JOURNAL OF VIROLOGY, Jan. 1981, p. 1-6
0022-538X/81/010001-06$02.00/0

Vol. 37, No. 1

Comparative Study of Rabies Virus Persistence in Human and Hamster Cell Lines

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Persistent infections by rabies virus in BHK-21/13S and HEp-2 cells were studied comparatively. No evidence of interferon production, selection of virus-resistant cells, or integration of the viral genome could be found. Persisting viruses replicated efficiently at 34, 36, and 40°C. Both persistently infected cultures released defective interfering virus particles. A cyclical pattern of infection, which was not characteristic of the persistently infected HEp-2 system, was observed in persistently infected BHK cultures. The virus from persistently infected BHK cultures lost its virulence for mice, whereas the virus from persistently infected HEp-2 cultures retained mouse-killing capacity for more than 3 years.

Persistent infection by rabies virus has been studied extensively in cell cultures of hamster origin (6, 8, 16); in general, the mechanism of rabies virus persistence is currently attributed to defective interfering particle (DIp)-mediated cell sparing, as evidenced from several studies with BHK-21 cells (6, 8, 17). However, a host effect with respect to DIp generation (7) or acquisition of virulence (3) has been observed when cells distinct from conventional BHK cells were used. This communication is an examination of the role of several factors that may contribute to rabies virus persistence in two different cell cultures. The results indicate that the host cell type may strongly influence the establishment and course of infection and the virulence of the persisting virus.

MATERIALS AND METHODS

Cells and viruses. Primary cultures of Japanese quail embryo (QE) cells were propagated as described previously (2). BHK-21/13S and HEp-2 cells were grown in Eagle and 199 media, respectively, supplemented with 10% bovine serum and antibiotics. A Pasteur strain of rabies virus that was adapted to growth in QE cells (2) was used for the initiation of persistent infection and as a control standard virus. Both persistently infected cell lines were maintained at 36°C.

Assay of viral infectivity. The infectious titer of rabies viruses was determined by the intracerebral inoculation of mice or by a plaque assay with CER cells (kindly provided by J. J. Holland and T. J. Wiktor) with a Sephadex overlay (10).

Direct fluorescent-antibody technique. The direct fluorescent-antibody technique was described previously (2).

Cell cloning experiments. Carrier cells were dispersed, counted, and plated at dilutions that provided very few cells per flask. Single cells were marked 3 to 4 days after seeding and observed until a colony had formed. These colonies were transferred to individual tubes, and the developed subcultures were examined.

Immunogenicity of persisting viruses. The immunogenic activity of the viruses was determined by the standard National Institutes of Health potency test in mice (11).

Concentration and purification of rabies virus. Rabies virus was pelleted from the culture fluid at 20,000 rpm for 2 h in a 6 × 250 ml rotor of an MSE SS 65 centrifuge, suspended in NTE buffer (13), and clarified through a 30% sucrose cushion at 61,000 × *g* for 40 min. The visible band at the interface was aspirated, diluted with NTE buffer, and pelleted at 45,000 rpm for 2 h in an SW50 rotor of a Beckman L5 65 centrifuge. Virus suspended in 0.5 to 0.8 ml was layered on a 5 to 30% linear sucrose gradient in NTE buffer made with the aid of an LKB Ultrograd gradient mixer. Centrifugation was performed in an SW40 rotor at 34,000 rpm for 60 min. Gradients were recovered from the bottom through a flow cell of a Gilford 2400-2 spectrophotometer, with a continuous monitoring of the optical density (OD) at 260 nm.

Polyacrylamide gel electrophoresis. Samples of purified virus were dissociated and separated on 7.5% cylindrical gels in a sodium dodecyl sulfate-phosphate system as described by Weber et al. (15). Electrophoresis was conducted in 14-cm tubes at 3 mA per tube for 20 to 24 h. Gels were fixed and stained in a methanol-glacial acetic acid-Coomassie brilliant blue R-250 mixture and after destaining were scanned in the Gilford spectrophotometer at 550 nm. The molecular weight standards were bovine albumin, ovalbumin, and myoglobin (Schwarz/Mann, Orangeburg, N.Y.; 68,000, 45,000, and 18,600, respectively).

Preparation of rabies virus [^{3}H]RNA and [^{3}H]-cDNA. For the preparation of [^{3}H]RNA, RNA was extracted from purified rabies virus (a total of 5×10^{10} 50% lethal doses [LD_{50}]) that had been propagated in QE cells by treatment with sodium dodecyl sulfate-phenol-chloroform (9). A portion of this RNA preparation was used for labeling by the direct introduction

of tritium into the polynucleotide in the course of RNA treatment with ^3H atoms (12). [^3H]RNA obtained in this manner was dialyzed against 25 volumes of 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate in an MMC Amicon system through a 10 PM membrane and stored under ethanol at −25°C. The specific activity of the preparation was 0.9×10^6 cpm/μg.

[^3H]complementary DNA ([^3H]cDNA) of rabies virus RNA was obtained essentially as described previously (1) with 50 U of *Escherichia coli* DNA polymerase (Sigma Chemical Co., St. Louis, Mo.) and ^3H-labeled deoxytriphosphates ([^3H]dGTP and [^3H]-dCTP [Amersham Corp., Arlington Heights, Ill.], specific activities, 10.3 and 25 Ci/mmol, respectively; and [^3H]dATP [Isotop, Leningrad, USSR], specific activity, 7.2 Ci/mmol). The composition of the reaction mixture, the isolation of cDNA, and the preparation of the hybrid were the same as previously described (1). The specific activity of cDNA was 6.7×10^7 cpm/μg as determined from the specific activities of the input ^3H-labeled deoxytriphosphates. After synthesis, cDNA sedimented in an alkaline sucrose gradient at a position corresponding to 8 to 9S and annealed to excess virion RNA to an extent of about 90%.

Hybridization of carrier cell DNA to rabies virus [^3H]RNA or cDNA. The DNA was isolated from carrier cultures at a level of 104 passages for persistently infected HEp-2 cultures (HEp-$2_{p.i.}$) and at a level of 117 passages for persistently infected BHK cultures (BHK$_{p.i.}$) by a chloroform-isoamyl alcohol (24:1) technique, including the steps of RNase and pronase digestions between subsequent deproteinizations, as described by Cooper and Temin (4). The DNA obtained in this manner had a ratio of absorbance at 260 nm to absorbance at 280 nm of 1.8 to 1.9, which was comparable to that of calf thymus DNA (Serva, Heidelberg, Germany). The composition of the annealing reaction mixtures and the protocol for the hybridization were identical to those described previously (1).

Transfection experiments with DNA from carrier cells. Confluent monolayers of 2×10^7 BHK cells were pretreated for 5 min with 300 μg of DEAE-dextran per ml in 0.15 M NaCl. After the aspiration of DEAE-dextran, cells were exposed to DNA dissolved in 0.15 M NaCl. The DNA exposure levels ranged from 0.5 to 7 mg per flask. After a 15-min adsorption at room temperature, growth medium was added and cells were incubated at 36°C. Transfected cultures were split twice weekly, with periodic assays of cultural fluid for infectious virus and of cells for viral antigen by immunofluorescence.

RESULTS

Establishment and time course of persistent infection. Confluent monolayer cultures of HEp-2 or BHK cells were infected with rabies virus at a multiplicity of 1 LD_{50} per cell. After a 1.5-h adsorption period, growth medium was added and the cultures were incubated at 36°C. No signs of cell destruction were observed in HEp-$2_{p.i.}$ at 7 to 10 days postinfection. In contrast, in BHK$_{p.i.}$, many cells died by postinfection day 4, and the addition of fresh BHK cells was required during eight subsequent passages to prevent complete cell destruction. After the persistent infection had stabilized, BHK$_{p.i.}$ were passed every 5 to 7 days, with no signs of visible cytopathology.

Different patterns of infection were found in the two persistently infected cultures (Fig. 1). For BHK$_{p.i.}$, cyclical fluctuations of antigen-positive cells ranging from 100 to less than 1% were observed, although the antigen never disappeared completely. The persisting virus gradually lost its virulence for mice; soon after 50 passages of BHK$_{p.i.}$, mouse-lethal virus was no longer detectable.

In contrast to BHK$_{p.i.}$, a significantly more constant production of infectious virus and viral antigen was found in HEp-$2_{p.i.}$ (Fig. 1).

Properties of persisting viruses. Starting from the early stages of infection, the persisting viruses from both cultures characteristically displayed incubation periods that were about twice as long in mice as those with standard virus (12 to 14 versus 6 to 8 days). The virus from HEp-$2_{p.i.}$ retained its virulence for mice for more than 3 years, whereas the virus from BHK$_{p.i.}$ lost its pathogenicity after 50 passages of the culture. This loss of mouse-killing ability remained after three intracerebral passages of the virus in suckling-mouse brains.

Persisting viruses could induce plaques on CER cell monolayers. The specificity of plaques was verified in a plaque reduction neutralization test with antiserum to original virus propagated in QE cells. BHK$_{p.i.}$ virus plaques were 0.1 to 0.15 cm in diameter, whereas HEp-$2_{p.i.}$ virus formed tiny pinpoint plaques.

The immunogenic ability of unconcentrated persisting viruses was poor. However, concen-

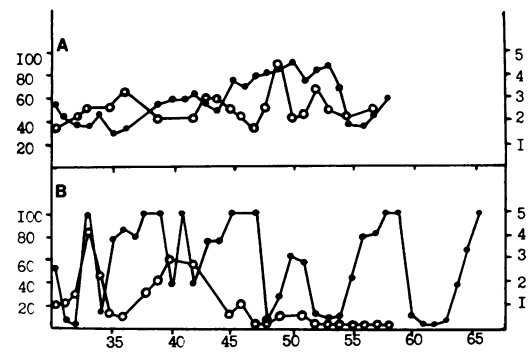

FIG. 1. *Time course of persistent infections by rabies virus in HEp-$2_{p.i.}$ (A) and BHK$_{p.i.}$ (B). Symbols: ○, infectivity; ●, percentage of antigen-positive cells. (Abscissa) Number of cell transfers; (left ordinate) percentage of fluorescing cells; (right ordinate) virus titer in log LD_{50} per milliliter.*

trated viruses displayed their immunogenicity even at late stages of persistence (Table 1).

To determine the effect of temperature on the multiplication of persisting viruses, BHK cells were infected with HEp-$2_{p.i.}$ virus from passage 134, $BHK_{p.i.}$ virus from passage 163, or standard virus and incubated at 34, 36, and 40°C. Samples of culture fluid were taken on 3, 4, and 5 days postinfection and titrated in mice or by plaquing. As shown in Table 2, the virus from HEp-$2_{p.i.}$ replicated almost equally well at different temperatures. The virus from $BHK_{p.i.}$ replicated somewhat better at 34°C than it did at 40°C. However, there were no significant differences in the numbers of antigen-positive cells when BHK cultures infected with $BHK_{p.i.}$ virus and incubated at various temperatures were compared (data not shown). The original virus displayed pronounced temperature sensitivity (Table 2).

In contrast to the original virus grown in QE cells, persisting viruses from HEp-$2_{p.i.}$ and $BHK_{p.i.}$ could readily establish a carrier state in BHK and Vero cell cultures, with a similarly fluctuating pattern of infection in BHK cells (data not shown).

Detection of rabies virus DIp in persistently infected cultures. Several lines of evidence suggested the presence of interfering activity in our persistently infected cultures: (i) a consistently observed autointerference phenomenon in initial virus dilutions when titrated by plaquing; (ii) a longer incubation period of persisting viruses in mice when compared with that of standard virus; and (iii) a cyclical pattern of infection in $BHK_{p.i.}$ that was reportedly due to DIp (8). To test directly for the presence of DIp,

TABLE 1. *Immunogenic properties of rabies viruses released by persistently infected cultures*[a]

Source of virus	Passage level of persistently infected cells[b]	Infectious virus titer (per ml)	Immunogenicity index
HEp-$2_{p.i.}$	29–31	3.1×10^6 PFU	2.4
	123–126	3.7×10^7 PFU	0.94
$BHK_{p.i.}$	36–39	2.1×10^8 PFU	2.2
	163–164	2.5×10^7 PFU	0.57
QE cells acutely infected		5.5 log LD_{50}	2.5

[a] Persisting viruses were concentrated 10^3-fold and injected intraperitoneally into mice. For each dilution of the virus, groups of 14 animals were used. The immunogenicity index is expressed as the ratio of the reciprocal of the highest dilution of the virus protecting 50% of the mice to that of the standard reference vaccine supplied by Tarasevich State Institute of Biological Standardization and Control, Moscow.

[b] A simultaneous comparison between viruses of different cell passages was possible due to the storage of the early passage carrier cell cultures in liquid nitrogen.

TABLE 2. *Replication of persisting viruses in BHK cells at different temperatures*

Source of virus	Time post-inoculation (h)	Titer[a] at following temp (°C):		
		34	36	40
HEp-$2_{p.i.}$	72	3.0	2.5	3.0
	96	4.25	3.5	2.75
	120	3.75	3.0	4.0
$BHK_{p.i.}$	72	4.5	4.0	3.0
	96	NT[b]	NT	NT
	120	NT	NT	NT
QE cells acutely infected	72	4.5	3.5	2.0
	96	6.0	3.5	2.0
	120	6.0	3.75	2.0

[a] All of the titers are expressed in log LD_{50} per milliliter, except for $BHK_{p.i.}$, where the titers are expressed in log PFU per milliliter.

[b] NT, Not tested.

persisting viruses were sedimented from 800- to 1,500-ml volumes and inoculated into mice in a mixture with 10-fold dilutions of standard virus. No differences were observed between the titers of the virus mixture and the standard virus only. However, after a preliminary one-step amplification on BHK cells (6), the titer of a mixture was 0.75 to 1.5 log LD_{50} per ml lower than that of similarly amplified standard virus. When BHK cells were infected with undiluted and 100-fold-diluted culture fluid from HEp-$2_{p.i.}$, the final virus titer was 1 log LD_{50} per ml higher in the latter case. Released viruses harvested from persistently infected cells at different passage levels were concentrated and analyzed in sucrose gradients. Viruses from HEp-$2_{p.i.}$ or $BHK_{p.i.}$ formed OD bands that could not be detected in concentrated materials from mock-infected or standard virus-infected QE cells (Fig. 2). $BHK_{p.i.}$ virus usually formed one broad band, whereas two bands could often be observed in HEp-$2_{p.i.}$ virus gradients (Fig. 2C). The infectivity peak sedimented ahead of the OD band (Fig. 2B), suggesting that OD structures were smaller in size than rabies virus virions. The polypeptide composition of purified OD structures was similar to that of standard virus propagated in QE cells (Fig. 3). The molecular weights of the polypeptides were 78,000, 62,000, 43,000, and 26,000, which are in agreement with reported data on rabies virus proteins (14). Thus, we concluded that these OD structures were true rabies virus defective particles. Purified defective particles were not infectious when injected intracerebrally into mice and did not protect animals against challenge with virulent rabies virus. However, when these particles were mixed with the infectious virus and amplified on BHK cells, a reproducible reduction in the virus titer, rang-

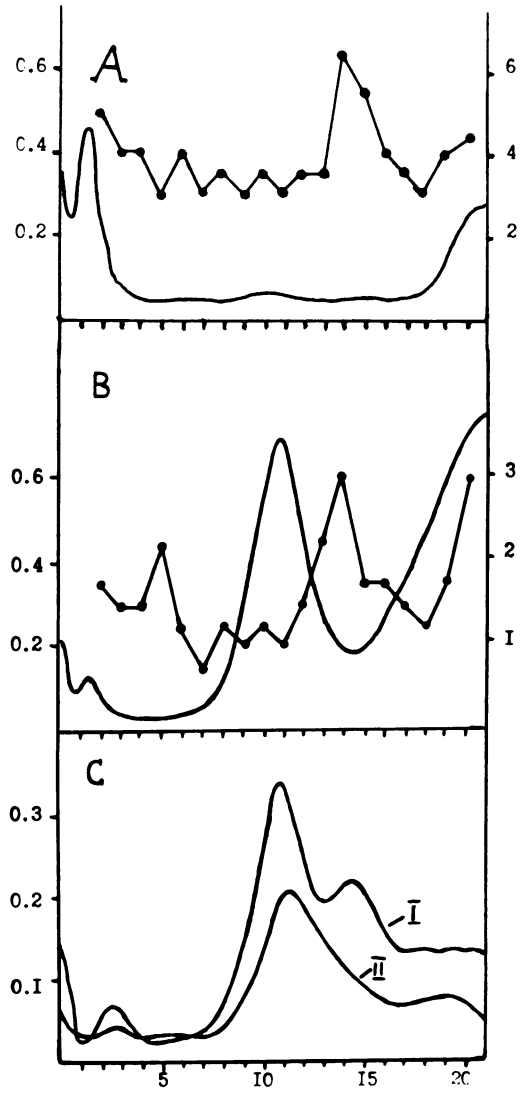

FIG. 2. *Sucrose gradient profiles of rabies viruses released from acutely infected QE cells (A) and HEp-$2_{p.i.}$ (B). (C) OD profiles of two parallel gradients (I, HEp-$2_{p.i.}$; II, $BHK_{p.i.}$) recorded on the same sheet of paper. (——) OD at 260 nm; (····) infectivity. (Abscissa) Fraction number; (left ordinate) OD at 260 nm; (right ordinate) virus titer in log LD_{50} per milliliter. Centrifugation was from left to right.*

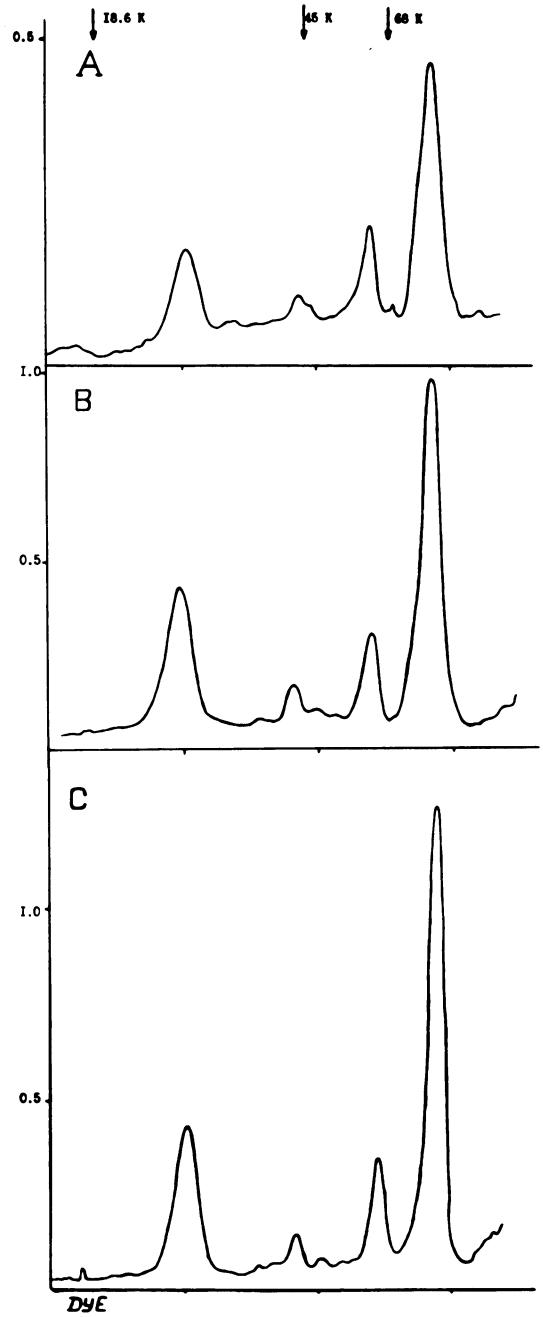

FIG. 3. *Polypeptide composition of QE cell-propagated standard rabies virus (A) and DIp from $BHK_{p.i.}$ (B) and HEp-$2_{p.i.}$ (C). (Ordinate) OD at 550 nm.*

ing from 0.5 to 1.25 log LD_{50} per ml, was observed.

Examination of cell clones isolated from persistently infected cultures. To determine whether the resistance of persistently infected cultures to homologous virus challenge was associated with the selection of rabies virus-resistant cells, we cloned $BHK_{p.i.}$ and HEp-$2_{p.i.}$ at the levels of 100 and about 70% of antigen-positive cells, respectively. A total of 32 clones were obtained from HEp-$2_{p.i.}$, 17 of which were antigen positive and virus producing. The antigen-free clones were susceptible to rabies virus to the same extent as were control uninfected HEp-2 cells. Of 26 $BHK_{p.i.}$ clones, 24 were antigen

negative and rabies virus susceptible. This result is intriguing because 100% of the cells in precloned $BHK_{p.i.}$ contained viral antigen. Moreover, two antigen-positive clones from $BHK_{p.i.}$ were spontaneously destroyed after four passages, whereas all antigen-negative clones were split at least another five times without noticeable changes.

Attempts to detect interferon production in $BHK_{p.i.}$ and $HEp\text{-}2_{p.i.}$ $BHK_{p.i.}$, $HEp\text{-}2_{p.i.}$, and control uninfected cells were infected with the Indiana serotype of vesicular stomatitis virus and an interferon-susceptible measles virus variant. At 72 h postinfection, challenge viruses were titrated by cytopathogenicity on BHK (vesicular stomatitis virus) or HEp-2 (measles virus) cells. Challenge viruses consistently multiplied equally well both in persistently infected cultures and in uninfected control cultures, suggesting the lack of detectable interferon production in persistently infected cultures.

Search for virus-specific sequences in DNA of the carrier cell cultures. At a C_0t ranging from 137 to 6,567 mol·s/liter and a carrier cell DNA concentration of 5 mg/ml, the percentage of hybridization was in the range of from 5 to 7% with either a [^{3}H]cDNA or a [^{3}H]-RNA probe. Control calf thymus DNA did not differ significantly from carrier cell DNA in its annealing rate. However, the cDNA probe present in the reaction mixture was still capable of specific annealing to excess virion RNA at the end of the hybridization period (59 to 62% at a C_0t of 3,283 mol·s/liter). Repeated transfection attempts with carrier cell DNA to induce infectious virus or viral antigen in BHK cells were also unsuccessful.

DISCUSSION

Persistent infections by rabies virus have been established in rabbit (5) and reptilian (16) cell cultures, but have been studied extensively in BHK-21 cells (6, 8, 16). However, an apparent host cell effect has been observed on rhabdovirus DIp generation in human cells (7) and on the virulence of rabies virus in neuroblastoma cells (3). We have studied the host cell effect on (i) the course of persistent rabies virus infection, (ii) properties of persisting virus, and (iii) the contribution of several factors currently regarded as the mechanisms of viral persistence into the establishment and maintenance of a carrier state. To this end, we have used a well-characterized $BHK_{p.i.}$ system and $HEp\text{-}2_{p.i.}$ of human origin. In contrast to $BHK_{p.i.}$, in which a cyclical pattern of infection has been observed, the course of infection in $HEp\text{-}2_{p.i.}$ was more constant. The persisting virus from $BHK_{p.i.}$ at the level of 50 cell passages lost its virulence for mice; this is in accord with the finding of Fernandes et al. (5). In contrast, the virus from $HEp\text{-}2_{p.i.}$ retained its virulence for mice for more than 3 years despite the production of DIp. Recently, Wunner and Clark have presented evidence that the virulence of rabies virus is not mediated by DIp (18).

An examination of persistently infected cultures with respect to a mechanism of virus persistence revealed that neither interferon nor integration of virus and cell genomes seemed to play a role. Temperature sensitivity of persisting viruses was poor and never reached the level of that of the original virus. A selection of virus-resistant cells is also unlikely due to the observed susceptibility of virus-negative clones to reinfection. Both carrier cell cultures were found to produce DIp, which is apparently inherent to rabies virus persistence (6, 8, 17). The viruses readily established persistent infections in Vero and BHK cells, in contrast to the QE cell-propagated non-DIp-containing virus, which caused regular cytopathic effect in the same cells. This finding suggests that in $BHK_{p.i.}$ the generation and protective effect of DIp are prerequisites for the initiation and maintenance of persistent infection. In $HEp\text{-}2_{p.i.}$, however, the role of DIp in virus persistence is questionable. In contrast to $BHK_{p.i.}$, persistent rabies virus infection could be readily and consistently instituted in HEp-2 cells, without any signs of initial cytopathic effect. We also did not find any evidence for the rapid generation of DIp within the first passage of rabies virus-infected HEp-2 cells (data not shown). These observations lead us to suggest that, in $HEp\text{-}2_{p.i.}$, DIp are produced as a by-product of rabies virus replication which is not necessary for HEp-2 cell sparing.

Thus, host cells may exhibit pronounced effects on the course of persistent rabies virus infection and the virulence of the virus, whereas inherent viral properties, e.g., the generation of DIp, could nevertheless be preserved in various cell systems.

ACKNOWLEDGMENTS

We are indebted to J. J. Holland, B. Janis, and T. J. Wiktor for an introduction into rabies virus plaque assays and to A. V. Shishkov for in vitro labeling of RNA. The expert technical assistance of I. Snyatkova, T. Luckjanova, and I. Kasatochkina is gratefully acknowledged.

LITERATURE CITED

1. **Andzhaparidze, O. G., I. D. Drynov, N. N. Bogomolova, N. V. Chelyapov, and Y. S. Boriskin.** 1979. Tick-borne encephalitis virus-specified sequences in persistently infected cell culture revealed by DNA-DNA hybridization. Experientia 35:601-602.
2. **Bektemirova, M. S., D. F. Osidze, E. R. Pille, L. V. Nadaichik, K. S. Matevosyan, F. G. Nagieva, N. N. Bogomolova, Y. S. Boriskin, and N. N. Yanova.** 1979. Properties of rabies virus (MNIIVP-74) strain

adapted to Japanese quail embryo cell culture. Arch. Virol. **61**:61–68.

3. **Clark, H. F.** 1978. Rabies virus increase in virulence when propagated in neuroblastoma cell culture. Science **199**: 1072–1075.
4. **Cooper, G. M., and H. M. Temin.** 1974. Infectious Rous sarcoma and reticuloendotheliosis virus DNAs. J. Virol. **14**:1132–1141.
5. **Fernandes, M. V., T. J. Wiktor, and H. Koprowski.** 1964. Endosymbiotic relationship between animal viruses and host cells. J. Exp. Med. **120**:1099–1116.
6. **Holland, J. J., and L. P. Villarreal.** 1975. Purification of defective interfering T particles of vesicular stomatitis and rabies viruses generated in vivo in brains of newborn mice. Virology **67**:438–449.
7. **Holland, J. J., L. P. Villarreal, and M. Breindl.** 1976. Factors involved in the generation and replication of rhabdovirus defective T particles. J. Virol. **17**:805–815.
8. **Kawai, A., S. Matsumoto, and K. Tanabe.** 1975. Characterization of rabies viruses recovered from persistently infected BHK cells. Virology **67**:520–533.
9. **Perry, R. P., J. La Torre, D. E. Kelly, and J. R. Greenberg.** 1972. On the lability of poly (A) sequences during extraction of messenger RNA from polyribosomes. Biochim. Biophys. Acta **262**:220–226.
10. **Schneider, L.** 1973. Cell monolayer plaque test, p. 339–342. *In* M. M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies. World Health Organization, Geneva.
11. **Seligmann, E. B.** 1973. The NIH test for potency, p. 279–286. *In* M. M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies. World Health Organization, Geneva.
12. **Shishkov, A. V., E. S. Filatov, E. F. Simonov, M. S. Unukovich, V. I. Goldansky, and A. N. Nesmeyanov.** 1976. Production of tritium-labelled biologically active compounds. Proc. Natl. Acad. Sci. USSR **228**: 1237–1239 (In Russian).
13. **Sokol, F., E. Kuwert, T. J. Wiktor, K. Hummeler, and H. Koprowski.** 1968. Purification of rabies virus grown in tissue culture. J. Virol. **2**:836–849.
14. **Sokol, F., D. Stanček, and H. Koprowski.** 1971. Structural proteins of rabies virus. J. Virol. **7**:241–249.
15. **Weber, K., J. R. Pringle, and M. Osborn.** 1973. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol. **26C**:3–27.
16. **Wiktor, T. J., and H. F. Clark.** 1972. Chronic rabies virus infection of cell cultures. Infect. Immun. **6**:988–995.
17. **Wiktor, T. J., B. Dietzschold, R. N. Leamnson, and H. Koprowski.** 1977. Induction and biological properties of defective interfering particles of rabies virus. J. Virol. **21**:626–635.
18. **Wunner, W. H., and H. F. Clark.** 1978. Study of virulent and avirulent rabies viruses and their defective RNA-containing particles, p. 599–606. *In* B. W. Mahy and R. D. Barry (ed.), Negative strand viruses and the host cell. Academic Press, Inc., New York.