Persistent infection of Vero cells by the flavivirus Murray Valley encephalitis virus

Michael Poidinger,* Robert J. Coelen and John S. Mackenzie

Department of Microbiology, Queen Elizabeth II Medical Centre, University of Western Australia, Nedlands, Western Australia 6009

Murray Valley encephalitis (MVE) virus strain OR2 was serially passaged on Vero cells to establish a persistent infection which was maintained for over 300 days. Supernatants from infected cells protected Vero cells from c.p.e. and caused up to a 95% reduction of wild-type virus yield. These protective and interfering effects suggest that defective interfering (DI) particles are responsible for the establishment and maintenance of the MVE virus persistent infection. The persistently infected cell supernatant preparations shared several features with DI particle preparations from other viral systems, such as their amplification to detectable levels after two to four passages of virus. However, results from this study suggest that DI particles of MVE virus differ from other studied systems in that they are able to affect only moderately the yield of infectious wild-type virus. The genetic drift of the parental virus during the course of a long term persistent infection *in vitro* appears to be minimal.

Introduction

Long term persistent infections of cultured cells provide an *in vitro* model for the study of the chronic, persistent disease state and the study of the processes by which persistent infections are established and maintained. The conversion of normally cytolytic viral infections to temperate persistent infections, both *in vivo* and *in vitro*, has been observed for many viruses. One of the mechanisms suggested in the establishment of persistent infections is the production of defective interfering (DI) virus particles, and indeed DI particles have been shown to be responsible for the establishment and maintenance of persistent infections with Sendai virus (Roux & Holland, 1979), Sindbis virus (Weiss *et al.*, 1983), Semliki Forest virus (Atkinson *et al.*, 1986; Barrett & Dimmock, 1984*a*) and vesicular stomatitis virus (VSV) and rabies virus (Cave *et al.*, 1985; Holland *et al.*, 1976).

DI particles are mutants of the wild-type (wt) virus, which are antigenically identical to the wt virus but contain deletions in the genome essential for replication. The particles interfere with the growth of virus in cell culture resulting in increased DI particle production at the expense of the wt virus growth (Huang & Baltimore, 1970; Perrault, 1981; Nayak, 1980).

Brinton (1986) reviewed the literature describing persistent infections with flaviviruses in both animals and cultured cells. DI particles of flaviviruses (Schmaljohn & Blair, 1977; Brinton, 1982) have been shown to be involved in the resistance induced by the murine resistance gene (RV gene; Brinton-Darnell & Koprowski, 1974; Brinton, 1983). These authors suggest that DI particles are responsible for the establishment and maintenance of flaviviral persistent infections. This report describes the establishment of a Murray Valley encephalitis (MVE) virus persistent infection in Vero cells and examines the hypothesis that DI particles are responsible for the creation and maintenance of the ersistent infection. Some of the biological properties of this infection and the DI particles derived from it are described.

Methods

Media. Vero cells were grown in medium consisting of Medium 199 supplemented with 10% foetal calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml), HEPES (4·77 mg/ml) and L-glutamine (14·6 mg/ml; pH 7·5) and maintained in maintenance medium consisting of growth medium supplemented with 2% foetal calf serum. The cells were subcultured weekly using conventional techniques.

Virus strains and cells. The flaviviruses Kunjin strain OR393 (KUN; Liehne *et al.*, 1981), MVE strain OR2 (Liehne *et al.*, 1976), MVE strain OR155 (Liehne *et al.*, 1976), West Nile strain Sarafend (WNV; Doherty *et al.*, 1959), yellow fever strain 17D (YFV; Commonwealth Serum Laboratories, Australia) and the alphaviruses Semliki Forest strain V13 (SFV; provided by Dr B. Dropulic) and Sindbis strain V623 (SIN; isolated from *Aedes normanensis* mosquitoes at Mataranka, Northern Territory, Australia, 1984) were used. Virus stocks were prepared by infecting Vero cell cultures in roller bottles with 10 ml of

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virus dilution at a multiplicity of 1 p.f.u./cell. The cells were incubated for 1 h at 37 °C, after which the supernatant was removed and replaced with 50 ml of maintenance medium. The infected cells were further incubated for 48 to 72 h, then the supernatant was collected, clarified by centrifugation at 13000 *g* for 1 h, and 1 ml aliquots were stored at −70 °C.

Virus passage. MVE virus strain OR2 was serially passaged in confluent Vero monolayers, in 80 cm² tissue culture flasks. The initial infection was at a multiplicity of 0·1 to 10. Cell culture supernatants were harvested every 3 days and half the volume (5 ml) was used to infect a fresh cell monolayer. The other half was stored in aliquots at −70 °C for determination of virus infectivity and interference activity. Surviving cells were routinely subcultured and supernatant fluids were removed at regular 3 day intervals.

To measure heterologous interference, persistently infected cells were superinfected with different viruses at a multiplicity of 10, and the supernatant was harvested 40 h (for the alphaviruses) and 60 h (for the flaviviruses) post-infection (p.i.). The supernatants were stored in 1 ml samples at −70 °C until assayed.

Infectivity assay. The infectivity of virus preparations was measured by $TCID_{50}$ assay, in serial 10-fold dilutions in Vero cells (Rosenbaum *et al.*, 1972). Titres were calculated according to the algorithm of Reed & Muench (1938).

Yield reduction assay. Interference activity was measured by a yield reduction assay adapted from Barrett *et al.* (1981). Aliquots of cell culture supernatant from serial passage and persistent infections were diluted twofold in maintenance medium. Samples (100 µl) of these dilutions were added to 100 µl of virus stock (diluted to 5 x 10^7 $TCID_{50}$/ml), and the resulting mixture was added to monolayers of Vero cells (5 x 10^5 cells) in 24-well plates (Falcon). The plates were incubated at 37 °C for 1 h, with occasional shaking. Maintenance medium was then added to each well and the plates were incubated for a further 47 h at 37 °C. Cell culture supernatant was harvested and stored at −70 °C until assayed for virus yield.

The reduction in virus yields was determined by plaque assay under methyl cellulose overlay (Schulze & Schlesinger, 1963).

Immunofluorescence studies. Immunofluorescence studies on infected Vero cell cultures were performed as described by Farrell & Shellam (1990), using monoclonal antibodies directed against NS1, pre-M and E proteins of MVE virus (Hall *et al.*, 1990).

RNase T_1 oligonucleotide fingerprinting. Oligonucleotide fingerprinting was performed as described by Coelen *et al.* (1988). Vero cell monolayers grown in roller bottles were infected with serially passaged MVE virus at a multiplicity of 10. Viral RNA was extracted from the cell supernatants, purified, digested with RNase T_1 and end-labelled with ^{32}P, and the oligonucleotides were separated on ultrathin gels.

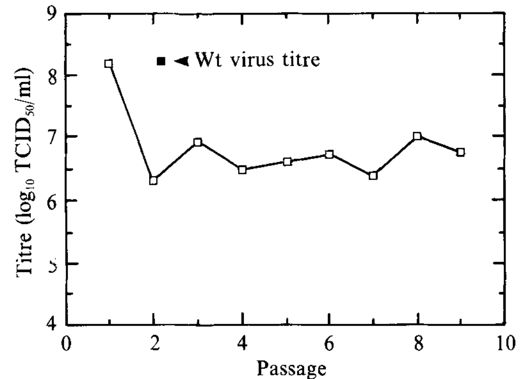

Fig. 1. Titration of a serial undiluted passage of MVE virus strain OR2 in Vero cells.

Results

Serial passage and assay of virus

Vero cells infected with MVE virus at a multiplicity of 0·1 to 10 underwent greater than 90% c.p.e. 72 h p.i. If the virus had been serially passaged at least two to four times it caused an infection which was less cytocidal, and only 10% to 50% of the cells underwent c.p.e. Virus titres in the serial undiluted passages exhibited a cycling of infectivity at a level 10- to 100-fold lower than that of a wt virus infection (Fig. 1). In addition in the $TCID_{50}$ assays a protective effect was observed in cells infected with 10^{-1} and 10^{-2} dilutions of passaged virus which was shown by reduced levels of c.p.e. At higher dilutions of the virus preparation, this protective effect was absent (Fig. 2*a*, *b*).

The capacity of the serially passaged virus preparation to cause a reduction in virus yield when co-infected with wt virus was quantitatively measured. In all assays the passaged virus preparation caused a yield reduction of 75% to 95% (Table 1). This effect could be titrated and followed a sigmoidal response (Fig. 3). The reciprocal of the log_2 dilution of a serially passaged virus preparation that caused a 50% reduction in virus yield was chosen as the interference titre, and is equivalent to 1 defective interfering virus unit (DIU) per 100 µl (Barrett *et al.*, 1981). The highest interference titre obtained in these experiments was 4 DIU/100 µl (Table 1).

The sigmoidal response of DI particles in MVE virus preparations suggests one-hit kinetics (Barrett *et al.*, 1981; McLain *et al.*, 1988; Sekellick & Marcus, 1980), which implies that one DI particle/cell is enough to cause interference. The number of DI particles present in the virus samples was statistically estimated from the interference titre (according to Barrett *et al.*, 1981) and found to range from $10^{6.6}$ to $10^{7.7}$ DI particles/ml.

Persistent infection

Ten to 30% of Vero cells survived infection with virus serially passaged two to four times. These cells were routinely subcultured and established a persistently infected culture which was maintained for over 300 days. During this period c.p.e. did not rise above 50%. The persistently infected cells grew at a rate similar to that of uninfected cells (Fig. 4).

Each time the cells were subcultured, the supernatant was titrated for infectious virus and sometimes for ability

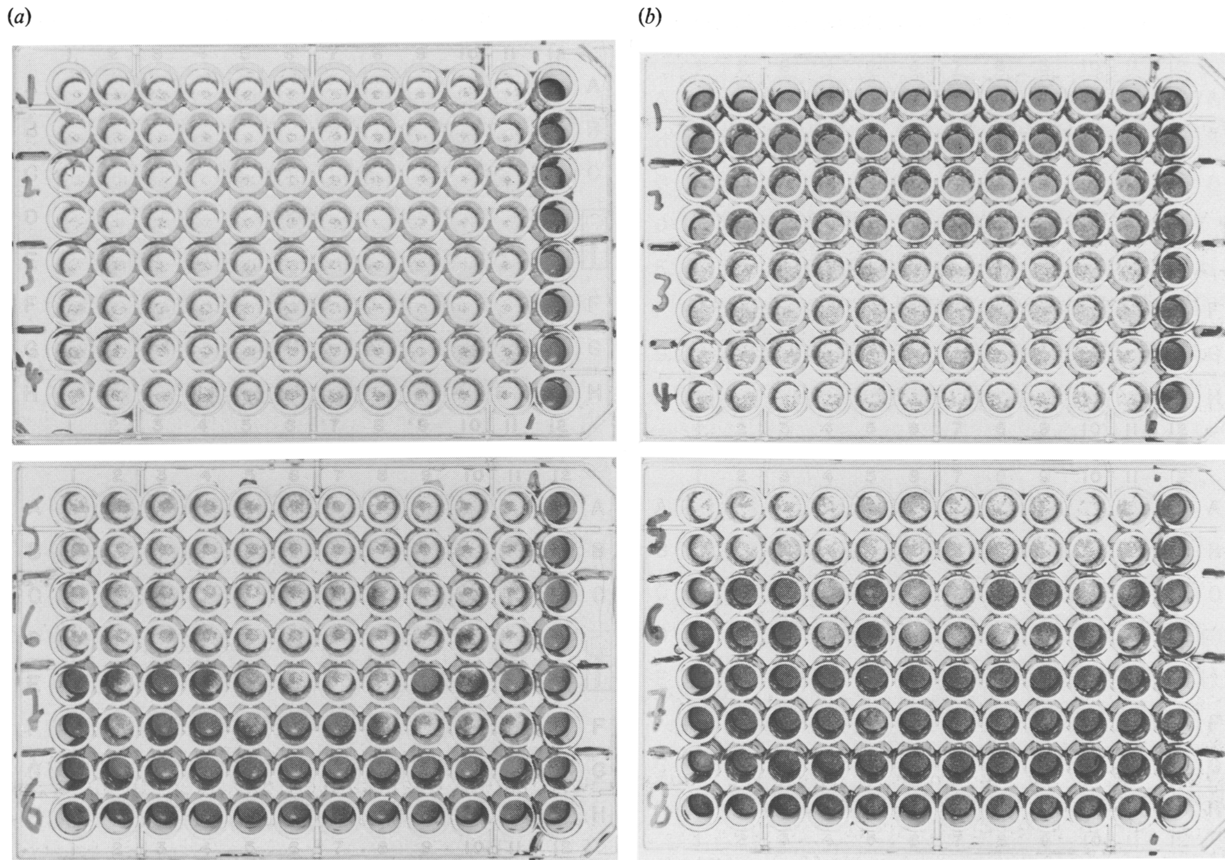

Fig. 2. $TCID_{50}$ assay of wt MVE virus (*a*) and MVE virus passaged eight times on Vero cells (*b*). The far right column is an uninfected cell control. Serial 10-fold dilutions are indicated on the left side of the plates. The plates are stained to determine the presence or absence of c.p.e. Inhibition of c.p.e. is evident in the 10^{-1} and 10^{-2} dilutions of the passaged virus (*b*).

Table 1. *Interference activity of serially passaged MVE virus preparations in Vero cells*

Passage number	Infectious titre*	Yield reduction†	Interference titre‡
1	8·2	0	0
2	6·3	81	2·3
3	6·9	75	1·2
4	6·5	75	1·2
5	6·6	93	3·1
6	6·8	78	0·6
7	6·4	90	3·9
8	7·0	95	2·9
9	6·8	86	2·4

* Infectious titre expressed as log $TCID_{50}$/ml.

† Yield reduction expressed as percent reduction in wt virus titre caused by co-infection of Vero cells with wt virus and an undiluted sample of the passaged virus.

‡ Interference titre expressed as the reciprocal of the $\log_2$ dilution of the serially passaged virus preparation that caused a 50% reduction in virus yield (Barrett *et al.*, 1981).

to reduce virus yield. Infectious virus yields in persistently infected cultures followed a cyclical pattern, with total virus yield at least 10-fold lower than that observed in wt virus infections (Fig. 5).

The Vero cell culture persistently infected with MVE virus strain OR2 was resistant to superinfection at a multiplicity of 10 with the homologous strain and with MVE virus strain OR155, WNV, YFV and KUN: in all of the superinfected cultures the cells appeared morphologically similar to uninfected cell monolayers, exhibiting little evidence of c.p.e. When measured by infectivity assay, virus titres from the superinfected cultures were generally lower than those from a wt infection of the superinfecting viruses in Vero cells (Table 2). In addition a protective effect was observed in the 10^{-1} and 10^{-2} dilutions of the infectivity assays of the supernatants from the superinfected cultures. No resistance was observed when the persistently infected cell cultures were superinfected with SFV or SIN, and in these cases the cell monolayers underwent complete c.p.e. (Table 2).

Immunofluorescence results

Immunofluorescence studies showed that 50% to 80% of the persistently infected cells contained MVE virus antigens (compared to 100% in a wt virus infection; results not shown). The immunofluorescence pattern in

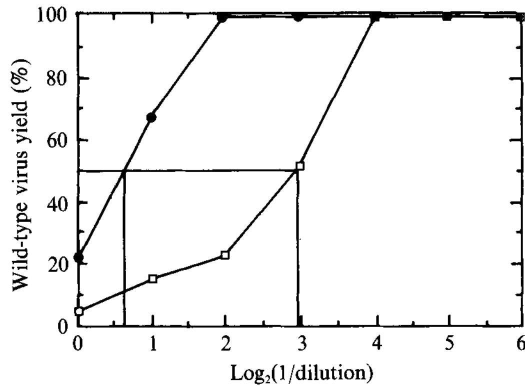

Fig. 3. Yield reduction assay for MVE virus passaged six (●) and eight (□) times on Vero cells. The $\log_2$ reciprocal dilution of passaged virus that caused a 50% reduction in virus yield is indicated (interference titre).

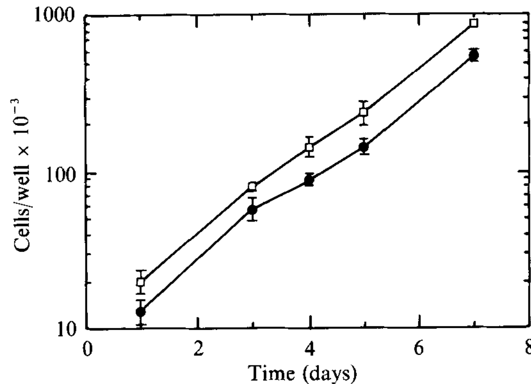

Fig. 4. Growth of uninfected (□) and persistently infected (●) Vero cells. Wells of a six-well plate were initially seeded with about 10^4 cells. Plates were harvested every 24 h and cells were counted with a haemocytometer.

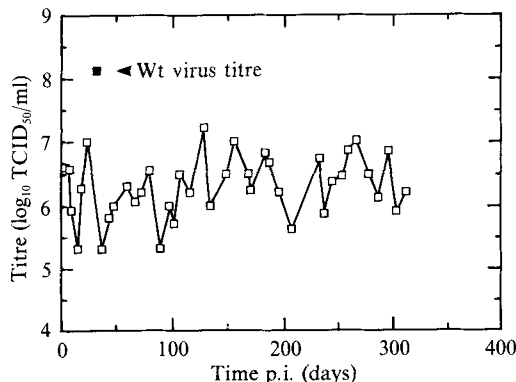

Fig. 5. Titration of the persistent infection of MVE virus strain OR2 on Vero cells. Supernatant from a persistent infection was regularly harvested and titrated by $TCID_{50}$ assay. Wt virus titre is the average of the assays of five independent infections.

Table 2. *Superinfection of a persistent infection of MVE virus strain OR2 on Vero cells with other viruses*

Virus	Normal infection*	Superinfection*
MVE OR2	8·3	6·7†
MVE OR155	8·5	7·1†
WNV	8·7	7·3†
KUN	7·9	7·7
YFV	6·6	6·6
SIN	8·5	8·6
SFV	8·9	8·8

* M.o.i. of 10. Titre is expressed as $\log_{10}$ $TCID_{50}$/ml.

† A protective effect was observed in these infections, resulting in negligible levels of c.p.e. during the course of the superinfections, and protection from c.p.e. in the 10^{-1} and 10^{-2} dilutions of the infectivity assays.

the persistently infected cells was uneven and patchy, as compared to cultures which underwent a wt virus infection.

Fingerprint analysis

MVE virus was isolated from the supernatant fluids of persistently infected cell cultures on days 150 and 300 p.i. The supernatants were used to infect Vero cell monolayers at a multiplicity of 0·001 to dilute out the interfering component. The infection resulted in 95% c.p.e. in the monolayers, and produced high titre virus with no interference activity.

Viral RNA from these virus preparations was fingerprinted and compared to the fingerprint of the MVE virus strain OR2 stock used to initiate the persistent infection. A difference of two oligonucleotides was found between the fingerprints of the stock virus and virus isolated at day 150 p.i. (Fig 6). Comparison of the fingerprint images was performed as described by Coelen *et al.* (1988). Only the oligonucleotides included in that study were used for analysis. The results of this comparison suggested that the viruses were related genetically by more than 99%. Fingerprints of viral RNAs at days 150 and 300 p.i. were found to be identical.

Discussion

MVE virus DI particles establish and maintain persistent infection in cell culture: supernatants from the persistent infection caused a 10- to 100-fold reduction in wt virus yield, which is similar to reports for other flaviviruses (Brinton, 1986). Persistent infection by MVE virus is similar to persistent infection described for Japanese encephalitis virus (JEV; Schmaljohn & Blair, 1977). Titration of the interference activity observed in the MVE virus persistent infections follows a pattern

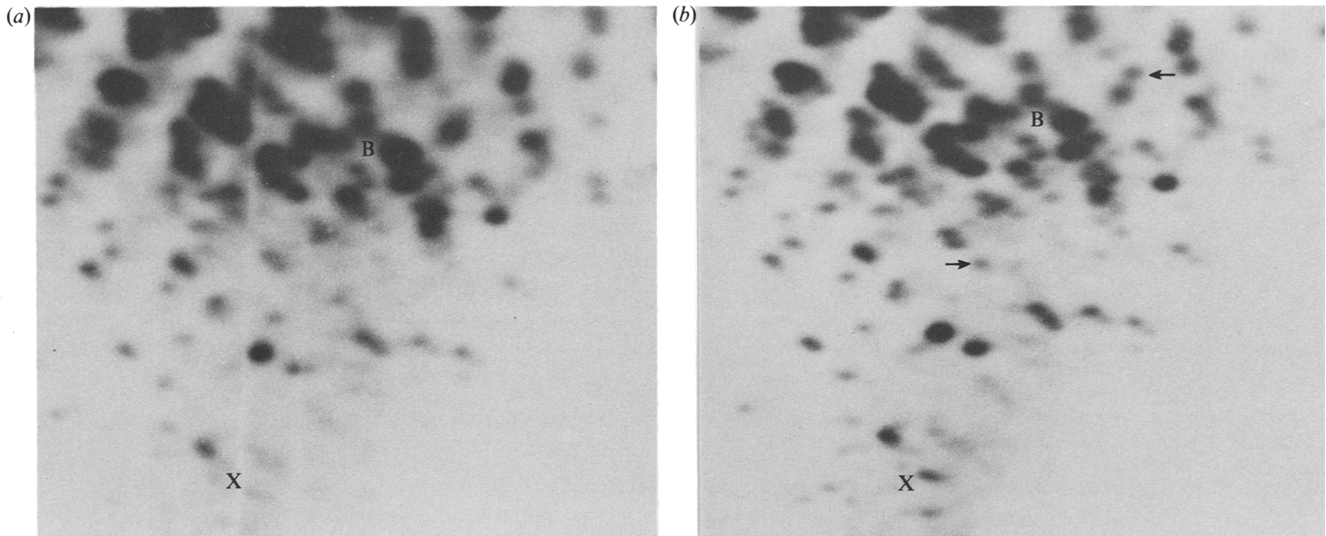

Fig. 6. RNase T_1 oligonucleotide fingerprints of wt MVE virus used to initiate a persistent infection (*a*) and MVE virus isolated from the persistent infection 150 days p.i. (*b*). The photographs are of the lower half of the fingerprints only. The position of the xylene cyanol FF is indicated by X, and the position of the bromophenol blue is indicated by a B. Two extra oligonucleotide spots were detected in the latter fingerprint (*b*); these are indicated by the arrows.

similar to that reported for DI particles of SFV (Barrett *et al.*, 1981), influenza virus (McLain *et al.*, 1988) and VSV (Sekellick & Marcus, 1980). Temperature-sensitive (ts) mutants and interferon production have also been suggested as mechanisms for the production of persistent infection in some viral systems. However, Schmaljohn & Blair (1977) reported that Vero cells persistently infected with JEV produced negligible levels of both ts mutants and interferon. Desmyter *et al.* (1968) reported that Vero cells produce negligible levels of interferon during viral infection.

The levels of DI particles generated in the persistent infection are consistent with the observation of protection from c.p.e. of cells in the 10^{-1} and 10^{-2} dilutions of the $TCID_{50}$ assays (Fig. 2*b*). DI particles were generally present in amounts greater than or equal to infectious virus particles (Table 1).

Cells persistently infected with MVE virus strain OR2 were found to be resistant to superinfection with four other flaviviruses, but susceptible to alphavirus superinfection. Schmaljohn & Blair (1977) reported that some of their persistent infections were resistant to superinfection by other flaviviruses, and interference by DI particles of SIN to SFV has also been reported (Barrett & Dimmock, 1984*b*; Weiss & Schlesinger, 1981).

The reduction in wt virus yield caused by supernatants from persistently infected MVE virus cell cultures is a low level of interference compared to that observed in other viral systems (Bellett & Cooper, 1959; Barrett *et al.*, 1981; Stark & Kennedy, 1978). Kowal & Stollar (1980) reported that some VSV DI particles caused complete disruption of infectious virus production. The low level of interference reported in this paper suggests that there may be less selective pressure on the MVE virus genome compared with other viral systems. Indeed, RNA fingerprint analyses of viruses isolated during the persistent infection suggested that the genomic changes were few over 300 days of the persistent infection. This is in marked contrast to results with SIN (Weiss *et al.*, 1983), WNV (Brinton, 1981), foot-and-mouth disease virus (de la Torre *et al.*, 1988), VSV (Depolo *et al.*, 1987) and influenza virus (Frielle *et al.*, 1984) in which considerable genetic drift was reported.

Determination of factors involved in the establishment and maintenance of persistent infections, especially in the MVE virus system in which persistent infections are rapidly generated, is essential to our understanding of viral replication, and important to our understanding of chronic and persistent disease states. Current results suggest that DI particles are responsible for the effects observed in MVE virus persistent infections. Hybridization and sequencing studies are currently being undertaken in an effort to characterize further the mechanism of interference in persistent infections, and the structure and function of DI particles of MVE virus.

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