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Rabies Serogroup Viruses in Neuroblastoma Cells: Propagation, "Autointerference," and Apparently Random Back-Mutation of Attenuated Viruses to the Virulent State

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Each of several strains of fixed rabies virus was found to replicate to high titers in C1300 mouse neuroblastoma (clone NA) cells, without adaptation. Rabies serogroup Lagos bat, Mokola, and Duvenhage viruses also replicated efficiently in NA cells. Kotonkan and Obodhiang viruses replicated efficiently after adaptation, to titers not previously obtained in vitro. Infection in NA cells was frequently more cytopathic than in BHK-21 cells, allowing titration of Kotonkan and Obodhiang viruses by plaque assay. Duvenhage virus caused syncytium formation. Serial propagation of rabies viruses at a high multiplicity of infection in NA cells led to a rapid decline in virus yields; similar "autointerference" has not previously been demonstrated with rabies virus in other cell systems. Rabies virus infection in NA cells exhibited extreme sensitivity to interference by experimentally added defective interfering virions. Although several strains of attenuated rabies virus consistently reverted rapidly to virulence after propagation in NA cells, other strains of attenuated rabies and rabies serogroup viruses acquired increased virulence at a more gradual rate or not at all, suggesting that diverse characters may control virulence. When attenuated Flury HEP rabies virus was serially propagated at a low multiplicity of infection in either NA cells or suckling mouse brain, virulence appeared at a very variable rate, indicating that these systems may selectively enhance replication of randomly occurring virulent virus mutants.

Rabies virus selectively infects the neurons of the central nervous system (23). Because of the lack of dramatic damage to central nervous system components often observed after fatal rabies encephalitis, the nature of the virus-neuron interaction is worthy of special attention. Experimental infection of neuroblastoma cells in culture, which possess many structural and physiological characters of neuronal cells (reviewed in reference 4), was studied as an initial step in the development of a model system for the study of this interaction.

We have investigated the replication of several strains of fixed rabies virus and several other rabies serogroup viruses in neuroblastoma cell cultures. The replication in neuroblastoma cells of two rabies subgroup viruses, Kotonkan (20) and Obodhiang (32), which have not readily been grown to high titer in cell culture, was characterized. We also report studies of an apparent special sensitivity of neuroblastoma cells to expression of "autointerference" with virus replication after infection at high multiplicity.

We have previously reported that several strains of attenuated rabies virus revert to a mouse-virulent state after a few passages in neuroblastoma cells (4). Evidence is presented that this conversion to virulence occurs with varying efficiency with different virus strains. Additional experiments indicate that the back-mutation to virulence is a randomly occurring event, either in neuroblastoma cells or in the brain of the newborn mouse.

MATERIALS AND METHODS

Cell cultures. Mouse neuroblastoma C1300, clone NA (hypoxanthine-guanine phosphoribosyl transferase deficient) was originally contributed by F. A. McMorris (25). CB3 cell line, a line of mixed cell composition derived from a normal cow brain (D. Santoli, unpublished data), was supplied by D. Santoli. ICR mouse fibroblasts are a cell strain established by the author from lung tissue explants of a normal adult ICR mouse.

Cell lines MBC-2 and MBC-3 were established from the cerebral and cerebellar gray matter, respectively, of an inbred 129 GIX$^-$ adult mouse and supplied to us by Y. Iwasaki (18). These cell lines are presumed to contain mixed fibroblasts and glial cells. Mouse glioma cell line G-26 clone (cl) 23 (44) was supplied by F. A. McMorris. Cell line MBC-1, established from the cerebral gray matter of a DBA/2J (18) mouse, is reported to be rich in astrocytes (Y. Iwasaki, personal communication) and was provided by Y. Iwasaki.

These cell lines, as well as BHK-21 and mouse L cells, were routinely propagated in Eagle minimal es-

sential medium containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Virus. BHK-21 cell-adapted fixed strains ERA, PM, CVS, and Flury HEP of rabies virus have been previously described (8, 9). BHK-21 cell-adapted Flury LEP virus was obtained from T. J. Wiktor. The rabies CVS *ts* mutant-revertant mixture, designated *ts* 2, and its *ts* revertant clone 4 were developed in this laboratory (6, 7). Clones selected from CVS virus stock on the basis of plaque morphology, L1 (large plaque, mouse virulent) and S1 (small plaque, mouse avirulent), were isolated in this laboratory (L. Stefanik and H F. Clark, unpublished data). The adaptation of Mokola and Lagos bat viruses (35) to BHK-21 cell culture and the characterization of derivative clones of each virus have been previously described (10). Duvenhage virus (26, 38) was obtained from F. A. Murphy, Center for Disease Control, Atlanta, Ga., and adapted to BHK-21 cells in our laboratory. Kotonkan and Obodhiang viruses (32) were obtained from R. E. Shope, Yale University School of Medicine, New Haven, Conn., as suckling mouse brain suspensions.

Of the nonrabies serogroup rhabdoviruses, the origin and cultivation in BHK-21 cells of Kern Canyon virus (28) have been previously described (1). Hart Park (Flanders) virus (27, 40) was obtained as suckling mouse brain suspension from the Center for Disease Control.

For propagation of virus stocks and for experimental tests of virus sensitivity, cell cultures were infected in an identical manner. Growth medium was decanted and virus was diluted to provide a multiplicity of infection (MOI) of approximately 1.0; it was then adsorbed for 30 min at 35°C. Inoculum was removed, the cell cultures were washed three times with phosphate-buffered saline (Dulbecco), and the cultures were fed with Eagle minimal essential medium supplemented with 0.2% bovine serum albumin. After 4 to 7 days of further incubation at 35°C, culture fluids were harvested for assay of released virus by the agarose-suspended BHK-21 13S cell technique (33).

Preparation of DIV. The defective interfering virus (DIV), characteristics of various rabies strains, and the method of purification will be described in detail elsewhere. (DIVs were identified by sedimentation characteristics, fine-structure morphology observed by electron microscopic examination, genomic ribonucleic acid size determination, and ultraviolet light-sensitive interfering capacity [H F. Clark, N. F. Parks, and W. H. Wunner, manuscript in preparation].) Briefly, to prepare DIV, stocks of rabies virus known to produce DIV were produced as described above using MOI ≥ 1.0. Virus fluids were clarified by low-speed centrifugation, and virus was subsequently sedimented by centrifugation for 2 h at 18,000 rpm in a Spinco no. 19 fixed-angle rotor. The virus pellet was suspended in sodium-tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid buffer (pH 7.8) and sedimented through a 5 to 30% (wt/vol) sucrose continuous density gradient at 54,000 × *g* for 45 min. Visible bands of defective virions were observed well separated and above the standard virion band. Samples were collected by side puncture of the tube and diluted in the buffer. Defective virions were again pelleted by centrifugation at 189,000 × *g* for 3 h. The resulting pellet was resuspended to a final protein concentration of 10 μg/ml and frozen at −70°C.

Animal studies. Assays of mouse virulence were performed by titration of virus in intracerebrally inoculated weanling (4 to 5 weeks of age) ICR outbred Swiss mice. The left cerebral hemisphere was inoculated with 0.03 ml of virus suspension; newborn mice were inoculated intracerebrally with a volume of 0.01 ml of virus suspension.

RESULTS

Sensitivity of NA cells to infection with rabies serogroup viruses. The special sensitivity of NA neuroblastoma cells to rabies serogroup viruses was demonstrated by parallel infections of several cell lines with two fixed strains of rabies virus and with the antigenically related (34, 35) Lagos bat virus (Table 1). It is apparent that the mouse neuroblastoma cells infected with low or high MOIs produced considerably higher titers of rabies virus than did either mouse L cells, a strain of nontransformed mouse fibroblasts, or the CB3 cell line derived from bovine brain. Lagos bat virus replicated only in the NA cells. Replication of the Flury HEP and ERA strain rabies viruses in NA cells was accompanied by marked cytopathic effect (CPE) characterized by cell pycnosis and death (Fig. 1A). Lagos bat virus caused less cell death but some syncytium formation (see below). Virus yields and the apparent rate of replication in NA cells were similar to those commonly obtained in BHK-21 cells (data not shown), the cell system most commonly used for propagation of rabies virus. However, the CPE induced by rabies virus in NA cells was much more severe than that induced in BHK-21 cells.

TABLE 1. *Replication of rabies serogroup viruses in C1300 neuroblastoma and other mammalian cell types*

Virus inoculum	MOI	Released virus yield (PFU/ml, $\log_{10}$)[a]			
		NA cells	L cells	CB3 cells	ICR mouse fibroblasts
Rabies ERA	10.0	8.1	5.2	5.7	4.4
	0.01	6.8	4.4	<3.0	<3.0
Rabies Flury HEP	10.0	7.6	6.1	6.7	<3.0
	0.01	7.3	4.0	4.5	<3.0
Lagos bat	10.0	7.5	<3.0	<3.0	<3.0
	0.01	6.8	<3.0	<3.0	<3.0

[a] Titers represent released virus harvested 3 days postinfection.

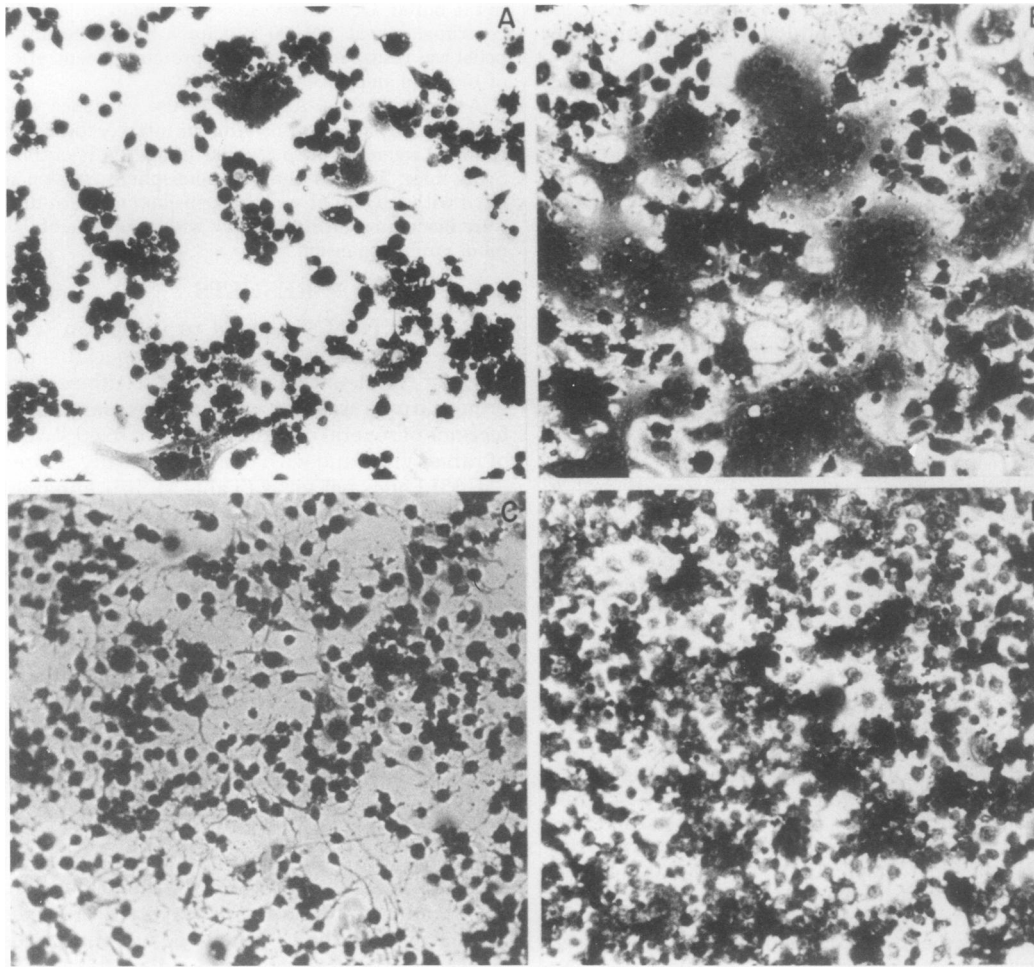

FIG. 1. *CPEs induced by rabies serogroup viruses in NA cells. All cultures were infected at an MOI of approximately 1.0, incubated 72 h at 35°C, and stained by the May-Giemsa-Greenwald technique. Photographed at 100× magnification. (A) Rabies virus (strain ERA). Pycnosis, cell death, and occasional syncytia are evident. (B) Duvenhage virus. Syncytial CPE predominates. (C) Kotonkan virus. Cell pycnosis is induced. Also noted is exaggeration of the differentiated cell phenotype indicated by pronounced extension of cell processes. (D) Normal control cells.*

The uniform sensitivity of NA cells to infection with rabies serogroup viruses is illustrated in Table 2. Fixed rabies viruses of strains ERA, Flury HEP, Flury LEP, and PM all replicated to a high titer while causing severe CPE. Surprisingly, virus of the highly mouse-pathogenic strain CVS and the less pathogenic derivative *ts* 2 virus neither replicated well in NA cells nor produced CPE. The apparent inability of CVS virus to replicate in NA cells has been shown to be an autointerference effect present at high MOI only (see below). Mouse-virulent clone CVS-L1 and avirulent clone CVS-S1 both replicated efficiently in NA cells, presumably because of their recent clonal (low MOI) origin.

The rabies serogroup Duvenhage virus replicated in NA cells to high titer. The CPE produced by this virus in NA cells is unique in that it consists almost entirely of syncytium formation (Fig. 1B). Mokola virus replicated in NA cells with minimal CPE; Lagos bat virus replicated more efficiently and produced moderate CPE. The CPE induced by Lagos bat virus consisted of a predominantly cytocidal pycnotic change accompanied by involvement of 10 to 25% of infected cells into syncytia.

Kotonkan and Obodhiang viruses were previously adapted to mammalian cell culture only by the use of susceptible mosquito helper cells (2). These viruses did not immediately yield high

TABLE 2. *Sensitivity of NA neuroblastoma cells to rabies serogroup viruses*[a]

Virus	Released virus yield (PFU/ml, $\log_{10}$)	CPE[b]
Rabies ERA	7.8	+++/++++
Rabies Flury HEP	7.8	+++
Rabies Flury LEP	7.9	++
Rabies PM	7.8	+++
Rabies CVS	3.3	—
Rabies CVS *ts* 2	<2.7	—
Rabies CVS, clone S1	8.0	++
Rabies CVS, clone L1	7.1	+++/++++
Duvenhage	8.0	+++/++++[c]
Mokola, clone 1	6.6	±
Mokola, clone 3	6.1	±
Lagos bat, clone 1	7.8	+++/++++
Lagos bat, clone 3	7.4	++[c]

[a] Cell cultures were infected on a multiplicity of ca. 1.0. Released virus was harvested 4 days postinfection.

[b] +, 0 to 25% cell destruction; ++, 25 to 50% cell destruction; +++, 50 to 75% cell destruction; ++++, 75 to 100% cell destruction; —, no effect; ±, minor changes of uncertain specificity.

[c] Partly or predominately syncytial CPE.

titers of released virus when stocks propagated in newborn mouse brain were inoculated onto NA cells. However, Kotonkan virus-inoculated NA cells developed CPE involving approximately 50% of the cell sheet at 11 days postinfection. Released virus harvested from this culture was subinoculated onto NA cell culture and caused 100% CPE within 48 h. This second NA cell passage virus could be readily titrated by assay of CPE in NA cells (titer, $10^{7.5}$ 50% tissue culture infective doses per ml, cytopathic endpoint within 4 days; Fig. 1C) or plaque assay in NA cells overlaid with Eagle minimal essential medium containing 5% fetal calf serum and 0.5% agarose (titer, $10^{6.2}$ plaque-forming units (PFU)/ml read at 5 days). This NA cell-adapted Kotonkan virus can also be plaque assayed on the agarose-suspended BHK-13S cell system used for rabies virus plaque assay.

Obodhiang virus-inoculated NA cells exhibited 50% CPE at 20 days postinfection. A second passage of fluids harvested at this time caused massive CPE within 4 days. This NA cell-adapted virus could also be assayed by titration of CPE. The identity of each virus was reestablished after propagation in NA cells by neutralization with specific antisera generously supplied by R. E. Shope, Yale University, New Haven, Conn.

Replication of Hart Park (Flanders) and Kern Canyon viruses in NA cells. Two nonrabies serogroup rhabdoviruses with limited host range in vitro, Hart Park (Flanders) and Kern Canyon viruses, both replicated in NA cells. Hart Park virus replicated causing severe CPE in NA cells within 4 days postinfection after two NA cell passages; the released virus titer assayed by CPE was $10^{5.5}$ 50% tissue culture infective doses per ml. Kern Canyon virus, previously known to replicate well only in BHK-21 cells, caused little CPE in NA cells but, at the second passage level, yielded $10^{7.2}$ PFU/ml within 4 days postinfection.

Autointerference: enhanced replication of CVS rabies virus after inoculation at low MOI in NA cells. Our original surveys employing virus inoculated at high concentration revealed little or no replication of CVS rabies virus in NA cells (see above). We had no reason to suspect autointerference, as several previous studies had revealed no inhibition of rabies virus yield after inoculation of BHK-21 cells at high multiplicity (7) (H F. Clark and T. J. Wiktor, unpublished data). Nevertheless, a striking autointerfering effect was noted when several dilutions of CVS virus were inoculated onto NA cells (Table 3): production of severe CPE and of released CVS virus in high titer occurred only when input MOIs were less than 1.0. A less marked autointerfering effect was noted with BHK-21 cells. Numerous other cell culture systems commonly exhibiting little or no susceptibility to infection with CVS virus did not exhibit increased susceptibility to virus inoculated at low multiplicity. These included cell lines of bovine and normal mouse brain origin, glioma cells and mixed cultures containing mouse glial cells, and a mouse cell line rich in astrocytes.

Autointerference: predilection of NA cells for expression of autointerference of rabies virus yield. We have observed (above) marked autointerference after inoculation of varying dilutions of CVS rabies virus onto NA cells. We further performed serial infections at a high and low (1,000-fold less) multiplicity of NA cells with Flury HEP and ERA viruses (Fig. 2). The original inocula were serially cloned virus stocks containing no demonstrable defective virions. In each high multiplicity passage series, a sharp reduction in virus titer was observed by the third to fourth passage level; the yield of each virus was at least 100-fold less in the high multiplicity series than in the low multiplicity series after only five serial passages. In parallel control series of infections of Flury HEP and ERA viruses in CB3 bovine cells, the released virus titer in the low multiplicity series never exceeded that of the high multiplicity series of infections (data not shown).

Rabies DIVs of a strain-characteristic size are produced in NA cells as previously noted in BHK-21 cell infections (Clark, Parks, and Wun-

TABLE 3. *Effect of MOI on the susceptibility to CVS virus of NA neuroblastoma and other cell lines*[a]

Cell type[b]	MOI	Released virus yield (PFU/ml, $\log_{10}$)	CPE[c]
NA	5.0	<3.7	+
	0.05	5.7	++
	0.0005	6.4	++
	0.00005	5.9	++
BHK-21	5.0	4.8	+++
	0.05	5.9	+++
	0.0005	7.0	+++
CB3	5.0	3.9	+
	0.0005	<1.7	—
L	5.0	<1.7	±
	0.0005	<1.7	—
ICR mouse fibroblasts	5.0	<1.7	—
	0.0005	<1.7	±
MBC-1	5.0	<0.7	—
	0.0005	2.0	+
G-26 cl 23	5.0	<1.7	—
	0.0005	<1.7	—
MBC-2	5.0	<1.7	—
	0.0005	<1.7	—
MBC-3	5.0	<1.7	±
	0.0005	<1.7	—

[a] Released virus was harvested 7 days postinfection except for NA cells which were harvested after 4 days.

[b] The origin and cell composition of the cow brain (CB3), mouse brain (MBC), and mouse glioma (G-26 cl 23) cultures are described in the text.

[c] +, 0 to 25% cell destruction; ++, 25 to 50% cell destruction; +++, 50 to 75% cell destruction; ++++, 75 to 100% cell destruction; —, no effect; ±, minor changes of uncertain specificity.

ner, in preparation). NA cell cultures infected at high multiplicity with DIV-free stocks of either ERA or Flury HEP viruses produced high concentrations of DIV within one or two passages (W. H. Wunner and H F. Clark, manuscript in preparation). When the protection afforded by purified ERA DIV against replication of infectious virus was studied in BHK-21 and NA cell cultures (Fig. 3), it was determined that NA cells were equally or more sensitive than BHK-21 cells to the interfering effects of rabies DIV.

Enhancement of rabies virus virulence after passage in NA cell culture. We have described the reacquisition of virulence for adult mice of attenuated rabies virus strains after passage in NA cells or in IMR-32 or SK-N-SH human neuroblastoma cells (4). A typical pattern of appearance of virulence for animals after sequential passage of Flury HEP rabies virus in NA cells is shown in Fig. 4. A limited increase in virulence was commonly noted after a single passage in NA cells and detected as a pattern of lethal infections following an irregular (or autointerfering) dose-response pattern. This phenomenon has been discussed in detail elsewhere (5). With sequential NA cell passage, virulence increases until the lethal dose for mice approximates the tissue culture infectious dose and follows a normal dose-response pattern. This pattern appears to develop most rapidly after passage at low MOI, presumably because such passage allows more total virus replication generations per passage. Acquisition of increased virulence in this manner has been characteristic of several substrains of Flury HEP virus and of Kelev and CVS clone S_1, S_2, and S_3 attenuated viruses (4).

However, certain viruses did not respond to passage in NA cells with dramatic increases in virulence. Strain ERA virus, which normally causes an autointerfering type death response in mice, continues to do so after either several low

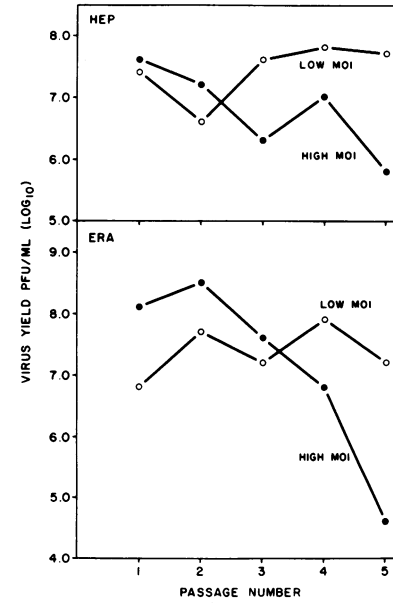

FIG. 2. *Effect of MOI on released rabies virus yields obtained during serial passage of virus of strains Flury HEP and ERA. Duplicate cultures of NA cells were infected with rabies virus at high (10.0) or low (0.01) MOIs. Released virus was harvested 3 days postinfection and subinoculated onto fresh NA cell cultures either undiluted (high MOI series) or diluted 1:1,000 (low MOI series). Released virus yields of five serial passages are shown.*

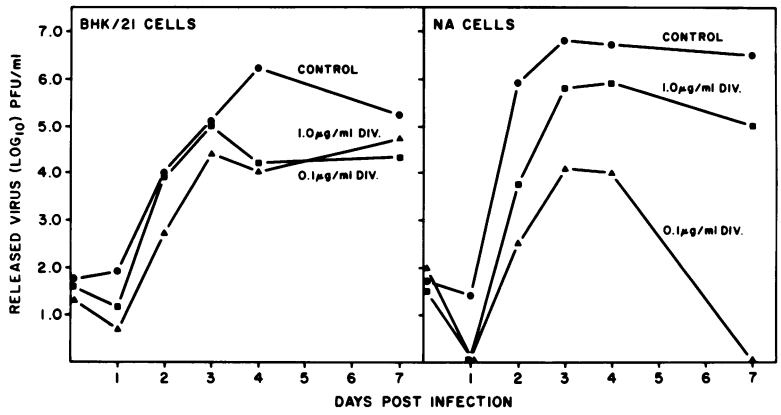

FIG. 3. *DIV-mediated interference of rabies virus replication in BHK-21 and NA neuroblastoma cells. Confluent cell monolayers in 24-well plastic (Linbro) plates were treated with 0.1 ml of rabies strain ERA DIV per well in the concentration indicated for 1 h at 35°C. Cultures were washed twice with phosphate-buffered saline and then challenged by infection at an input multiplicity of 0.05 with purified ERA standard virions adsorbed for 30 min at 35°C. Cultures were again washed twice with phosphate-buffered saline, fed with Eagle minimal essential medium containing 0.2% bovine serum albumin, and incubated at 35°C. Culture supernatant fluids were harvested for assay of infectious virus at the postinfection intervals indicated.*

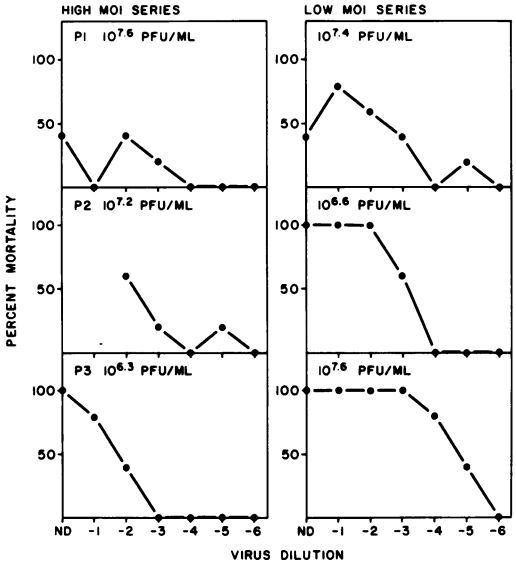

FIG. 4. *Results (mortality) of titration in weanling mice of released virus harvested from serial passage in NA cells of BHK-21 cell-adapted Flury HEP virus. High and low multiplicity passages were performed as described in Fig. 2.*

MOI passages in NA cells or after passage in suckling mouse brain (Fig. 5). Similarly, CVS *ts* 2 virus, a mutant-revertant mixture that rapidly acquired increased virulence after passage in suckling mouse brain, exhibited no increase in virulence after six serial passages in NA cells (data not shown). The *ts*-revertant clone CVS *ts* 2 cl 4 exhibited complete reversion to mouse virulence after three passages in newborn mouse brain, but no increase in virulence after nine passages in NA cells (Fig. 6).

Nonrabies viruses of the rabies serogroup did not exhibit rapid increases in virulence after passage in NA cells. Mokola virus clone 1, an avirulent large plaque variant, acquired only partial mouse virulence after eight passages in NA cells. This was expressed as a capacity to cause deaths giving an autointerfering type of death curve: nondiluted eighth NA cell-passage Mokola virus caused no deaths. The rabies serogroup Kotonkan virus, which is normally avirulent for adult mice (20), exhibited no increase in virulence after nine NA cell passages. Obodhiang virus exhibited no pathogenicity for weanling mice after three passages in NA cells; an avirulent clone of Lagos bat virus developed no mouse virulence after two NA cell passages. Among the nonrabies serogroup viruses, Hart Park virus developed no mouse virulence after three NA cell passages and Kern Canyon virus did not exhibit mouse pathogenicity after two NA cell passages.

Appearance of reversion to virulence of attenuated rabies viruses is apparently a randomly occurring event. Reversion to full virulence for adult mice commonly required several passages of rabies viruses such as Flury HEP in NA cells, regardless of the MOI. Yet Flury HEP virus was repeatedly noted to acquire a high degree of virulence for adult mice after a single passage in newborn mice. Such passage has usually involved high MOI and a harvest of pooled littermates (4, 21; unpublished data).

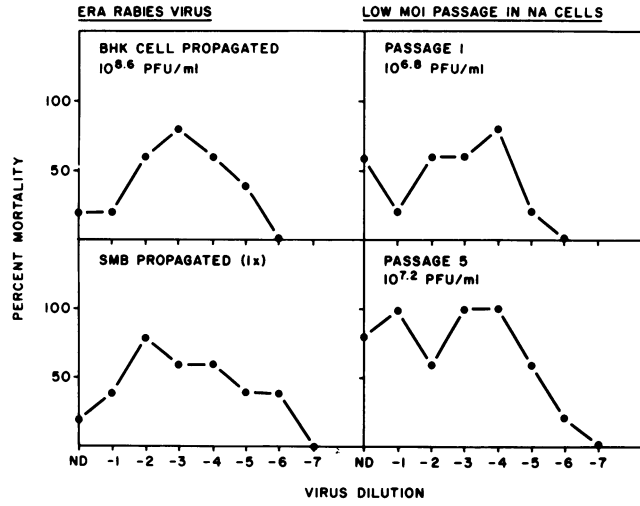

FIG. 5. *Mortality patterns induced by titration in weanling mice of ERA strain rabies virus propagated in BHK-21 cells, in suckling mouse brain (SMB), or NA cells (one, and five serial low MOI passages).*

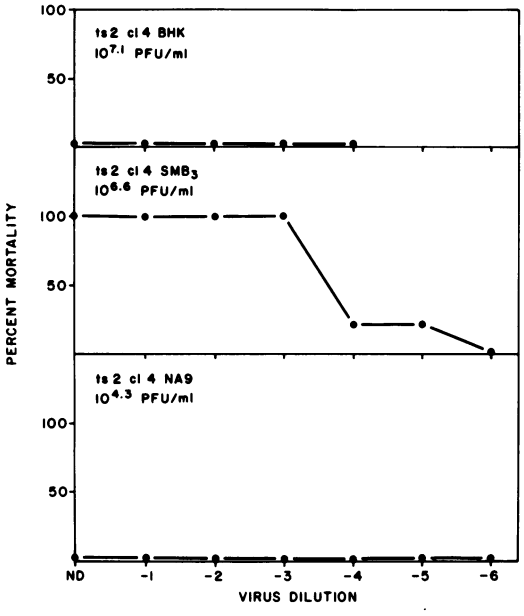

FIG. 6. *Mortality induced in weanling mice by CVS ts 2 cl 4 rabies virus as BHK-21 cell-propagated stock virus and after serial passage in either suckling mouse brain (three passages) or NA cell culture (nine passages).*

Since the most reasonable explanation for reversion to virulence in either system seems to be selectively enhanced replication of randomly occurring back mutants to virulence, an experiment was designed to assess whether development of virulent virus populations from attenuated Flury HEP parental virus occurred in a random manner after NA cell or suckling mouse brain passage.

Six clones of Flury HEP rabies virus were harvested directly from plaques in agarose-suspended BHK-21 13S cells. Virus from each plaque suspension was first determined to be avirulent for adult mice and then inoculated directly into NA cell cultures (MOI, ca. 0.001 PFU/cell) and into newborn mice (suckling mouse inoculum, ca. 100 PFU/mouse).

Virus from each clone was passaged five times (undiluted) in NA cells; each passage harvest was tested for virulence by undiluted inoculation into adult mice (Table 4). One single clone (cl 6) developed full mouse virulence after one NA cell passage. Other clones did not acquire this degree of virulence until the fourth or fifth passage level or later. Selected clone passage harvests that killed no mice when inoculated at undiluted concentration at early passage levels were titrated to endpoint in adult mice. Acquisition of partial virulence at these passage levels was often indicated by the induction of mouse deaths following an autointerfering pattern (data not shown). It was not possible to correlate the eventual appearance of full virulence with adaptation to very efficient replication in neuroblastoma cells, as virus yields declined with the serial undiluted NA cell passages (data not shown), a phenomenon presumably caused by autointerference mediated by DIV accumulation.

In studies of newborn mice inoculated with each clone of Flury HEP rabies virus, a single moribund mouse was selected from each litter. Virus recovered from these brain suspensions

TABLE 4. *Serial passage of HEP Flury virus clones in NA cells*[a]

Passage no.	Mouse mortality					
	Cl 1[b]	Cl 2	Cl 3	Cl 4	Cl 5	Cl 6
1	0/5	0/5	0/5	0/5	0/5	5/5
2	1/5	0/5	0/5	1/5	1/5	5/5
3	1/5	3/5	1/5	3/5	3/5	ND[c]
4	4/5	1/5	5/5	3/5	5/5	ND
5	5/5	5/5	5/5	4/5	5/5	ND

[a] Separate plaques of virus were suspended in medium and inoculated into NA cell cultures. Released virus was harvested at 4 days postinfection and passed serially undiluted in NA cell cultures. Each harvest was assayed for virulence by inoculation undiluted into five weanling mice.

[b] Clone number.

[c] ND, Not determined.

was inoculated undiluted into adult mice (Table 5). As in the case of NA cell-passaged virus, first suckling mouse brain passage virus varied from fully virulent to totally avirulent for adult mice. Each first-passage virus was again inoculated into a litter of newborn mice; second-passage virus was harvested from either individual mice or pooled brains (Table 5). Increase in virulence was evident after the second passage, but either fully virulent, partially virulent, or avirulent virus populations were recovered from different individual mice in the same second-passage litters. Virus titration of second-passage mouse brain suspensions gave results suggesting that the ability of a virus clone to reach high concentration in the suckling mouse brain may be correlated with its ability to cause lethal infection in adult mice. As in the case of the NA cell passages, the results were totally compatible with the theory of selectively enhanced replication of randomly occurring virulent virus mutants.

DISCUSSION

Neuroblastoma cells in culture have been known for many years to possess a variety of the phenotypic characters of differentiated neurons and, thus, to provide a potential model for the in vitro study of diseases of the central nervous system. Yet, although neurotropic viruses are numerous and varied, only limited studies of virus replication in neuroblastoma cells have been reported (13, 31, 37, 39). We have previously reported that certain attenuated strains of rabies virus reacquire virulence after passage in neuroblastoma cells (4). A comparison of the replication pattern of attenuated Flury HEP and revertant (after suckling mouse passage) virulent Flury HEP in NA neuroblastoma cells suggested a possible correlation of attenuation in vivo with rapid virus maturation and rapid appearance of virus antigens on the cell surface (17). Limited studies at the physiological level have suggested that lymphocytic choriomeningitis virus infection of C1300 mouse neuroblastoma is associated with reduced concentrations of enzymes associated with acetylcholine metabolism (29) and that Flury HEP rabies virus infection of a C1300 neuroblastoma X rat glioma hybrid cell line is associated with alterations in the intracellular cyclic adenosine monophosphate response to prostaglandin and isoproterenol (22).

We report here an apparent exquisite sensitivity of C1300 neuroblastoma cells to productive infection with a variety of neurotropic rhabdoviruses, including several (Hart Park, Obodhiang, Kotonkan) that have not previously been cultivated efficiently in cell culture (3, 40). This

TABLE 5. *Mortality in adult mice inoculated with HEP rabies virus clones passaged in suckling mouse brain*[a]

Clone	SMB passage	SMB no.	Mortality	Titer (PFU/ml)
1	1	1	1/10	ND[b]
	2	1	5/5	5.4
	2	2	3/5	4.7
	2	3	2/5	3.9
	2	Pool	5/5	6.0
2	1	1	2/10	ND
	2	1	5/5	4.6
	2	2	0/5	4.1
	2	3	5/5	4.5
	2	Pool	5/5	6.2
3	1	1	1/10	ND
	2	Pool	2/5	ND
4	1	1	4/5	ND
	2	1	5/5	6.1
5	1	1	0/10	ND
	2	1	2/5	3.2
	2	2	0/5	3.2
	2	3	5/5	3.7
6	1	1	3/10	ND
	2	Pool	5/5	5.7

[a] The six virus clones (individual plaque harvests) tested in NA cells (Table 4) were inoculated into newborn mouse litters (passage 1). Single moribund mice were sacrificed; their brain suspensions were assayed for virulence in weanling mice and also subinoculated into a second litter of newborn mice (passage 2). Single and pooled brain suspensions from passage 2 were titrated for infectivity in plaque assay and assayed for mortality in weanling mice. SMB, Suckling mouse brain.

[b] ND, Not determined.

sensitivity is not shared by several other cell culture systems of central nervous system origin. Studies are in progress to determine whether only neuroblastoma cells possess receptor sites for these viruses or if virus permissiveness may be regulated at a later stage of the replicative cycle. Neuroblastoma cell hybrids (24, 25), which express varying spectra of neuronal characters, may provide a powerful tool with which to approach this problem.

All strains of fixed rabies virus tested replicated in NA cells without adaptation at rates similar to or sometimes exceeding those obtained in BHK-21 cells, previously the optimum system for cultivation of these viruses. However, although neuroblastoma cells have been previously reported to be highly susceptible to infection with feral rabies street virus strains (36), we were unable to produce high yields of rabies street virus in NA cells (unpublished data).

Kotonkan and Obodhiang are among the least pathogenic of rabies serogroup viruses. However, after a brief period of adaptation to cell culture, these viruses caused a much more cytolytic infection than rabies viruses. Thus, Kotonkan and Obodhiang viruses could be readily plaqued on C1300 NA cells. Plaque assay of rabies virus in this system was possible but not practical as the pH range supporting both cell viability and rabies virus replication (favored by relatively alkaline pH [41]) is extremely narrow.

NA cells are apparently unique in frequently developing syncytia in response to infection with rabies serogroup viruses. Duvenhage virus causes an exclusively syncytial CPE in NA cells; this virus can be conveniently assayed by determining the syncytial CPE endpoint of titrations of infectivity. It has been suggested that nerve tissue-derived cells are particularly prone to form syncytia in response to infection by the vesicular stomatitis rhabdovirus. Fusion induced by *ts* mutants of vesicular stomatitis rhabdovirus at nonpermissive temperatures was reported only in neuroblastoma cells (16).

Although the vesicular stomatitis rhabdovirus and numerous other viruses (reviewed in reference 15) have been shown to produce sharply reduced infectious virus yields after serial passage at a high MOI, repeated attempts to demonstrate this effect with rabies viruses in BHK-21 cells have failed (see above). Indeed, a high yield of rabies virus has been associated with high MOI (42).

Studies of autointerference readily induced in neuroblastoma cells by serial passage at a high MOI with cell-free rabies virus contradict previous observations in which such autointerference was detected only after serial subcultivation of infected cells (19, 43). That such autointerference in NA cells is DIV mediated is strongly suggested by observations that rabies DIV are efficiently generated in NA cells and that NA cells are equally or possibly more sensitive to DIV-mediated interference of rabies virus replication than BHK-21 cells. Generation of rabies virus DIV in newborn mouse brain has been reported after only a single passage of Flury HEP virus (14), although DIV appearance was not noted after many passages of ERA rabies virus in newborn mice in our laboratory (Wunner and Clark, in preparation).

The implications of these observations for determining the possible role of DIV in rabies infections in vivo are uncertain. DIV added to standard virions of the vesicular stomatitis rhabdovirus have been reported to either protect mice or convert acute infections to chronic disease (12, 30). However, different investigators have made contradictory interpretations of such findings that disease modification results from classical DIV-mediated interference (12) or, on the contrary, from interferon and immune responses induced by DIV (11). Studies in progress directed towards characterizing DIV production and DIV-mediated interference in experimentally differentiated NA cells may shed new light on this problem.

Finally, although we have previously reported uniformly rapid conversion of several attenuated rabies viruses to a virulent state after passage at high multiplicity in neuroblastoma cell cultures, we now report that such reversion to virulence does not occur readily with all attenuated rabies viruses or low virulence rabies serogroup viruses. This observation suggests that several mechanisms may govern attenuation; perhaps neuroblastoma cell passage selects for virus revertants in only one of several characters necessary for virulence.

Observations that infection at a low multiplicity of either NA cell cultures or newborn mice with attenuated Flury HEP virus leads to the appearance of virulent virus at a very variable rate support the theory that both systems selectively support replication of randomly occurring back mutants to virulence. The fact that such back mutants are, nevertheless, consistently detected with serial passage should justify continued caution in the administration of such viruses to domestic or wild animals.

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