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Induction and Biological Properties of Defective Interfering Particles of Rabies Virus

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A method for obtaining large quantities of defective interfering (DI) rabies virus particles that fulfill all the criteria delineated by Huang and Baltimore (1970) is described. The purified rabies DI virion was found to be much shorter (60 to 80 nm) than the complete virion (180 nm) and to have a viral genome of about half the size of normal rabies RNA but with all of the structural proteins of standard virions. Rabies DI virions were noninfectious for both cells in culture and for animals. As determined by in vitro and in vivo techniques, interference with the replication of standard virus was specific to rabies virus. The possible role of rabies DI virion in the pathogenicity of rabies virus infection and in the establishment of attenuated strains for use as live rabies vaccines is discussed.

Defective interfering (DI) viral particles that possess only a portion of a viral genome have been described for nearly all classes of animal viruses (9). DI particles of vesicular stomatitis virus (VSV) have been the most extensively studied, and nearly all basic information about the autointerference phenomenon has been derived from the VSV model (9).

Only limited information concerning induction and the biological properties of DI rabies virus particles is available. The autointerference phenomenon in both hamsters and guinea pigs infected with Flury HEP (high egg passage) virus was reported by Koprowski (12). In that research, undiluted HEP failed to kill either hamsters or guinea pigs, yet dilutions of up to 1:5,000 killed some animals. Autointerference in vitro by HEP virus was reported by Yoshina et al. (22) and by Matsumoto and Kawai (18) in the reduction of plaques in chicken fibroblasts. In our studies, several cell systems chronically infected with rabies virus resisted superinfection with another strain of rabies (6, 24, 25).

DI particles of Flury LEP (low egg passage) rabies virus isolated from BHK-infected cells (3) were only one-third the standard length, contained shorter than normal genomic single-stranded RNA, and interfered with virus replication. Kawai et al. (10) demonstrated that rabies DI particles in vitro from BHK cells were infected with HEP virus and attributed their presence to the persistence of rabies infection of the cell cultures. Production of DI particles in vivo in HEP-infected newborn mice was demonstrated by Holland and Villarreal (8). Finally, Clark and Ohtani (2) observed that a temperature-sensitive (ts 2) mutant of challenge rabies virus (CVS) that was propagated in newborn mouse brains exhibited a strong autointerference when it was inoculated intracerebrally (i.c.) into adult mice.

In the present study we devised a procedure for obtaining large quantities of purified rabies DI virus, characterized them biochemically, and compared their biological properties in vitro and in vivo according to the general criteria for DI particles as defined by Huang and Baltimore (9). DI particles should: (i) contain a part of the viral genome; (ii) contain normal viral structural proteins; (iii) reproduce only in the presence of helper virus; and (iv) interfere specifically with the intracellular growth of homologous standard virus.

MATERIALS AND METHODS

Animals. Five-week-old random-bred ICR female mice were obtained from Flow Laboratories, Inc., Rockville, Md. Young adult New Zealand rabbits (body weight, 2 to 3 kg) were used for the preparation of anti-rabies serum.

Virus strains. Plaque-purified ERA and CVS strains of rabies virus (12) and a plaque-purified strain of VSV (Indiana serotype) (1) were prepared in monolayer cultures of BHK cells as previously described (18). Standard CVS was prepared in mouse brain cells and stored as a 10% mouse brain suspension. The AF street rabies virus was isolated in our laboratory from an infected, wild red fox salivary gland suspension courteously supplied by L. Andral, (Nancy, France). The virus was adapted to growth in BHK cells and propagated in this cell host for 31 passages.

Virus assays. Infectivity was determined by i.c. inoculation of mice (0.03 ml/mouse) and expressed as the mean lethal dose, or by either the plaque assay technique in agarose-suspended S-13 cells (23)

or in monolayers of CER cell cultures (obtained from Tsunemasa Motohashi, Tokyo, Japan), with the results expressed as PFU per milliliter. Anti-rabies serum was prepared in rabbits (19). Virus-neutralizing antibody (VNA) titers were measured by a plaque reduction assay technique (23). The fluorescent antibody (FA) test for the detection of viral antigen in either brain or tissue culture preparations was performed as described (23). Complement fixation tests were conducted according to previously described procedures (13, 14).

Virus purification and separation of DI particles. Virus from infected tissue culture fluid was sedimented for 2 h at 18,000 rpm in a Beckman 19 rotor at 4°C. The resulting pellet was resuspended in NTE buffer (20) and sedimented a second time for 40 min at 20,000 rpm in an SW50.1 rotor at 4°C. The pellet was again resuspended in a small amount of NTE buffer, layered on top of a 5 to 30% continuous sucrose gradient, and centrifuged for 30 min at 21,000 rpm in an SW41 rotor at 4°C. Visible bands were separately fractionated and further purified by a second cycle of sedimentation through a 5 to 30% sucrose gradient. The final bands of virus were collected in a small volume of NTE buffer.

Electrophoretic analysis of viral RNA. Purified [^{3}H]uridine-labeled ERA virus and ERA-DI particles were disrupted in a solution of 1% sodium dodecyl sulfate (SDS) in 0.01 M Tris-hydrochloride, pH 7.8, and the RNA was isolated by phenol extraction according to the method of Kirby (11). The ethanol precipitate containing the viral RNA was dissolved in water and analyzed on 2.5% polyacrylamide gels (16). The gels were cut into 1-mm slices, solubilized overnight in a Protosol (New England Nuclear Corp., Boston Mass.)-Liquifluor (New England Nuclear Corp.)-toluene mixture, and counted in a Beckman scintillation counter.

Electrophoretic analysis of viral polypeptides. Rabies polypeptides were subjected to electrophoresis in SDS-polyacrylamide gels as described by Laemmli (15). Purified virions of either ERA and ERA-DI or lysates of infected and control cells were precipitated with 5 volumes of ethanol and solubilized with 1% SDS (wt/vol) and 1% (wt/vol) 2-mercaptoethanol. Prior to electrophoresis, the samples were boiled for 1 min. After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue in 40% methanol and 10% acetic acid for 5 h, destained with 40% methanol and 10% acetic acid, and scanned using a Gilford spectrophotometer at 575 nm. For the determination of radioactivity, gels were cut into 1-mm slices and treated for 18 h with a Protosol-Omnifluor (New England Nuclear Corp.)-toluene mixture at 37°C before counting.

RESULTS

When BHK cells infected with ERA virus at a high multiplicity of infection (20 PFU/cell) were maintained in culture by cell transfer, a drastic decrease in the yield of infectious virus was observed during four consecutive cell transfers (Fig. 1). Titers of released virus dropped from $10^{8.2}$ PFU/ml on the first cell transfer to $10^{4.4}$ PFU/ml by the fourth cell transfer. However, when cells were infected at a low multiplicity of infection ($10^{-4.3}$ PFU/cell), the released virus titer remained at $10^{8.0}$ PFU/ml during the four consecutive cell transfers (Fig. 1).

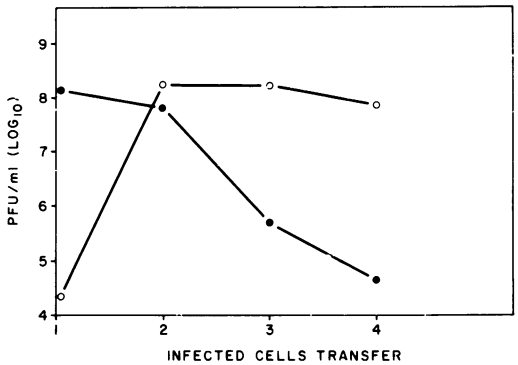

FIG. 1. *Monolayers of BHK cells were infected with ERA virus at a multiplicity of infection of 20 PFU/cell* (●) *and* $10^{-4.3}$ *PFU/cell* (○). *Every 3 or 4 days, infected cells were dispersed and transferred into new vessels in a split ratio of 1:4. Titers of released virus were evaluated for four consecutive cell transfers.*

With a culture infected at a high multiplicity of infection, cells were transferred 32 times with the virus population periodically evaluated for the presence of DI particles. The percentage of FA-positive cells and the amount of released virus were determined at each passage level (Fig. 2), with the number of FA-positive cells fluctuating between 70 and 100%. After the first drop in virus production, a new peak of infectivity was observed at the sixth passage level, followed by a progressive decline in the production of infectious virus. The size of plaques in agarose-suspended S-13 cells decreased greatly by the 20th passage, an observation also reported by Kawai et al. (10); these plaques remained small during the next 12 passages.

Released virus harvested from persistently infected cells at the 1st, 11th, 13th, 18th, and 25th to 32nd passage levels was purified and analyzed in 5 to 30% sucrose gradients (Fig. 2). Only one band of virus was present in the gradient on the first passage; two bands were clearly seen at the 11th and 13th passage levels; in all further preparations, a dense upper band and a faint lower band were seen.

Nucleic acid and infectivity of the virus from the 18th cell transfer were analyzed by labeling cultures for 24 h in the presence of 10 μCi of [^{3}H]uridine per ml. The virus was purified and sedimented through a 5 to 30% sucrose gra-

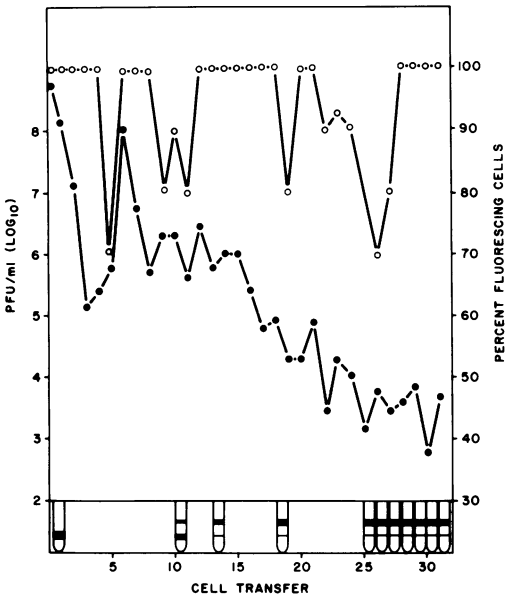

FIG. 2. *BHK cell monolayers were infected with ERA virus at a multiplicity of infection of 20 PFU/cell. Every 3 to 4 days, cells were dispersed and transferred into new vessels in a split ratio of 1:4. The percentage of FA cells* (○) *and infectivity of released virus* (●) *(PFU per milliliter) were determined at each passage level. At different passage levels purified virions were sedimented in sucrose velocity gradients, and virus bands were collected and purified further by a second sucrose gradient sedimentation.*

dient. Two bands were visible after centrifugation at 21,000 rpm for 30 min in an SW41 rotor, with the upper band being much larger. The peak of infectivity corresponded to the lower band, whereas most of the radioactivity was found in the top band which contained only 1% of the infectivity (Fig. 3). The virus collected in the region of the peak of radioactivity was examined by electron microscopy (Fig. 4) after negative staining. All of the viral particles in this region were short and truncated and measured 60 to 80 nm in length.

The material from top and bottom bands from passages 25 to 31 was pooled, diluted with phosphate-buffered saline supplemented with 1% bovine serum albumin, portioned in small amounts, and stored at −70°C. The top band material was designated ERA virus DI stock (ERA-DI). The bottom bands were similarly collected and designated "B"-enriched population (ERA-BEP). A culture at the 32nd passage level was [^{3}H]uridine-labeled, and the viral particles were isolated and subjected to an RNA analysis.

Chemical properties of ERA-DI. The protein content of ERA-DI was 0.116 mg/ml, and the number of physical particles was estimated to be 4×10^{11}/ml. This was calculated from the amount of viral protein present in the DI particle preparation and the molecular weight of the rabies virion (21). The polypeptide profile of ERA-DI virus and of ERA stock was evaluated using 10% SDS polyacrylamide gels. Figure 5 shows no difference in the distribution and proportions of the four major viral polypeptides of either viral populations. Phenol-extracted viral RNA from ERA-DI and from ERA stock were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 6). From extrapolations of the assigned molecular weights of 1.7×10^6 and 5.0×10^5 for 28 and 18*S* rRNA, respectively, the molecular weight of the complete virion genome was estimated to be 4.2×10^6 daltons and closely corresponded to the values obtained with other strains of rabies (21), whereas that of the principal species of the ERA-DI was estimated at 2.5×10^6.

Biological properties of ERA-DI virus. (i) Infectivity by plaque formation. No plaques could be obtained with ERA-DI in agarose-suspended S-13 cells or in monolayer cultures of CER cells infected with undiluted ERA-DI (4×10^{11} particles/ml) and with serial 10-fold dilutions up to 10^{-7}. Four plates per dilution were used and infected with 0.1 ml of inoculum.

(ii) Infectivity in BHK cells. Equal amounts (10^{10} particles) of ERA-DI and ERA stock and their 10-fold dilutions were mixed in suspension with BHK cells and seeded into eight-well chambers (Lab-Tek Products, Div. Miles Laboratories, Inc., Westmont, Ill.) (5×10^4 cells/

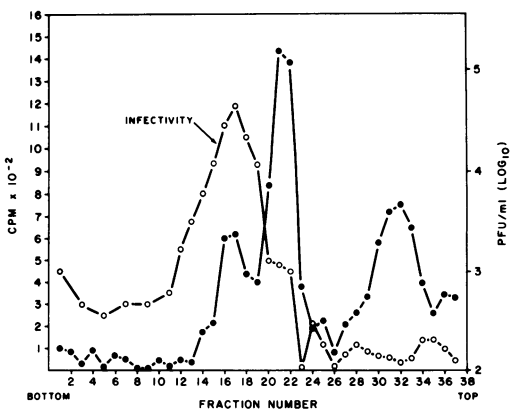

FIG. 3. *Sedimentation profile of ERA virus from persistently infected cells (18th cell passage level) in a 5 to 30% sucrose gradient. Symbols: Infectivity* (○; *PFU per milliliter); radioactivity ([^{3}H]uridine labeled,* ●; *counts per minute).*

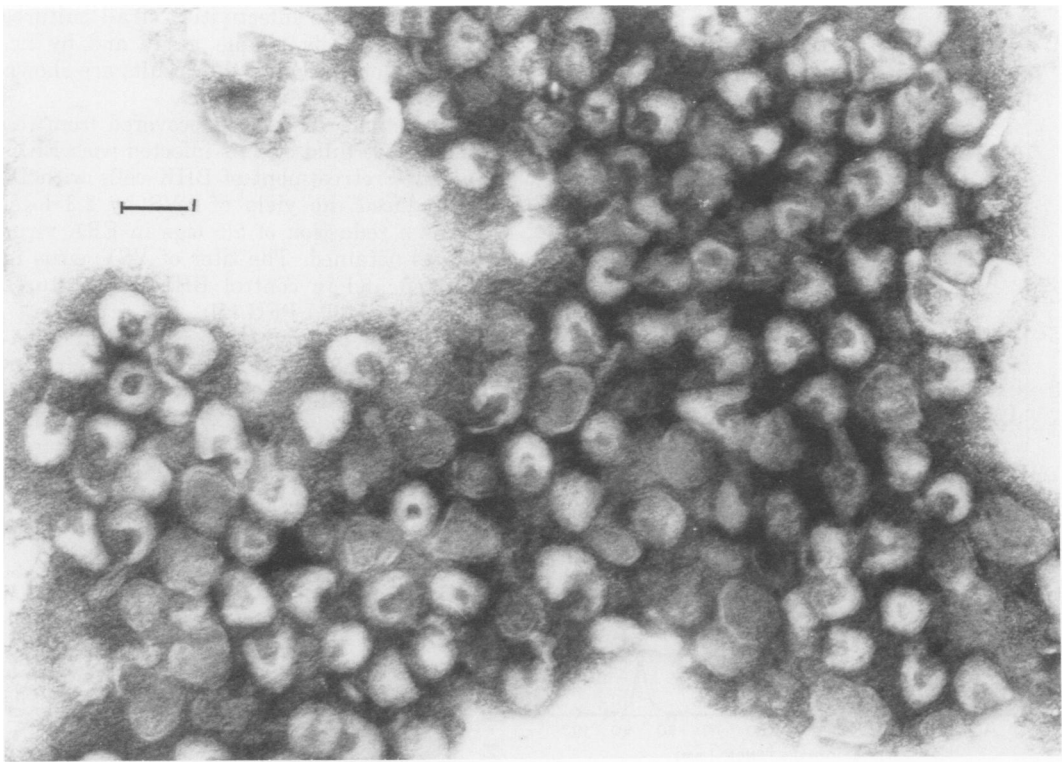

FIG. 4. *ERA rabies virus DI particles. Virus from the 18th passage of persistently infected BHK cells was purified and sedimented through a 5 to 30% sucrose gradient. The upper band of virus was negatively stained for electron microscope observation. Scale, 100 nm.*

well). After incubation at 37°C for 24 and 72 h, the slides were fixed and stained with FA. The percentage of FA-positive cells for each virus dilution was calculated with a stock ERA virus tested under similar conditions as a control. After 24 h of incubation, viral antigen was detected in cells exposed to ERA-DI virus particles (Fig. 7a), with the number of FA-positive cells decreasing with viral dilution from 80% for undiluted virus to less than 5% for virus diluted 1:1,000. There was a small increase in the number of FA-positive cells in cultures incubated for 72 h. In contrast, the difference between readings at 24 and 72 h in control cultures infected with stock ERA virus (Fig. 7b) indicated active viral replication and spread in the culture at dilutions up to 10^{-6}.

No infectious virus was produced in cultures exposed to ERA-DI inoculum (10^{10} particles), as determined by plaque assay, by infection of BHK cells, or by i.c. inoculation of mice. When rabies protein synthesis was analyzed by the high-salt technique (17), no virus-specific protein synthesis could be detected in 10^6 BHK cells infected with 10^{11} particles of ERA-DI virus (Fig. 8), whereas protein synthesis in BHK cells infected with ERA stock virus was observed.

(iii) Inoculation of mice. No signs of disease or mortality were observed in groups of eight mice injected i.c. with 10-fold dilutions of ERA-DI virus particles ranging from undiluted concentrations up to 10^{-6}.

Evaluation of the interfering activity of rabies ERA-DI. (i) Plaque reduction method. Monolayers of CER cells were exposed for 60 min to serial 10-fold dilutions (8×10^{10} to 8×10^7 particles/10^6 cells) of ERA-DI at 37°C. The inoculum was removed, and the cells were infected with 100 PFU of homologous ERA, CVS, and heterologous VSV. After another 60-min incubation, the plates were rinsed twice with minimal essential medium, overlayed with nutrient medium containing agarose, and incubated at 35°C. Rabies plaques were counted after 6 days, and VSV plaques after a 24-h incubation. A 1:10 dilution of ERA-DI particles completely inhibited the formation of rabies virus plaques; a 50% reduction was noted with DI particles diluted 1:100, whereas no inhibition was observed with DI particles diluted 1:1,000; and the growth of VSV was not in-

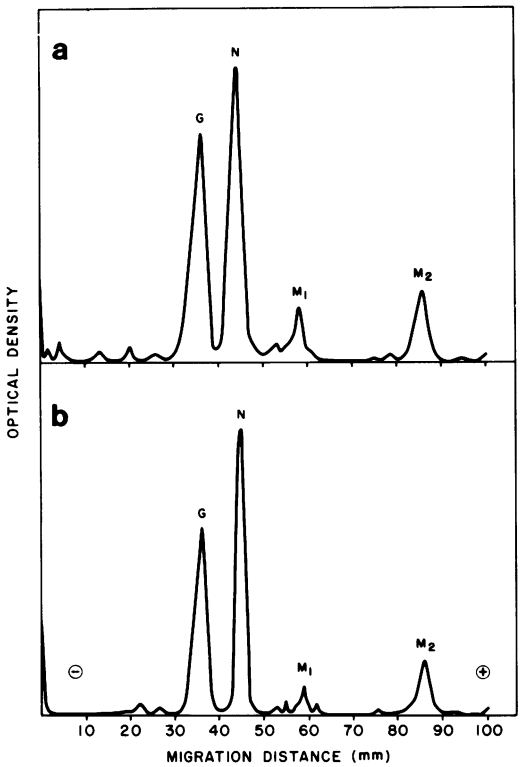

FIG. 5. *Polypeptide pattern of ERA stock virus (a) and ERA-DI particles (b).*

hibited by the DI particles at any dilution used (Fig. 9). These results suggest that the interfering activity was specific for rabies virus. In addition, exposure of ERA-DI to ultraviolet light (7×10^3 ergs/cm^2 per sec^{-1}) for 4 min, a period sufficient to inactivate at least 10^7 PFU/ml of standard virus (26), completely abolished interfering activity of ERA-DI in vitro.

(ii) Virus yield reduction method. Monolayers of BHK cells were treated for 60 min with 0.5 ml of a 1:10 dilution of ERA-DI (2×10^{10} particles). The ERA-DI inoculum was removed, and the cultures were infected with a low multiplicity of infection of ERA, CVS, or VSV virus (see Table 1). Untreated control cultures were similarly infected with ERA, CVS, and VSV without prior treatment with ERA-DI. After a 60-min incubation at 37°C, the cultures were washed twice, and the unadsorbed virus was inactivated with anti-rabies serum (see Materials and Methods) and extensively washed before the addition of minimal essential agar. No antibody treatment was used on cultures infected with VSV. Rabies virus-infected cultures were incubated for 3 days at 33°C, whereas the incubation time for VSV-infected cultures was 20 h at 37°C. The infectivities of all cultures were evaluated by plaque assay and by i.c. inoculation of mice, and the results are shown in Table 1.

No infectious virus was recovered from the tissue culture fluid of cells infected with ERA-DI alone. Pretreatment of BHK cells with DI virus reduced the yield of CVS by 2.3 logs, whereas a reduction of 5.0 logs in ERA virus titer was obtained. The titer of VSV virus in DI-treated and in control BHK cell cultures was identical ($10^{8.7}$ PFU/ml).

Evaluation of protective activity of ERA-DI. Serial 10-fold dilutions of ERA-DI virus (4×10^{11} to 4×10^8 particles/ml) were mixed with equal volumes containing 20 to 50 mean lethal doses per 0.03 ml of CVS, ERA, AF street virus, or VSV and inoculated i.c. into groups of eight mice. The observed mortalities (Fig. 10) indicate complete protection for mice inoculated

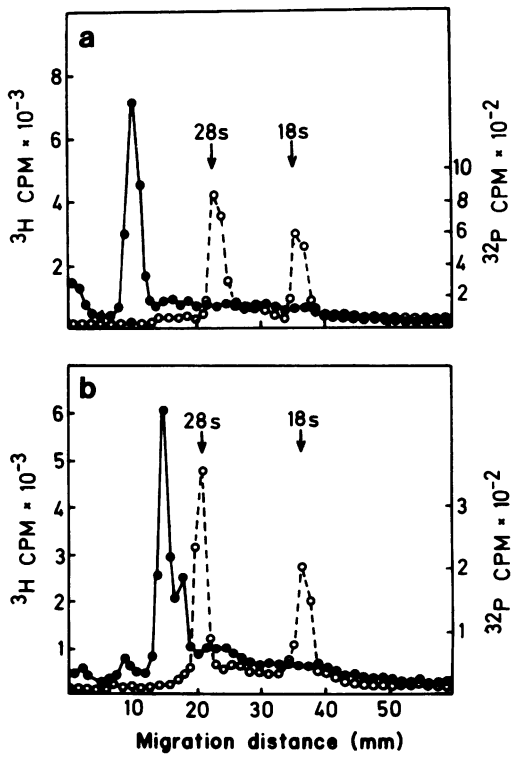

FIG. 6. *Electrophoretic migration of ERA virions (a) and ERA-DI RNA (b) on 2.5% polyacrylamide gels. Infected cells were grown in the presence of [^{3}H]uridine, and particles were purified and extracted as in Materials and Methods. rRNA labeled with ^{32}P (gift of Roberto Weinmann) was mixed with the viral or DI-RNA just before electrophoresis. Symbols: ^{3}H (●); ^{32}P (○).*

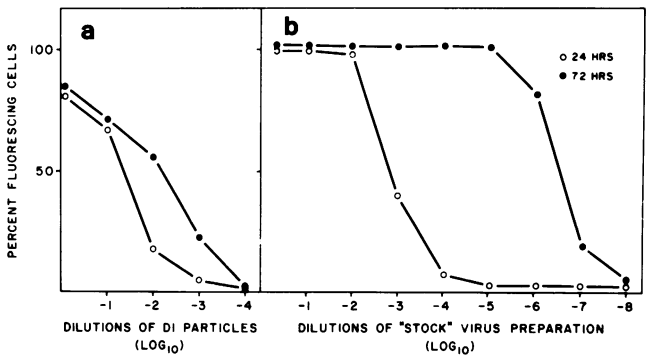

FIG. 7. *Serial 10-fold dilutions of purified ERA-DI particles (a) and ERA stock virus (b) were mixed with BHK cells and incubated for 24 and 72 h, at which time the percentage of FA-positive cells was determined. The undiluted inoculum (ERA-DI and ERA stock) contained 10^{10} particles.*

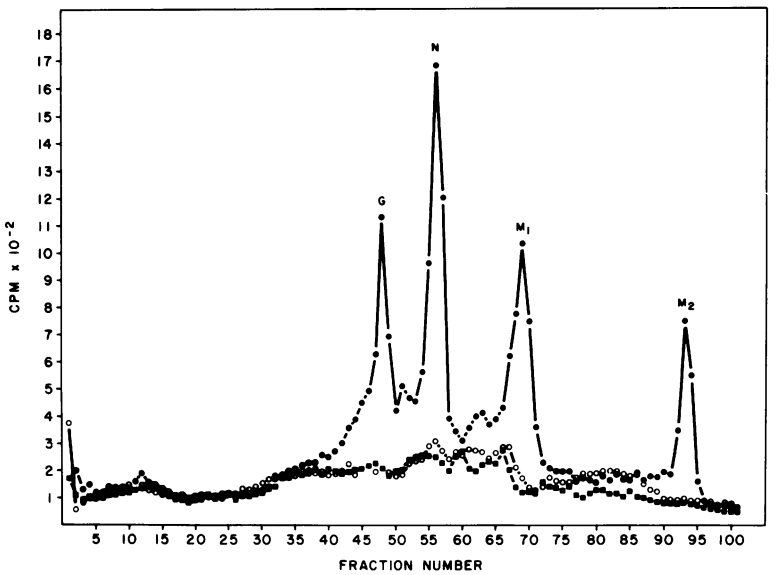

FIG. 8. *Protein synthesis in uninfected, ERA-virus-infected and ERA-DI particle-infected BHK cells under high-salt conditions. Symbols: ERA stock (●); DI particles (■); uninfected cells (○).*

with a mixture of undiluted DI preparation and either ERA or street rabies virus as well as a partial protection of mice inoculated with lower dilutions of DI virus in the presence of either ERA or street rabies virus. Protection of mice after DI-CVS inoculation was less effective, correlated with lower interference with CVS in the yield reduction assay, and a shorter incubation time of CVS virus in mice when compared with the incubation time observed for the ERA or street viruses. The specificity of the DI rabies protective effect was further demonstrated by the fact that rabies ERA-DI did not protect mice after challenge with VSV (Fig. 10).

No protection could be demonstrated when ERA-DI was mixed with undiluted ERA stock virus and inoculated into the footpads of 5-week-old mice. Both DI-treated and control mice showed signs of paralysis of the inoculated limb but survived. Thus, the protective effect of ERA-DI was observed in vivo only when a DI-infectious virus mixture was inoculated i.c., but not in the footpads of mice. Survival of mice injected i.c. with concentrations of virus pathogenic by footpad inoculation was previously reported (2) (see Discussion).

Comparative pathogenicity and immunogenicity of ERA-DI and ERA-BEP virus. A pooled sample of unconcentrated tissue culture fluid from the 25th to 31st passages of persistently infected BHK cell cultures (ERA-PIC), and two virus preparations (ERA-DI and ERA-

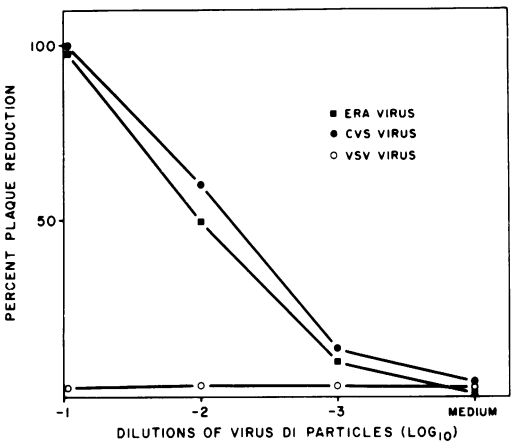

FIG. 9. *Monolayers of CER cells were treated for 60 min with 10-fold dilutions of purified ERA-DI particles (8×10^{10} to 8×10^{7} particles/10^{6} cells). After washing, cells were superinfected with 100 PFU of ERA, CVS, or VSV viruses and overlayed with agarose-containing medium. The percentage of plaque reduction was calculated after 6 days of incubation for rabies virus and after 24 h for VSV.*

TABLE 1. *Interfering activity of DI rabies virus particles on replication of CVS, ERA, and VSV viruses in BHK cells*[a]

Viral inoculum	Infectivity titers	
	PFU/ml	LD_{50}/ml[b]
DI only	0	0
DI + CVS	5.3	5.0
CVS only	7.6	7.5
DI + ERA	3.5	1.7
ERA only	8.5	7.4
DI + VSV	8.7	NT
VSV only	8.7	NT

[a] Monolayers of BHK cells were treated for 60 min with 1:10 dilution of ERA-DI preparation and then infected with CVS, ERA, or VSV viruses. After a 60-min incubation, unadsorbed virus was inactivated by antibody treatment, and cultures were incubated at 33°C for 3 days. In cultures infected with VSV virus antibody treatment was omitted, and cultures were incubated at 37°C for 24 h.

[b] LD_{50}, Mean lethal dose.

BEP) obtained after purification and separation of this virus in sucrose gradient were evaluated for their pathogenicity and immunogenicity in mice. Groups of eight animals were injected i.c. with serial 10-fold dilutions of these three viral preparations. After 4 weeks of observation, surviving mice were tail bled and challenged i.c. with CVS virus. VNA levels in pooled serum samples were determined for each group of mice, and the percentage of animals surviving challenge in each of the three groups was calculated after a 4-week incubation period.

In Fig. 11a and b, all animals inoculated with serial dilutions of either ERA-PIC or ERA-DI virus survived the initial inoculation without any sign of active disease. Most of the animals injected with either undiluted virus or with 1:10 virus dilutions survived CVS challenge. Mice injected i.c. with a 1:100 dilution of ERA-DI virus resisted challenge better than animals injected with a similar dilution of ERA-PIC virus. The level of VNA induced by ERA-DI was directly dependent on the dose of virus inoculum. Serum antibody titers of greater than 1:1,000 were found in mice injected with an undiluted ERA-DI preparation, whereas titers of 1:800 and 1:200 were observed in mice inoculated with 1:10 and 1:100 dilutions of ERA-DI, respectively. Thus, the resistance of mice to CVS correlated with the antibody level present at the time of challenge. The ERA-DI particles were shown to induce VNA in mice and protect against active disease when challenged with infectious virus.

In the mice injected with serial dilutions of ERA-BEP, all animals inoculated with the three highest virus concentrations remained well during the first 20 to 22 days of observation (Fig. 11c), while animals inoculated with virus dilutions of 10^{-3} to 10^{-5} developed symptoms characterized by rough fur, increased excitability, paralysis, and death. The highest mortality was observed in mice inoculated with virus diluted 10^{-3}. Rabies antigen was demonstrated in brain preparations of dying animals by FA staining. In animals that had been injected i.c. with undiluted ERA-BEP virus and virus diluted 1:10, the VNA levels in survivors were

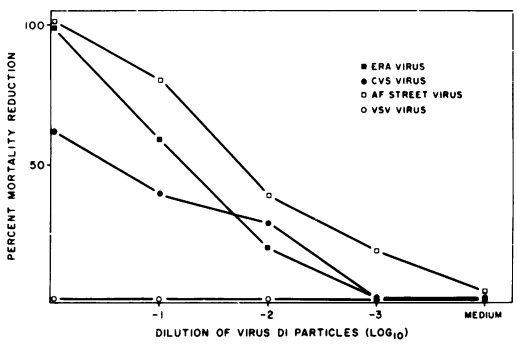

FIG. 10. *Serial 10-fold dilutions of purified ERA-DI particles (4×10^{11} to 4×10^{8} particles/ml) were mixed with 10 to 50 mean lethal doses of ERA, CVS, and AF street rabies viruses or with 10 mean lethal doses of VSV virus and inoculated i.c. into ICR mice. The percent reduction of mortality, as compared with mortalities after inoculation of these viruses alone, was calculated after 30 days of observation.*

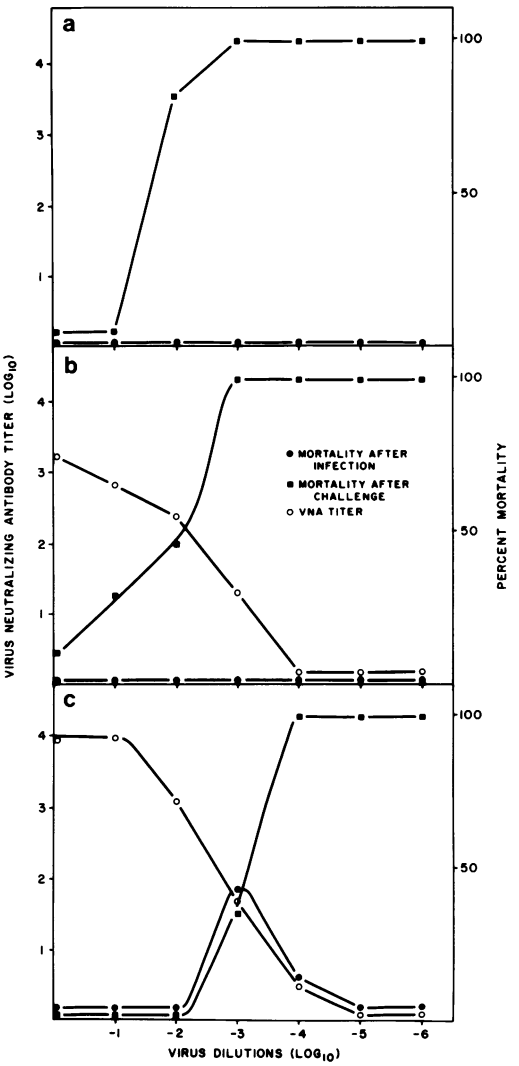

FIG. 11. *Serial 10-fold dilutions of ERA-PIC (a), of purified ERA-DI particles (b), and virus populations obtained from the lower band of sucrose gradient (ERA-BEP) (c) were inoculated i.c. into ICR mice. Four weeks later, VNA levels in surviving animals were determined, and animals were challenged i.c. with 100 mean lethal doses of CVS virus. Symbols: Percent mortality after primary inoculation (●); percent mortality after challenge inoculation (■); VNA level (○).*

extremely high, with titers over 10,000. A 10-fold reduction in VNA titer was detected in animals at each subsequent viral dilution, with no antibody detectable at higher viral dilutions. After challenge with CVS, all animals that had been primed with either undiluted virus or with viral dilutions of up to 10^{-2} survived. Partial protection was found in mice inoculated with the 10^{-3} dilution of virus, but no protection was accorded animals injected with higher dilutions of virus when challenged with CVS. Thus, the presence of ERA-DI particles in the ERA-BEP preparation protected mice from rabies infection without interfering with host antibody production. After dilution of the ERA-DI population and reduction in antibody levels, the virus was lethal for a substantial number of animals.

These results indicate that the presence of ERA-DI particles in a virus preparation (ERA-PIC) can in some as yet unknown manner effectively modulate the virus' pathogenicity in vivo. Removal of the ERA-DI particles can at least partially restore pathogenicity, as in ERA-BEP, but mortalities are observed only in animals injected with high virus dilutions, whereas animals receiving low virus dilutions survive, develop VNA, and become resistant to subsequent challenge.

DISCUSSION

The production of high concentrations of DI particles was due to the success in establishing a persistently infected culture of BHK cells that yielded large quantities of DI particles. The DI particles were isolated from sucrose gradients after rate zonal centrifugation.

The rabies DI particles bear a remarkable similarity both physically and chemically to the DI particles isolated from another rhabdovirus, VSV (9). As with VSV, the rabies DI particles are much shorter than the complete virions and maintain the bullet shape of rabies virus with its surface spikes and hollow axial. The estimated molecular weight of the DI genome of rabies is 2.5×10^6, as compared to a value of 4.2×10^6 estimated for the complete rabies virion. These molecular weights are nearly identical for values obtained for the genome of the Prevec long T particle and for the genome of VSV (16). Further studies on the RNA of rabies DI particles may show particles that contain varying genome lengths, as is the case with VSV (9). In spite of the difference in the size of the rabies DI particle genome, compared to whole virus, the rabies DI particles seem to have all of the structural proteins of the infectious particles in the same ratio.

After exposure of BHK cells to rabies DI particles, a rabies antigen was detected; however, no infectious progeny virus was produced. Since the mechanism by which DI particles actually interfere with viral replication is still not well understood, even in the case of VSV, the positive FA response to rabies DI particles in infected cells defies easy explanation. As the

antibody used is highly specific for rabies nucleocapsid (RNA bound to the N protein), the antigen detected could be due to the uncoated input particles and cannot be unequivocally eliminated. Also, DI preparations are seldom free of infectious virus, and synthesis of nucleocapsid due either to the replication of the contaminating infectious virus or DI particle synthesis using helper functions provided by contaminating infectious virus remains a possibility. The hypothesis suggesting new protein synthesis is not supported by the protein synthesis experiment (Fig. 8).

Like the DI particles of VSV (5), rabies DI particles are not infectious for mice injected i.c. but protect mice when challenged with infectious rabies. These same DI particles are capable of interfering specifically with the replication of the standard rabies virus in tissue culture. This sparing effect in mice may be mediated by an immune response to the DI particles, which are as capable of inducing antibody as the standard virus because of their similar protein composition.

Isolation and identification of rabies DI particles permits a reconstruction of previously unexplained events. In 1953 the Flury strain of rabies was serially passaged in developing chicken embryos (12), with the infectivity of the virus for adult mice injected i.c. checked at each passage level. An abrupt (and permanent) loss of infectivity for adult mice was observed at the 180th passage. When virus stock that had been frozen at an early passage level was repassaged in developing chicken embryos, loss of infectivity for adult mice occurred in each experimental series at almost the same passage level, as in the case of the original series. This phenomenon may be explained by the fact that in each passage series of the Flury strain, the concentration of DI particles increased and modulated the lethal effect of the LEP Flury virus in adult mice. This attenuation of the LEP Flury virus due to DI particles led to the development of a HEP Flury virus that could be used as a live virus vaccine for many animal species. The HEP Flury strain still remained pathogenic for suckling mice and adult monkeys injected i.c., but lost its pathogenicity at any given dilution for either rabbits or dogs injected i.c. Hamsters and guinea pigs injected i.c. with low dilutions of HEP Flury virus remained healthy, but if the virus was diluted a few of the inoculated animals died (12).

With a stock of purified DI particles, the experiments may be reconstructed in research that would compare to that conducted in vitro with influenza and VSV (7) to determine whether interference of DI particles with standard virus in vivo is host dependent. It would be interesting also to investigate whether, similar to the findings with West Nile virus, the genetic makeup of the host of the same species may determine the rate of replication of the DI particles and their capacity for interference with the standard virus (4).

The fact that the DI particles seem unable to interfere with the spread of the standard rabies virus from the periphery to the central nervous system may explain the puzzling results obtained by Clark and Ohtani (2) when working with the ts 2 mutants of rabies virus. This virus, when injected in low dilution into the footpads of mice, caused either paralysis or death of the infected animals, whereas mice injected i.c. with the same dilution showed no signs of disease. When the ts 2 virus was diluted apparently beyond the concentration of the DI particles, inoculation of mice (i.c.) caused paralysis and death.

In nature, rabies is usually transmitted by bite, and the virus, after spreading along the nerve trunks, seems to replicate first in the neurons of the corresponding ganglia of the spinal cord. Even if DI particles were to be present in the saliva of the biting animal, it seems unlikely, in light of the results obtained with mice injected into the footpads, that the DI particles would interfere with the spread of the virus from the site of infection to the central nervous system. On the other hand, replication of the virus in neurons may lead to the production of DI particles, and their capacity to interfere with the spread of the virus within the central nervous system may not only determine the ultimate outcome of the infection but may also explain the latency of the infectious process, which may extend over months or even years.

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