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NOTES

Restricted Growth of Attenuated Poliovirus Strains in Cultured Cells of a Human Neuroblastoma

VADIM I. AGOL,[1,2]* SERGEI G. DROZDOV,[1] TATYANA A. IVANNIKOVA,[1] MARINA S. KOLESNIKOVA,[1] MIKHAIL B. KOROLEV,[1] AND ELENA A. TOLSKAYA[1]

Institute of Poliomyelitis and Viral Encephalitides, the USSR Academy of Medical Sciences, Moscow Region 142782,[1] and Moscow State University, Moscow 119899,[2] USSR*

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Cultured cells of a human neuroblastoma, SK-N-MC, were found to be highly resistant to Sabin attenuated poliovirus types 1 and 2 strains; no appreciable cytopathic effect was observed, and the total harvest was generally in the order of 1 PFU per cell or less. On the other hand, related neurovirulent strains of these antigenic types produced a relatively good (2 orders of magnitude higher) yield in a markedly protracted infectious cycle. The limited growth of the attenuated virus in the neuroblastoma cells appeared to be confined to a minor cell subpopulation. Experiments with intratypic (type 1) poliovirus recombinants suggested that the major genetic determinants limiting reproduction of the attenuated polioviruses in the neuroblastoma cells are located in the 5′ half of the viral RNA, although the 3′ half also appears to contribute somewhat to this phenotype. The possibility that neuroblastoma cells may represent an in vitro model for studying poliovirus neurovirulence is briefly discussed.

Poliovirus strains are known to vary markedly with respect to their pathogenicity; wild-type strains generally exhibit a high neuropathogenic potential, while strains empirically selected by A. Sabin are in wide use as an efficient and safe live-virus vaccine (13). The molecular basis for attenuation of the Sabin strains has recently been a matter of considerable interest from a number of laboratories (for reviews, see references 1, 4, 9, and 12). Several attenuating mutations have been identified at the nucleotide level, and mechanisms underlying phenotypic expression of some of them have been suggested. Further progress in exploring the nature of poliovirus neurovirulence and attenuation is, however, hindered by the lack of adequate in vitro host cell systems that will be able to clearly distinguish among attenuated and neurovirulent virus strains. Such a model would also be very useful in more applied studies, e.g., for the neurovirulence testing of poliovirus strains circulating within human populations and, possibly, even for the biological control of live vaccine production.

To be more exact, some in vitro markers, like *rct/40* (temperature sensitive [*ts*]) or *d* (small plaques under an acidic agar overlay due to sensitivity to sulfated polysaccharides [2]) have long been known to correlate with the attenuated phenotype of Sabin strains, but this correlation is not rigorous enough (8) and is not necessarily related to the major attenuating mutations. Thus, for example, mutations within the 5′-untranslated region of the Sabin vaccine types 1 and 3 RNAs, while contributing greatly to attenuation, are neither *ts* (4) nor responsible for the *d* phenotype (10).

An analysis of the existing data allowed us to speculate that the biological differences among attenuated and neurovirulent strains might be more evident in cells of neural origin compared with commonly used host cell systems, like HeLa or monkey kidney cells (1). Such a possibility has been investigated, and the first results reported here appear to show that, at least for the type 1 and perhaps for type 2 Sabin vaccine strains, our expectations have come true.

Three lines of human neuroblastoma cells obtained from the American Tissue Culture Collection, IMR-32 (ATCC CCL 127), SK-N-MC (ATCC HTB 10), and SK-N-SH (HTB 11), were investigated with respect to their ability to support the growth of attenuated (LSc 2ab) and neurovirulent (Mahoney) representatives of poliovirus type 1 (actually, clonal derivatives of these two strains, LSc-1 and M-1-2p [3], respectively, were used throughout this study). SK-N-MC cells, originally derived from a supraorbital metastasis of a human neuroblastoma, exhibited the most explicit ability to discriminate between the strains. Upon infection with the attenuated strain (an input multiplicity of 30 to 100 PFU per cell), the infected cultures exhibited no appreciable cytopathic effect for at least 3 days (the time of observation). On the other hand, the overwhelming majority of the Mahoney-infected SK-N-MC cells degenerated; many of them rounded up and detached from the glass by 13 h postinfection. As few as 1 PFU per cell or less were produced upon LSc 2ab infection, whereas the yield of Mahoney-infected cells was reasonably high (generally more than 100 PFU per cell). Another characteristic feature of the poliovirus infection of the neuroblastoma cells was a considerably retarded infectious cycle compared with that in HeLa cells (Fig. 1).

At relatively early stages (e.g., 7 h), electron microscopy revealed ultrastructural changes typical of poliovirus infection (in particular, the appearance of small vesicles and the enlargement of smooth endoplasmic reticulum elements) in cells treated with either of the strains (Fig. 2). However, such changes have been observed in a much larger proportion of cells during the Mahoney infection compared with those during the LSc 2ab infection. By 24 h, when the cultures infected with the neurovirulent strain appeared to be nearly completely degenerated, no detectable virus-spe-

* Corresponding author.

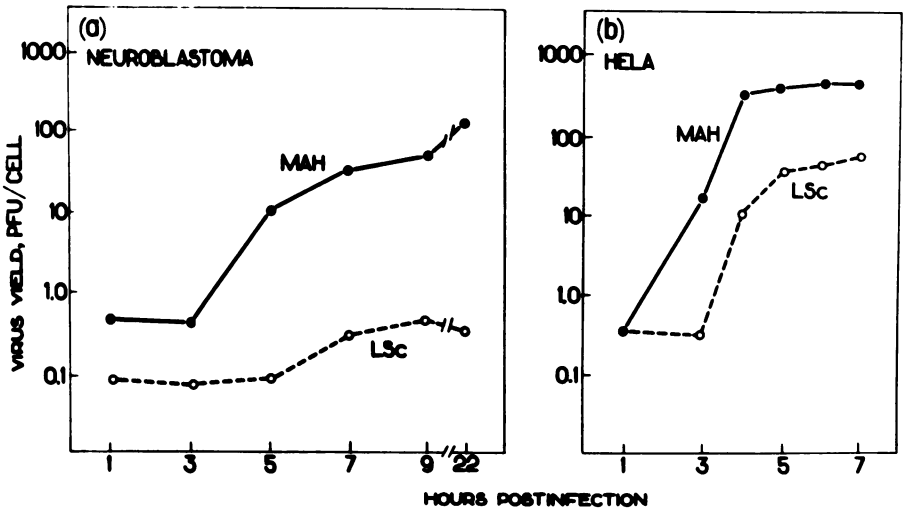

FIG. 1. Reproduction of attenuated (LSc) and neurovirulent (MAH) poliovirus type 1 strains in cultures of SK-N-MC (a) and HeLa (b) cells. The cultures, grown on the bottom of antibiotic vials (neuroblastoma cells were grown in Eagle minimum essential medium with nonessential amino acids in the presence of 10% fetal bovine serum, and HeLa cells were grown in Eagle medium with 10% bovine serum), were infected with the appropriate virus strains at an input multiplicity of 100 PFU per cell and incubated in the serum-free Eagle medium at 36.5°C. The duplicate cultures were frozen at the times indicated, and the virus yield was determined in the mixed pool by plaque titrations in primary monkey kidney cell cultures.

cific ultrastructural alterations were found in the cultures infected with the attenuated counterpart.

The proportion of the poliovirus antigen-containing neuroblastoma cells, as revealed by immunofluorescence, steadily increased from 13% at 5 h to 31% at 13 h of infection with the virulent strain Mahoney; by contrast, this index in the LSc 2ab-infected cultures diminished from 4.7% (5 h) to 1.6% (9 h), and very few, if any, antigen-containing cells could be detected later on. The nearly complete disappearance of the antigen-containing and structurally altered cells by 24 h, and the time course of infectious virus accumulation (Fig. 1) suggested that the productive infectious cycle occurred in only a minority of the LSc 2ab-infected neuroblastoma cells and that these affected cells were destined to die and to detach from the glass by day 2 of infection. Uninfected cultures of SK-N-MC cells appeared to be heterogeneous with respect to morphological cell types; they contained polygonal or epitheliumlike (67%), spindleform or fibroblastlike (28%), and starlike (5%) cells when investigated under the conditions used in virus growth experiments, that is, without fetal bovine serum. The viral antigen was predominantly detected in the prevailing polygonal cells during the Mahoney infection; antigen was detected in the starlike cells during the LSc 2ab infection (Fig. 3).

Reproduction of the neurovirulent strain in neuroblastoma cells, as opposed to HeLa cells, was highly sensitive to treatment with dactinomycin, being completely abolished in the presence of 2 μg of the drug per ml (the only concentration tested). The same was also true for the limited reproduction of the attenuated strain in these cells (data not shown).

Cultures of SK-N-MC cells did not support the growth of Sabin type 2 strain P712 ch 2ab either; no cytopathic effect was visible, and the harvest was scarce. In contrast, a ts^+ derivative of P712 ch 2ab (P40) produced relatively abundant progeny (Fig. 4a) and induced a marked cytopathic effect. Interestingly, a wild neurovirulent type 2 strain, Neva, while exhibiting comparatively efficient reproduction, failed to induce appreciable morphologic damage to the neuroblastoma cells (data not shown). As far as poliovirus type 3 is concerned, the situation appeared to be more complicated. Upon receipt of SK-N-MC cells from the United States, the culture discriminated the Sabin vaccine strain Leon 12 a_1b from its neurovirulent predecessor Leon/37 relatively well; the discriminatory capacity was, however, diminished and nearly lost during subsequent passages of the neuroblastoma cells in this laboratory.

The accumulation of infectious RNA of the attenuated polioviruses of type 1 (data not shown) and of type 2 (Fig. 4b) was also severely restricted in SK-N-MC cells.

As a preliminary step to mapping the viral genetic determinants responsible for the limited growth of attenuated strains in the neuroblastoma cells, the efficiency of reproduction in these cells of two intratypic (type 1) poliovirus recombinants was investigated. The genome of one recombinant, v1/a1-6, has the Mahoney-derived 5′ half and the LSc 2ab-derived 3′ half, whereas the opposite is true for a1/v1-7 (the latter has also a relatively short centrally located insertion of the poliovirus type 3 origin) (3). As shown in Table 1, both recombinants grew to levels intermediate between those of LSc 2ab and Mahoney, but the growth of a1/v1-7 appeared to be markedly more restricted compared with that of v1/a1-6. It can be concluded that the main determinant(s) responsible for the poor reproduction of LSc 2ab in neuroblastoma cells is located in the 5′ half of its genome, though the 3′ half also contributes somewhat to this phenotype.

The above results pose several important questions which cannot yet be answered definitely. First, is the inability of the poliovirus strain to grow efficiently in SK-N-MC cells determined by attenuating mutations? Our experiment with the intratypic recombinants v1/a1-6 and a1/v1-7 appeared to be compatible with an affirmative answer. Indeed, these experiments demonstrated that the major determinant(s) of the limited growth of LSc 2ab in the neuroblastoma cells is confined to the 5′ half of the viral genome, with the 3′ half also contributing somewhat to this phenotype. A very similar pattern was observed when determinants of neurovirulence for monkeys were investigated by using the same pair of recombinants (3). Nevertheless, a finer mapping of mutations that restrict the growth of attenuated strains in the neuroblastoma cells is needed for a definite conclusion on this point. Another promising approach may consist in analyzing the

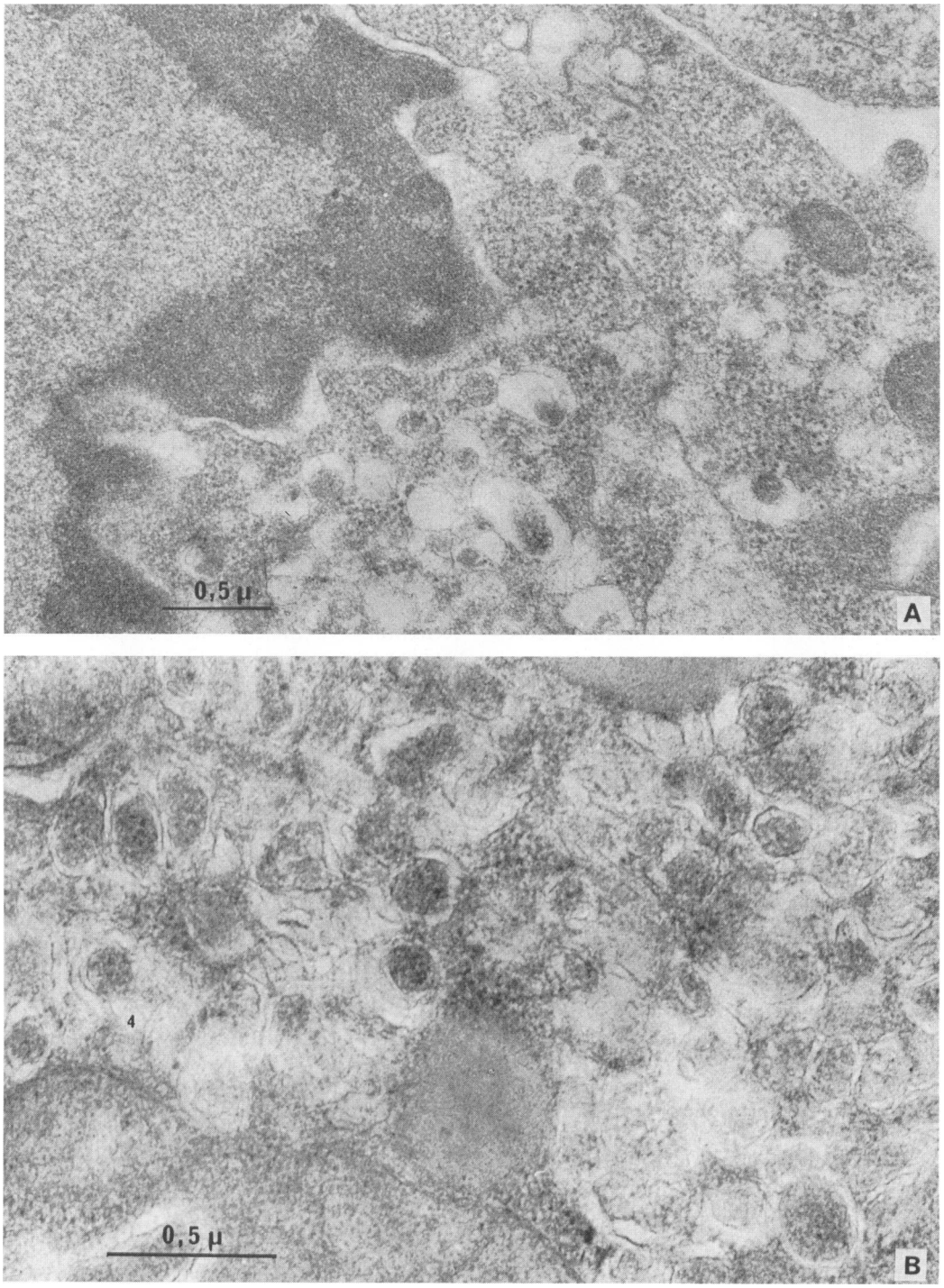

FIG. 2. Ultrastructure of SK-N-MC cells infected with Mahoney (A) and LSc 2ab (B) poliovirus strains. Uranyl acetate-lead acetate-treated ultrathin preparations were investigated under a JEM-100B electron microscope at 80 kV.

genetic structure and phenotypic properties of mutants of the attenuated strains which acquire an ability to grow efficiently in SK-N-MC cells (in fact, such mutants have already been selected by consecutive passages in these cells). Furthermore, one should investigate whether a similar correlation between the two biological properties, attenuation of neurovirulence and growth restriction in the neuroblastoma cells, does exist in other, not necessarily Sabin-related, poliovirus strains (preliminary data suggested that poliovirus strains isolated from healthy persons and patients with poliomyelitis did vary with respect to the ability to grow in SK-N-MC cells). The uncertainty with the poliovirus type 3 attenuated strain ought to be solved as well. The variation in susceptibility of different neuroblastoma cell lines to attenuated poliovirus strains and the apparent tendency of LSc 2ab to grow in a certain morphologic type of cells hint at possible ways of

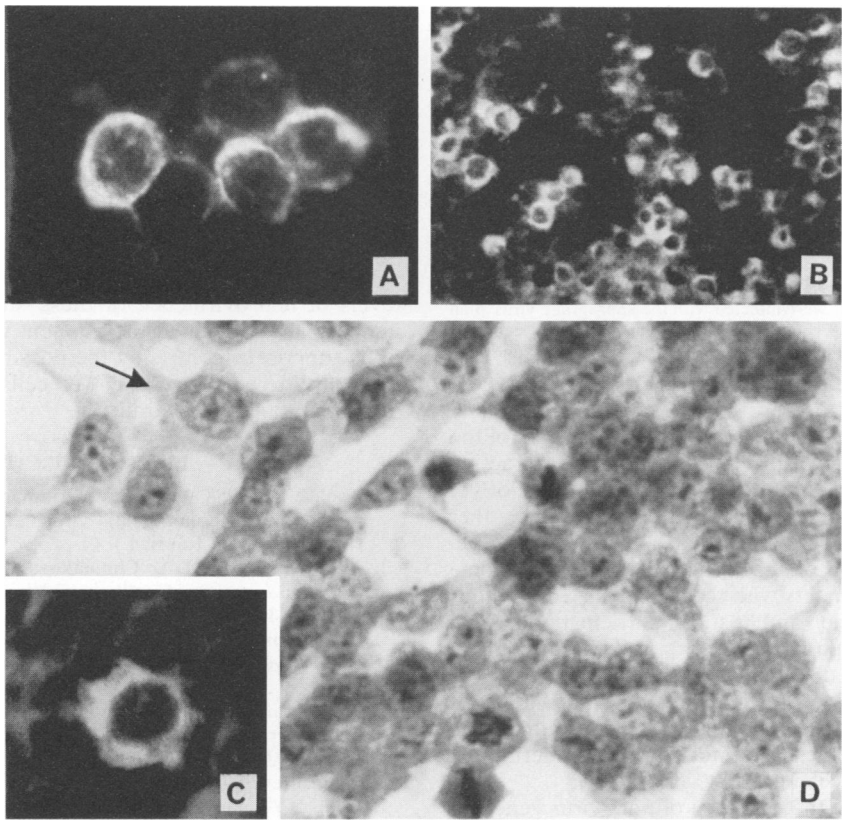

FIG. 3. Accumulation of poliovirus antigens in SK-N-MC cells. Specific indirect immunofluorescence in Mahoney-infected cells at 5 (A) and 13 (B) h postinfection and in LSc-infected cells at 5 h postinfection (C). A hematoxylin-eosine-stained LSc 2ab-infected culture at 5 h postinfection is shown in panel D; a starlike cell is indicated (arrow). Magnification, ×900 (A and C); ×200 (B); ×800 (D). The cover slip cultures were infected as specified in the legend to Fig. 1.

finding a culture with an even better discriminating capacity, e.g., by cloning the uninfected neuroblastoma cells. In this regard, it is important to elucidate whether the apparent ability of only a minor SK-N-MC subpopulation to support the reproduction of attenuated polioviruses has a truly genetic basis or merely reflects some undefined physiological conditions of the cells.

The second major question to be resolved concerns the molecular mechanism(s) of the restriction. We know that the attenuating mutations in the 5′-untranslated region, specifically, in positions 472 to 480 of the poliovirus RNA affect initiation of viral polyprotein synthesis (14, 15); we know also that initiation is accomplished with dissimilar efficiencies in cell-free systems of different origin (11, 14, 15). On the basis of these facts, it was proposed that the translation machinery of human neural cells may be especially sensitive to the attenuating mutations in the 472 to 480 region of poliovirus RNA (1). In fact, just these consider-

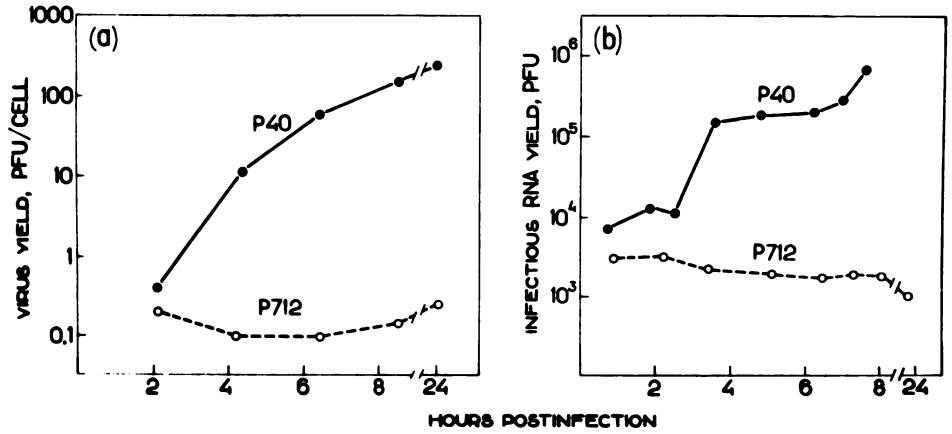

FIG. 4. Virus (a) and infectious RNA (b) yields in SK-N-MC cells infected with the attenuated poliovirus type 2 strain P712 ch 2ab (P712) and its ts^+ derivative (P40). The virus yield was determined as indicated in the legend to Fig. 1. The infectious RNA yield was determined by transfection of DEAE-dextran-treated monolayers of African green monkey kidney cells.

TABLE 1. Reproduction of intratypic poliovirus recombinants in SK-N-MC and HeLa cells[a]

Infecting virus	Virus yield (PFU/ml × 10^6) in indicated cells	
	SK-N-MC	HeLa
Mahoney	210	460
LSc 2ab	1	76
v1/a1-6	70	194
a1/v1-7	14	72

[a] Test tube cultures of SK-N-MC and HeLa cells grown under conditions specified in the legend to Fig. 1 were infected with the appropriate virus at an input multiplicity of about 50 PFU per cell. The progeny were harvested at 24 h postinfection and assayed by plaque titration in primary monkey kidney cell cultures.

ations prompted us to undertake this study. In the light of the present findings, the above proposal remains an attractive possibility. It should be admitted, however, that it is not yet substantiated by any direct evidence. Until this is done, other possibilities should not be disregarded, all the more so because some contribution of the 3′ half of the viral genome follows from the experiments with recombinants. Initial steps of the virus-cell interaction and host cell participation in viral RNA replication are among these other possibilities (although it may be noted that the discriminating capacity of SK-N-MC cells was retained if the cells were infected with the RNAs from attenuated and virulent poliovirus strains rather than with the viruses themselves [data not shown], rendering the former of the above possibilities not very likely).

As a matter of fact, in the early era of poliovirus genetics, Kanda and Melnick (5) reported that reproduction of attenuated strains of all three poliovirus serotypes was greatly restricted in a continuous line, MS, of monkey kidney cells, though, in contrast to the present system, a typical cytopathic effect was induced. Although attracting the interest of several investigators (6, 7), this phenomenon was poorly investigated. Very recently, Tershak and Makkar (16) reported that the Sabin poliovirus strains produced somewhat less progeny in another monkey-derived cell culture, Vero, compared with HeLa cells, and that this difference was greatly augmented in the presence of dactinomycin. The authors attributed the inefficiency of attenuated viruses primarily to their impaired adsorption to, and uncoating by, Vero cells as well as to their failure to induce, in these cells, a strong shutoff of host protein synthesis. The cell-virus system described in the present report differs sharply from those just mentioned not only by a significantly lower yield of attenuated viruses but first and foremost by the amazing absence of any appreciable cytopathic effect in the cultures infected with attenuated strains.

Summing up, cultured cells of a human neuroblastoma were found to be highly resistant to the Sabin strains of poliovirus types 1 and 2. The limited growth of attenuated strains in these cultures appeared to be confined to a minor cell subpopulation of this culture. The main genetic determinant(s) responsible for this viral phenotype was located in the 5′ half of the poliovirus genome. The prospect of finding an adequate in vitro model of poliovirus neurovirulence warrants further study of this and related host cell systems.

Addendum. Since the submission of the original version of the manuscript, it was found that even closely related poliovirus strains might somewhat differ from each other with respect to their capacity to grow in SK-N-MC cells. This finding may be illustrated by experiments with two poliovirus strains kindly donated by Akio Nomoto, PV1(M)pDS306 and PV1(Sab)1C-0; these strains originated from infectious plasmids derived from Mahoney and LSc 2ab, respectively (10). However, PV1(M)pDS306 appeared to yield, in SK-N-MC cells, less infectious progeny compared with our Mahoney, whereas PV1(Sab)1C-0 proved to be more active in this system than our LSc 2ab (it may be noted that the virus derived from the Mahoney-related infectious plasmid, obtained through the courtesy of David Baltimore, exhibited no appreciable difference from our Mahoney). Similar variability was also observed among strains called Sabin type 2 virus but obtained from different sources. Inasmuch as no information about comparative levels of neurovirulence of these strains is available, it is impossible to decide at the moment whether these observations support or contradict the possible correlation between the ability to grow in SK-N-MC cells and the pathogenicity of a given poliovirus strain.

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