK-21 cell monolayers at the passage levels indicated below in order to label RNA of standard virions and DI particles with <sup>3</sup>H-uridine. The virus inoculum for the first passage was diluted 1:1000 from stock ERA<sub>pp</sub> virus and each mouse was given a dose of 6·5×10⁸ p.f.u.; thereafter, virus was serially passed both undiluted and at a 10<sup>-3</sup> dilution. Animals inoculated with either virus dose usually became sick and were sacrificed on day 4. After 1, 2, 3, 4, 5, 9 and 13 passages with undiluted and 10<sup>-3</sup> diluted brain homogenate, only standard size (B band) virions were produced in BHK-21 cells. The RNA extracted from gradient-purified virions was characterized by velocity sedimentation as 42S RNA (data not shown). No DI particles were visible and no labelled DI/RNA was detected in sucrose gradient fractions.
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Fig. 7. Comparative analysis of regenerated ERA DI/RNA with parental ERA virus DI/RNAs. 

The titre of virus in newborn mouse brain at each passage level was virtually the same
\((10^8 \text{ p.f.u./g})\) whether an undiluted or a \(10^{-3}\) dilution was used. Titres in newborn mice were 2 to 3 log units higher than titres found after inoculation of ERApp virus into brains of 4-week-old mice. Virus inoculated i.c. into adult mice not only replicated with low efficiency but also failed to kill animals by the second virus passage in adult brain. This phenomenon may be accounted for by the auto-interfering death phenomenon in ERA-inoculated adult mice as described earlier.

Intracerebral inoculation with parental ERA virus in 4-week-old mice and subsequent inoculation of infected mouse brain homogenates (diluted 1:5) on to BHK-21 cell monolayers produced T, M and B bands of particles that corresponded in size to the T, M and B band particles of the parental virus (data not shown). The adult in vivo system was therefore capable of maintaining DI particles present in the inoculum.
DISCUSSION

The defective particles of the Flury HEP and ERA strains of rabies virus studied here represent the classical type of deletion mutant that has been described for many animal virus groups (Huang & Baltimore, 1970).

It would appear that the regeneration of DI/RNA is virus strain-specific. This is illustrated by the selective regeneration of ERA T1 DI/RNA (≈ 0.9 × 10⁶ mol. wt.) and Flury HEP T DI/RNA (≈ 0.6 × 10⁶ mol. wt.) during serial passage of cloned DI particle-free virus in tissue culture. The regenerated DI particles are distinguishable by the small DI/RNAs that differ in mol. wt. by only approx. 0.3 × 10⁶. Furthermore, these data illustrate that virulence and DI particles are not interdependent in the pathogenesis of rabies virus since it has not been possible to correlate production of one or more DI particles with the virulence of fixed rabies virus strains.

The DI/RNA produced by Flury HEP virus in BHK-21 cells (HEP/BHK) included both RNase-sensitive and -resistant RNA species. The population of RNA molecules derived from a single velocity gradient band of presumably homogeneous DI particle size was, in fact, heterogeneous. The possibility of variable RNA degradation during preparation was unlikely since DI/RNA profiles were reproducible over many experiments. The RNA component that migrated slowly (3 × 10⁶ mol. wt. ssRNA) in the gel was not entirely sensitive to pancreatic-plus-T₁ RNase. Its partial resistance could have resulted from the hybridization of genomic (−) with complementary (+) strand RNA, possibly covalently linked, as is the RNA of DI particle VSV-DI-oII (Lazzarini et al. 1975). The nucleotide sequence of this RNA component is being studied to determine whether it has regions identical with the faster migrating ssRNA co-extracted from the slow sedimenting T band DI particles. Its biological relevance as an interferon inducer is also being examined. It is worth noting that after two serial passages in either NA cells or BHK-21 cells, the HEPpp virus produced a single band of DI particles that contained a ssRNA species of mol. wt. 0.62 × 10⁶ and 0.65 × 10⁶, respectively. Since the electrophoretic mobility of the regenerated DI/RNAs and their sensitivity to RNase were similar to those of the ssRNA from T band DI particles of parental HEP/BHK virus, this DI/RNA species serves as a subgenome RNA marker for the small Flury HEP DI particle.

DI/RNA from T₂ and M band DI particles produced in NA or BHK-21 cells following the third HEPpp virus passage also revealed distinctive characteristics that corresponded to the DI particle size and sedimentation rate. Neither DI particle population was present in the parental Flury HEP/BHK stock virus.

The three DI/RNA sizes produced by the ERA strain of rabies virus identify characteristically three DI particle populations. The reappearance of a single DI particle population following serial passage of the ERApp virus in BHK-21 cells substantiates the observation with HEPpp virus in BHK or NA cells that suggests that DI particles are produced sequentially in order of size, beginning with the smallest. Although ERA T₂ and M band particles were not detected by the fifteenth serial passage at low m.o.i. in BHK-21 cells, they were detected following passages at high multiplicity. This suggests that they would eventually emerge if virus were serially passaged for an extended time.

Holland & Villarreal (1975) have reported production of defective particles generated by i.c.-inoculated newborn mice during the first passage of cloned rabies (Flury HEP) B virions. ERApp virus inoculated i.c. into newborn or adult mice failed to produce DI particles during fifteen passages. One possible explanation for this difference is that DI particles were detected in Flury HEP-infected mouse brain by an amplification test in which support cloned B virions were added at high m.o.i. Since adult mice inoculated with
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ERA virus, which contains T, M and B particles, produced and maintained the different-sized particles, we concur with Holland & Villarreal (1975) that DI particles, if present in mouse brain, can be replicated in vivo. Whereas it is not clear from these studies that regeneration of DI particles in vivo is virus strain-specific, expression of virulence by these same strains is clearly not altered by the presence or absence of DI particles.

This study was supported by U.S. Public Health Service research grants AI-09706 from the National Institute of Allergy and Infectious Diseases and RR-05540 from the Division of Research Resources. The authors gratefully acknowledge the excellent technical assistance of Sally Shane and Nancy F. Parks.

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(Received 10 March 1980)