Interferon-mediated Persistent Infection of Saint Louis Encephalitis Virus in a Reptilian Cell Line

By J. H. MATHEWS* and A. V. VORNDAM
Vector-Borne Viral Diseases Division, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services P.O. Box 2087, Fort Collins, Colorado 80522, U.S.A.

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SUMMARY

A persistent infection with Saint Louis encephalitis (SLE) virus in a poikilothermic cell line TH-1 (turtle heart cells) was studied. Infected TH-1 cells were subcultured weekly at 31 °C for 1 year and continued to produce low levels (10^2 to 10^3 p.f.u./ml) of virus without obvious cytopathic effects or marked cyclic events. Indirect fluorescent antibody and infectious centre assays indicated that <1% of the cells were producing detectable virus proteins or infectious virus. Defective-interfering particles, temperature-sensitive mutants and DNA provirus were not detected. Interferon (IFN) mediation of the persistent infection was considered since the persistently infected cells (PIC) and normal TH-1 cells were resistant to heterologous virus challenge after treatment with virus-free culture fluid from PIC. A direct relationship was found between the m.o.i. and the amount of IFN produced, plateauing at an m.o.i. of approx. 10. The reptilian IFN was physically and chemically similar to mammalian and avian IFN. Certain biological markers of the SLE virus changed during the persistent infection. It was less virulent for mice, showed distinct differences in cell culture host range and had increased thermal lability.

INTRODUCTION

Since chronic disease states and slow virus infections that occur in humans and animals are not well understood, the study of the nature and mechanisms of persistent virus infections with normally cytolitic viruses in cell culture has received considerable attention (Friedman & Ramseur, 1979). Viruses from the Togaviridae and Rhabdoviridae families have been used to study the persistent state in cell cultures of mammalian and arthropod origin. Examples of such infections are vesicular stomatitis virus (VSV) in LY and BHK cells (Ramseur & Friedman, 1977; Holland & Villarreal, 1974), rabies and Sindbis viruses in BHK and Aedes albopictus cells (Kawai et al., 1975; Weiss et al., 1980; Riedel & Brown, 1977), Semliki Forest virus (SFV) in mouse L929 cells and Japanese B virus in Vero and MA-111 cells (Meinkoth & Kennedy, 1980; Schmaljohn & Blair, 1977).

The establishment and maintenance of the persistent state may involve: (i) defective-interfering (DI) particles (Huang & Baltimore, 1970; Graham, 1977); (ii) temperature-sensitive (ts) mutants (Igarashi et al., 1977; Preble & Youngner, 1975); (iii) interferon (IFN) (Sekellick & Marcus, 1980; Friedman, 1977); (iv) integration as a provirus into the host genome via activity of endogenous RNA-dependent DNA polymerase activity (Zhdanov & Parfanovich, 1974; Haase et al., 1977). These factors may act in concert; DI particles and ts mutants may be involved in establishing the persistent state, but IFN is responsible for the long-term maintenance of the infection (Meinkoth & Kennedy, 1980).
Most persistent virus infections are studied in cells of homeothermic origin; poikilothermic cells have not been well-utilized despite their ability to produce IFN and to support the replication of a variety of viruses (Galabov et al., 1973; Falcoff & Fauconnier, 1965). A chronic infection of rabies virus in viper cells has been described by Wiktor & Clark (1972), but IFN was not detected. Likewise, a persistent fish rhabdovirus infection in Chinook salmon embryo cells was not mediated by IFN (Engelking & Leong, 1981). Clark & Karzon (1967a, b) developed a continuous cell line (TH-1) derived from the heart of a box turtle (Terrapene carolina), which supports the replication of vaccinia virus, herpes simplex virus, pseudorabies virus and VSV. In this report we describe a prolonged persistent infection of the TH-1 cells with an arbovirus, Saint Louis encephalitis (SLE) virus. This persistent infection was mediated by IFN, and biological changes in the virus were observed.

METHODS

Cells, viruses and immune sera. TH-1 cells were obtained from Dr H. F. Clark. They were routinely grown at 31 °C in Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% (v/v) foetal calf serum (FCS), and subcultured weekly with a 1:3 split ratio. Primary duck embryo cell culture (DECC) and Vero cells were grown at 37 °C in the same medium with 8% and 5% FCS respectively. Virus stock of a virulent strain of SLE virus (MSI-7) (Monath et al., 1980) was prepared in DECC from the third suckling mouse brain passage. VSV (Indiana) and Sindbis virus (Ar 339) were also grown in DECC. Hyperimmune ascitic fluid (HIAF) to SLE was obtained from the Arbovirus Reference Branch of this laboratory.

Plaque and infectious centre assays. All plaque assays were done in DECC. The agarose overlay consisted of 0.5% agarose in MEM with 2% FCS, without phenol red dye. SLE plaques were observed at 3 days without neutral red dye. Infectious centre assays with TH-1 cells were performed as described by Meinkoth & Kennedy (1980) but in DECC.

Detection of virus proteins. An indirect fluorescent antibody (IFA) technique was used to detect virus antigens in acetone-fixed TH-1 cells (Lyerla & Forrester, 1979). The fluorescein conjugate (Miles-Yeda, Rehovot, Israel) was anti-mouse IgG prepared in rabbits.

Interference. Interference of wild-type (wt) SLE virus replication by virus from persistently infected cells (PIC) was determined in DECC and Vero cells. SLE virus from PIC was concentrated with polyethylene glycol (PEG) 6000 (Union Carbide; 8 g/100 ml). After 1 h at 4 °C, the precipitate was collected by centrifugation at 10000 g for 1 h and resuspended in MEM. A standard concentration of wt SLE was mixed with varying amounts of virus from PIC before inoculation. Culture fluids were sampled for virus at 72 h.

Curing and cloning. The curing procedure used was similar to the one described by Nishiyama (1977). PIC were passaged every 5 to 7 days into media with and without SLE HIAF. The latter were checked for viruses after the cells had grown to confluency. TH-1 PIC were cloned by terminal dilution in 96-well Linbro plates with 48 h conditioned MEM. After the clones had grown to confluency, the medium was checked for virus by plaque assay.

Provirus induction. Inhibition of protein, RNA and DNA synthesis in cured and cloned cells using 5-iodo-2'-deoxyuridine, cycloheximide, mitomycin C, and actinomycin D was done according to the procedure of Schmaljohn & Blair (1979). After 48 h in the presence of these drugs, the media and fluid from several rapid freeze-thaws of the cells were assayed for virus in DECC. Normal DECC and Vero cells were co-cultivated with cured or cloned TH-1 cells from PIC (1:1) at 31 °C and 39 °C. Culture fluids were assayed for virus at 6 days.

Interferon assay. IFN activity was determined by inhibition of cytopathic effect (c.p.e.). Culture fluids were either acidified with 1 M-HCl to a pH of 2, stored overnight at 4 °C and then neutralized with 1 M-NaOH or were centrifuged at 40000 g for 6 h to pellet residual virus. Uninfected TH-1 cells, grown to confluency in 24-well Linbro culture plates, were
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![Fig. 1. Replication of SLE (MSI-7) virus (m.o.i. of 1) in DECC, Vero and TH-1 cell cultures. The latter were then subcultured weekly and assayed for the presence of virus. O, DECC; A, Vero; □, TH-1.](image)

exposed overnight to 1 to 2 ml twofold dilutions of culture fluids. The media was removed; cells were rinsed with phosphate-buffered saline (PBS) and were challenged with 100 TCID₅₀ of VSV. The reciprocal of the last dilution not showing c.p.e. at 72 h was considered to be the titre. IFN protection was also measured by reduction in virus yield after heterologous virus challenge. VSV (10⁵ TCID₅₀) was inoculated on to TH-1 PIC or normal cells that had been treated with IFN culture fluids in 25 cm² tissue culture flats, adsorbed and washed thoroughly with PBS; the media was sampled at 24 to 48 h and assayed for virus in DECC.

**Interferon characterization.** IFN specimens without FCS were treated with 100 μg/ml trypsin (Grand Island Biological, 1:300) at 37 °C for 1 h (Galabov et al., 1973). Trypsin activity was stopped by adding FCS to a final concentration of 10%. IFN specimens were also treated with RNase and DNase (Galabov et al., 1973b). As a control to ensure that excess RNase had been used, RNase activity was detected by adding purified [³H]uridine-labelled SLE RNA (Pedersen & Hazeltine, 1980) to an aliquot of supernatant fluid. After incubation at 37 °C for 1 h, the RNA was centrifuged into a 15 to 30% sucrose gradient for 18 h at 35 000 g. All of the virus RNA was degraded and remained at the top of the gradient. IFN was treated with ether according to the procedure of Falcoff & Fauconnier (1965).

**Biological characterization of SLE virus from persistently infected TH-1 cells.** Three-week-old outbred Swiss mice were used for the SLE virus virulence studies. The mice were inoculated intraperitoneally (i.p.) (10⁶ to 10⁵ p.f.u.) and observed for 2 weeks. Growth curves of wt SLE and PIC viruses were done in DECC and Vero cells. Cells grown in 150 cm² flasks were inoculated with an m.o.i. of 0.001. Samples were taken over a 3 day period for plaque assay. Thermal stability tests on viruses from PIC and wt (10⁴ and 10⁵ p.f.u./ml respectively) were accomplished by diluting the virus suspensions into prewarmed MEM with 20% FCS and sampling periodically over 2 h. Controls at 4 °C were also included.

**RESULTS**

**Induction of the persistent state**

TH-1 cells are epithelial in nature but when adapted to grow at 31 °C the cells develop a fusiform morphology and are not contact-inhibited (Clark & Karzon, 1967 b). SLE virus will replicate in a variety of cell cultures. When growth curves with wt SLE were done in DECC,
Vero and TH-1 cells, no c.p.e. and low virus titres were observed using the TH-1 cells; however, both DECC and Vero cells were lytically infected (Fig. 1). The replication pattern of SLE virus in TH-1 cells suggested that a persistent infection may have been established.

Subsequent weekly subculturing of the TH-1 cells for an extended period of time showed that infectious SLE virus levels in the culture supernatants remained between $10^2$ and $10^4$ p.f.u./ml. There were no marked virus cyclic events, and no c.p.e. has been observed.

**Evaluation of PIC for the production of virus or virus antigens**

Infectious centre assays (ICA) and IFA were used to determine the percentage of cells expressing infectious virus or virus antigens. ICA showed that 25% of the cells contained infectious virus 24 h after a primary infection, whereas only 0.2% of the PIC at the 10th passage level contained infectious SLE virus. It is known that cells can produce togavirus antigens without producing infectious virus (Weiss *et al.*, 1980; Schmaljohn & Blair, 1977), but IFA showed that less than 1% of the PIC were expressing cytoplasmic virus antigens, and only isolated positive cells were found. A solid-phase radioimmunoassay (Tew *et al.*, 1977) confirmed that few PIC were expressing virus antigens (data not shown). Therefore, the PIC did not harbour a detectable percentage of non-yielding cells.

**Influence of ts mutants and DI particles on the persistent state**

It is known that *ts* mutants can play a role in persistent infections; therefore, ICA and routine plaquing techniques were performed with SLE virus from PIC and from culture fluids at the commonly used permissive and restrictive temperatures of 31 °C and 39 °C. In all cases the results from the plaque titrations of cells and fluids were the same at both temperatures. These data indicate that *ts* viruses were not present in significant numbers.

There was no evidence of a role for DI particles in this persistent infection, as is indicated by the relatively low titres of virus produced without marked cyclic virus replication. Nevertheless, we searched for DI particles by mixing in varying proportions of PEG-concentrated SLE virus from PIC with the wt SLE. These were then inoculated on to DECC and Vero cell cultures. In neither case did the presence of virus from PIC affect the final titre of the wt SLE. The virus from the PIC did not replicate well in Vero cells.

**Cloning and curing of PIC**

Fifty cell clones from PIC were grown to confluency in 24-well Linbro cell culture plates. In all cases the culture supernatants were negative for infectious SLE virus by plaque assay.

It is characteristic of persistent infections that are maintained by horizontal transmission of the virus that they can be 'cured' by passaging the cells in the presence of antiviral sera (Friedman & Ramseur, 1979). To determine if this was the case in TH-1 cells, PIC were passaged in the presence of SLE HIAF. We found that by the sixth passage in the presence of antibody the cultures no longer produced infectious virus as detected by plaque assay. When the antibody was removed, the 'cured' cultures remained virus-free for six subsequent passages. Parallel cultures, grown in the presence of normal HIAF, continued to replicate SLE virus throughout the experiment.

To determine if virus was still present in the cured or cloned cultures but not fully expressed, the following tests were performed: (i) when the cells were tested by IFA for the presence of virus antigens, the tests were uniformly negative; (ii) when the cells were tested for heterologous interference by challenging them with VSV, the replication of the rhabdovirus was not affected; (iii) when the cells were co-cultivated with Vero and DECC, no infectious virus was produced; (iv) when the cells were treated with inhibitors of protein, RNA and DNA synthesis to induce a possible provirus, no infectious virus was produced. These results indicated that the cured or cloned PIC contained no inducible latent SLE virus.
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Table 1. Challenge of normal and SLE persistently infected TH-1 cells with homologous and heterologous virus

<table>
<thead>
<tr>
<th>Cells</th>
<th>SLE</th>
<th>Sindbis</th>
<th>VSV</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistent TH-1†</td>
<td>3.3±</td>
<td>3.4</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>TH-1</td>
<td>4.6</td>
<td>6.3</td>
<td>5.9</td>
<td></td>
</tr>
</tbody>
</table>

* Cells challenged at an m.o.i. of 1.
† Culture fluids from PIC had an IFN titre of 1:4 by the c.p.e. method.
‡ Log_{10} p.f.u./ml. Control value represents the background SLE virus in persistent cell culture media.

Influence of interferon on the persistent state

Normal TH-1 cells were treated for 24 h with culture fluids from various passages (10 to 30) of PIC and challenged with VSV. The yield of virus after 48 h was reduced by more than 90% in cells treated with culture media from PIC as compared to untreated cultures. This indicated that a reptilian IFN-like substance was present in culture fluid from PIC.

When IFN levels in culture fluids are low, Sekellick & Marcus (1980) suggest that challenge of PIC may be more useful than assay of culture fluids as an indicator of IFN activity. When our IFN titres were low, as measured by the c.p.e. method, the virus yield from PIC after homologous wt or heterologous virus challenge was sharply reduced (Table 1). These data indicated that an IFN-like substance was playing a role in the persistent infection.

Most studies on IFN induction show a typical dose-response curve until a plateau of 5 to 10 m.o.i. is reached (Marcus et al., 1978; Fleischman & Simon, 1974). Other studies with certain classes of DI particles from VSV and Sindbis show that maximum IFN production occurs at lower m.o.i. (<1), higher m.o.i. drastically reducing IFN output (Sekellick & Marcus, 1980). When culture fluids from wt SLE-challenged normal TH-1 cells were evaluated over a 2-week period for IFN production by the c.p.e. method (Fig. 2a, b), several features could be observed. (i) The p.f.u./ml in the supernatant fluid on days 1 and 2 was related to the initial m.o.i., i.e. higher m.o.i. produced higher virus titres (Fig. 2b). (ii) No detectable virus was found in the higher m.o.i. sample by day 7 (Fig. 2b). (iii) Higher m.o.i. induced greater levels of IFN than the lower m.o.i. (Fig. 2a). (iv) The lower m.o.i. challenges resulted in IFN production, but to lower titre; initial virus titres were reduced, but residual virus was still present at day 12 (Fig. 2a, b). (v) Unchallenged PIC had maximum IFN production on days 2 and 3 after subculturing. Virus titre was the highest on day 1, and then decreased until day 8 when it remained constant (Fig. 2a, b).

Mammalian and avian IFN are characteristically resistant to or degraded by various physical and chemical treatments (Bellanti, 1978). SLE-induced IFN from TH-1 cells behaved similarly to other IFN (Table 2). TH-1 IFN was species-specific, since it would not protect Vero or DECC from VSV challenge (data not shown).

Biological characterization of the SLE virus isolated from PIC

The wt MSI-7 is a virulent strain of SLE virus (Monath et al., 1980). To determine if it had undergone any changes in virulence during 35 passages, pooled culture fluids from various passage levels were inoculated i.p. into 3-week-old mice (Fig. 3). After the third passage virus from PIC was considerably less virulent than the wt. By the 35th passage, the LD_{50} had increased approx. 10000-fold. Sera from animals inoculated with 100 p.f.u. of either the wt or the 35th passage cultures were found to contain similar levels of neutralizing antibody (data not shown).
Fig. 2. (a) Induction of IFN in TH-1 cells using various m.o.i.s of wt SLE virus. ○, 100:1; △, 10:1; □, 1:1; ●, 0.1:1; ▲, IFN production in PIC after subculturing. (b) Levels of infectious SLE virus present in culture fluid of the challenged normal TH-1 cells and the SLE PIC. For symbols, see (a).

Table 2. Various physical and chemical treatments of TH-1 reptilian interferon

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IFN titre*</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>256</td>
</tr>
<tr>
<td>56 °C†</td>
<td>32</td>
</tr>
<tr>
<td>Ether</td>
<td>128</td>
</tr>
<tr>
<td>Trypsin‡</td>
<td>16</td>
</tr>
<tr>
<td>DNase and RNase§</td>
<td>256</td>
</tr>
<tr>
<td>Dialysis</td>
<td>256</td>
</tr>
</tbody>
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* As determined by the c.p.e. method.
† 1 h.
‡ 100 µg/ml, 1 h, 37°C.
§ 100 µg/ml, 2 h, 37 °C, pH 7.
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The decay curve for wt SLE is linear at a temperature of 48 °C. Thermal stability studies at 48 °C showed that the SLE virus from PIC was more heat-labile than the wt SLE (Fig. 4). Growth curves of the two viruses were done in both DECC and Vero cell culture (Fig. 5). We found that the virus from PIC did not replicate as well as the parent virus in DECC and failed to replicate altogether in Vero cells.

SLE wt virus in DECC routinely produces plaque sizes ranging from ≤1 mm to 3 mm in diam. The plaquing characteristics of the SLE virus from PIC and wt virus were similar.

DISCUSSION

In the present study we have found that the persistent infection of SLE virus in a turtle heart cell line was mediated by an interferon-like substance. Since the cells never went through a virus-mediated crisis period, the TH-1 cells appeared to be persistently infected from the time of infection. This infection met all the criteria set forth by Walker (1964) for a virus-carrier culture mediated by IFN, including: (i) virus was cured from the culture with
homologous virus antibody; (ii) clones from PIC or cured cells were not resistant to heterologous challenge, whereas PIC were resistant to homologous and heterologous challenge; (iii) only a small percentage of the cell population is infected or expressing virus antigens when the carrier state is stable. Although factors other than IFN can make cells refractory to heterologous challenge (Igarashi, 1979; Eaton, 1979), we found no ts mutants or evidence for a putative interfering factor. Mediation of persistent virus infections with other flaviviruses has involved DI particles with Japanese B encephalitis virus (Schmaljohn & Blair, 1977) and IFN with tick-borne encephalitis virus (Stancek, 1965). Sekellick & Marcus (1980) described the selection of certain classes of DI particles and small plaque ts mutants that acted to maintain the persistent state. The role of DI particles and ts mutants in our study was not apparent.

Sekellick & Marcus (1980) have proposed a model for a regulatory role for IFN in persistent virus infections through the phenomenon of cell sparing, where a delicate balance arises between virus replication (cell killing) and virus inhibition (cell sparing). Our results with SLE IFN induction in TH-1 cells support this model. The initial m.o.i. determined, in general, the magnitude of the IFN response and whether or not the culture would be cured of virus or develop a persistent-like state.

SLE-induced IFN was species-specific and was physically and chemically similar to higher phylum IFN. TH-1 IFN is heat-labile, but there is much diversity among IFN in susceptibility to heat (Galabov et al., 1973).

Biological variation in the SLE virus did occur. There were changes in virulence for mice, thermal stability, and ability to replicate well in Vero cell culture. SLE viruses isolated from nature are a biologically diverse group. Naturally occurring persistent infections such as we have described may be a source for such variation.

It is interesting to speculate whether such a persistent infection of reptiles might play some role in the overwintering of arboviruses. Experimental inoculation of the alphaviruses western equine encephalomyelitis virus and eastern equine encephalomyelitis virus into snakes, with subsequent infection of mosquitoes after hibernation, has been reported by Thomas & Ecklund (1960). However, Reeves (1974), in a review, concluded that the evidence for the isolation of arboviruses in field circumstances, from poikilothermic reservoirs, has been conflicting.
REFERENCES


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