Defective Interfering Particles of Fixed Rabies Viruses:
Lack of Correlation with Attenuation or Auto-interference in Mice

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SUMMARY

Six different fixed strains of rabies virus were analysed for their capacity to produce defective particles following acute infection of BHK-21 cells. Five of the six strains produced one or more defective particle populations with strain-specific sedimentation properties, particle length and abbreviated RNA genome size. These defective particles varied in their capacity to interfere with replication of standard rabies virus in cell culture. Each virus strain characteristically either killed adult mice according to a normal dose-response pattern or to an auto-interference type of pattern, or failed to kill mice. Different strains also varied in their capacity to induce a cytopathic effect in cell culture. However, there was no apparent correlation between the presence of defective particles and the pathogenic potential of rabies virus in mice or in cell culture.

INTRODUCTION

The possibility that defective interfering (DI) particles may play a significant role in determining the outcome of virus infections in vivo has been strongly advocated (Huang & Baltimore, 1970). There is indirect evidence that DI particles may be associated with attenuation of virus infection in some animal systems (Darnell & Koprowski, 1974; Woodward & Smith, 1975). The DI particles of vesicular stomatitis virus (VSV) are the most thoroughly characterized and have been tested for their potential protective role by intracerebral (i.c.) inoculation into mice. The presence of excess DI particles in the VSV inoculum was correlated with conversion of an acute viral encephalitis, which is usually quickly fatal, into an infection with a greatly prolonged course, or survival (Doyle & Holland, 1973; Rabinowitz et al., 1977). Such protection has been attributed, however, solely to immunological factors (Crick & Brown, 1977). Subsequently, it was also reported that exogenously administered DI particles of Semliki Forest virus (SFV) protected mice against fatal encephalitis induced by infectious SFV given intranasally (Dimmock & Kennedy, 1978). This same route has also been used to demonstrate apparently antibody-mediated protection against VSV (Wagner, 1974).

Fixed rabies viruses provide a particularly attractive system for the testing of the role of DI particles in vivo because: (i) rabies virus causes a fatal encephalitic disease that is of public health importance; (ii) rabies infections are frequently characterized by very long incubation periods for which the explanation is unknown; (iii) fixed virus strains with widely divergent virulence characters are available for study (Clark & Wiktor, 1972a; Clark, 1979); and (iv) the DI particles of rabies virus can be readily separated physically from standard virus and characterized.

Prior to this report, production of DI particles following acute infection of cell culture was observed only with fixed virus of strain Flury LEP (Crick & Brown, 1974). However, there has been interest in the possible role of DI particles in rabies pathogenesis based on...
the early observations (Koprowski, 1954) of irregular lethal-dose responses [or 'auto-interfering' (AI) death patterns] in hamsters and guinea-pigs inoculated with Flury HEP virus. We have noted an AI death pattern following titration of the temperature-sensitive CVS mutant, ts2 (Clark & Koprowski, 1971) and certain derivative virus clones (Clark & Ohtani, 1976). We have also reported that an AI death pattern is often induced by rabies virus populations which undergo an increase in virulence caused by passage in newborn mouse brain or in neuroblastoma cells (Clark 1978, 1979, 1980) or a decrease in virulence concurrent with virus passage in chronically infected cell cultures (for review, see Clark, 1979).

Whereas certain fixed rabies virus strains that are adapted to BHK-21 cells cause an AI death pattern, other fixed strains either kill mice following i.c. inoculation according to a normal dose-response or cause no deaths at any dilution. We have examined stocks of several virus strains for the presence of DI particles, and have investigated the possible role of such particles in strain-characteristic patterns of pathogenicity.

**METHODS**

*Cell culture.* BHK-21 cells were maintained in Eagle's minimal essential medium supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin (MEM-FCS10). BHK S13 cells were maintained in Stoker's BHK cell medium (MacPherson & Stoker, 1962) with 10% FCS and antibiotics. Human neuroblastoma cell lines SK-N-SH and SK-N-MC (Biedler et al., 1973) were kindly supplied by Dr J. Biedler, Sloan-Kettering Memorial Cancer Institute, N.Y., U.S.A., and human neuroblastoma cell line IMR-32 (Tumilowicz et al., 1970) was obtained from the American Type Culture Collection; all were grown in MEM-FCS10 with Eagle's non-essential amino acids added. Mouse neuroblastoma C1300 clone NA (NA) cells (McMorris & Ruddle, 1974) were supplied by Dr F. A. McMorris, The Wistar Institute, Philadelphia, Penn., U.S.A. A mixed cell type, cell line CB3, from normal bovine brain was derived and supplied by Dr D. Santoli, The Wistar Institute. The latter two cell lines were propagated in MEM-FCS10.

*Virus*

The origin of the six strains (or substrains) of fixed rabies virus used and their original adaptations to a laboratory host(s) are described elsewhere (Clark & Wiktor, 1972b). The history of each strain at the Wistar Institute laboratories before use is listed below.

**Pitman–Moore (Pasteur strain).** Pasteur strain virus designated PM 11 was obtained from the National Institutes of Health, Bethesda, Md., U.S.A. on 28/1/60. Virus was passaged twice in rabbit brain, 54 times in human diploid cell strain WI-38 (HDCS), and once in hamster cell line Nil 2 (Diamond, 1967). It was then cloned twice in agarose-suspended BHK S13 cells (Sedwick & Wiktor, 1967) and subsequently passed 12 times in BHK-21 cells infected at a multiplicity of infection (m.o.i.) of about 1.

**CVS.** Strain CVS-11 which had been passaged 112 times in primary hamster kidney cells (Kissling & Reese, 1963) was obtained from the Center for Disease Control, Atlanta, Ga., U.S.A. on 13/4/67. The virus was passaged twice in BHK-21 cells, cloned three times and then passaged seven times in BHK-21 cells infected at an m.o.i. of 1.

**Flury low egg passage (LEP).** This virus was obtained from Dr R. Barth, Behringwerke, Marburg, F.R.G. This virus, originally from the American Type Culture Collection, was passaged at Behringwerke 31 times in primary hamster kidney cells, 12 times in HDCS cells, 20 times in chick embryo fibroblasts, and once in Nil 2 cells. In our laboratory, the virus was passaged once in BHK-21 cells, cloned and then passaged twice in BHK-21 cells at an m.o.i. of 1.
DI particles of fixed rabies viruses

Flury high egg passage (HEP). This virus was obtained from Dr H. Koprowski, The Wistar Institute. It was passaged three times in chick embryos, 16 times in HDCS, cloned three times, and then passaged eight times in BHK-21 cells at an m.o.i. of 1. ERA (WT). This virus identified as Wistar Institute stock was obtained as commercial vaccine from the World Health Organization, Geneva. It was passaged twice in BHK-21 cells, cloned three times and then passaged 14 times in BHK-21 cells. Kelev. This virus was obtained from Haifa, Israel in 1968 as 108th chick embryo passage stock. It was passaged four times in BHK-21 cells at an m.o.i. of 1.

Virus propagation. Rabies virus was routinely propagated in BHK-21 cells infected at an m.o.i. of about 1. Infected cell cultures were fed with MEM supplemented with 0-2% bovine serum albumin (Sokol & Clark, 1973) and incubated at 33 °C. Virus was harvested on day 3 post-infection (p.i.), fresh maintenance medium was added to cell cultures and virus was harvested again on day 7 p.i. The yield of total virus and of DI particles was maximum in the day 7 harvest.

Virus infectivity and pathogenicity assays. Plaque assay was performed on cultures of agarose-suspended BHK S13 cells (Sedwick & Wiktor, 1967). The pathogenic potential of virus preparations was determined by titration in 4- to 6-week-old Swiss ICR outbred mice inoculated i.c. (0-03 ml in the left cerebral hemisphere). Inoculated mice were observed daily for 30 days p.i.

DI particle isolation. Infected cell culture supernatant fluids were clarified by centrifugation for 30 min at 600 g. Virus was sedimentsed by centrifugation at 48 000 g for 2 h in a Beckman no. 19 rotor. Virus pellets were resuspended to 1% of the original volume in 0-2 ml 0-13 M-NaCl, 0-05 M-tris-HCl, 1 mM-EDTA (NTE buffer, pH 7-8) (Michalski et al., 1976). The concentrated virus was layered on to sucrose density-gradients (5 to 30%, w/v) and centrifuged for 45 min at 54 000 g. Visible bands of particles were collected by side-puncture. The harvested particles were diluted in NTE, sedimented either at 110 000 g for 5 h in an SW41 rotor or at 189 000 g for 3 h in an SW50.1 rotor, and resuspended in NTE for further analysis.

Electron microscopy. Virus preparations were mixed with an equal volume of 2% phosphotungstic acid and examined with an Hitachi HS8 electron microscope at a magnification of approx. ×33 000. Sizes were determined from the mean dimensions of at least 100 particles.

Virus RNA labelling and SDS–polyacrylamide gel electrophoresis. Infected BHK-21 cells were labelled with 10 μCi/ml 3H-uridine (New England Nuclear, Boston, Mass., U.S.A.) as described previously (Wunner & Clark, 1980). The extraction of genomic RNA from standard and DI particles and electrophoresis in SDS–polyacrylamide gel have been described (Wunner & Clark, 1980).

Protein determinations. Virus protein concentrations were determined by the xylene brilliant cyanin G colorimetric method described by Bramhall et al. (1969). A standard curve was constructed using concentrations of bovine serum albumin of 0-1 to 2-5 mg/ml.

Ultraviolet (u.v.) light inactivation. Particles were exposed for double the time required to inactivate completely the infectivity of standard virus particles: 2 ml of a test particle preparation in a 6 cm Petri dish was exposed to 60 ergs/mm²/s of u.v. energy for 10 min.

RESULTS

Defective particle production characteristic of different fixed rabies viruses

Rabies viruses were passaged from 2 to 14 times in BHK-21 cells since their most recent clone purification. Fresh virus stocks prepared in this way were titrated in i.c.-inoculated mice and analysed in parallel by rate-zonal sucrose density-gradient centrifugation for identification of DI particles (results are shown in Fig. 1). The characteristic particle size distribution of each strain was consistently observed following repeated passages at an
m.o.i. of 1. Three strains of rabies virus (CVS, PM and Flury LEP) killed mice according to a normal dose response; all strains produced a single defective particle population with characteristic sedimentation properties. Strain ERA virus, which characteristically caused a marked AI death pattern in mice, produced three distinct populations of DI particles. Flury HEP virus, previously reported to be completely avirulent for adult mice (Koprowski, 1954; Koprowski et al., 1954) was frequently found to kill 5 to 40% of mice inoculated with near terminal dilutions of virus (see also Clark, 1979). This virus strain also produced a characteristic DI particle population that sometimes could be resolved into two closely sedimenting populations. Only the Kelev virus strain, which appears to be fully attenuated, produced no detectable defective particles.

DI particle bands were examined by electron microscopy; each was found to be composed predominantly of truncated particles. Mean particle lengths and other characterizations of DI particles are shown in Table 1. DI particles consistently exhibited substandard particle lengths corresponding to their relative positions in density gradients. Five defective particle populations, from CVS, ERA and HEP strains, were demonstrated to contain subgenomic
**DI particles of fixed rabies viruses**

### Table 1. Preliminary characterization of rabies DI particles*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Particle</th>
<th>Mean particle length (nm)</th>
<th>RNA mol. wt. (x 10^-6)</th>
<th>Relative particle concentration (T/B)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>µg protein (%)</td>
</tr>
<tr>
<td>CVS</td>
<td>T</td>
<td>ND</td>
<td>0.6</td>
<td>59</td>
</tr>
<tr>
<td>ERA</td>
<td>T_1</td>
<td>64.3</td>
<td>0.9</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>T_2</td>
<td>83.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>96.8</td>
<td>2.6</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>138.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEP</td>
<td>T</td>
<td>ND</td>
<td>0.6</td>
<td>32</td>
</tr>
<tr>
<td>PM</td>
<td>T</td>
<td>55.9</td>
<td>ND</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>163.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEP</td>
<td>T</td>
<td>53.8</td>
<td>ND</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>144.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Virus was propagated and progeny particles separated by rate-zonal density-gradient centrifugation as described in Methods. The relative distribution of each particle class (T = defective; B = standard) is given for a single experiment typical of each virus strain.

† Standard virus RNA, mol. wt. approx. 4 x 10^6 (Brown *et al.*, 1979).

‡ ND, Not determined.

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**Fig. 2.** Fixed rabies virus of strain CVS and derivative substrains analysed for mouse-pathogenic potential and particle size distribution as described in Fig. 1. Upper band is T and lower band is B; N.D., non-diluted. (a) CVS, 10^6.5 p.f.u./ml; (b) ts2, 10^6.3 p.f.u./ml; (c) Cl 2, 10^6.7 p.f.u./ml; (d) Cl 4, 10^6.7 p.f.u./ml; (e) Cl 5, 10^6.6 p.f.u./ml; (f) G26a, 10^5.9 p.f.u./ml.
single-stranded RNA of a characteristic size which also corresponded to their relative sedimentation properties. DI particle yields (μg of particle protein) varied from approx. one-third of standard virus production to amounts exceeding that of standard virus. Infectious virus was recovered from DI particle bands in amounts less than 5% of the standard virus yield.

**Defective particle production by CVS virus compared to derivative variants**

Although strain CVS was our most efficient lethal virus for mice (mouse LD_{50}/p.f.u. ratios are consistently ≥1.0; Clark & Wiktor, 1972a; Clark, 1978), several variant viruses derived from CVS exhibited reduced virulence accompanied by an AI dose response. These variant viruses were compared with the parental CVS virus by parallel titration in mice and analysis of defective particle production (Fig. 2). Virus ts2, a ts revertant virus mixture (Clark & Ohtani, 1976), three clones derived from that mixture (ts viruses Cl 2 and Cl 5, ts revertant virus Cl 4), and a CVS virus variant (G26a), modified by 26 serial passages of infected CB3 cells (H F. Clark, unpublished data), each produced a single population of defective particles that sedimented at a similar position (T band) in the sucrose gradient.

**Effect of depletion of DI particles on the pathogenicity of fixed rabies viruses**

It has been observed that the capacity of viruses to produce DI particles is reduced or lost following serial passage at very low m.o.i. (Huang et al., 1966). Similarly, strain ERA (WI) and Flury HEP viruses were passaged five or six times at very low multiplicity by serial cloning, i.e. plaques were harvested, plaque suspensions were titrated directly by plaque assay, and plaques derived from those titrations were again harvested and the resultant suspensions titrated directly. This approach gave a final effective m.o.i. of <1.0 × 10^{-6} and consistently yielded virus after three to six passages that produced no visible bands of DI particles.

ERA (WI) stock virus consistently produced the largest number of DI particle classes (T₁, T₂ and M band particles) and a dramatic AI dose response in mice (Fig. 3a). A stock of plaque-purified ERA (WI) virus was prepared that produced only standard virus but nevertheless retained the property of causing a definite AI pattern of deaths in mice (Fig. 3b). Similarly, when standard ERA (WI) virions of uncloned stock were partially purified by two cycles of gradient centrifugation, standard (B band) particles also retained the capacity to cause an AI death pattern in mice (Fig. 3c). It appeared very unlikely that contaminant defective particles in the standard virion preparation mediated the AI effect in mice, as mouse protection experiments showed that a very great excess of rabies DI particles was required in order to provide even minimum protection (see below). Titration in mice of the separate bands of ERA DI particles that occasionally retained up to 1% of the infectivity of standard virus (as determined by plaque titration) also caused an AI death pattern which was indistinguishable from that associated with untreated virus preparations (data not shown).

The association of DI particles with an AI death pattern was also analysed with Flury HEP rabies virus. Flury HEP virus caused a minimal mouse mortality response near terminal dilutions and typically produced a broad T band of DI particles (sometimes appearing as two closely adjacent bands). A Flury HEP virus with full virulence for mice may be obtained by a single passage at high m.o.i. in suckling mouse brain (Koprowski, 1954; Clark, 1978). Fully virulent virus developed in this way from our BHK-21 cell-adapted stock contained a population of defective particles with sedimentation characteristics indistinguishable from those of the parent virus.

We also studied the effect of either physically separating standard virions or of serial cloning upon the lethal potential of BHK-21 cell-adapted Flury HEP virus. Partially purified
Fig. 3. The Wistar Institute (WI) substrain of ERA-fixed rabies virus was tested for lethal potential in mice and particle size distribution, as described in Fig. 1, (a) before and (b) after five low m.o.i. passages in BHK cells by serial cloning of harvested plaques and (c) purification of standard virions by two cycles of rate-zonal centrifugation in sucrose density-gradients; N.D., non-diluted. (a) $10^8.6$ p.f.u./ml; (b) $10^{8.2}$ p.f.u./ml; (c) $10^{8.8}$ p.f.u./ml.

standard Flury HEP virions produced an AI death pattern in mice indistinguishable from that caused by an unfractionated parent virus stock. Virus stocks prepared from serially cloned Flury HEP virus produced no DI particles but retained the capacity for killing a few mice according to an AI pattern.

After serial cloning, BHK-21 cell-adapted CVS virus also failed to produce defective particles. CVS stocks prepared from serially cloned or partially purified CVS standard virus caused mouse deaths according to the same normal dose response curve and with the same high efficiency (mouse LD$_{50}$/p.f.u.) as the parent uncloned virus.

AI cytopathic effect pattern in cell culture associated with rabies standard and defective particles

Although rabies virus is not normally highly cytolytic in cell culture, considerable cytopathic effect (c.p.e.) is usually induced in C1300 NA mouse neuroblastoma cells (Clark, 1980). When fixed rabies viruses were titrated in NA cell monolayers, they caused a marked AI c.p.e. pattern that did not correspond to the death pattern observed in titrations of the same virus in mice. Strain CVS, which was uniformly lethal for mice, caused the most extreme AI c.p.e. pattern in cell culture; strain ERA virus which caused a definite AI death pattern in mice showed only slight evidence of AI c.p.e. induced in NA cells.

To determine whether defective particles play a role in mediating this c.p.e. phenomenon in NA cells, separate populations of standard and defective particles of ERA, CVS, and Flury HEP viruses were titrated in parallel in NA cell culture and in mice (Fig. 4). Like the unfractionated ERA virus populations, ERA standard particles (B band) induced only a minimal AI c.p.e. pattern in NA cells despite causing a very extreme AI death pattern in mice. ERA DI particles of combined $T_1$ and $T_2$ bands retaining approx. 0.1% of the
Fig. 4. Comparison of lethal potential in mice and cytopathic potential in vitro of representative defective particle populations with standard virions of rabies virus strains ERA (WI), CVS and Flury HEP. Cytopathic effect was assayed by inoculation of 0.1 ml of serial 10-fold dilutions of test preparations into individual wells of 24-well plastic tissue culture trays (Linbro) containing confluent monolayers of NA cells or a 0.03 ml inoculum/mouse brain. Infected cultures were incubated at 35 °C and observed daily. The % c.p.e. is the % of cell destruction observed in virus-infected tissue culture wells on either day 6 or 8 p.i. (the last day that control cell monolayers retained 100% integrity), and % mortality is described in the legend to Fig. 1. (a) ERA T band particles, $10^{6.2}$ p.f.u./ml; (b) ERA M band particles, $10^{6.5}$ p.f.u./ml; (c) ERA B band virions, $10^{9.5}$ p.f.u./ml; (d) CVS T band particles, $10^{8.7}$ p.f.u./ml; (e) CVS B band virions, $10^{8.3}$ p.f.u./ml; (f) HEP B band virions, $10^{6.7}$ p.f.u./ml. •—•, Mortality (%); △—△, cytopathic effect (%).

The results suggested that c.p.e. induced by standard rabies virus may be reduced in the presence of excess DI particles. However, it also appeared that a dramatic AI c.p.e. pattern may be induced by standard CVS rabies virus depleted of defective particles.
**DI particles of fixed rabies viruses**

### Table 2. *ERA virus T band DI particle-mediated interference in diverse host cell systems*

<table>
<thead>
<tr>
<th>Host cell</th>
<th>Challenge virus titre (log&lt;sub&gt;10&lt;/sub&gt; p.f.u./ml) on day</th>
<th>DI particle-induced reduction in titre (log&lt;sub&gt;10&lt;/sub&gt; p.f.u./ml) on day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>BHK-21</td>
<td>4.0</td>
<td>6.2</td>
</tr>
<tr>
<td>C1300 (NA)</td>
<td>5.9</td>
<td>6.7</td>
</tr>
<tr>
<td>IMR-32</td>
<td>2.1</td>
<td>6.1</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>2.7</td>
<td>5.7</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>&lt;0.7</td>
<td>3.6</td>
</tr>
<tr>
<td>CB3</td>
<td>&lt;0.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Confluent cell cultures in individual wells of 24-well plastic trays were washed once with PBS, treated with 0.1 ml ERA T (T1 and T2) DI particles at the concentration indicated or with control diluent for 30 min at 35 °C, washed twice with PBS and challenged by inoculation with standard ERA virus at an m.o.i. of 0.01. Challenge virus was adsorbed for 30 min at 35 °C. Cells were then washed twice with PBS and fed with 1 ml/well cell maintenance medium and incubated at 35 °C. At the indicated intervals p.i. (days 2, 4 or 7), 0.1 ml cell supernatant fluid was harvested from each well and titrated for virus infectivity.

† ND, Not determined.

### Demonstration of interfering capacity of rabies DI particles in cell culture

To determine whether the truncated particles produced by fixed rabies strains were indeed DI particles, the interfering capacity of selected particle populations was tested. BHK-21 cells were treated with truncated particles at a concentration of 10 μg/ml (about 3500 particles/cell; Wiktor et al., 1977). Similar results were obtained using either CVS or ERA strain standard particles (m.o.i. 0.01 to 0.02) as challenge virus. Particles of either ERA T (combined T1 and T2 or M bands led to reduction in virus yield (measured at day 2 to day 7 p.i.) varying in magnitude from 10-fold to 1000-fold. Little or no interference was demonstrated with the CVS T band particles. Flury HEP T band particles interfered with the replication of infectious rabies virus, but with slightly less efficiency than ERA T and M band particles (data not shown).

The effect of u.v. irradiation on the interfering activity of DI particles was determined. Strain ERA T and M band particles, irradiated under conditions that completely inactivated infectious rabies virus, lost all capacity to interfere with the replication of standard ERA virions. Thus, DI particle interference requires the molecular integrity of the DI particle genome, a definitive characteristic of DI particles (Huang & Wagner, 1966; for review, see Bay & Reichmann, 1979).

Additional experiments were performed to measure quantitatively the interfering efficiency of the ERA T band DI particles by titration and to assess the capacity of different host cell systems to support DI particle-mediated interference. We have reported elsewhere that NA cells support DI particle-mediated interference with an efficiency greater or equal to that of BHK-21 cells (Clark, 1980). The results of representative experiments are presented in Table 2. DI particle-mediated interference was consistently detected throughout the course
Table 3. Effect upon mouse mortality of addition of DI particles or standard rabies virions to lethal CVS virus preparations

<table>
<thead>
<tr>
<th>CVS challenge virus</th>
<th>Test additive</th>
<th>Mortality</th>
<th>Mean day of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Mouse brain-origin virus (non-purified)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>Diluent</td>
<td>5/5</td>
<td>10.0</td>
</tr>
<tr>
<td>1000</td>
<td>T</td>
<td>5/5</td>
<td>9.6</td>
</tr>
<tr>
<td>1000</td>
<td>M</td>
<td>5/5</td>
<td>8.6</td>
</tr>
<tr>
<td>1000</td>
<td>B</td>
<td>5/5</td>
<td>7.5</td>
</tr>
<tr>
<td>100</td>
<td>Diluent</td>
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<td>11.0</td>
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<td>100</td>
<td>T</td>
<td>5/5</td>
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<td>B</td>
<td>3/5</td>
<td>8.3</td>
</tr>
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<td>T</td>
<td>1/20</td>
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<tr>
<td>1</td>
<td>B</td>
<td>3/10</td>
<td>9.3</td>
</tr>
<tr>
<td>(b) Partially purified standard CVS virions</td>
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<tr>
<td>330</td>
<td>Diluent</td>
<td>5/5</td>
<td>9.6</td>
</tr>
<tr>
<td>330</td>
<td>T</td>
<td>5/5</td>
<td>11.8</td>
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<tr>
<td>330</td>
<td>M</td>
<td>5/5</td>
<td>10.8</td>
</tr>
<tr>
<td>330</td>
<td>B</td>
<td>ND†</td>
<td>—</td>
</tr>
<tr>
<td>33</td>
<td>Diluent</td>
<td>8/10</td>
<td>10.2</td>
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<td>(c) Diluent control</td>
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<tr>
<td>0</td>
<td>T</td>
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<td>0</td>
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</tr>
<tr>
<td>0</td>
<td>B</td>
<td>2/10</td>
<td>13.5</td>
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</table>

* To infectious preparations of CVS rabies were added equal quantities of strain ERA T band DI particles (T), M band DI particles (M), B band standard virions (B) or as control, BHK-21 cell medium containing 2% foetal calf serum (diluent). Preparations of T, M and B contained respectively 10 μg protein and <7 p.f.u./ml, 10 μg protein and 7 x 10⁵ p.f.u./ml, and 20 μg protein and 3.1 x 10⁸ p.f.u./ml. The mixed virus suspensions were immediately inoculated in a dose of 0.03 ml into the left cerebral hemisphere of 4- to 6-week-old ICR female Swiss mice. The results of several experiments have been collected.

† ND. Not determined.

of a 7-day growth cycle and was clearly DI particle dose-dependent. It was consistently detected at a DI particle concentration of 1 μg/ml and often at a concentration of 0.1 μg/ml representing about 35 particles/cell. Efficient DI particle-mediated interference was induced consistently in BHK-21 cells, in NA cells, and in the human neuroblastoma cell lines IMR-32, SK-N-SH and SK-N-MC. The slight deficiency in sensitivity apparent in SK-N-MC cells may reflect the low efficiency of replication of rabies virus in this system. Cell line CB3 did not support DI particle-mediated interference.
Attempts to demonstrate DI particle-mediated protection against rabies infection in vivo

To determine whether rabies DI particles could protect against standard lethal rabies virus, strain CVS virus which killed mice at the lowest concentration of any of our fixed strains (Clark & Wiktor, 1972a), was mixed in serial dilutions to near endpoint with 10 μg/ml (approx. 3.5 × 10^10 particles/ml) of ERA T band or M band DI particles (the M band DI particles are most efficient in interfering in cell culture assays). This resulted in a dose for mice of about 5 × 10^8 DI particles. The mixtures were inoculated i.c. into adult mice and the results are shown in Table 3.

When the challenge virus was a non-purified infected mouse brain suspension (Table 3a), no protection was afforded by DI particles against virus doses of > 10 LD_{50}. Indeed, the addition of ERA DI particles (or standard ERA virus) to CVS virus was often associated with a reduced incubation period before death (Table 3a). Apparent partial protection against 1 LD_{50} of CVS challenge virus was associated with DI particles but also with standard ERA virus.

When density gradient-purified CVS standard virions of BHK-21 cell culture origin were used as challenge virus (Table 3b), the addition of either ERA M or T band DI particles was associated with a slight reduction in the mouse mortality caused by a challenge dose of 33 LD_{50} and a marked reduction in the mortality caused by a challenge dose of 3.3 LD_{50}. However, more pronounced protection against either dose of challenge virus was provided by addition of standard ERA virions. The reduction in incubation period often noted when DI particles were added to non-purified mouse brain-origin CVS virus was not noted when DI particles were added to CVS standard virions of tissue culture origin.

A further attempt was made to determine whether rabies DI particle-mediated protection might be demonstrated more efficiently against infections initiated by peripheral intraplantar or intranasal routes of inoculation. This approach is complicated by the fact that large doses of challenge virus are required to cause a uniform death response following peripheral inoculation. No protection was induced when ERA T band DI particles (as described in Table 3) were added to CVS rabies virus administered by the intranasal or intraplantar route (data not shown). However, ERA standard (B band) virions did induce some protection against a CVS challenge given by the intraplantar route, but not by the intranasal route.

We determined the effect of u.v. irradiation of ERA M and T band DI particles upon protection afforded against standard CVS virus given i.c. in low dose and the effect of u.v. irradiation of ERA standard B particles upon the protection they confer against CVS intraplantar challenge. In both cases, protection associated with the added particles was lost following u.v. irradiation (data not shown). Since standard virus was clearly shown to possess a u.v.-sensitive interfering activity, the possibility that contaminant standard virus might play a role in DI particle-mediated protection in vivo could not be eliminated.

DISCUSSION

Five of the six BHK-21 cell-adapted fixed strains of rabies virus characteristically produced one or more distinct populations of DI particles. The DI particles of these strains were shown to fulfil the established criteria for DI virus particles (Huang & Baltimore, 1970). The rabies virus DI particles contain part of the virus genome (Wunner & Clark, 1978, 1980) and all of the virion structural polypeptides (W. H. Wunner & H F. Clark, unpublished data); the DI particles replicate in the presence of standard virus and interfere with the replication of homologous standard virus.

Although DI particles were previously reported in the virus progeny of acute infections of BHK-21 cells with Flury LEP virus (Crick & Brown, 1974), other investigators have detected rabies DI particles only following prolonged transfer of cells persistently infected
with either strain ERA (Wiktor et al., 1977) or strain Flury HEP (Kawai et al., 1975; Holland & Villarreal, 1975; Kawai & Matsumoto, 1977) rabies virus. Our virus stocks producing detectable concentrations of DI particles were each obtained after two to 14 serial passages at an m.o.i. of 1 following repeated clone purification; the failure of the Kelev strain to produce DI particles may be due to its brief total passage history in cell culture (four passages) since adaptation to chick embryos. (A Kelev defective particle has recently been detected in chronically infected rat myeloblasts of cell line L6; H F. Clark & N. F. Parks, unpublished data.) We have demonstrated that high concentrations of DI particles were regenerated within three passages when DI particle-free serially cloned ERA (WI) virus was inoculated into BHK-21 cells at an initial m.o.i. of 13.5, whereas at least six passages were required to detect DI particles when serial passage was initiated at an m.o.i. of 1 (Wunner & Clark, 1980).

The DI particles of different strains of fixed rabies virus were of varying size, characteristic of each strain, as indicated by sedimentation properties in velocity gradients, measurement of negatively stained particles observed by electron microscopy and determination of genome size. Comparison with DI particles of rabies virus reported by other investigators working with several of the same strains (Wiktor et al., 1977; Kawai et al., 1975; Crick & Brown, 1974; Holland & Villarreal, 1975) is difficult because genome characterization has been reported previously only in the single case of DI particles recovered from cells chronically infected with ERA virus (Wiktor et al., 1977). The mol. wt. of the RNA recovered from those DI particles was reported to be $2.5 \times 10^6$, comparable to the $2.6 \times 10^6$ mol. wt. RNA from ERA M band DI particles found in our studies (Table 1). Our results, coupled with the reports of multiple bands of DI particles recovered from BHK-21 cells chronically infected with Flury HEP virus (Kawai & Matsumoto, 1977) and from progeny of the Flury HEP propagated in newborn mice (Holland & Villarreal, 1975), indicate that the rabies virus, like VSV (Leamnson & Reichmann, 1974), can generate many diverse sizes of DI particles.

Preliminary experiments have indicated that both virus and host cell-mediated effects can affect the efficiency of rabies DI particle-related interference with homologous virus in cell culture. The CVS defective particles are very inefficient at causing interference, strain ERA T1 plus T2 and M band DI particles cause marked interference, and strain Flury HEP DI particles express intermediate interfering activity. The single type of ineffective CVS defective particle has a small genome size ($0.6 \times 10^6$ mol. wt.); nevertheless, it is equal to that of the more effective Flury HEP virus DI particle.

Of six host cell types tested, only cell line CB3 was refractory to expression of rabies DI particle-mediated interference. Interference mediated by rabies DI particles was readily demonstrated by reconstitution experiments in BHK-21 cells and in several neuroblastoma cell systems. We have no explanation for the paradoxical observation that it is extremely difficult to demonstrate DI particle-associated reduction of standard virus yield following serial high m.o.i. passage in BHK-21 cells (Clark & Ohtani, 1976), but that such effects are readily demonstrated in NA cells (Clark, 1980). Particularly, we have been unable to correlate the presence or absence of DI particles in rabies virus stocks with virulence or with AI patterns of pathogenesis in adult mice. Both virulent CVS virus and fully or partially attenuated derivatives of CVS virus produced similar concentrations of DI particles with apparently CVS-specific sedimentation properties. Kelev virus, the most attenuated fixed rabies virus in our collection, produced no detectable DI particles. Serial clone purifications of Flury HEP virus and of our ERA (WI) virus led to loss of DI particle-producing potential with retention of full potential for causing an AI death pattern in mice.

Studies in cell culture revealed that the c.p.e. induced by rabies virus may also proceed according to an AI-dose-response pattern. This host response, like the in vivo host response, does not seem to require the presence of detectable concentrations of DI particles in the
DI particles of fixed rabies viruses

inoculum. Furthermore, no correlation is observed between AI patterns of c.p.e. in cell culture and AI death patterns in vivo.

Attempts to demonstrate a definitive capability for DI particles to provide protection against rabies virus in vivo were inconclusive. We were able to demonstrate protection only when massive concentrations of DI particles were administered i.c. into mice along with challenge rabies virus doses of less than 10 LD\textsubscript{50}. We were not able to induce DI particle-mediated protection against rabies virus infection initiated at the intraplantar site or intranasally. It seems unlikely that large excesses of DI particles occur in naturally induced rabies infections, in view of the electron microscopic observations of Dierks et al. (1969) which show that a remarkably uniform standard virus is produced in salivary glands. The possibility remains that de novo synthesis of DI particles may partially account for the consistent observation in brains of rabid animals that infectious virus titres are much lower than expected on the basis of particles observed by electron microscopic examination (Murphy, 1977; Iwasaki & Clark, 1975). For continued testing of the role of DI particles in vivo, rabies virus should provide a superior system as: (i) the longer incubation periods associated with rabies would allow more time for DI particle interference to act and (ii) DI particles from attenuated rabies virus strains can be used, obviating the problem of lethal effects of contaminating infectious standard virions in DI particle preparations.

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REFERENCES


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