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Reversible repression and activation of measles virus infection in neural cells

(paramyxovirus/persistent infection/membrane protein/cyclic nucleotide)

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ABSTRACT **Conversion of acute measles virus infection to an indolent state has been achieved by treatment of infected cells of neural origin with agents that affect cyclic nucleotide metabolism. Striking results were obtained with papaverine, an inhibitor of cAMP phosphodiesterase that is capable of enhancing neural differentiation. In papaverine-treated cultures, decreased production of infectious virus was accompanied by selective disappearance of intracellular matrix protein, as detected by immunofluorescence. Viral nucleocapsid protein was enhanced in the cytoplasm while three other structural proteins—polymerase, hemagglutinin, and fusion protein—showed little change in distribution or intensity of staining. These results were specific for cells of neural origin and not observed in CV-1 or Vero cultures. cAMP, dibutyryl cAMP, 8-bromo-cAMP, and isobutylmethylxanthine all inhibited replication of virus but less so than did papaverine. Inhibition of virus replication by any of these agents was rapidly reversible, either by removal of the agent or by addition of cGMP to the culture medium and was accompanied by reappearance of the matrix protein. These results suggest that measles virus replication in neural cells depends on host factors, particularly those affecting endogenous cAMP and cGMP. Viral persistence may thus be related to the state of neural differentiation. This model system may yield information on mechanisms of recrudescence observed in some chronic diseases of the nervous system.**

Measles virus has been shown to be the cause of human subacute sclerosing panencephalitis (SSPE), a slowly progressive usually fatal disease of the central nervous system, with onset of symptoms late in the first decade of life. A high percentage of patients are known to have had acute measles virus infection prior to age two (1), when the brain is still undergoing maturation. Little is known about the mechanism of conversion of the acute infection to the persistent form or the recrudescence many years later. Recent investigations of both acute and persistent measles virus infection have focused primarily on comparison of viral structure and composition (2–5) and on possible effects of the immune system (6–8). Studies of defective interfering virus (9) and temperature-sensitive mutants (10, 11) have also not yet yielded complete descriptions of either normal or aberrant viral replication. Several studies of neural tissue in man (3, 12–14), in animal models (15), and in continuously infected cell lines (16–19) of nonneural origin have implicated changes in the expression of the viral matrix or M protein. However, a clear relationship to viral persistence has not yet been established.

Because of the complexity of the central nervous system, examination of host cell factors has been difficult. There is little information regarding specific effects of neural cell growth and differentiation on measles virus replication. Age-related effects on the establishment of persistent infections in central nervous system tissues (20, 21) may reflect changing susceptibilities to infection of neural tissues during development. Neural cells contain abundant endogenous cAMP, with levels closely correlated with terminal differentiation, cessation of cell division, and induction of neural specific function (22, 23).

Recently, Robbins and Rapp (24) described a decrease in M protein in human amnion cells (AV-3) infected with measles virus in the presence of exogenous cAMP. We have examined measles virus replication in two kinds of neural cells cultured in the presence of agents known to increase intracellular cAMP. The regulation of cAMP and its role in neural specific function in these cells have been well described (25, 26). Our studies provide a model for reversible conversion of an acute to an indolent or chronic infection. We have also examined the expression of the individual viral structural proteins during this change of state.

MATERIALS AND METHODS

Cells and Viruses. A clonal line of mouse neuroblastoma N2A (27), originally derived from the C-1300 tumor, and TE671, human neural cells derived from a medulloblastoma (28), were used. Nonneural cells included Vero and CV-1 cultures (both monkey kidney cells). All cultures were grown as monolayers in complete medium [Eagle's minimal essential medium (GIBCO)/10% fetal calf serum] at 37°C. Cells were routinely monitored for mycoplasma contamination (29).

The Edmonston strain of measles virus, twice cloned by plaque purification, was obtained from low-passage stock grown in Vero cells. For experiments, we used a multiplicity of infection of 0.5–1 plaque forming units (pfu) per cell. Infected cultures were scraped into supernatant fluid with a rubber policeman and stored at −90°C for subsequent assay. The semimicrotiter method of Rager-Zisman and Merigan (30) was used for titration of infectious virus on Vero cells.

Immunofluorescence Microscopy. Infected cells were analyzed for the presence of viral antigens by an indirect immunofluorescence antibody procedure. Cultures grown on glass coverslips were washed in phosphate-buffered saline, pH 7.3, fixed for 15 min in cold acetone, and then stained with mouse hybridoma antibodies specific for the antigens followed by fluorescein-conjugated anti-mouse IgG (31). A 1% rhodamine solution was added as a nonspecific fluorescent counterstain. The hybridoma antibodies monospecific for the HA, NP, F, P, and M polypeptides were the gift of Erling Norrby (Karolinska Institute, Stockholm, Sweden), who has described their preparation, characterization, and specificity (32).

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Abbreviations: IBMX, isobutylmethylxanthine; SSPE, subacute sclerosing panencephalitis; pfu, plaque-forming unit(s); M, F, P, and NP, matrix, fusion, polymerase, and nucleocapsid (protein), respectively; HA, hemagglutinin.

RESULTS

Inhibition of Measles Virus Replication. To evaluate effects of endogenous cAMP on measles virus replication, $\approx 2 \times 10^5$ uninfected cells (TE671, N2A, CV-1, or Vero) were planted in 35-mm dishes (Corning) and incubated in complete medium at 37°C. After 24 hr, cultures were refed with complete medium or complete medium/7 μM papaverine (6,7-dimethoxy-1-veratrylisoquinoline; Sigma), an inhibitor of cAMP phosphodiesterase that would be expected to increase the level of intracellular cAMP. After an additional 72 hr, the cells, now nearly confluent at $\approx 5 \times 10^5$ per dish, were infected and maintained in medium/inhibitor until harvest 48 or 96 hr later.

As shown in Table 1, in both neural cell cultures, the yield of infectious virus was reduced by a factor of 10^4 in the presence of papaverine. However, TE671 cells showed slightly higher yields in untreated control cultures than N2A cells. In contrast, there was little or no reduction in virus yields in companion papaverine-treated CV-1 and Vero cells, which are of nonneural origin. Papaverine-treated TE671 and N2A cultures manifested virtually no cytopathic changes while, in untreated cultures, there was fusion and cytolysis. Both papaverine-treated and control CV-1 and Vero cells showed marked cytopathic effects with destruction of cell monolayers. The treatment of cultures with the agent for at least 72 hr prior to infection and continued treatment during the course of infection by refeeding cultures every third day were necessary to produce maximal suppression of replication in the neural cells. It was important to use fresh papaverine as stock solutions of papaverine were no longer maximally effective after 4 weeks of storage at 4°C.

To test for the possibility of inactivation of released virus by papaverine, virus was incubated in medium at 37°C with 7 μM papaverine and sampled at 15-min intervals. There was no significant difference in decay of infectivity compared with controls; over a 3-hr period, the number of pfu decreased by a factor of 10.

The effects of isobutylmethylxanthine (IBMX), cAMP, dibutyryl cAMP, and 8-bromo-cAMP (Sigma) are also shown in Table 1. At 1 mM, each agent reduced yields of infectious virus by factors $\geq 10^2$, indicating that the effects of papaverine may be due to cyclic nucleotide changes. These effects are less than in papaverine-treated cultures, perhaps due to low cellular permeability to these agents.

As dibutyryl cAMP contains some free butyrate, cultures were also tested with medium containing Na butyrate. Rather than suppression, enhancement of yield of infectious virus occurred under these conditions in two of the four cell types. As Na butyrate is an inhibitor of cell division (25), correction for lower cell densities in treated cultures actually results in increased yields of infectious virus per cell over control levels. The most striking difference occurs in N2A cultures, in which 400-fold more virus is produced per cell in the presence of the agent (not shown).

Table 1. cAMP-related inhibition of measles virus

	Infectivity, pfu/ml			
	Neural cultures		Nonneural cultures	
Treatment	TE671	N2A	Vero	CV-1
Control	5×10^5	2×10^5	2×10^5	6×10^5
Papaverine	8×10^1	8×10^1	6×10^5	1×10^4
IBMX	4×10^3	1×10^1	1×10^4	2×10^4
cAMP	6×10^2	6×10^1	NT	4×10^4
8-Br-cAMP	2×10^3	3×10^2	2×10^4	7×10^4
Dibutyryl cAMP	2×10^3	3×10^2	8×10^4	1×10^5
Na butyrate	7×10^5	3×10^6	2×10^5	4×10^6

Cultures were treated for 3 days, infected, maintained in the same agent, and harvested 3 days later. All agents were 1 mM, except papaverine, which was 7 μM. NT, not tested. Data are averages of 12 assays from three separate experiments (two cultures each experiment, two assays per culture).

Expression of Viral Antigens in Papaverine-Inhibited Infections. The effect of papaverine on the expression of individual viral antigens was examined in infected N2A, TE671, and CV-1 cells. Monolayer cultures grown on glass coverslips were treated for 72 hr, infected, refed with papaverine-containing medium, and harvested 12, 16, 24, 48, and 72 hr after infection. Viral HA, P, F, NP, and M protein antigens were assayed in the infected cells by immunofluorescence. Viral antigens became visible in all three cell lines as early as 12 hr after infection and maximal expression of all five antigens occurred by 48 hr. The entire TE671 culture contained viral antigen, while only a third of the N2A cells were visibly stained.

In both neural cell lines (N2A and TE671), papaverine caused significant changes in the amounts of M protein and NP antigens in infected cells. In contrast, CV-1 cells showed no such changes. None of the three cell lines showed any change in intensity or distribution of viral HA, P, and F antigens with papaverine.

Infected TE671 cells (without papaverine) contain viral NP antigen diffusely distributed in the cytoplasm (Fig. 1*A*). When papaverine-treated, they show an increased number of antigen-containing inclusion bodies and also redistribution of the antigen into focal aggregates (Fig. 1*B*). On prolonged (4 days) incubation of the infected cells in the presence of papaverine, the staining for NP antigen continued to increase in intensity with a striking accumulation into even larger intracellular inclusions (not shown).

Although a minor structural protein, the M antigen was de-

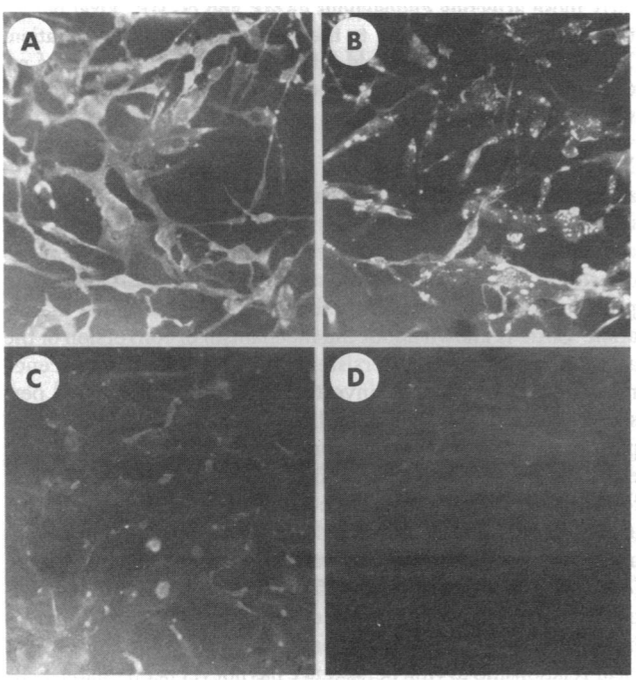

FIG. 1. Fluorescent-labeled monoclonal antibodies show expression of viral NP and M proteins in measles virus-infected TE671 cells. (*A*) Viral NP antigen in control cultures 48 hr after infection. (*B*) Viral NP antigen in cells treated with 7 μM papaverine for 3 days, infected, and then maintained in papaverine until harvest 48 hr later. (*C*) Untreated culture stained for viral M antigen; note diffuse staining 48 hr after infection. (*D*) Companion papaverine-treated culture; staining for M protein is virtually absent. (×120.)

tected in infected cells (without papaverine) as both cytoplasmic inclusions and diffuse staining of membrane structures (Fig. 1C). However, in papaverine-treated cells (Fig. 1D), virtually no M antigen was detectable. The M antigen remained undetectable even after prolonged (7 days) incubation of the infected culture with papaverine.

Reversal of Inhibition. We next attempted to reverse inhibition of viral replication by withdrawal of the blocking agents. Duplicate TE671 cultures were treated with papaverine and infected as above, and samples were harvested daily (Fig. 2). Two, five, and seven days after infection, one set of the cultures was refed with complete medium (minus papaverine), and other sets were refed with complete medium or complete medium/papaverine. Over the subsequent 4–7 days, those cultures continuously maintained in papaverine did not release any infectious virus. However, in papaverine-treated cultures refed with complete medium only, yields of infectious virus increased progressively, maximal yields being slightly greater than those previously attained by infected cultures without papaverine. Viral yields were maximal 2 days after papaverine removal; the titers then decreased as in the untreated cultures. Cultures blocked for a longer time (5 or 7 days) showed the same reversal but at a slightly slower rate of increase in titers of infectious virus when the papaverine was removed. A similar pattern of release of infectious virus was also observed in cultures incubated with cAMP or IBMX after refeeding with medium free of the blocking agent (data not shown).

To test whether inhibition of viral replication involves cGMP-related mechanisms, cells were incubated for 3 days with papaverine, IBMX, or cAMP and then infected and maintained in medium containing the blocking agent. Forty-eight hours after infection, 1 mM cGMP was added to the medium. Marked cytopathic effect was observed within 2 days, and there was an increase in titer of infectious virus equal to that in infected cultures grown in the absence of any agent (Table 2). Thus cGMP counteracted the effects of cAMP-stimulatory agents. Paradoxically, in cultures treated for 3 days with 1 mM cGMP only and then infected, yields of infectious virus decreased by a factor of 10^2, compared with controls without cGMP. These results suggest a possible complex interrelationship between the cyclic nucleotides.

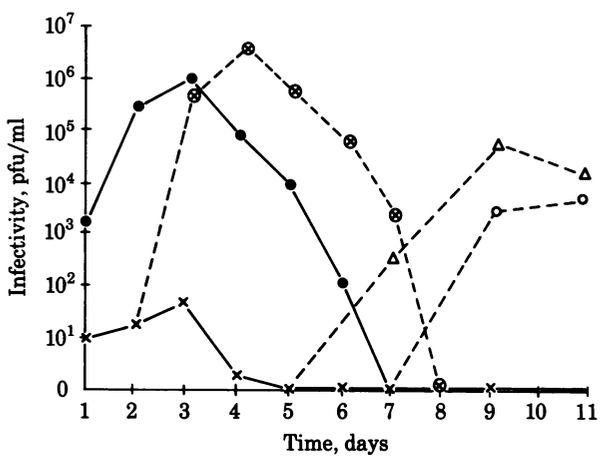

FIG. 2. Reversal of papaverine inhibition of measles virus replication. TE671 cells were either untreated (control) or treated for 72 hr with 7 μM papaverine. Two, five, or seven days after infection with measles virus, medium containing papaverine was removed, and the cultures were refed with complete medium. Infectivities were determined for duplicate samples; data are means of three separate experiments. ●, Control (no papaverine); ×, continuous papaverine; ⊗, △, and ○, papaverine removed 2, 5, and 7 days, respectively, after infection.

Table 2. cGMP and measles virus replication

Before infection	After infection	Infectivity, pfu/ml
Control	Control	5×10^5
cGMP	cGMP	3×10^3
Papaverine	Papaverine	<5
Papaverine	Papaverine/cGMP	1×10^4
IBMX	IBMX	3×10^2
IBMX	IBMX/cGMP	4×10^3
cAMP	cAMP	6×10^2
cAMP	cAMP/cGMP	2×10^5

TE671 cultures were treated as indicated for 3 days, infected, and refed with the same agent(s). As indicated, cGMP was added 48 hr after infection. Cultures were harvested when cytopathic effect was maximal: 48 hr for control, 96 hr for treated cultures. All agents were 1 mM except papaverine, which was 7 μM. Data are averages of 12 assays from three separate experiments. (Two cultures each experiment, two assays per culture).

Expression of Viral Antigens After Reversal of Inhibited Viral Replication. As described above, no staining for M protein was visible in papaverine-treated infected cultures; however, within 24 hr of removal of papaverine, this polypeptide was again present (Fig. 3).

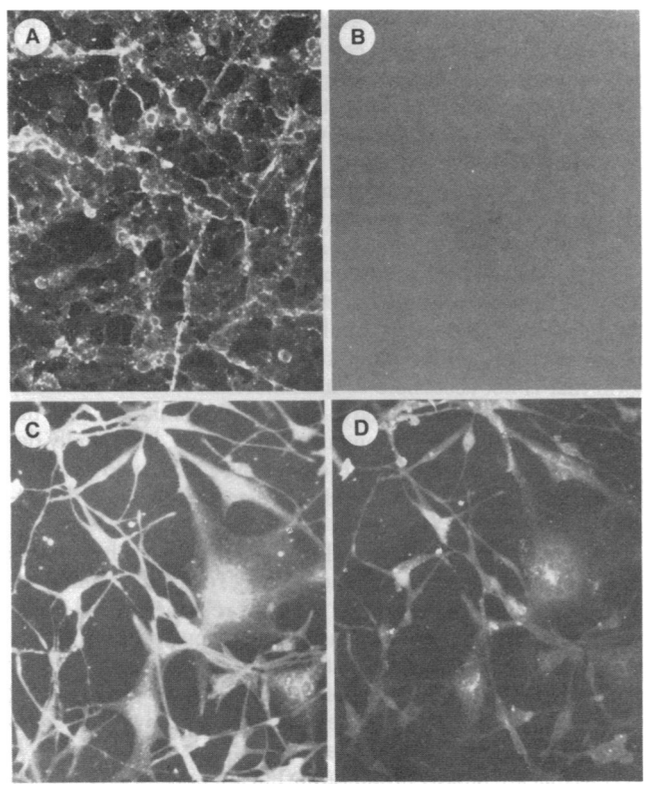

FIG. 3. Immunofluorescent staining with monoclonal antibody to viral M protein of measles virus-infected cells in the papaverine-blocked state. TE671 cultures were treated with 7 μM papaverine for 3 days and then infected with measles virus. (A) Cells continuously incubated in papaverine 5 days after infection were stained nonspecifically with rhodamine. (B) The same field stained with antiserum specific for M protein shows no immunofluorescence despite long exposure. (×120.) In other cultures, the papaverine was removed 3 days after infection, and the cells were harvested 2 days later. (C) Slightly higher (×235) magnification shows that cells are stained nonspecifically with rhodamine. (D) The same field, showing return of staining of M protein after reversal, with accumulation of antigen in areas adjacent to the clustered nuclei.

DISCUSSION

Our studies indicate that, in cell lines of neural origin, measles virus replication is dependent on certain cellular functions. Examples of host control of replication of various viruses have been described (33, 34). Understanding host control is important because, in many instances, it determines the potential of a virus for malignant transformation of the cell (35) or the virulence of the virus in an animal host (36). The results presented suggest that the shift in the state of the cell from acute to persistent measles virus infection may depend on the intracellular level of cyclic nucleotides. In several other virus–host cell systems, cAMP exerts a variety of effects on viral replication (37, 38), the precise effects depending on the specific virus and host cell. We have used our neural cell cultures for preliminary testing of infections with type 1 herpes simplex virus, Theiler murine encephalomyelitis virus, and vesicular stomatitis virus. No significant inhibition of replication by cAMP was observed with these viruses.

Recently, Robbins and Rapp (24), in contrast to our results with measles virus, demonstrated that exogenous cAMP partially inhibited replication of measles virus in AV-3 cells, a nonneural human amnion cell line. Using polyacrylamide gel electrophoresis, they reported a decrease in both M and P viral polypeptides. Our immunofluorescence studies showed a marked decrease in M protein but no significant decrease in P protein in the neural cells. An explanation for this discrepancy may be a difference in host cell modulation of expression of the two proteins. Robbins and Rapp also did not observe dibutyryl cAMP inhibition of viral replication. Again, host cell differences in response, as well as the stimulatory effects of butyrate, may have produced their results.

Recently, Graves has demonstrated, by polyacrylamide gel electrophoresis, that M protein occurs in two forms in measles-infected Vero cells, the more slowly migrating species being phosphorylated (2). Furthermore, Carrigan has observed that M protein in persistently infected cell lines consists mainly of the phosphorylated form (unpublished data). It is possible that, in our papaverine-blocked cultures, M protein is present in a form that is not accessible or immunoreactive with our highly specific monoclonal antibody. Another possibility is that the polypeptide is being continuously synthesized, but rapidly degraded. Removal of the cAMP-enhancing agent could alter this degradation, with the M protein then accumulating to detectable levels.

In general, levels of intracellular cAMP vary with the state of cell growth and differentiation, being relatively high in stationary cells and decreasing as the cells reenter the cell cycle. Full expression of measles virus infection appears to be closely associated with the metabolic state of the infected cell, possibly through the inhibitory action of cAMP. Ehrnst *et al.* (39), using a human nonneural cell line persistently infected with measles virus, showed that viral antigens are expressed at high levels in cells that are rapidly proliferating but are present at low levels in stationary cells. By using peripheral blood lymphocytes from children with rubeola, Osunkoya *et al.* (40) demonstrated that measles virus antigens and cytopathic effects were expressed only after mitogenic stimulation with phytohemagglutinin. Sullivan *et al.* (41) analyzed *in vitro* infection of human peripheral blood lymphocytes with measles virus and found that unstimulated cells replicated the virus very poorly, whereas phytohemagglutinin-stimulated lymphocytes supported high levels of viral replication. Similar results were obtained by Lucas *et al.* (42).

cGMP often manifests a reciprocal relationship to cAMP, both in intracellular levels and specific physiologic effects. In the central nervous system, it is primarily located within the neurons (43). In our studies, cGMP reverses the inhibition of virus replication by agents that elevate intracellular cAMP. This may reflect regulation that is dependent on the ratio of intracellular levels of these cyclic nucleotides. Phosphorylation of viral proteins occurring by cAMP-independent mechanisms (44) may change the ratio of intracellular forms of M protein. This premise could be examined by direct measurement of intracellular cGMP and cAMP.

This experimental model carries with it implications concerning the pathogenesis of diseases associated with persistent viral infection. Persistent central nervous system infection by measles virus has been shown to be central to the etiology of SSPE (45). The viral M protein does not appear to be expressed, which may be responsible for a block in the release of viral progeny. The virus, in most cases, does possess the genetic information for the M protein, as shown by the reversion of most strains of SSPE virus to a productive form, with concomitant expression of M protein, after passage in tissue culture (46). In an experimental model of SSPE (47) in hamsters, expression of M protein selectively disappears during the transition from acute to subacute infection and is correlated temporally with reduced release of infectious virus and appearance of a detectable immune response by the animal against the virus. In those studies, it was postulated that immune factors, possibly antiviral antibodies, interact with the infected cell surface to cause either rapid degradation or loss of expression of M protein. Of interest in this context is the finding by Fujinami *et al.* (48) that growth of measles virus-infected cells in the presence of antiviral antibody leads to a several-fold increase in phosphorylation of the viral M protein.

While there are similarities between our findings and possible pathogenetic mechanisms involved in SSPE, certain precautions must be taken in the interpretation of these observations. The cell lines used in our studies, N2A and TE671, are tumor cells and may have altered regulatory mechanisms compared with normal neural tissue. However, they are capable of expressing neural-specific differentiated function. Papaverine may also have effects beyond those associated with inhibition of cAMP phosphodiesterase. Finally, the immunofluorescence technique used in these studies is relatively insensitive and poorly quantitative, and more sophisticated analysis using biochemical techniques will be necessary to further define the extent of and mechanisms behind the apparent selective regulation of the viral M protein.

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