Host Effect on Arbovirus Replication: Appearance of Defective Interfering Particles in Murine Cells

JUDITH G. LEVIN,¹ JANET M. RAMSEUR, AND PHILIP M. GRIMLEY

Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20014, and Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

Received for publication 21 August 1973

Serial passage of Semliki Forest virus (SFV) in chicken embryo cells had little effect on SFV yield; however, high multiplicity infection of murine cells with one of the late passage pools (passage 9 SFV) resulted in a virus yield 10- to 20-fold lower than that obtained with earlier passage virus and 80-fold lower than the corresponding yield in chicken cells. This effect was accompanied by a striking decrease in the levels of 42S and 26S RNA and by increased proportions of a small single-stranded viral RNA (molecular weight, 9×10^6) and of a low-molecular-weight replicative form. There was also a reduction in the number of specific membranous structures previously associated with the group A arbovirus replication complex. These results suggested that passage 9 SFV contained defective interfering particles which were detected more readily after one passage in a murine indicator host cell. Identical results were obtained with two different murine cell lines: one a leukemia virus-free clone of AKR cells and the other JLS-V9 cells chronically infected with Rauscher leukemia virus. Host production of RNA tumor virus particles apparently did not affect arbovirus replication.

Biochemical studies of the replication of Semliki Forest virus (SFV), a group A arbovirus, have been carried out almost entirely in chicken embryo (CE) cells (3-7, 9, 12, 13, 18). A few experiments have suggested that infection of mammalian cells can result in an altered pattern of viral RNA synthesis (12) or differences in virus yield (9; G. Burleson, P. Jameson, and S. E. Grossberg, *Bacteriol. Proc.*, p. 214, 1971). The present investigation was initiated as part of efforts to define host factors which might influence arbovirus infection of murine cells. To exclude possible effects of endogenous murine leukemia virus (MuLV), arbovirus infection of a recently described clone of leukemia virus-free AKR cells (15) was compared to infection of another murine line (JLS-V9) known to produce Rauscher leukemia virus (21). Parameters examined included arbovirus growth and viral RNA synthesis. No differences could be correlated with the presence or absence of C-type particles; however, the studies led to an unexpected observation that murine cells

allow the expression of defective interfering (DI) particles formed during serial passage of the arbovirus in CE cells.

MATERIALS AND METHODS

Materials. Actinomycin D was a gift from Merck, Sharp, and Dohme Research Laboratories (Rahway, N.J.). Adenosine-2,8-³H (15 to 35 Ci/mmol) and uridine-5-³H (35 to 50 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, Mass.). Modified McCoy 5a medium and RPMI-1640 medium were purchased from Grand Island Biological Co. (Grand Island, N.Y.).

Cell lines. Primary CE cells were prepared from 8- to 10-day-old chicken embryos as previously described (7). The AKR mouse embryo line (15) was the generous gift of Natalie Teich and Wallace Rowe, National Institute of Allergy and Infectious Diseases. JLS-V9 cells (21), chronically infected with Rauscher leukemia virus, were graciously provided by Nelson Wivel, National Cancer Institute. The AKR cells were grown in modified McCoy medium supplemented with 10% fetal bovine serum and the JLS-V9 cells were grown in RPMI-1640 supplemented with 15% calf serum.

SFV pools. A plaque-purified clone (RWGI) of the SFV Kumba strain, originally obtained from Joseph Sonnabend, Mill Hill, London, England, was used.

¹ Present address: Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 20014.

SFV pools were prepared in CE cells as described previously (7). Except where noted otherwise, the number of serial passages after plaque purification was seven or less.

Infection procedures. Cells were infected with SFV in the presence of 1 μ g of actinomycin D per ml. The multiplicity of infection was 10 to 20 PFU per cell, or as specified. After 1 h at 37 C, the appropriate medium supplemented with 2.5% serum (AKR cells) or 10% serum (JLS-V9 cells) was added and incubation was continued at 37 C. In experiments which measured growth of SFV, the virus inoculum was removed after the 1-h adsorption period. The cells were washed three times with warm serum-free medium, and then medium containing serum and 0.1 μ g of actinomycin D per ml was added. Virus titer was determined by plaque assay on monolayers of CE cells (20).

Isolation and analysis of SFV RNA. Monolayer cultures on 100-mm Falcon petri plates (4×10^7 cells) were infected with SFV as described above except that the concentration of actinomycin D in the medium was maintained at 1 μ g/ml. At 1 or 2 h postinfection, fresh medium containing 3 H-adenosine and 3 H-uridine (each 25 μ Ci/ml) was added and incubation was continued until 5 h postinfection. RNA was extracted at room temperature with 0.5% sodium dodecyl sulfate (SDS) and phenol according to procedures described previously (12). Samples were analyzed on composite 2.0% polyacrylamide-0.5% agarose gels run for 3.5 h (12).

RESULTS

Growth of SFV in MuLV producer and nonproducer lines. In assessing possible host influences on SFV infection, we initially considered the possibility that chronic infection of murine cells with C-type virus might interfere with arbovirus replication. SFV growth was therefore examined in a leukemia virus-free line of AKR cells (15) and in JLS-V9 cells (21) which have been shown to produce MuLV (Levin, *et al.*, manuscript in preparation). As illustrated in Fig. 1, SFV yield and kinetics of growth were similar in the two lines. In addition, other experiments showed that SFV-infected AKR and JLS-V9 cells contained identical virus-specific RNA (Fig. 2) and protein species, as well as high levels of SFV RNA polymerase (J. G. Levin, unpublished observations). These results indicated that arbovirus replication was unaffected by chronic infection and active production of MuLV by a murine host cell.

Effect of serial passage of SFV in CE cells on virus yield in murine cells. In view of recent reports suggesting that the host cell exerts control over synthesis of DI particles (1, 10, 11a, 14), it was also of interest to us to determine whether defective virus is produced during SFV infection and, in particular, to see whether a

FIG. 1. Growth of SFV in JLS-V9 and AKR cells. Replicate cultures of JLS-V9 and AKR cells were grown in 16-mm tubes (10^6 cells per tube) and were infected with SFV as described in Materials and Methods. Virus titers were determined by plaque assay on monolayers of chicken embryo fibroblast cells. (20). All points represent the average of duplicate determinations. Similar results were obtained when SFV was grown in the absence of actinomycin D. Symbols: ●, AKR; Δ , JLS-V9.

host effect can be observed. Although previous studies from this laboratory had indicated that defective SFV was not present in our virus pools (9, 12), we found that, after nine serial passages of plaque-purified SFV in CE cells, there was a small but reproducible decrease in virus yield (Table 1, CE). Interestingly, at low multiplicity of infection, the virus yield from passage 9 SFV was increased approximately threefold. Further passage of SFV, i.e., beyond passage 9, led to a similar rise in titer. Although these differences were not too outstanding, they appeared consistent with the possibility that some DI particles might have accumulated during SFV passage in CE cells.

Using the murine lines as indicator cells we next determined the effect on virus yield when each of the successive SFV passages from CE cells was passaged one time in AKR or JLS-V9 cells. As shown in Table 1 (AKR, JLS-V9), infection with passage 7 SFV at high or low multiplicity gave somewhat lower yields in murine

cells than in CE cells. However, infection with passage 9 SFV gave more striking results. At high multiplicity, virus yield in murine cells was 10- to 20-fold lower than that obtained with earlier passage SFV and 80-fold lower than the corresponding yield in CE cells; at low multiplicity, the titer was consistently higher. In agreement with the results obtained in CE cells, the yields from passages 10 to 13 in AKR and JLS-V9 cells were increased relative to the yield from passage 9 SFV, and by passage 11 the titer was close to that observed in CE cells.

The data presented in Table 1 suggested that passage 9 SFV contained DI particles which could be detected more readily after infection of a murine host. Further evidence for the existence of the DI particles was obtained by measuring the ability of passage 9 SFV or the virus produced from passage 9 SFV (passage 1, AKR) to interfere with the replication of a high-titer pool (passage 13 SFV). As may be seen from Table 2, co-infection of AKR cells with passage 13 and passage 9 SFV at high multiplicity of

infection reduced the yield from passage 13 SFV by 60-fold. Addition of passage 9 or passage 1, AKR SFV at very low multiplicities of infection inhibited infectious virus production to almost the same extent.

Effect of serial passage of SFV in CE cells on viral RNA synthesis and morphogenesis in murine cells. SFV RNA from JLS-V9 and AKR cells infected with early passage virus was characterized by polyacrylamide gel electrophoresis (Fig. 2). As may be seen, the distribution of viral RNA species was fairly similar to the pattern obtained with the ^{32}P -labeled marker RNA from CE cells. A strikingly different pattern resulted when AKR cells were infected with passage 9 SFV at high multiplicity (Fig. 3A). The proportion of 26S RNA was drastically reduced, and the relative amounts of the low-molecular-weight replicative form (RF) and the small single-stranded RNA (molecular weight 9×10^6) were greatly increased. There was also a two- to threefold reduction in the total amount of 42S RNA. Similar observations were made in

FIG. 2. Polyacrylamide gel electrophoresis of ^3H -labeled RNA from JLS-V9 and AKR cells infected with early passage SFV. At 2 h postinfection, fresh medium containing ^3H -adenosine and ^3H -uridine (each 25 $\mu\text{Ci/ml}$) was added, and the cells were incubated for an additional 3 h. RNA was extracted and analyzed on composite 2.0% polyacrylamide-0.5% agarose gels as described in Materials and Methods. A, Sample contained 40 μl of ^3H -labeled SFV RNA from JLS-V9 cells and 20 μl of SFV RNA from chicken embryo cells labeled with ^{32}P between 1 and 6 h postinfection (12). B, Sample contained 30 μl of ^3H -labeled SFV RNA from AKR cells and 30 μl of the ^{32}P marker. The ^3H values were corrected for 4% crossover of ^{32}P , and the data were plotted by computer as percentage of total radioactivity (counts per minute) recovered from the gels. A, JLS-V9 cells; B, AKR cells. Symbols: $\bullet$, ^3H ; $\circ$, ^{32}P . Abbreviations: RI, replicative intermediate; RF's, replicative forms.

TABLE 1. Relationship of serial passage of SFV in chicken embryo cells to virus yield in AKR and JLS-V9 cells

SFV passage no. ^a	Virus titer after one additional passage at high and low multiplicity of infection ^b (PFU/ml)					
	CE		AKR		JLS-V9	
	high	low	high	low	high	low
7	1.1×10^8		1.5×10^8	8.0×10^7	7.5×10^7	4.0×10^7
8	8.8×10^8		1.5×10^8			
9	6.5×10^8	1.6×10^8	8.0×10^8	7.5×10^7	8.5×10^8	1.8×10^8
10	1.9×10^9		8.5×10^7			
11	1.7×10^9		1.8×10^8		1.1×10^9	
13	5.5×10^9		4.0×10^8		1.9×10^9	

^a The passage number refers to the number of times the virus was passaged at high multiplicity in CE cells after plaque purification. The yields from each of the resulting SFV pools after one additional passage in CE, AKR, or JLS-V9 cells are given in the columns to the right.

^b Duplicate cultures on 60-mm dishes were infected with SFV at a multiplicity of 25 PFU/cell (high multiplicity) or 10^{-2} PFU/cell (low multiplicity) as described in Materials and Methods except that actinomycin D was maintained in the growth medium at $0.5 \mu\text{g/ml}$ after the 1-h absorption period. The cultures were harvested at 16 h postinfection.

TABLE 2. Effect on virus yield in AKR cells as a function of co-infection with passage 13 SFV^a and other SFV pools

SFV pool added with passage 13 SFV	Virus-to-cell ratio of added SFV pool (PFU/cell)	Virus yield (PFU/ml)
None		40×10^8
Passage 9, CE ^b	25	0.65×10^8
Passage 9, CE ^b	0.25	1.4×10^8
Passage 1, AKR ^c	0.28	0.95×10^8
Passage 1, AKR ^c	0.028	4.0×10^8

^a Passage 13 SFV was grown in CE cells (see Table 1). Infection of AKR cells was always at a virus-to-cell ratio of 25 PFU/cell.

^b Passage 9, CE SFV was obtained by infecting CE cells with passage 8 SFV at a high multiplicity of infection (see Table 1).

^c Passage 1, AKR SFV was obtained by infecting AKR cells with passage 9, CE at a high multiplicity of infection (see Table 1).

JLS-V9 cells (not shown). These results are in agreement with recent studies on viral RNA synthesis in BHK cells infected with a late passage pool of Sindbis virus (2, 17).

As might be expected, when AKR cells were infected with passage 9 SFV at a virus-to-cell ratio of 10^{-2} , the viral RNA pattern (Fig. 3B) was similar to the one illustrated in Fig. 2B. There was still a very small enrichment of ³H-labeled counts in the region of the low-molecular-weight RF (2% in the peak fraction compared to 6% in Fig. 3A). However, the low-molecular-weight, single-stranded RNA was no longer a major species, and the 26S and 42S RNA molecules were now present in equal proportions.

To learn whether the infectivity and RNA

data might correlate with any ultrastructural changes, JLS-V9 and AKR cells infected with passage 7 or passage 9 SFV were examined in the electron microscope at 8 h postinfection. Attention was focused on the numbers of specific membranous structures (CPV-1) present in the cells, since these structures have been shown to be loci of viral RNA synthesis (8) and are associated with the SFV replication complex (6). In every experiment, regardless of the passage number of the virus inoculum, numerous structures were observed. At high multiplicity, cells infected with passage 7 SFV contained approximately 120 CPV-1 per 100 cell sections. This number was consistently lower in cells infected at high multiplicity with passage 9 virus, with a reduction in counts of about two- to threefold. Cytoplasmic nucleocapsids and budding virus were also observed in these experiments.

DISCUSSION

The data reported in this study indicate that, although growth of SFV in murine cells was unaffected by chronic infection of the host with C-type particles (Fig. 1), differences in SFV yield could be related to the number of times the virus inoculum had been passaged in CE cells (Table 1). Thus, high multiplicity infection of murine cells with one of the late passage pools from CE cells (passage 9 SFV) led to a considerable reduction in SFV titer and a dramatic shift in the distribution of intracellular viral RNA species (Fig. 3A). These observations suggested that DI particles were present in passage 9 SFV and, as might be expected, both passage 9 SFV and the progeny virus produced after one passage in AKR cells (passage 1, AKR)

FIG. 3. Polyacrylamide gel electrophoresis of ^3H -labeled RNA from AKR cells infected with SFV passaged nine times in CE cells. A, Cells were infected with SFV at a virus-to-cell ratio of 25. B, Cells were treated with actinomycin D ($5 \mu\text{g}/\text{ml}$) for 30 min at 37°C prior to infection with SFV at a virus-to-cell ratio of 10^{-2} . Procedures for labeling and electrophoresis were carried out as described in the legend to Fig. 2 except that in A the cells were labeled between 1 and 5 h postinfection. The samples used for electrophoresis contained $40 \mu\text{liters}$ (A) or $30 \mu\text{liters}$ (B) of ^3H -labeled SFV RNA from AKR cells and $20 \mu\text{liters}$ (A) or $30 \mu\text{liters}$ (B) of ^{32}P -labeled SFV RNA from CE cells (12). A, High multiplicity of infection; B, low multiplicity of infection. Symbols: $\bullet$, ^3H ; $\circ$, ^{32}P . Abbreviations as in Fig. 2.

had the capacity to interfere with the replication of a normally high-titer SFV pool (Table 2).

The present findings are consistent with the picture which has emerged from characterization of other DI particle-producing systems, including the extensive studies on defective particles of vesicular stomatitis virus (10, 11, 19) and more recent reports on production of defective Sindbis virus (2, 16, 17). Furthermore, in our own studies on SFV infection in HeLa cells, we have found that serial passage of the virus in HeLa cells markedly reduces SFV yield and alters viral RNA synthesis in a manner virtually identical to that shown in Fig. 3A (J. G. Levin, P. M. Grimley, J. M. Ramseur, and I. K. Berezsky, *Abstr. Annu. Meet. Amer. Soc. Microbiol.*, p. 240, 1973).

The observation that particles produced in CE cells with potential interfering activity were detected after one additional passage in a murine host (Table 1) emphasizes the importance of the host in determining the extent of DI particle formation and expression. In previous investigations, it was demonstrated that synthesis of DI particles is dependent upon the particular

host cell used for serial passage of the virus (1, 10, 11a, 14); however, the use of an indicator cell to uncover the presence of defective virus produced in a different host is somewhat novel. This approach could have some practical application as a general method for screening other virus systems with low levels of DI particles.

ACKNOWLEDGMENTS

We thank Wallace Rowe, Natalie Teich, and Nelson Wivel for providing us with the cell lines employed in this study. We are also grateful to Florence K. Millar for generous help in setting up the computer program and Mary Jan Rosenak for processing the polyacrylamide gel data. We also thank Irene Berezsky for expert assistance with the electron microscopy. J. G. L. is an Established Investigator of the American Heart Association.

LITERATURE CITED

1. Choppin, P. W. 1969. Replication of influenza virus in a continuous cell line: high yield of infective virus from cells inoculated at high multiplicity. *Virology* **39**:130-134.
2. Eaton, B. T., and P. Faulkner. 1973. Altered pattern of viral RNA synthesis in cells infected with standard and defective Sindbis virus. *Virology* **51**:85-93.
3. Friedman, R. M. 1968. Protein synthesis directed by an arbovirus. *J. Virol.* **2**:26-32.

4. Friedman, R. M. 1968. Structural and nonstructural proteins of an arbovirus. *J. Virol.* 2:1076-1080.
5. Friedman, R. M., and I. K. Berezsky. 1967. Cytoplasmic fractions associated with Semliki Forest virus ribonucleic acid replication. *J. Virol.* 1:374-383.
6. Friedman, R. M., J. G. Levin, P. M. Grimley, and I. K. Berezsky. 1972. Membrane-associated replication complex in arbovirus infection. *J. Virol.* 10:504-515.
7. Friedman, R. M., H. B. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus: three forms of viral RNA produced during infection. *Proc. Nat. Acad. Sci. U.S.A.* 56:440-446.
8. Grimley, P. M., I. K. Berezsky, and R. M. Friedman. 1968. Cytoplasmic structures associated with an arbovirus infection: loci of viral RNA synthesis. *J. Virol.* 2:1326-1338.
9. Grimley, P. M., J. G. Levin, I. K. Berezsky, and R. M. Friedman. 1972. Specific membranous structures associated with the replication of group A arboviruses. *J. Virol.* 10:492-503.
10. Huang, A. S., and D. Baltimore. 1970. Defective viral particles and viral disease processes. *Nature (London)* 226:325-327.
11. Huang, A. S., and R. R. Wagner. 1966. Defective T particles of vesicular stomatitis virus. II. Biologic role in homologous interference. *Virology* 30:173-181.
- 11a. Kingsbury, D. W., and A. Portner. 1970. On the genesis of incomplete Sendai virions. *Virology* 42:872-879.
12. Levin, J. G., and R. M. Friedman. 1971. Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. *J. Virol.* 7:504-514.
13. Martin, E. M., and J. A. Sonnbend. 1967. Ribonucleic acid polymerase catalyzing synthesis of double-stranded arbovirus ribonucleic acid. *J. Virol.* 1:97-109.
14. Perrault, J., and J. Holland. 1972. Variability of vesicular stomatitis virus autointerference with different host cells and virus serotypes. *Virology* 50:148-158.
15. Rowe, W. P., J. W. Hartley, M. R. Lander, W. E. Pugh, and N. Teich. 1971. Noninfectious AKR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus. *Virology* 46:866-876.
16. Schlesinger, S., M. Schlesinger, and B. W. Burge. 1972. Defective virus particles from Sindbis virus. *Virology* 48:615-617.
17. Shenk, T. E., and V. Stollar. 1972. Viral RNA species in BHK-21 cells infected with Sindbis virus serially passaged at high multiplicity of infection. *Biochem. Biophys. Res. Commun.* 49:60-67.
18. Sonnbend, J. A., E. M. Martin, and E. Mécs. 1967. Viral specific RNA's in infected cells. *Nature (London)* 213:365-367.
19. Stampfer, M., D. Baltimore, and A. S. Huang. 1969. Ribonucleic acid synthesis of vesicular stomatitis virus. I. Species of ribonucleic acid found in Chinese hamster ovary cells infected with plaque-forming and defective particles. *J. Virol.* 4:154-161.
20. Taylor, J. 1965. Studies on the mechanism of action of interferon. I. Interferon action and RNA synthesis in chick embryo fibroblasts infected with Semliki Forest virus. *Virology* 25:340-349.
21. Wright, B. S., P. A. O'Brien, G. P. Shibley, S. A. Mayyasi, and J. C. Lasfargues. 1967. Infection of an established mouse bone marrow cell line (JLS-V9) with Rauscher and Moloney murine leukemia viruses. *Cancer Res.* 27:1672-1677.