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Postinfection Treatment with Antiviral Serum Results in Survival of Neural Cells Productively Infected with Virulent Poliovirus

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The death of human neuroblastoma cells undergoing productive infection with virulent poliovirus was prevented by addition of antiserum against the virus a few hours after the onset of infection; this treatment, however, did not prevent reproduction of the virus. Despite the presence of the viral antigen, the cells retained the ability to divide. Upon further cultivation in the absence of antiserum, the cells developed specific postinfection immunity or resistance to superinfection with poliovirus.

Poliovirus is usually regarded as a cytocidal agent whose efficient reproduction is strictly coupled to cell death. Survival of poliovirus-infected cultures having a lowered level of permissiveness has, however, been described. For example, we have shown recently that nearly all cells of a human neuroblastoma line (SK-N-MC; ATCC HTB 10) survived treatment with attenuated poliovirus (strain Sabin 1 or Sabin 2; input multiplicity of infection, 10 to 100 PFU per cell); the harvest of infectious progeny under these conditions was only about 1 PFU per cell (1; see also reference 5). By contrast, the same cells, upon infection with a virulent poliovirus, e.g., type 1 strain M-I-2p (a close relative of Mahoney), experienced a more or less normal, although somewhat protracted, infectious cycle, resulting in a relatively good harvest; most of the infected cells exhibited typical signs of the cytopathic effect (CPE), with nuclear pyknosis, rounding up, and detachment from the glass by 24 h (1).

Upon further investigation of this system, an unexpected phenomenon, prevention of cellular death by postinfection (p.i.) addition of antiviral serum to virulent poliovirus-infected neuroblastoma cells, was discovered; a description of this phenomenon is the aim of this report.

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When SK-N-MC cells in suspension were infected with M-I-2p and then planted, very few spread cells or growing colonies could be observed at 24 to 48 h p.i. (Fig. 1A and C). However, at least half of the cells did survive and eventually most of the survivors formed colonies if the specific hyperimmune antiserum was added at 2 h p.i. (Fig. 1B and D). (The proportion of surviving cells in infected, antiserum-treated cultures was always much higher than that in antiserum-free cultures; nevertheless, it varied somewhat in different experiments. This variation was possibly related to the number of passages that SK-N-MC cells experienced in our laboratory. A critical condition which allowed most of the surviving cells in such cultures to stick to the glass surface was the presence of fetal calf serum; otherwise, the cells, still viable, readily detached from the substrate.) A trivial explanation of the protective effect, namely, prevention by the antiserum of viral genome entry into the cells, was unambiguously ruled out by two independent assays.

First, as shown in Table 1, essentially the same, and quite large (>350 PFU per cell), amounts of virus did accumulate within the cells by 6 h p.i. in samples incubated with or without antiserum; the extracellular virus was inactivated by the antiserum, as expected. It should perhaps be noted that monolayer cultures, rather than suspensions, were infected in this experiment; under such conditions, a slightly larger proportion of infected cells appeared to survive without addition of antiserum, and nearly no CPE was observed at 24 h p.i. in antiserum-containing samples (see also Fig. 3).

Second, the virus-specific antigen did accumulate in due course within antiserum-treated virus-infected cells, as revealed by the immunofluorescence assay; at 6 h p.i., that is, before the appearance of any marked CPE, the proportions of antigen-containing cells in antiserum-treated and non-treated samples showed no significant differences (85 to 90%) and the intensities of the signals were also similar in the two cultures (Fig. 2A and B) (at even earlier stages, e.g., 2 h p.i., no appreciable signal was detected in any cultures [data not shown]). Thus, antiserum addition did not seem to interrupt the formation of infectious virus and virus-specific products. Later on, e.g., at 24 h p.i., the immunofluorescence faded somewhat, but the antigen was still present, typically perinuclearly, within the overwhelming majority (~90%) of the cells that survived in the presence of the antiserum (Fig. 2D). The abundant presence of small blobs, markedly enriched in the viral antigen, was a highly characteristic feature of such surviving cultures (Fig. 2H); these eosinophilic entities were found intra- and extracellularly, as well as adjoining (or budding from) the cellular surface at different stages of poliovirus infection of neuroblastoma cells, both in the presence and in the absence of the antiserum. It is tempting to speculate that generation and export of the antigen-accumulating blobs represent a kind of cellular self-clearance mechanism, but this idea has to be supported by more direct evidence.

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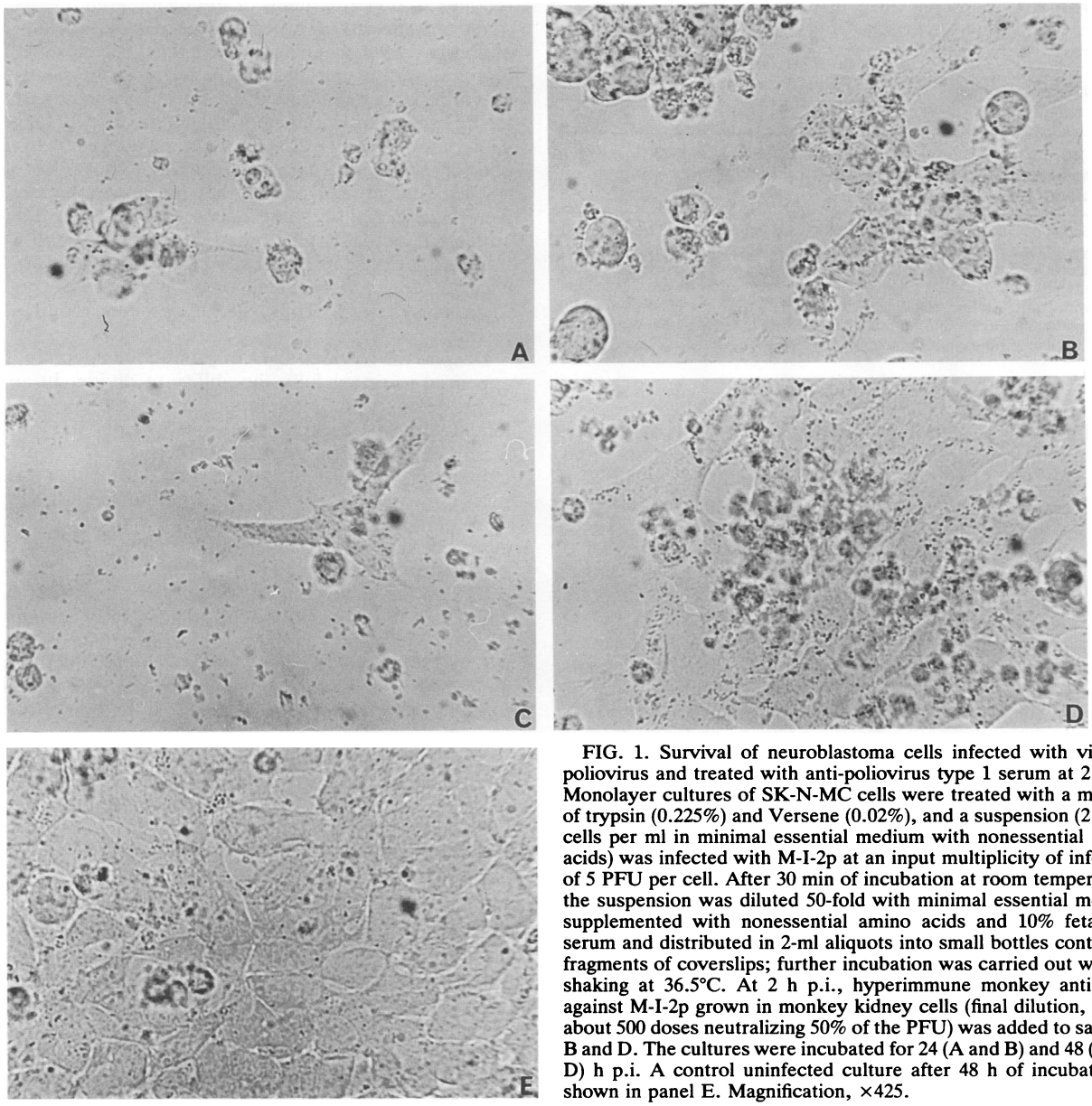

FIG. 1. Survival of neuroblastoma cells infected with virulent poliovirus and treated with anti-poliovirus type 1 serum at 2 h p.i. Monolayer cultures of SK-N-MC cells were treated with a mixture of trypsin (0.225%) and Versene (0.02%), and a suspension (2×10^7 cells per ml in minimal essential medium with nonessential amino acids) was infected with M-I-2p at an input multiplicity of infection of 5 PFU per cell. After 30 min of incubation at room temperature, the suspension was diluted 50-fold with minimal essential medium supplemented with nonessential amino acids and 10% fetal calf serum and distributed in 2-ml aliquots into small bottles containing fragments of coverslips; further incubation was carried out without shaking at 36.5°C. At 2 h p.i., hyperimmune monkey antiserum against M-I-2p grown in monkey kidney cells (final dilution, 1:100; about 500 doses neutralizing 50% of the PFU) was added to samples B and D. The cultures were incubated for 24 (A and B) and 48 (C and D) h p.i. A control uninfected culture after 48 h of incubation is shown in panel E. Magnification, ×425.

The protective effect of the antiserum was due to the presence of serotype-specific antibodies, in particular, antibodies directed against capsid proteins. Thus, treatment of SK-N-MC cells with antiserum against poliovirus type 2 did not prevent reproduction of and CPE induction by M-I-2p, nor was anti-poliovirus type 1 serum effective against poliovirus type 3 (data not shown). Involvement of anticellular antibodies seemed highly unlikely, because the hyperimmune sera were prepared by immunizing monkeys with virus grown in monkey kidney cells. Moreover, a similar protective effect was observed in an experiment with a mixture of human convalescent-phase sera (data not shown). But unambiguous proof that just the anticapsid antibodies were responsible for the effect was obtained with monoclonal neutralizing antibodies. Three such antibodies, 234, 427, and 431, kindly donated by P. D. Minor, were investigated. Each of them, added at 2 h p.i. to M-I-2p-infected SK-N-MC monolayers, protected most of the cells from death. The greatest protective activity was exhibited by antibody 234: at a dilution of 1:100, it prevented the CPE nearly completely (Fig. 3). Only a very slight CPE developed upon addition of the two other antibodies at a dilution of 1:20 (data not shown). The protective activity of the antibodies correlated only partially, if at all, with their neutralizing activity (234 and 427 had comparable neutralizing powers, whereas 431 was about fourfold less active [data not shown]). It should be noted that neither of the antibodies prevented intracellular accumulation of infectious virus during the first 6 h of infection; thus, monoclonal antibodies mimicked perfectly the effects of hyperimmune sera.

Interesting conclusions could be drawn from the known specificities of the monoclonal antibodies used (6a, 7, 8). Antibodies 427 and 431 are antigenic site 2 antibodies; they

TABLE 1. Antiserum effect on poliovirus reproduction in neuroblastoma cells[a]

Conditions of infection	Intracellular/extracellular virus yield (PFU/cell) at:		CPE at:	
	6 h	24 h	6 h	24 h
Standard	370/40	280/970	−	++++
Anti-poliovirus type 1 serum at 2 h p.i.	360/<0.001	0.9/<0.001	−	−

[a] Monolayer cultures of SK-N-MC cells were infected with virulent poliovirus type 1, strain M-I-2p, at a multiplicity of infection of about 20 PFU per cell. After 2 h of incubation at 37°C, anti-poliovirus type 1 serum was added at a final dilution of 1:100. At the time indicated, the cultures were washed five times with Earle's saline, the cells were detached from the glass by freezing and thawing, and intra- and extracellular infectious virus yields were determined by plaque titration in monkey kidney cells.

failed to react with mutants having substitutions at residues 270 of VP2 and 220 of VP1, respectively; on the other hand, antibody 234 selected for mutations at position 72 of VP2 and is, therefore, a site 4 antibody. Site 4 appears to be a conformational site formed by adjacent pentamers. Therefore, whatever the mechanism of the preventive effect of monoclonal antibodies, it should involve assembled capsids rather than individual structural polypeptides or promoters.

The mechanism of prevention of cellular death by p.i. addition of antibodies is unknown. Obviously, the phenomenon is not caused by neutralization of extracellular virus. Various effects of exogenous antiviral antibodies on the intracellular stages of ongoing reproduction of different viruses have been documented; although no detailed explanations for these effects were proposed, interaction of the antibodies with membrane-bound viral antigens was considered to be a likely possibility (3, 9). Well-documented studies of this kind, however, concerned infections with enveloped viruses, like herpesvirus or measles virus, in which integration of virus-specific proteins into cellular membranes is a common feature. Irrespective of the correctness of such explanations, they could hardly be extrapolated to nonenveloped poliovirus, even less so if the target for the antibodies is represented by assembled viral particles. We do not know what relationship, if any, the protective effect of the antibodies described here has to antibody-mediated inhibition of encephalomyocarditis virus release from infected cells (11) because of the insufficiency of the information reported.

We prefer to speculate that the preventive effect was due, in our case, to antiserum penetration into the cells at a relatively late step of the infectious cycle. We propose that the penetrated antibodies interact with assembled viral or subviral particles, thereby inhibiting an unknown reaction responsible for development of irreversible cell killing. The data in Table 1 strongly suggest that significant penetration did not occur until at least 6 h p.i., when no neutralization of intracellular infectivity by external antiserum could be detected. The presence of antiserum from 2 to 6 h p.i. did not interfere with subsequent growth of the virus and the virus-induced cell injury (data not shown). Similarly, by 6 h p.i., no appreciable increase in cellular permeability was registered by counting of methylene blue-stained cells; later on, however, the staining assay demonstrated significant enhancement of permeability (data not shown). Concomitantly, a drop in intracellular infectivity was registered, and this drop was, at least in part, due to neutralization of the viral progeny inside the cells, since some infectivity was recovered after treatment of the disintegrated cells at pH 3 (data not shown), a procedure used to dissociate antigen-antibody complexes (see, for example, reference 10). When antiviral serum was present between 2 and 24 h p.i., the surviving cells exhibited, after thorough washing, resistance to superinfection with a homotypic (specifically tagged, e.g., guanidine resistant) virus but not to superinfection with a heterotypic virus (data not shown). These findings also suggest, but do not prove, that the superinfecting viruses were neutralized within the cells. On the other hand, if the antiserum actually penetrated into the infected cells, its intracellular concentration should be relatively low, since it was not detected by using relevant fluorescent antibodies (data not shown). Thus, the hypothesis requires further testing.

Despite the obvious signs of productive infection, the cells appeared to retain fully the ability to divide. In an experiment with sparse monolayer neuroblastoma cultures infected with M-I-2p in the presence of antiserum (added at 2 h p.i.), the mitotic index at 24 h p.i. was about 11.8 (in uninfected control cultures, it was 10.6). Such sparse cultures grew nearly to confluence by 48 to 72 h. Similar results were obtained when mitotic activity was assayed in monolayers of the cells infected and treated with antiserum in suspension (data not shown). In both cases, the daughter cells continued to contain the viral antigen (perinuclearly and within the blobs), although at apparently declining levels (Fig. 2). Further passages in the presence of antiserum resulted in complete convalescence of the infected cultures, as judged by their morphological appearance and susceptibility to infection. However, when cultivation of the surviving cells was carried out in the absence of antiserum, a kind of persistent infection developed after a few passages. The cultures continued to produce the infectious virus in the medium and became resistant to superinfection with poliovirus, e.g., a heterotypic (type 3) virulent strain that grew well and induced a nearly complete CPE in uninfected neuroblastoma cells (Table 2). Some features of this resistance to superinfection will be reported separately. Development of persistent poliovirus infection of human neuroblastoma cells has also been described by others (2).

Perhaps it is appropriate to note that attempts to prevent,

TABLE 2. Development of resistance to superinfection in poliovirus-infected neuroblastoma cells[a]

No. of passages after primary infection	Virus titer (PFU/cell)		CPE after superinfection
	Type 1 virus before superinfection	Superinfecting type 3 virus	
2	375	49	+
3	58	40	±
4	20	<1	−
2 with antiserum	0.01	268	+++
2 with antiserum + 2 without antiserum	38	<1	−

[a] Primary infection of SK-N-MC cells with M-I-2p and subsequent treatment with anti-poliovirus type 1 serum were performed as described in the footnote to Table 1. The cultures were grown in minimal essential medium with nonessential amino acids supplemented with 10% fetal calf serum and, after 24 h of infection, were passaged every week without the antiserum, if not indicated otherwise. After the number of passages indicated, the cultures were washed five times with Earle's saline and superinfected with type 3 virulent strain Leon/37 at 20 PFU per cell. The yield of superinfecting virus at 24 h thereafter was determined by plaque titration. For differential assay of type 1 and 3 viruses, appropriate antisera were added to the agar overlay.

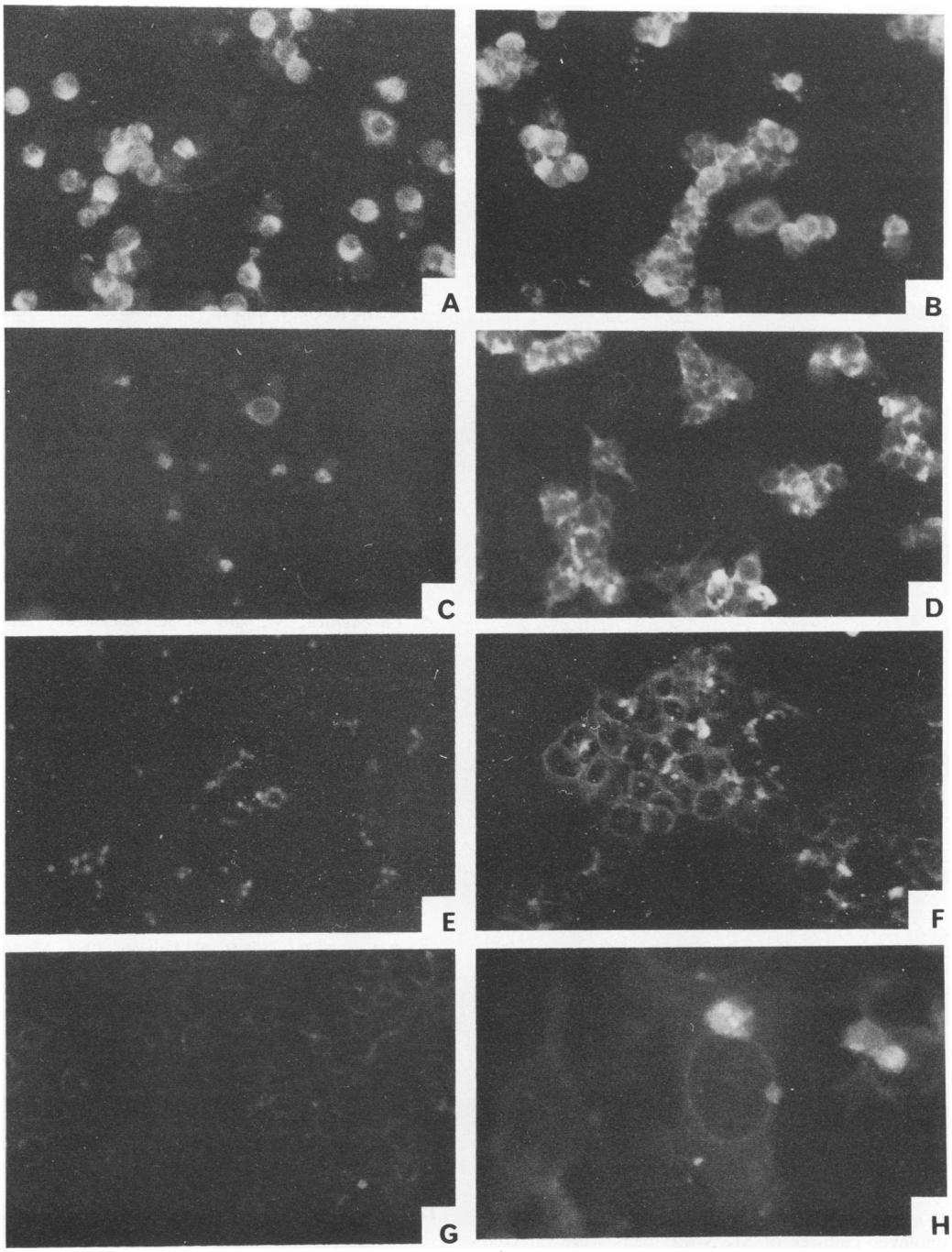

FIG. 2. Indirect immunofluorescence assay for poliovirus antigen. The assay was carried out as described previously (1). The conditions of infection and p.i. treatments were as described in the legend to Fig. 1. Cultures incubated in the absence (leftward column) or in the presence (rightward column) of antiserum at 6 (A and B), 24 (C and D), and 48 (E and F) h are shown. Magnification, ×250. Sample G is an uninfected control culture. Panel H is the same as panel D but is shown at a higher magnification (×800) to illustrate the accumulation of antigen-containing blobs.

by p.i. antiserum treatment, the poliovirus-induced CPE in HeLa cells have failed; preliminary data suggest that in this respect, monkey kidney cells occupy an intermediate position between neuroblastoma and HeLa cells.

The data reported here demonstrate that productive infection of neural cells with virulent poliovirus was uncoupled from the fatal CPE by externally present antibodies; eventually, the infected cells were cured. We propose that these phenomena, as well as the development of specific resistance to superinfection, contribute to the pathogenesis of poliomyelitis and, in particular, to clearance of the virus from the central nervous system. It is tempting to speculate that a similar mechanism is responsible for antibody-mediated clearance from neural cells of Theiler's murine enceph-

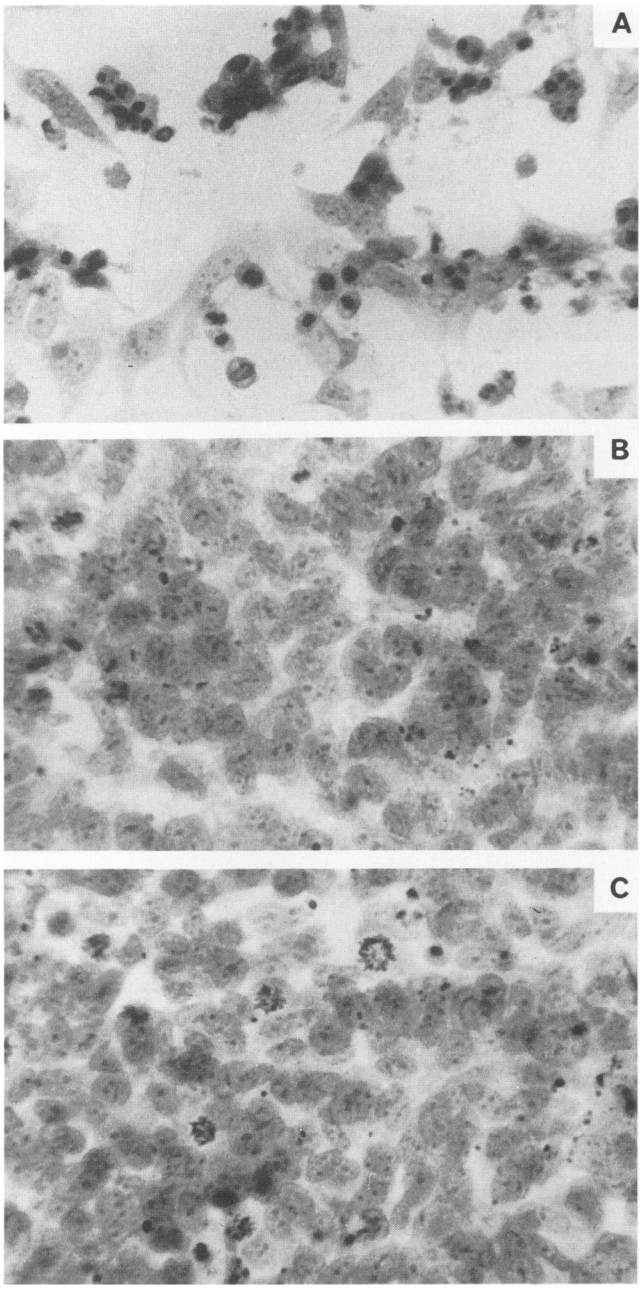

FIG. 3. Survival of neuroblastoma cells infected with a virulent poliovirus strain and treated with anti-poliovirus type 1 monoclonal antibodies. Monolayer cultures of SK-N-MC cells were infected with M-I-2p at multiplicity of infection of 20 PFU per cell, and 2 h later monoclonal antibody 234 was added to sample B at a final dilution of 1:100 (~1,000 doses neutralizing 50% of the PFU). Panels: A, infected cells at 24 h p.i.; B, infected cells incubated with the monoclonal antibody from 2 to 24 h p.i.; C, uninfected control. Hematoxylin-eosin staining was used. Magnification, ×450.

alomyelitis virus (4), reovirus (12, 13), Sindbis virus (6), and perhaps some other viruses. Future experiments should show to what extent the possibility of such curing is specific for neural cells.

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