Interferon Induction by Viruses. IV. Sindbis Virus: Early Passage Defective-Interfering Particles Induce Interferon

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SUMMARY
We have shown that a single defective-interfering (DI) particle of early (5th) passage Sindbis virus induces maximal amounts of interferon in an ‘aged’ primary chick embryo cell. The capacity of such DI particles to induce interferon is inactivated by small amounts of u.v. radiation (1/e dose = 232 ergs/mm²). The 1/e dose for inactivation of the interferon-inducing capacity of infectious virus particles is 399 ergs/mm² and for infectivity is 101 ergs/mm². Pre-treatment with interferon blocks formation of interferon in response to either DI or infectious virus particles. Our results suggest that Sindbis virus genes must be expressed to form the interferon inducer, which is presumably a molecule of double-stranded (ds)RNA. We postulate that for interferon induction, the genomic RNA which codes for genes G and A must be translated into products whose concerted action produces a dsRNA molecule upon synthesis of a segment of RNA complementary to the genome. The RNA from early passage DI particles is sufficiently large (25S, 1.6 × 10⁸ mol. wt.) to accommodate these genes, whereas the RNA from the late passage DI particles (20S, 1.0 × 10⁸ mol. wt.) is not. Late (15th) passage DI particles do not induce interferon formation.

INTRODUCTION
We have presented evidence that interferon induction results from the appearance of a single molecule of double-stranded (ds)RNA in the cell (Marcus & Sekellick, 1977, 1980; Marcus & Fuller, 1979). With Sindbis virus, the formation of dsRNA appears to require the expression of that quarter of the virus genome most proximal to the 5′ terminus, which seems to code for genes G and A (our unpublished observations). These genes may code for polymerase units comparable to those found in the closely related Semliki Forest virus (Clewley & Kennedy, 1976). We have hypothesized that the Sindbis virus polymerase generates a helical structure at the 3′ terminus of the infecting genome, thus forming the putative interferon-inducing dsRNA (Marcus & Fuller, 1979). In addition, Johnston et al. (1975), Guild & Stollar (1977) and Stark & Kennedy (1978) have presented evidence that, in general, during the generation of defective-interfering (DI) particles of Sindbis and Semliki Forest viruses, the RNA becomes smaller as the passage level increases: nucleotide sequences from the 3′ end of the genome are deleted first, followed by a progressive loss of sequences from the 5′ end. According to our hypothesis, genes G and A must be retained and expressed in DI particles of Sindbis virus for them to function as interferon inducers.

In this communication we have examined the interferon-inducing capacity of early- and late-passage DI particles of Sindbis virus and demonstrated that (i) a single early passage...
DI particle, in the absence of helper virus, functions as an excellent inducer of interferon, whereas those from late passages induce little or no interferon, and (ii) the interferon-inducing capacity of these early passage DI particles is sensitive to both u.v. radiation and interferon action. We also suggest how infectious particles and DI particles of Sindbis virus give rise to interferon-inducing dsRNA.

METHODS

Cells and medium. Primary chick embryo cells from 9- to 10-day-old embryos were plated into 50 mm plastic dishes containing NCI medium and 6% calf serum as described previously (Marcus & Fuller, 1979). Confluent cell monolayers were established within 1 or 2 days of plating and were ‘aged’ in vitro for 6 to 9 days. Cells cultured and ‘aged’ under our conditions produce large amounts of interferon upon appropriate treatment with inducers and are more responsive to the action of interferon (Carver & Marcus, 1967; Marcus & Sekellick, 1977). Baby hamster kidney (BHK-21) cells were also grown at 37.5 °C, but in Dulbecco’s modified Eagle’s medium plus 10% tryptose phosphate broth and 10% calf serum.

Virus stocks and the generation of defective-interfering particles. Wild-type Sindbis virus obtained from David H. Carver was used as the standard helper virus to generate DI particles. Stock virus was grown in BHK-21 cells inoculated at m.o.i. < 0.005 and incubated at 30 °C for 36 to 40 h (one p.f.u. equals one plaque-forming particle). These stocks usually contained 0.5 × 10^10 p.f.u./ml with very few, if any, DI particles (Schlesinger et al. 1972; Shenk & Stollar, 1972). Defective-interfering particles were generated by serial undiluted passage in BHK-21 cells at 37.5 °C (Schlesinger et al. 1972). Each passage was harvested 24 h p.i. and stored at −70 °C.

Defective-interfering particle assay. Defective-interfering particles were assayed quantitatively by the method used by Bellett & Cooper (1959) for vesicular stomatitis virus (VSV) and described in detail previously (Marcus & Sekellick, 1974), except that all assays were carried out on primary chick embryo cells in the presence of 0.1 to 0.2 μg/ml actinomycin D. This treatment prevented interferon-mediated interference (Heller, 1963; Carver & Marcus, 1967). In a typical assay (Fig. 1) there was a linear relationship between the log fraction of maximum yield of virus (p.f.u.) and the relative DI particle concentration. From this we infer that a single DI particle suffices to block totally the yield of a co-infecting infectious (helper) virus. We assume that the concentration of DI particles (lower abscissa) that produces a 63% reduction in yield of wild-type Sindbis p.f.u. contains, on average, 1 DI particle per cell, and that cells infected with DI particles at m.o.i. > 1 do not yield virus (upper abscissa). These assumptions appear valid in view of the one-particle-to-interfere kinetics observed here and also for the loss of infectious centres as reported by Johnston et al. (1975). Defective-interfering particle titres can be calculated in the same way as the number of cell-killing particles in a virus preparation (Marcus, 1959). Fifth-undiluted passage stocks contained 3.3 × 10^10 DI particles and 2.8 × 10^8 p.f.u./ml, whereas preparations of 15th-undiluted passage contained 2.1 × 10^8 DI particles and 2.1 × 10^7 p.f.u./ml.

U.v. irradiation of virus. Stocks of Sindbis DI particles and p.f.u. were inactivated with u.v. radiation (254 nm) as described previously (Marcus & Fuller, 1979). The germicidal lamp produced a flux of 83 ergs/mm²/10 s.

Interferon induction and assay. The methods used to induce and assay interferon were described by Marcus & Fuller (1979). In our assay 1 PR50 unit was equivalent to 10 to 25 units, in terms of the research reference chick interferon standard 63/A: also, 1 PR50 (VSV) unit = 4.7 PR50 (Sindbis) units.
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Fig. 1. Defective-interfering (DI) particle assay. The procedure of Bellett & Cooper (1959) was used and modified slightly as described in Methods. Maximum control yields were routinely \(0.5 \times 10^{10}\) to \(1 \times 10^{10}\) p.f.u./ml.

Sizing of virus-specific RNA. Monolayer cultures of primary chick embryo cells in 50 mm plastic dishes were used to study the RNA species formed in cells infected with various passage levels of Sindbis virus. Cells were infected at a m.o.i. of 10 to 20 p.f.u. After virus attachment for 30 min at 37.5°C, the inoculum was removed and replaced with NCI medium plus 6% calf serum and 1.0 \(\mu\)g/ml actinomycin D. After incubation for 3 h, the medium was removed, the cells were washed three times with NCI medium (calf serum, a rich source of RNase (Fuller & Marcus, 1979), was omitted) and 1.0 ml of NCI medium containing 20 \(\mu\)Ci \(^3\)H-uridine and 1.0 \(\mu\)g of actinomycin D was added. The cells were incubated for another 3 or 4 h at 37.5°C prior to extraction of RNA. At the end of the labelling period, the cultures were washed three times with chilled saline D (a balanced salt solution which lacks Ca\(^{2+}\) and Mg\(^{2+}\)). The cells, manipulated as described by Brzeski & Kennedy (1978), were scraped off the plate into 2.0 ml of chilled 50 mm-tris (pH 7.4) containing 100 mm-NaCl and 5 mm-EDTA (TNE) centrifuged at 10000g and resuspended in 2.0 ml of TNE. Then 0.5 ml of 10% Triton N-101 in TNE was added and after 2 min at room temperature, the nuclei were removed by centrifuging at 1000g for 10 min at 4°C. The resultant supernatant fluid was made 2% in sodium dodecyl sulphate and the RNA was extracted with phenol/chloroform/isoamyl alcohol as described in Fuller & Marcus (1979). The extracted RNA was precipitated with ethanol and resuspended in TNE, and samples were added to a solution of formamide in gel buffer, heated to 70°C for 30 s and placed into slots of a 2% (w/v) acrylamide, 0.1% \(NN'\)-methylenebisacrylamide and 0.6% agarose slab gel. Electrophoresis was at 100 V for 6 to 8 h. The gel was then stained with 0.1% toluidine blue to reveal the unlabelled ribosomal RNA markers, impregnated with 22% 2,5-diphenyloxazole in dimethylsulphoxide, dried under vacuum and autoradiographed on Kodak X-Omat R X-ray film. This procedure follows closely that described by Collins et al. (1978).
RESULTS

Early (5th) passage defective-interfering particles induce interferon

The upper curve in Fig. 2 demonstrates, from two representative experiments, the capacity of a 5th undiluted passage of Sindbis virus to induce interferon in 'aged' primary chick embryo cells. The lower abscissa reveals that the maximum yield of interferon was induced when, on average, one DI particle was attached per cell. This dose-response curve is typical of six others with 5th passage Sindbis DI particles and is like that obtained under similar conditions with the [+]RNA interferon-inducing DI particle of VSV (Marcus & Sekellick, 1977) and the standard infectious Sindbis virus (Marcus & Fuller, 1979). From such curves we deduced that attachment of a single particle per cell sufficed to induce a maximum yield of interferon. We term these particles interferon-inducing particles.

The upper abscissa in Fig. 2 labelled 5th pass. indicates the multiplicity of residual plaque-forming particles in that stock. Note that with DI (5th pass.) particles at a m.o.i. of 1 (the optimum for interferon induction) the m.o.i. for infectious virus was 0.007, i.e. the ratio of DI to infectious particles was 128:1. Dose-response curves previously reported (Marcus & Fuller, 1979) and from the present study (data not shown) show that standard infectious virus does not contribute significantly to the induction of interferon by stock preparations of 5th passage DI particles (p.f.u.) in this test range of multiplicities. It is possible that the high proportion of DI particles masks the detection of plaque-forming particles and that the latter are the actual stimulus for the induction of interferon. This, however, is unlikely for with every 100 p.f.u. of the 5th passage virus stock, there were about $10^4$ DI particles. Thus, with a monolayer of $10^6$ cells, the probability of simultaneous infection of cells, and hence interference with the scoring of p.f.u., in the preparation is nil. Furthermore, the use of Vero cells in these assay experiments, or small amounts of actinomycin D (0.01 μg/ml) in the overlay for chick embryo cells obviates any interference mediated by the interferon system (Marcus & Sekellick, 1977; Sekellick & Marcus, 1978, 1979). Nonetheless, we tested for the presence of p.f.u. directly with a reconstruction experiment. The data in Table 1 show that simultaneous addition of $10^4$ DI particles (5th passage) had no effect on the expression of 100 p.f.u. per monolayer. Thus, we feel confident that the observed ratio
Table 1. *Sindbis* virus plaquing efficiency is unaffected by a high ratio of defective-interfering (DI) particles in stock preparations

<table>
<thead>
<tr>
<th>Number of particles added per plaque plate*</th>
<th>DI</th>
<th>P.f.u.</th>
<th>Observed plaque count</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>None</td>
<td>78</td>
<td>78</td>
</tr>
<tr>
<td>None</td>
<td>60</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td>60</td>
<td>142</td>
<td></td>
</tr>
</tbody>
</table>

* One day after seeding, monolayers of primary chick embryo cells were infected with 10⁴ DI particles (5th passage). An average of 78 plaques from residual p.f.u. was observed (line 1). A stock of diluted standard virus that contained little or no DI particles produced an average of 60 plaques (line 2). When the inocula of each were combined an average of 142 plaques was observed (line 3). This value is considered the same, within sampling error, as that expected if the 10⁴ DI particles had no effect on the plaquing efficiency of residual p.f.u. i.e. 78 + 60 = 138.

of DI particles to p.f.u. of 128 is real. Nevertheless, although we presume that the DI particles in these preparations were indeed those responsible for inducing interferon, we cannot rule out the possibility that there were an equal number of non-interfering defective particles detectable only through their capacity to induce interferon.

Interferon-inducing capacity of 5th passage defective-interfering particles

Sensitivity to u.v. radiation

The survival curves for wild-type virus infectivity and for the interferon-inducing particle activity of 5th passage DI particles were determined by exposing virus stocks for various intervals to u.v. radiation. In Fig. 3, the survival curve for infectivity represents the average of 10 different experiments, with individual points varying less than ±15% from the mean value. The linear curve indicates that a single u.v. 'hit' to the virus genome inactivates infectivity. As reported previously (Marcus & Fuller, 1979) the u.v. dose for 37% (1/e) survival was 101 ergs/mm² [this value agrees within 10% with that reported previously by Carver & Marcus (1968)].

The curve for surviving interferon-inducing particle activity was obtained by infecting cells with a constant amount of virus, equivalent to a m.o.i. of 1 for DI particles (i.e. interferon-inducing particles) in the unirradiated sample. The 24 h yields of interferon induced are plotted in Fig 3 as the fraction of surviving activity, with the experimental points representing the average of three different experiments in which individual determinations varied by ±40% from the mean value. The peak yields of interferon induced by DI particles were usually about one-half or two-thirds of those induced by p.f.u. (Table 2). Again, the linear nature of the survival curve indicates that a single hit suffices to inactivate an interferon-inducing DI particle but the 37% (1/e) survival dose of about 232 ergs/mm² shows that 2-3 times more u.v. radiation is required compared with inactivation of infectivity (p.f.u.). The significance of the difference between the 37% survival dose for the interferon-inducing particle activity of infectious virus (399 ergs/mm²; Marcus & Fuller, 1979) and of DI particles (232 ergs/mm²) is considered in the Discussion.

Sensitivity to the effects of interferon action

The high sensitivity of the interferon-inducing activity of DI particles to u.v. radiation suggests that gene expression is required for the formation of an interferon inducer moiety, which is, presumably, dsRNA. To test this point further we used the action of interferon as a probe, reasoning that virus gene expression would be sensitive to an interferon-mediated
antiviral state. Primary chick embryo cells ‘aged’ in vitro for 7 to 8 days were pre-treated with various low concentrations of chicken interferon for 24 h. The interferon was removed from the plate, and the cell monolayers were washed three times, then infected with interferon-inducing DI particles at a m.o.i. of 1. The yields of interferon were measured 24 h later and plotted as a percentage of that from control (untreated cells) v. the interferon pre-treatment dose. Fig. 4 demonstrates that interferon induction by DI particles is very sensitive to interferon action: 2 units sufficed to reduce interferon induction by 50%. Interferon induction by infectious virus is also sensitive to interferon action (our unpublished observations). Clearly, ‘aged’ chick embryo cells displayed no evidence of ‘priming’ (Stewart, 1979) but do show blocking (Vilcek & Rada, 1962).

**Late (15th) passage defective-interfering particles fail to induce interferon**

Continued serial passage of Sindbis virus (Guild & Stollar, 1977) or of Semliki Forest virus (Stark & Kennedy, 1978) leads to DI particles which contain RNA with increasingly large deletions of ribonucleotide sequences proximal to the 5' end of their RNA. We therefore continued to passage Sindbis virus DI particles in order to test whether the progressive loss of nucleotides [by our hypothesis (Marcus & Fuller, 1979), those specifically coding for genes G and A] would produce a functional DI particle devoid of interferon-inducing activity. The data in the lower curve of Fig. 2 demonstrate that this predicted behaviour
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Table 2. Comparative efficiency of interferon induction by Sindbis virus plaque-forming and DI particles

<table>
<thead>
<tr>
<th>Virus particles</th>
<th>M.o.i.</th>
<th>Interferon yield at 24 h (units/10^7 cells)</th>
<th>(% maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.f.u.</td>
<td>1</td>
<td>10200</td>
<td>100</td>
</tr>
<tr>
<td>U.v.-p.f.u.*</td>
<td>1</td>
<td>&lt; 30</td>
<td>&lt; 0.3</td>
</tr>
<tr>
<td>DI (5th pass.)</td>
<td>1</td>
<td>4620</td>
<td>45</td>
</tr>
<tr>
<td>U.v.-DI (5th pass.)</td>
<td>1</td>
<td>&lt; 30†</td>
<td>&lt; 0.3</td>
</tr>
<tr>
<td>DI (15th pass.)</td>
<td>10</td>
<td>240</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>132</td>
<td>1</td>
</tr>
</tbody>
</table>

* U.v. dose of 1240 ergs/mm².
† As extrapolated from Fig. 3 at a dose of 1240 ergs/mm² for u.v.-irradiated DI particles.

Fig. 5. Sindbis virus RNA species from cells infected with 5th (—), or 15th (-----) passage DI particles analysed by polyacrylamide-agarose gel electrophoresis. An autoradiograph of the dried gel was traced on a Joyce-Loebl densitometer. Monolayers of control cells which had received only standard virus and actinomycin D, and uninfected cells in the presence or absence of actinomycin D served as sources for virus 42S and 26S RNA (not shown) and ribosomal 28S and 18S RNA which were used as markers.

was attained with 15th passage DI particles: these were virtually devoid of interferon-inducing particle activity while retaining high homotypic interfering activity (DI particles = 2.1 x 10⁹/ml; no interferon-inducing particles present). Table 2 compares the interferon-inducing activity of early and late serially passaged DI particles and summarizes all of the classes of Sindbis virus particles used so far to induce interferon. These data, from the same experiment, illustrate the relative efficiency of the various inducers. Table 2 also includes data comparing the u.v. sensitivity of the interferon-inducing particle activity of infectious and DI particles of Sindbis virus, and shows that a u.v. dose of 1240 ergs/mm² completely inactivates their interferon-inducing capacity. This same dose has virtually no effect on the interferon-inducing capacity of a DI particle which contains a 'pre-formed' inducer moiety (Marcus & Sekellick, 1977). These results suggest that the interferon-inducing capacity of both infectious and DI particles of Sindbis virus requires gene expression.
The RNA in DI particle-infected cells from the 5th and 15th undiluted passages was labelled with $^3$H-uridine, extracted and analysed by polyacrylamide gel electrophoresis as described in Methods. Fig 5 illustrates the migrations, with standards of 28S and 18S ribosomal RNA, shown for comparison and as size markers. The RNA from 5th passage DI particles migrated predominantly as 25S (estimated as representing 36% of the genome, $1.6 \times 10^6$ mol. wt.) and is sufficiently large to accommodate genes G and A. In contrast, the RNA extracted from 15th passage DI particles migrated predominantly as 20S (estimated as representing 23% of the genome, $1.0 \times 10^6$ mol. wt.) and is probably too small to code for both these genes.

DISCUSSION

Primary chick embryo cells ‘aged’ in vitro respond in a very sensitive and unique manner to a single interferon-inducing particle (Marcus & Sekellick, 1977, 1980; Marcus & Fuller, 1979). The experimental system is sensitive enough to detect interferon induction in the absence of readily detectable RNA synthesis and enables induction by one molecule of dsRNA per cell to be registered (Marcus & Sekellick, 1977).

In a previous paper (Marcus & Fuller, 1979) we demonstrated that each standard infectious particle (p.f.u.) of Sindbis virus was capable of inducing interferon and thus functioning as an interferon-inducing particle. We now describe a new class of interferon-inducing particles for Sindbis virus, namely early passage DI particles.

In contrast to the interferon-inducing activity of the [±]RNA DI particles of VSV (described in Marcus & Sekellick, 1977), which contain a pre-formed interferon inducer (dsRNA) in the form of a covalently linked self-complementary RNA (Lazzarini et al. 1975), the induction of interferon by Sindbis virus DI particles is sensitive to u.v. radiation ($D_0 = 232$ ergs/mm$^2$). On this basis we postulate that induction of interferon requires expression of some of the DI particle genome. This view is strengthened by our observation that induction of interferon is also sensitive to the action of low doses of interferon [about 2 PR$_{50}$ (Sindbis) units/ml reduced the yield of interferon by 50%].

The linear nature of the u.v. survival curve for the interferon-inducing particle activity of a DI particle indicates that a single hit to its genome renders it non-functional in this respect. [This also appears to be the case for DI particle activity (Kowal et al. 1979; our unpublished observations).]

The difference in target size of the interferon-inducing particles in standard virus (399 ergs/mm$^2$) and in 5th passage virus (232 ergs/mm$^2$) perhaps can be attributed to the configurations of their RNA, since DI particles probably contain multiple copies of RNA (Bruton & Kennedy, 1976; Kennedy et al. 1976). We note that even though multiple copies of RNA may be packaged in a DI particle, the linear nature of the u.v. inactivation kinetics indicates that only one copy is active. Conceivably, only the 5' end of one of the several DI particle RNAs functions in translation as our model requires (see below). The added complications of base pairing, and the possibility of RNA–capsid protein interaction, might have the effect of this single molecule presenting a disproportionately large target to u.v. radiation.

We have previously demonstrated that genes G and A are required for interferon induction by infectious virus and postulated that synthesis of the products of genes G and A are required for the formation of virus dsRNA as the proximal inducer of interferon (Marcus & Fuller, 1979). We now propose that the interferon-inducing activity of Sindbis virus DI
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Fig. 6. A model for the formation of interferon inducer (dsRNA) by standard infectious Sindbis virus and 5th passage DI particles (d.i.p.). A detailed explanation of this model is provided in the Discussion. The placement of genes G and A at the 5' end of the genome is based upon our unpublished observations on the relative order of loss of translation of wild-type (W+) genes following u.v. radiation. Other aspects of the model are derived in part and are consistent with schemes published by Simmons & Strauss (1972), Guild & Stollar (1977) and Stark & Kennedy (1978); i.f.p., interferon-inducing particle.

The model we propose for formation of the proximal interferon inducer (dsRNA) of wild-type and early passage Sindbis virus is shown in Fig. 6. Translation is required to synthesize the products of genes G and A (presumably polymerase; Brzeski & Kennedy, 1978), which in turn initiate synthesis of RNA at the 3' end of the genome (the 26S region where genes C, E and D are located). We postulate that this synthetic event generates a single dsRNA molecule in the form of a transcriptive intermediate for the synthesis of the 26S mRNA or the 42S genome strand (Simmons & Strauss, 1972) and that this intermediate is the actual interferon-inducing moiety. Late (15th) passage DI particles, lacking the capacity to induce interferon, presumably fail to generate this dsRNA intermediate due to the deletion of some bases for genes G and/or A. However, this internal deletion apparently does not affect the ability of late passage DI particles to function as an interfering particle and the decrease in size of the RNA may actually offer a selective advantage over larger DI particles during replication (Johnston et al. 1975; Stark & Kennedy, 1978).

The model presented in Fig. 6 requires that the DI particle genome functions as mRNA and moreover that 5th passage DI particles synthesize polypeptides G and A. The presence of poly(A) tracts at the 3' terminus of DI particle RNA (Weiss et al. 1974) is consistent with this function, although information regarding the presence of a methylated guanosine cap at the 5' end seems lacking. Most investigators have reported a failure to detect protein synthesis directed by DI particle RNA in cell-free translation systems (Weiss et al. 1974; Bruton et al. 1976). However, two groups have reported the appearance of a new poly-
peptide in cells infected with DI particles only (Weiss & Schlesinger, 1973; Weiss et al. 1974; Guild & Stollar, 1975). It remains unresolved whether the new polypeptide was translated from DI particle RNA. The results do not disprove the model, since only a few polymerase molecules (products of genes G and A) may suffice to synthesize the interferon inducer.

It may prove difficult to detect the low levels of polypeptide needed to produce dsRNA by our model. Consequently, we have conducted experiments to construct a DI particle with a ts lesion in gene A, and have tested this type of particle for interferon-inducing activity at permissive and non-permissive temperatures. Construction of such ts-DI particles has allowed precise definition of the kind of genetic information carried in interferon-inducing DI particles (Fuller & Marcus, 1980).

The discovery of defective-interfering particles with interferon-inducing activity in a member of the togavirus family extends our early observation with a rhabdovirus interferon-inducing DI particle (Marcus & Sekellick, 1977; Sekellick, & Marcus 1978; Marcus & Sekellick, 1980) and suggests an alternate mechanism involving the role of interferon for persistent infection with togaviruses in some cell and animal systems (for review, see Sekellick & Marcus, 1979; Stollar, 1979).

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