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Isolation of Cold-Sensitive Mutants of Measles Virus from Persistently Infected Murine Neuroblastoma Cells

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Clone NS20Y of the mouse neuroblastoma C1300 was infected with wild-type Edmonston measles virus, and, after a transition to a carrier culture, became persistently infected. Persistently infected clones were derived and characterized morphologically by the appearance of multinucleate giant cells and nucleocapsid matrices in cytoplasm and nucleus, but very few budding virus particles. Antimeasles antibodies markedly suppressed the expression of viral antigens and giant cells, and the effect was totally reversible. When the cells were cultured at 33°C, the number of giant cells began to diminish and ultimately disappeared; in contrast, when cultured at 39°C, the cultures invariably lysed. Yields at 33°C were ca. 2 logs lower than those at 39°C. Cells cultured at 33°C produced relatively high levels of interferon, whereas those at 39°C produced little or no interferon. When the persistently infected cultures were exposed to anti-interferon α/β serum at a nonpermissive temperature, there was a marked increase in multinucleate cells, suggesting that maintenance of the persistence state and its regulation by temperature may be related to the production of interferon. Viral isolates from cells cultured at 39°C were obtained, and 90% of viral clones were found to be cold sensitive. Complementation studies with different viral clones indicated that the cold-sensitive defect was probably associated with the same genetic function. Western blot analysis of the persistently infected cells indicated a significant diminution and expression of all measles-specific proteins at a nonpermissive temperature. Infection of NS20Y neuroblastoma cells with the cold-sensitive virus isolates resulted in the development of an immediate persistent infection, whereas infection of Vero or HeLa cells resulted in a characteristic lytic infection, suggesting that the cold-sensitive mutants may be selected or adapted for persistent infection in cells of neural origin.

The nature of viral persistence in the central nervous system (CNS) has become crucial to the understanding of a variety of human demyelinating diseases. Measles variants have been isolated, with some difficulty, from patients with subacute sclerosing panencephalitis (SSPE) (12, 13, 26), and measles virus genome has been reported to be present in lesions of multiple sclerosis (9). Measles virus can infect a variety of cell types in vitro and in vivo, usually producing acute, lytic infection, although in certain circumstances persistent infections can be established (31). In studies of persistently infected cell cultures, both temperature-sensitive (*ts*) mutants (17) and defective interfering particles (30) have been obtained. The mechanisms by which host cell functions affect the persistence of measles virus remain poorly understood. There is evidence that cells of the CNS may restrict the replication of measles virus. Particularly important are studies of Hall et al. on SSPE patients, in which the sera of patients were found to be deficient in antibodies to the matrix (M) protein, and CNS tissue explants showed no evidence of M protein synthesis (10, 11). Additionally, acute infection of murine spinal cord cultures with measles virus led to selective but nonlytic infection of neuronal cells (29). It has recently been reported that agents which raised cyclic AMP levels restricted the production of M protein in murine and human neuroblastoma cells (21).

The present experiments were undertaken to ascertain whether cell lines derived from the CNS would allow measles virus persistence and whether there might be a host selection for a peculiar type of viral mutant. The results indicate that murine neuroblastoma cells rendered persistently infected with measles virus produce cold-sensitive (*cs*) mutants, a type not previously described for this virus.

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MATERIALS AND METHODS

Cells. The mouse neuroblastoma C1300, clone NS20Y, cells of A strain mouse (32) were kindly supplied by Peter Davies of this institution. Cells were propagated as loosely adherent monolayers on RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine, and antibiotics. Cells were passaged when confluent by trypsinization or by shaking the culture vigorously.

Monolayer cultures of Vero cells, derived from green monkey kidney cells, were used for the growth of viral stocks and for titration assays. They were grown in Dulbecco modified Eagle medium (DME) supplemented with 10% FCS, glutamine, and antibiotics. Mouse fibroblasts (L929) used for interferon (IFN) assays were grown on DME supplemented with 10% FCS, antibiotics, and glutamine. All cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C, unless otherwise stated.

Viruses. The Edmonston strain of measles virus was doubly plaque purified on Vero cells, and stock virus was produced by growth in Vero cells at a multiplicity of infection (MOI) of 0.01 PFU per cell (17).

Antisera. Antimeasles hyperimmune serum was prepared by immunizing rabbits with purified measles virus grown on Vero cells. Rabbits were immunized and boosted by intravenous injections of virus. Monoclonal antibodies against measles virus hemagglutinin (HA) and nucleocapsid (NP) were prepared and characterized in this laboratory (4). Monoclonal antibodies against measles fusion and matrix (M) polypeptides were the generous gift of Erling Norrby (Karolinska Institute, Stockholm, Sweden), who has described their preparation previously (24).

Anti-IFN serum. Sheep anti-mouse IFN serum (globulin fraction) was prepared as previously described (8). This

serum, at a dilution of 1:1,000, completely neutralized 200 U of mouse IFN-α. (Anti-IFN serum was kindly provided by I. Gresser, Institut de Recherches Scientifique sur le Cancer, Villejuif, France.)

Virus titrations. The titers of virus suspensions or culture supernatants were determined by a semiquantitative plaque assay (27). Vero cell monolayers were trypsinized and plated in 96-well plastic trays (Linbro Scientific Co., Hamden, Conn.); ca. 2×10^5 cells were seeded in each 16-mm well and incubated at 37°C overnight. Tenfold dilutions of virus samples were prepared in DME supplemented with 2% FCS. The growth medium from each well was aspirated, and duplicate wells were inoculated with 0.2 ml of viral dilutions. After an adsorption period of 2 h at 33°C, 1 ml of growth medium containing 0.75% carboxymethyl cellulose was added. The monolayers were then incubated at the appropriate temperature for 5 days and fixed with 10% Formol-saline and stained with crystal violet.

Infectious centers assay. Persistently infected NS20Y cells were treated with rabbit antimeasles serum for 30 min at 4°C, washed, and resuspended, and various numbers of viable cells were assayed on Vero cell monolayers (25).

Cloning by limiting dilution. NS20Y/MS cell suspensions (containing 1 to 10 cells per 0.1 ml) were seeded in 96-well microcytotoxicity plates (Linbro Scientific Co.) and incubated in a humidified atmosphere of 10% CO_2 in air at 37°C. Ten days later, cells from wells containing only a single colony were transferred to 24-well tissue culture plates (Linbro Scientific Co.).

IFN titrations. Mouse IFN titers were measured by the microtiter method, determining 50% virus plaque reduction of encephalomyocarditis virus on L929 cell monolayers. In these assays, 1 U was approximately equal to 1 National Institutes of Health mouse IFN standard reference unit.

Immunofluorescent staining. An indirect immunofluorescence assay was used to visualize viral antigen expression. The specific antisera and fluorescein isothiocyanate-conjugated antibody (the latter purchased from Cappel Laboratories, Cochranville, Pa.) were absorbed with an equal volume of uninfected NS20Y cells for 30 min at 4°C and subsequently centrifuged (15,000 rpm) to remove cells and aggregates. For cytoplasmic immunofluorescent staining, cells were grown in Lab-Tek tissue culture chamber slides (Lab-Tek Scientific, Div. of Miles Laboratories, Inc., Naperville, Ill.) until nearly confluent. Monolayers were washed, air dried, and fixed in cold acetone. Unfixed cell suspensions were used for membrane fluorescence.

Preparation of cells for electron microscopy. Cells were washed in serum-free medium for 5 min and then fixed as a monolayer in 2.5% gluteraldehyde in Millonig phosphate buffer for 20 min at pH 7.3 (22). The monolayer was then gently scraped off the culture dish, and the cell suspension was centrifuged into a pellet at 1,000 rpm for 5 min. After 3 washes in Millonig phosphate buffer, the cell pellet was postfixed in 1% OsO_4 in Millonig phosphate buffer for 30 min, dehydrated in upgraded ethanol, and embedded in Araldite-Epon. Thin sections were stained in uranyl acetate for 15 min, followed by lead citrate for 2 min. The sections were examined in a Siemens 101 electron microscope at 80 kV. For quantitative studies, at least 200 cells per grid were scored for each sample.

Plaque purification of viral isolates from NS20Y/MS and screening for *ts* mutants. NS20Y/MS cultures were grown at 39°C for 24 to 48 h. The supernatants from these cultures were harvested, filtered through a 0.45-μm Millipore filter to remove viral aggregates, and plated on Vero monolayers in 60-mm petri dishes (Becton Dickinson and Co., Paramus, N.J.). An appropriate dilution of the filtered supernatants was used to give 25 to 50 plaques per dish. After 2 h of adsorption at 33°C, the monolayers were overlaid with 4 ml of DME containing 10% FCS and 0.75% agarose. Plates were incubated at 39°C for 5 to 7 days. Well-isolated plaques were selected and inoculated onto individual Vero monolayers in 16-mm Linbro tray wells, adsorbed for 2 h at 33°C, and then overlaid with 1 ml of growth medium. The plaque-purified isolates were incubated at 39°C and harvested when 90% of the monolayer showed cytopathic effects (CPE).

Preparation of viral subclones. The entire culture fluid (with cell debris) was then inoculated onto Vero monolayers in 25-cm^2 Falcon flasks (Becton Dickinson and Co.), incubated at 39°C, and harvested when 90% of the monolayer showed CPE. Each isolate was then screened for temperature sensitivity by a plaque assay at 33 and 39°C. A plating efficiency (PE) ratio for each isolate was calculated as follows: PE ratio = titer at 33°C/titer at 39°C.

Thermolability of *cs* mutant isolates. Thermolability was determined by measuring the rate of the reduction in PFU per milliliter at 39°C after the incubation of virus samples at 45°C. Undiluted virus stocks were incubated in a 45°C water bath. Samples (0.2 ml) were withdrawn in 1.8 ml of ice-cold DME containing 2% FCS at 0, 15, 30, 45, 60, and 120 min. Residual infectivity was determined by plaque assay at 39°C.

Complementation. Complementation experiments were carried out by the procedure of Bergholz et al. (3), by infecting Vero cell monolayers either with individual mutants at a MOI of 2 to 3 or with pairs of mutants, each at a MOI of 1 to 1.5. After 2 h of adsorption at 33°C, growth medium was added, and the cultures were further incubated at 33°C.

When doubly infected cultures showed measles virus CPE, the contents of each culture were harvested. At the same time, the corresponding singly infected wells were also harvested. Virus yields were determined by a plaque assay on Vero monolayers. The index of complementation was calculated according to the following formula: complementation index = [(yield of $x + y$ at 39°C) − (yield of $x + y$ at 33°C)]/[(yield of x at 39°C) (yield of y at 39°C)].

Immunochemical analysis of measles polypeptides. Confluent monolayers of Vero cells (3×10^6 cells per 60-mm tissue culture dish) were infected at a MOI of 1 with wild-type Edmonston virus or cloned *cs* isolates. Infection was allowed to proceed for 48 to 72 h in parallel at 33 and 39°C, after which the monolayers were solubilized with sodium dodecyl sulfate extraction buffer (0.02 M Tris-hydrochloride, 0.05 M NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.3% sodium dodecyl sulfate, 2 mM EDTA, 2,000 Kallikrein inactivator units per ml of aprotinin [pH 7.5]) (ca. 10^7 cells per ml). Subconfluent cultures of NS20Y/MS and uninfected NS20Y cells (10^6 cells per 60-mm Falcon tissue culture dish) were incubated in parallel at 33°C for 5 days. The cells were then solubilized with sodium dodecyl sulfate extraction buffer (ca. 10^7 cells per ml). Samples (ca. 25 μl each) were subjected to polyacrylamide gel electrophoresis in 10% gels and transferred to nitrocellulose paper as described by Towbin et al. (35). The measles-specific proteins were stained with hyperimmune rabbit antimeasles antiserum and developed with peroxidase-conjugated goat anti-rabbit immunoglobulin reagent.

RESULTS

Establishment of neuroblastoma line NS20Y/MS persistently infected with measles. Clone NS20Y of the mouse neuro-

blastoma C1300 was infected with wild-type Edmonston measles virus at a MOI of 1. After 2 h of adsorption at 33°C, cultures were incubated at 37°C. By 4 to 5 days, cultures manifested CPE, which included cell fusion, multinucleated giant cells, dendrite extensions, and cytolysis (Fig. 1A and B). After the acute crisis, there remained a small number of cells, which grew into colonies after 10 days. These colonies had a markedly reduced growth rate. During the culture of these cells, three cycles of crisis and recovery were observed before the cultures stabilized as persistently infected cells after 1 year in culture.

Cloning of the NS20Y/MS line. After the establishment of a persistent infection in the neuroblastoma cells, repeated attempts were made to isolate single cell clones from the parental culture. All such attempts were unsuccessful until the NS20Y/MS line had been carried for 1 year. We infer that the initial failures to obtain clones expressing viral antigens indicated that first a carrier culture was produced, in which there was a continuous low level of production of infectious virus which was able to infect new cells in the culture. After 1 year, cloning for virus-infected cells was accomplished initially by limiting dilutions, and more than 50% of the clones isolated were clearly positive for measles virus antigens and production of infectious measles virus. One positive isolate was recloned, and 185 subclones were isolated, of which 124 were strongly positive for measles antigen. Since the vast majority of the clones derived from single cells were positive for viral antigens, we infer that the

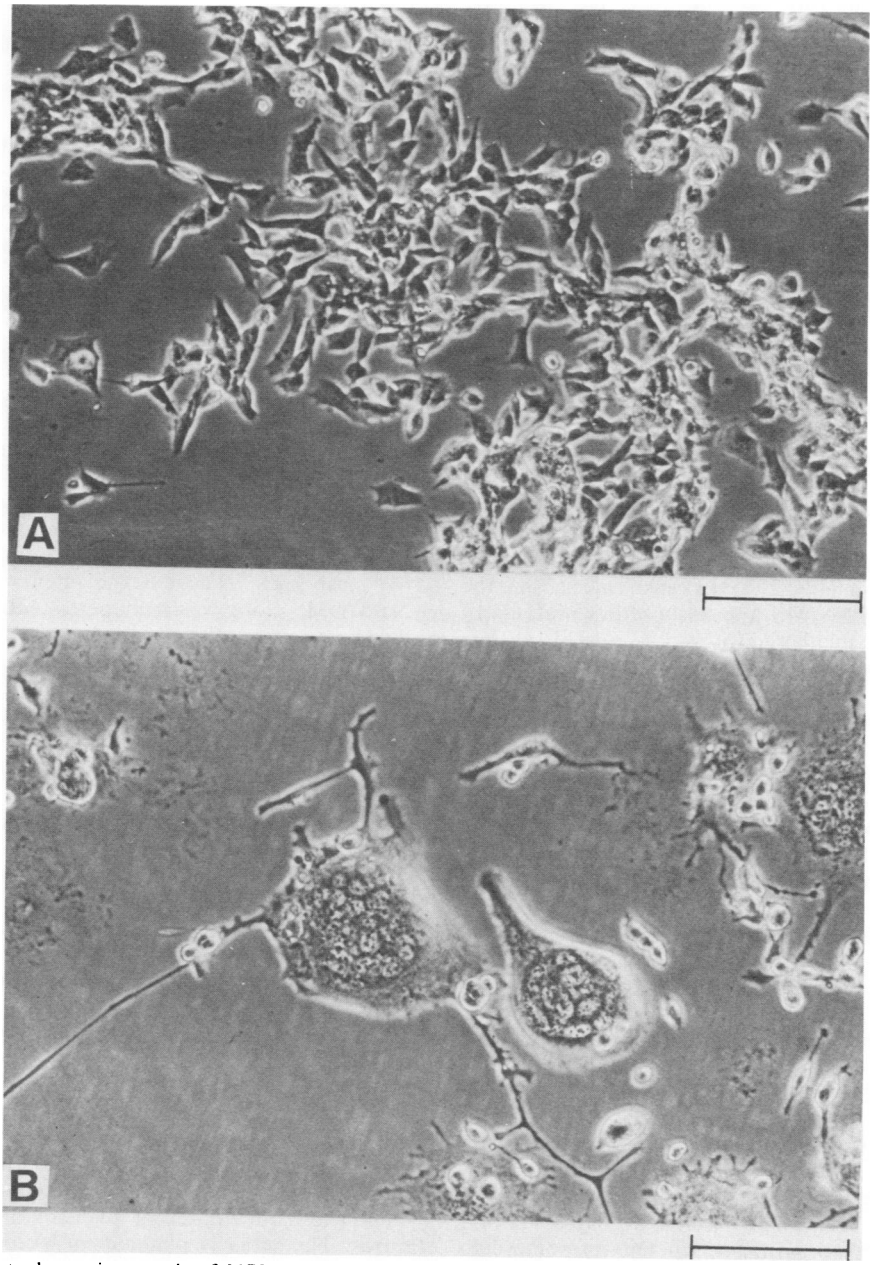

FIG. 1. Phase-contrast photomicrograph of NS20Y neuroblastoma cells. (A) Control uninfected cells; (B) NS20Y cells 5 days after infection with wild-type Edmonston measles virus. Bar, 100 μm.

cultures had converted to a persistently infected line. Infectious measles virus was isolated from all antigen-positive clones. All further experiments were carried out with a doubly cloned isolate, clone 2.7.16, which is referred to as the NS20Y/MS line.

Characteristics of the NS20Y/MS cell line (clone 2.7.16). The persistently infected cloned cell line appeared morphologically normal until the cells reached confluency, upon which cell fusion and formation of multinucleate giant cells (polycaryocytes) containing up to 20 nuclei were observed. Immunofluorescent staining by using polyclonal rabbit antimeasles and monoclonal antimeasles antibodies indicated that 50 to 80% of the cells produced detectable measles virus antigens. Single cells and polycaryocytes were both positive for membrane immunofluorescence by using monoclonal antimeasles HA antibodies or polyclonal rabbit antimeasles serum, although the intensity of staining varied. Staining with monoclonal antibodies directed against measles virus NP showed the formation of inclusions characteristic of measles virus NP matrices (23). Comparisons of intensities of cytoplasmic staining by monoclonal antibodies directed against measles virus HA, M, and fusion proteins indicated that the largest number of cells showed positive staining with the anti-HA antibodies, whereas more M protein was found in polycaryocytes than in single cells, and the staining with anti-fusion antibodies was rather weak.

Electron microscopic examination of the NS20Y/MS cell line indicated that the majority of either polycaryocytes or single cells contained small crystalline arrays of measles virus NPs in the cytoplasm and nucleus. Of particular interest was the finding that relative to the large numbers of NPs observed in the cytoplasm, very few budding virus particles could be detected (Fig. 2).

General characteristics of the NS20Y/MS cells are summarized in Table 1. It is clear that many more cells produced viral antigens than infectious centers or infectious virus, and it is estimated that each infectious center produced ca. 1 to 10 PFU of infectious virus.

Effects of temperature on NS20Y/MS cells. To examine the possibility that the NS20Y/MS line harbored *ts* mutants of measles, the cells were incubated for various periods of time at 33 or 39°C, and the effects of temperature shifts were compared relative to those of the cultures maintained at 37°C. After a shift down for 4 days to 33°C, the typical multinucleate giant cells began to diminish, and then totally disappeared after 6 days. When the cultures were shifted up to 37°C, multinucleate cells were once again observed in cultures, and their number increased when the cultures were shifted to 39°C for 4 days. The majority of cells lysed after culture for ca. 2 weeks at 39°C.

The yields of infectious measles virus in the culture supernatants at various temperatures showed significant differences. After 4 days of incubation at 33°C, 7×10^1 PFU were obtained, whereas at 39°C, virus yields were ca. 1.5×10^4 PFU. Overall changes in the intensity of immunofluorescent staining for viral antigens at different temperatures were similarly observed; whereas 80 to 85% of the cells showed positive staining for measles antigens upon incubation at 39°C, only 20 to 25% of the cells expressed measles virus antigens at 33°C. By electron microscopy, cultures incubated at 39°C showed relatively large numbers of budding viruses, whereas essentially no budding virus was detected after incubation at 33°C, although massive aggregates of NPs accumulated in both cytoplasm and nucleus of some cells (Fig. 3).

Effects of antibody treatment on viral persistence. When NS20Y/MS cells growing at 37°C were cultured with polyclonal rabbit antimeasles serum, the giant multinucleated cells disappeared within 3 days. No giant multinucleated cells or infectious virus could be demonstrated in cultures treated with the antiserum for up to 4 weeks, and only occasional measles-positive cells were seen by immunofluorescence (Fig. 4). When the antiserum was removed, multinucleate giant cells reappeared along with the production of infectious virus (Table 2). These experiments indicated that the culture was not cured by antimeasles antibodies, although the expression of viral antigen was modulated by antibodies. These experiments confirmed that the NS20Y/MS clone was truly persistently infected rather than being a carrier culture.

TABLE 1. Characteristics of a neuroblastoma cloned cell line (NS20Y/MS) persistently infected with measles

Characteristic	Characteristic in NS20Y/MS
Morphologic appearance (37°C)	Polycaryocytes and single cells
Immunofluorescent staining (37°C)	50 to 80% of the cells; positive for NP, HA, M, and fusion measles virus polypeptides
Infectious centers (37°C)[a]	1 to 2%
Intracellular virus yield (37°C)	1 to 10 PFU/10^6 cells per ml
Virus yield in supernatants	
(33°C)	7×10^1 PFU/ml
(39°C)	1.5×10^4 PFU/ml
Electron microscopy	N; Ms in cytoplasm and nucleus; virus budding at 39°C; no budding but M accumulation at 33°C
IFN production	
IU of IFN/ml (39°C)	1/20
IU of IFN/ml (33°C)	1/80–1/320

[a] Percentage of cells producing infectious virus.

Role of IFN in maintenance of the persistent state. IFN levels detected in supernatants of the NS20Y/MS cells cultured at different temperatures indicated that cultures maintained at 33°C produced relatively high levels of IFN, ranging from 1/80 to 1/320 per ml (Table 1). In contrast, cultures maintained at 37 or 39°C produced no IFN or very low levels. These results suggested that IFN might be involved in maintaining the persistent state and in the inhibition of expression of infectious virus production at low temperature. Consequently, NS20Y/MS cultures were exposed to sheep anti-mouse IFN-α/β serum at a nonpermissive temperature. Within 24 h after the addition of the anti-IFN serum, there was a marked increase in the number of multinucleate cells, and by 4 days, the majority of cells in the cultures were giant (Fig. 5). Morphologically, the appearance of the anti-IFN-treated cultures was very similar to that of cultures of neuroblastoma cells acutely infected with measles virus, and infectious virus titers were generally 100-fold higher in the presence of anti-IFN. It should be noted that the cold sensitivity of the mutants is an intrinsic property of the viruses and not merely the result of their ability to induce different levels of IFN at the two temperatures. The virus is plaqued on Vero cells, which are well known to be unable to produce or respond to IFN. We have examined the medium from Vero cells infected at both

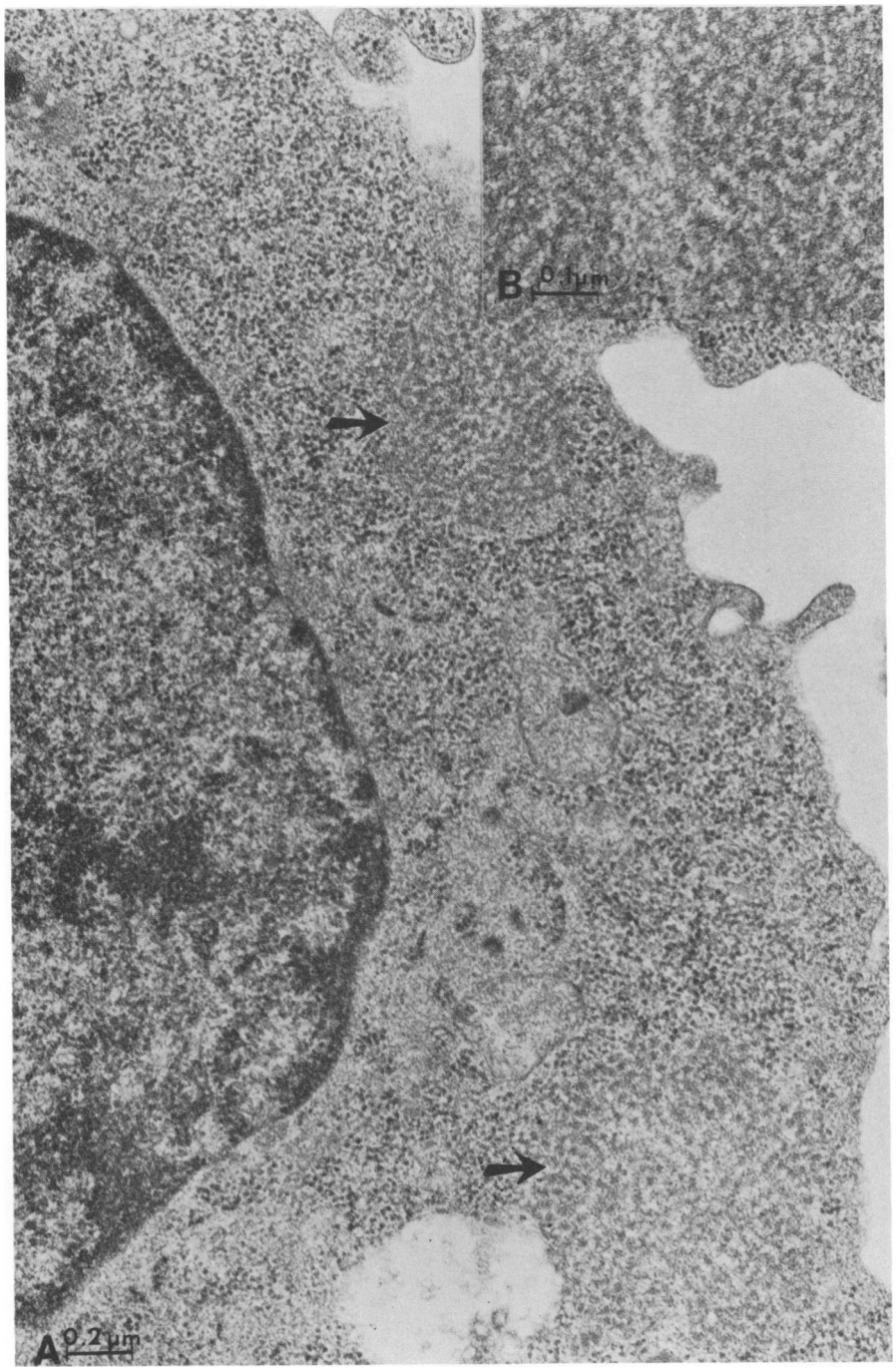

FIG. 2. Electron micrograph of neuroblastoma cells PI with measles virus. (A) Aggregates of measles virus nucleocapsids in the cytoplasm (arrows); (B) higher magnification of nucleocapsid matrix.

temperatures and found no IFN active on either Vero or a human trisomic cell line. Conversely, treatment of Vero cells with 1,000 U of human IFN-α failed to alter the number of plaques of the mutant or wild-type viruses on Vero cells at either temperature. In summary, these experiments strongly suggest that maintenance of the persistent state in neuroblastoma cells and its regulation by temperature are related to the production of IFN.

Isolation of *cs* mutants. Because of the decreased expression of measles virus at 33°C and the increase in CPE and virus yields at 39°C, the possibility that the cells contained *cs* mutants was explored. NS20Y/MS cells were cultured for 5 to 7 days at 39°C and then plaqued on Vero cells. Individual plaque isolates were picked at 5 to 7 days and then propagated on Vero cells at 39°C. The individual isolates were titrated for PFU on Vero cells at both 39 and 33°C. The PE (PE ratio = titer at 33°C/titer at 39°C) was determined for a total of 40 plaque purified isolates. A representative titration is shown in Table 3; It was found that 90% of the isolates were *cs*. In previous studies (17), 29 clones of the parental Edmonston virus were plaque purified and examined for the presence of *ts* or *cs* mutants, and the PE of all clones was very close to 1.

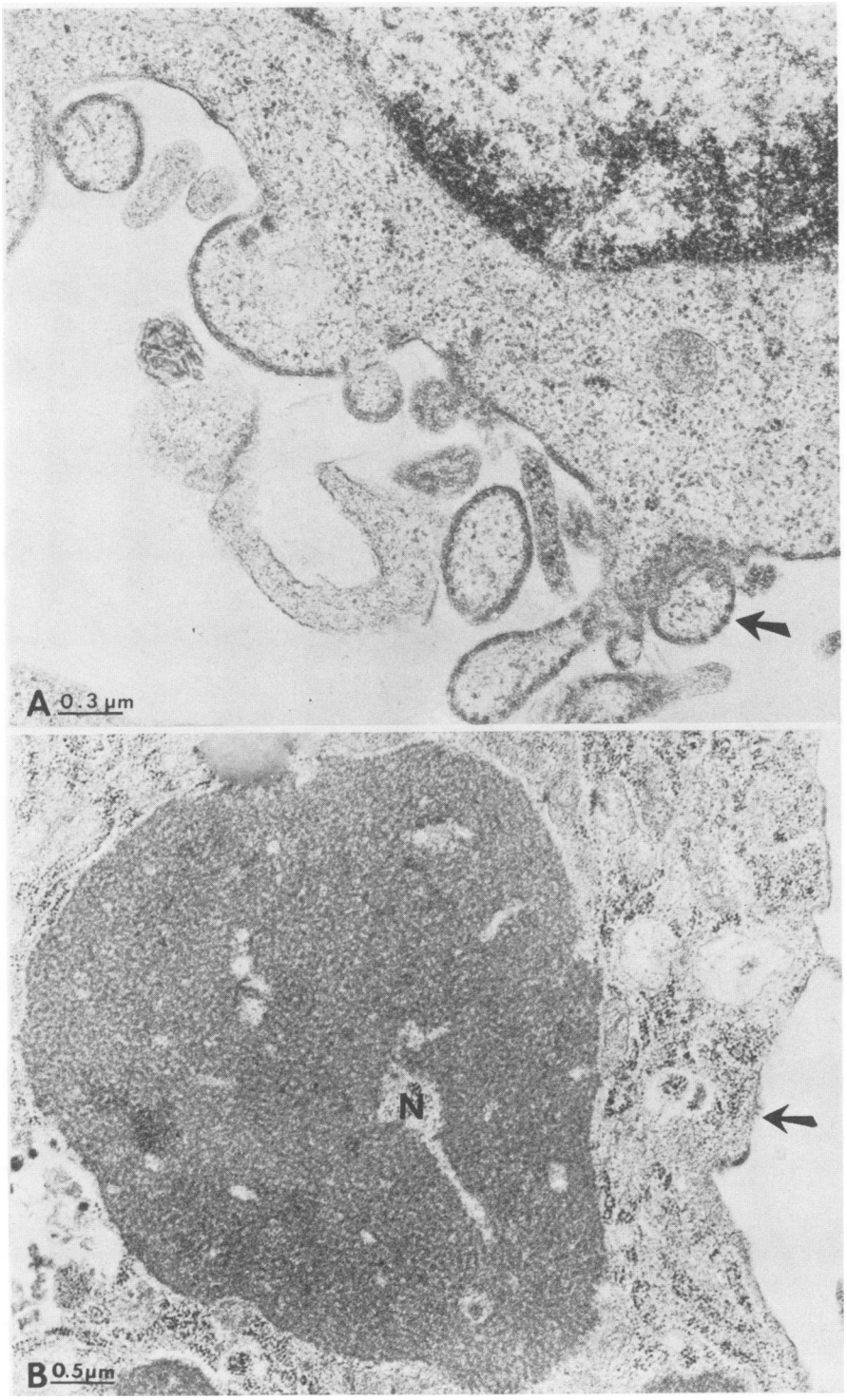

FIG. 3. Electron micrograph of NS20Y/MS cells. (A) After incubation at 39°C, a large number of budding measles virus particles are seen (arrow). (B) After incubation at 33°C, massive aggregates of nucleocapsids are accumulated in the cytoplasm of some cells. No budding virus particles along the membrane (arrow) were observed.

cs isolates were obtained first from the NS20Y/MS cells in passage 16 and were maintained through passage 86.

Genetic complementation studies were carried out to explore whether the *cs* isolates from the NS20Y/MS cells possessed lesions in different genes. Pairs of isolates were coinfected onto Vero cell monolayers, and the yield of virus at a restrictive temperature (33°C) was measured. As shown in Table 4, the complementation index was less than 1 for all six combinations tested, indicating that the *cs* defect in all of the isolates was probably associated with the same genetic function.

Since it is recognized that *ts* mutations affecting a structural component of the virion frequently result in altered thermolability of the mutant virus, the kinetics of heat

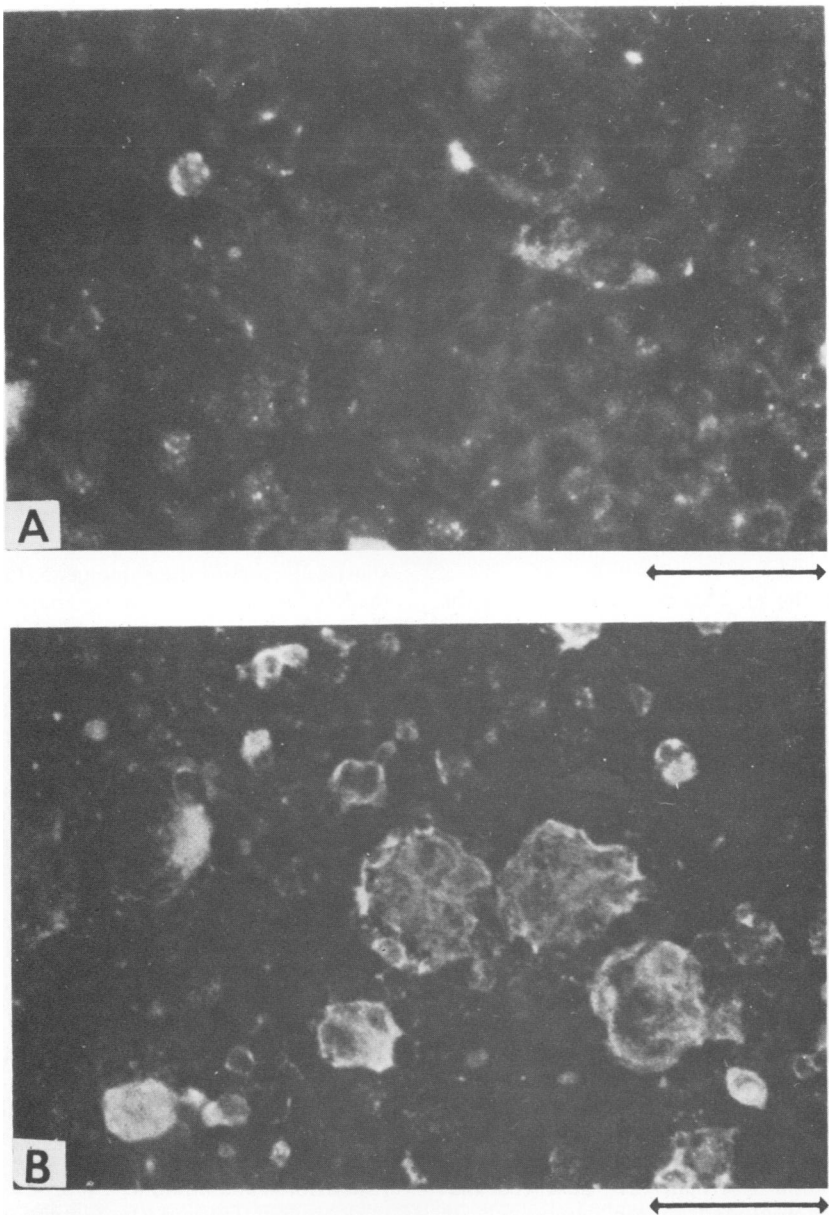

FIG. 4. Modulation of measles virus antigen expression by antimeasles antibody; immunofluorescent staining of NS20Y/MS cells. (A) Cells after 4 weeks of treatment with polyclonal rabbit antimeasles. For details, see the legend of Table 2. Acetone-fixed monolayers were indirectly stained with polyclonal rabbit antimeasles serum and fluorescein isothiocyanate goat anti-rabbit serum. Bar, 50 μm. (B) NS20Y/MS cells.

inactivation of *cs* mutants were compared with those of the parental virus. Parental measles virus was rapidly inactivated at 45°C, displaying linear inactivation kinetics (Fig. 6). *cs* viral isolates were considerably more resistant than the wild-type virus and retained 75% of their activity after 2 h at 45°C. Conversely, maintaining virus at 4°C for various periods of time or freezing the virus failed to indicate any differences in cryolability.

Although the nature of the lesion in the *cs* mutants will require more detailed molecular analysis, extracts of Vero cells infected with the cloned *cs* isolates at 33 and 39°C were subjected to polyacrylamide gel electrophoresis and transfer (Western blotting), and a representative result is shown in Fig. 7. At the permissive temperature, all measles polypeptides could be stained with rabbit antimeasles serum, whereas at 33°C, very few measles-specific proteins could be detected. Polyacrylamide gel electrophoretic analysis of the persistently infected cells indicated that both identifiable measles-specific proteins and a large number of breakdown products were found at all temperatures (data not shown). Although not definitive, the results suggest that the *cs* mutants might have a mutation in the viral polymerase.

Rapid development of persistent infection of neuroblastoma but not of Vero or HeLa cells by *cs* mutant. Infection of NS20Y neuroblastoma cells at 37°C with *cs* virus isolates resulted in the development of an immediate persistent infection. No CPE, crisis, or lysis characteristic of acute infection by wild-type measles virus could be detected. Virus isolated from the supernatants of infected cultures retained cold sensitivity. In contrast, infection of Vero or

TABLE 2. Effects of antimeasles antibody treatment on viral persistence

Cell line (treatment)	% IFA positive[a]	% Infectious centers[b]	PFU/ml[c]
I. NS20Y/MS	80	0.1	1×10^3
NS20Y/MS[d] (RαMS, 4 weeks)	Occasional single cells	0.001	0
NS20Y/MS[e] (RαMS, +4 weeks, −2 weeks)	40–50	NT[f]	5×10^1

[a] Percentage of cells in culture containing cytoplasmic measles virus antigens by indirect immunofluorescent staining.
[b] Percentage of cells producing infectious virus.
[c] Virus yields in culture supernatants at 37°C.
[d] NS20Y/MS cells were cultured with 50 μl of polyclonal rabbit antimeasles serum per ml for 4 weeks. The amount of antimeasles serum used was sufficient to neutralize 10^4 PFU of measles virus.
[e] NS20Y/MS cells were cultured with anti-serum for 4 weeks, and then the serum was removed. Two weeks later, cells were stained by indirect immunofluorescent staining, and viral titration of the supernatant was performed.
[f] NT, Not tested.

TABLE 3. Isolation of cold-sensitive mutants from NS20Y/MS supernatants

Isolate no.	Virus titer (PFU/0.2 ml)[a]		PE[b]
	33°C	39°C	
5	5×10^1	3×10^3	0.016
6	5×10^2	5×10^4	0.001
7	5×10^1	1×10^4	0.005
16C	1×10^1	3×10^4	0.0003
14C	1×10^1	4×10^4	0.00025
10	2×10^2	2×10^3	0.1
11	2×10^2	7×10^3	0.028
5B	1×10^1	7×10^3	0.00014
9B	1×10^1	2×10^3	0.005
32A	1×10^2	2×10^3	0.05

[a] Individual isolates were titrated for PFU on Vero cells at 33 and 39°C.
[b] The PE ratio = titer at 33°C/titer at 39°C. PE ratios of 10 of 40 isolates tested are presented. Ninety percent of isolates had PE ratios greater than 10^{-1}.

HeLa cell cultures with the *cs* mutants obtained from the NS20Y/MS cells resulted in a characteristic lytic infection leading to complete destruction of the host cells. These results suggest that the *cs* mutants are selected or adapted for persistent infection in the murine neuroblastoma cells, although not in more permissive tissue culture cell lines.

DISCUSSION

There is considerable evidence that measles viruses can persist in cells of the CNS in humans and in experimental animals (9, 12, 15, 26, 28, 36). From the study of culture cells which are persistently infected with virus, a number of mechanisms, by no means exclusive, have been described that might be relevant to persistence in vivo, including the development of viral mutants, host cell restrictions, immunological restrictions, defective interfering particles, and IFN (6, 10, 11, 16, 30, 33, 38).

The elegant studies on SSPE by Hall and co-workers (10, 11) indicated that host restrictions on viral replication indeed may exist in cells of the CNS. In this disease, the sera of patients were deficient in antibodies to the M protein, and in explants of CNS tissue, synthesis of M protein was selectively deficient. Miller and Corrigan (21) observed that although relatively undifferentiated neuroblastoma cells in culture underwent lytic infection with measles virus, papaverine- or cyclic AMP-treated cells, which appeared morphologically more differentiated, restricted the replication of measles virus. The present experiments were undertaken to explore the possibility that measles virus persistence in cells of CNS origin may have unique genetic aspects.

Clone NS20Y of the murine neuroblastoma cells C1300 was infected with measles virus and, after a transition to a carrier culture, became persistently infected. These cultures could not be cured by exposure to antimeasles antibodies for over 1 month, and single cell clones and subclones expressing measles antigens were obtained and characterized. In contrast to other reported cells persistently infected with measles virus (17), viral yields diminished when the cells were cultured at 33°C and increased when the cells were shifted to 39°C. Characterization of plaque-purified virus produced by these cells at 39°C indicated that the majority of isolates were *cs* in their replication, i.e., produced greater virus yields at 39 than at 33°C. None of these isolates complemented one another, suggesting that all isolates shared a lesion in the same gene. Of particular interest was the finding that the isolated *cs* mutants, although producing a typical measles lytic infection in Vero or HeLa cells at 37°C, induced persistent infection in the neuroblastoma cell line with no evidence of cythopathic changes or crisis. This result suggests that these mutants may be peculiarly adapted to persistence in the neuroblastoma cell line.

Although the factors governing measles virus persistence remain unclear, it was striking that cells maintained at a nonpermissive temperature produced more IFN than did those maintained at a permissive temperature. When cells at 33°C were cultured in the presence of anti-IFN antibodies, the majority of cells transformed into multinucleate giant cells within 96 h and assumed a morphological appearance of cells maintained at a permissive temperature, and infectious virus yields were generally 100-fold higher. Consonant with the work of Sekellick and Marcus (33), these experiments suggest that IFN and, in this case, its regulation by temperature are related to the maintenance of the persistent state in these cells.

It is not an unreasonable possibility that the nature and outcome of viral persistence in vivo are determined by multiple factors, including possible viral mutants, host cell restrictions, and immune responses. It was early established by Rustigian that persistent infections with measles virus in highly permissive cells could be established when the cultures were maintained in the presence of antimeasles anti-

TABLE 4. Complementation analysis of measles *cs* isolates

Isolate nos.[a]	Complementation index[b]
5 + 6	0.20
5 + 7	0.15
5 + 10	0.46
5 + 11	0.14
6 + 7	0.83
6 + 10	0.50
6 + 11	0.25
7 + 10	0.03
7 + 11	0.51
10 + 11	0.03

[a] Pairs of isolates tested.
[b] For an index of the complementation formula, see the text. A complementation index of less than 1 indicates no complementation.

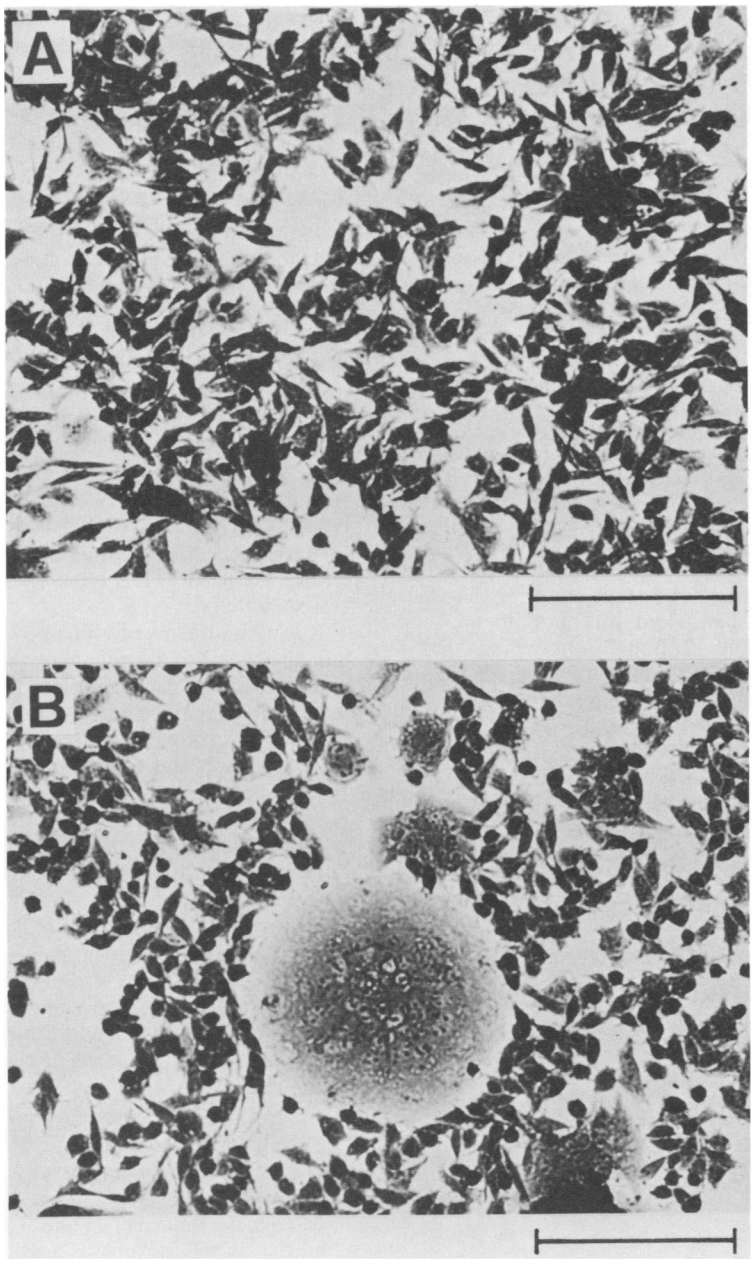

FIG. 5. Effect of anti-IFN serum on NS20Y/MS cells. (A) NS20Y/MS cells persistently infected with measles virus. (B) NS20Y/MS cells 2 days after incubation at 33°C with sheep anti-mouse IFN globulin and after Giemsa staining. Bar, 100 μm.

bodies (31). Joseph and Oldstone (16) have noted that antibody can modulate the expression of measles virus antigens in persistently or acutely infected cells, thus rendering them more resistant to immunological attack. Fujinami and Oldstone (7) have recently shown that antimeasles antibody can affect the synthesis of intracellular proteins as well as the expression of the surface HA. Rammohan et al. (28) have shown that the mouse-adapted HNT strain of measles virus produced persistent infections in the mouse CNS only when the animals were treated with antibody to measles virus HA. In the present experiments, we similarly observed that antibody to measles can markedly modulate the expression and production of measles virus in neuroblastoma cells in a reversible fashion. When persistently infected cells were cultured at 37°C in the presence of antibody, within 24 h no giant or multinucleated cells could be seen, and over the period of 1 month only very few cells expressing any cytoplasmic measles protein could be detected.

Although *cs* mutations in bacteriophage and yeasts have been very useful in genetic experiments (14), *cs* mutants of normally lytic mammalian viruses have not commonly been described (37). There are only two reports of *cs* mutants being isolated from persistently infected cells. In the case of reovirus type 2, this mutation was found to be associated with the s1 gene but did not predispose for establishing persistent infections in the absence of defective interfering particles (1, 18). Of particular interest are the studies of Stohlman et al. (34), who isolated *cs* mutants of the JHM

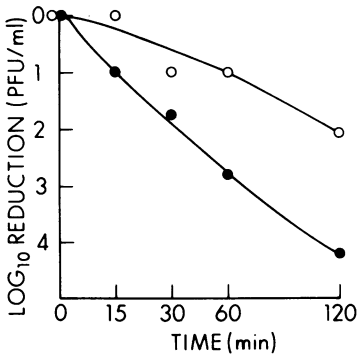

FIG. 6. Thermolability of parental and *cs* isolate of measles virus at 45°C. Undiluted virus stocks were incubated in a 45°C water bath. Samples were withdrawn into ice-cold medium at different times. Residual infectivity was determined by plaque assay at 39°C. Symbols: ○, parental virus; ●, *cs* isolate (*cs* 7).

strain of the murine hepatitis virus, a coronavirus related to the morbillivirus group, of which measles is a member. What makes this report of particular interest is the fact that this virus was isolated from a persistent infection in another murine neuroblastoma cell line. Although it may be a coincidence, it is striking that both *cs* isolates were derived from persistently infected neuroblastoma cells. The molecular nature of the lesion in the JHM isolate has not been elucidated; nevertheless, Stohlman et al. suggest that it may be defective in viral polymerase, a result quite consistent with our findings on the measles *cs* mutants, which produce few viral polypeptides in acute infection of Vero cells at a nonpermissive temperature.

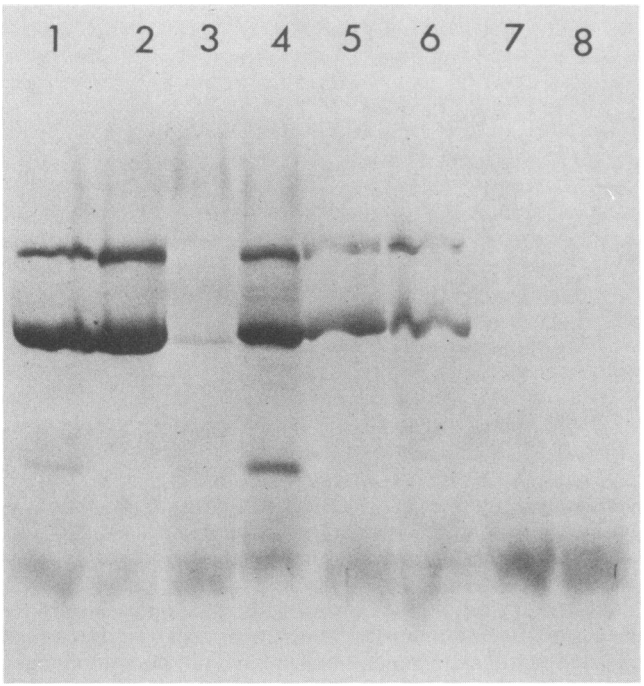

FIG. 7. Western blot of acute infections in Vero cells at two temperatures. Lanes stained with rabbit antimeasles and, peroxidase-conjugated goat anti-rabbit immunoglobulin, were prepared as follows (lane): 1, Edmonston at 33°C; 2, Edmonston at 39°C; 3, *cs* mutant (clone 6) at 33°C; 4, *cs* mutant at 39°C; 5, NS20Y/MS at 33°C for 5 days; 6, NS20Y/MS at 39°C for 5 days; 7, Uninfected NS20Y at 33°C; 8, Uninfected NS20Y at 39°C.

Consistent with the possibility that growth and replication of measles virus may be restricted selectively by different cells of the CNS are the observations of Lucas et al. (19), who found that measles virus replicated at 33°C in a rat schwannoma cell line (RN2-2) without causing overt CPE. Upon a temperature shift from 33 to 40°C, the production of measles virus from the persistently infected cells rapidly diminished, suggesting the persistence of *ts* mutants in schwannoma cells. Viral mutants with different tropisms to cells in the CNS have been generated in studies with Semliki Forest virus in mice and in neuroblastoma cells (2), as well as with reovirus (5). Infection of mice with wild-type Semliki Forest virus produced a lethal infection resulting from the destruction of neurons; in contrast, demyelination was seen in animals receiving mutant Semliki Forest virus which produced destruction of oligodendrocytes. In vitro studies indicated that neuroblastoma cells infected with Semliki Forest virus mutants survived, whereas those infected with wild-type virus were lysed. Conversely, glioma cells were lysed by all mutants as well as by wild-type virus. In the case of reovirus, antigenic mutants selected by monoclonal antibodies in the viral HA have been shown to determine target cell specificity.

It is by no means obvious how *cs* mutants of measles virus can be related to chronic neurological diseases of humans. A great deal more has to be learned about the nature of the *cs* lesion, its specificity or selectivity for cells of the CNS, particularly primary cells, and the factors which regulate their selection and growth of *cs* mutants. Nevertheless, that this novel type of mutant could be involved in human disease is suggested by the results of McKimm-Breschkin et al. (20), who observed that the Hallé isolate of SSPE virus was composed of two stable plaque variants. One, termed SYN^+, was found to be similar to laboratory-adapted measles virus; however, the second, SYN^-, plaqued and formed syncytia more efficiently at 39 than at 33°C. It is probable that this SSPE isolate was indeed a *cs* mutant. It will clearly be of interest to explore whether the measles *cs* mutants, in the presence or absence of antibodies, are capable of establishing persistent infection of cells of the CNS in experimental animals.

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LITERATURE CITED

1. **Ahmed, R., R. S. Kauffman, and B. N. Fields.** 1983. Genetic variation during persistent reovirus infection isolation of cold-sensitive and temperature-sensitive mutants from persistently infected L cells: role in maintenance of the persistent state. Virology **131**:79–87.
2. **Atkins, G. J., and B. J. Sheahan.** 1982. Semliki Forest virus neurovirulence mutants have altered cytopathogenicity for central nervous system cells. Infect. Immun. **36**:333–341.
3. **Bergholz, C. M., M. P. Kiley, and R. E. Payne.** 1975. Isolation and characterization of temperature-sensitive mutants of measles virus. J. Virol. **16**:192–202.
4. **Birrer, M. J., B. R. Bloom, and S. Udem.** 1981. Characterization of measles polypeptides by monoclonal antibodies. Virol. **108**:381.

5. **Dale, R., R. T. Spriggs, T. Bronson, and B. N. Fields.** 1983. Hemagglutinin variants of reovirus type 3 have altered central nervous system tropism. Science, **220**:505–507.
6. **Fisher, L. E., and F. Rapp.** 1979. Role of virus variants and cells in maintenance of persistent infection by measles virus. J. Virol. **30**:64–68.
7. **Fujinami, R. S., and M. B. A. Oldstone.** 1980. Alterations in expression of measles virus polypeptides by antibody: molecular events in antibody induced antigenic modulation. J. Immunol. **125**:78–85.
8. **Gresser, I., M. G. Tovey, M. T. Bandu, C. Maury, and D. Brouty-Boye.** 1976. Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by use of anti-interferon serum. J. Exp. Med. **144**:1305–1315.
9. **Haase, A. T., P. Ventura, C. J. Gibbs Jr., and W. A. Tourtellotte.** 1981. Measles virus nucleotide sequences: detection by hybridization *in situ*. Science **212**:672–675.
10. **Hall, W., and P. Choppin.** 1979. Measles and subacute sclerosing panencephalitis virus proteins: lack of antibodies to the M protein in patients with subacute sclerosing panencephalitis. Proc. Natl. Acad. Sci. U.S.A. **76**:2047–2051.
11. **Hall, W., R. Lamb, and P. Choppin.** 1981. Measles virus proteins in the brain tissue of patients with subacute sclerosing panencephalitis: absence of the M protein. N. Engl. J. Med. **304**:1152–1155.
12. **Haspel, M. V., P. R. Knight, R. G. Duff, and F. Rapp.** 1973. Activation of a latent measles virus infection in hamster cells. J. Virol. **12**:690–695.
13. **Horta-Barbosa, L., D. A. Fuccillo, W. T. Lonclon, J. T. J. Jabbove, and J. L. Sever.** 1969. Isolation of measles virus from brain cell cultures of two patients with subacute sclerosing panencephalitis. Proc. Soc. Exp. Biol. Med. **132**:272–277.
14. **Jarvik, J., and D. Botstein.** 1973. A genetic method for determining the order of events in a biological pathway. Proc. Natl. Acad. Sci. U.S.A. **70**:2046–2050.
15. **Johnson, R. T.** 1975. The possible viral etiology of multiple sclerosis. Adv. Neurol. **13**:1–46.
16. **Joseph, B. S., and M. B. A. Oldstone.** 1975. Immunologic injury in measles virus infection. II. Suppression of immune injury through antigenic modulation. J. Exp. Med. **148**:864–873.
17. **Ju Grace, S. Udem, B. Rager-Zisman, and Barry R. Bloom.** 1978. Isolation of a heterogeneous population of temperature-sensitive mutants of measles virus from persistently infected human lymphoblastoid cell lines. J. Exp. Med. **147**:1637–1652.
18. **Kauffman, R. S., R. Ahmed, and B. N. Fields.** 1983. Selection of a mutant s1 gene during reovirus persistent infection of L cells: role in maintenance of the persistent state. Virology **131**:79–87.
19. **Lucas, A., M. Coulter, R. Anderson, S. Dales, and W. Flintoff.** 1978. *In vivo* and *in vitro* models of demyelinating diseases. II. Persistence of host regulated thermosensitivity in cells of neural derivation infected with mouse hepatitis and measles viruses. Virology **88**:325–327.
20. **McKimm-Breschkin, J. L., A. M. Breschkin, and F. Rapp.** 1982. Characterization of the Halle SSPE measles virus isolate. J. Gen. Virol. **58**:57–64.
21. **Miller, C., and D. R. Corrigan.** 1982. Reversible expression and activation of measles virus infection in neuronal cells. Proc. Natl. Acad. Sci. U.S.A. **79**:1629–1633.
22. **Millonig, G.** 1961. Advantages of a phosphate buffer for OsO_4 solutions in fixation. J. Appl. Physiol. **32**:1637–1643.
23. **Minagawa, T., T. Sakuma, S. Kuwajima, T. K. Yamamota, and H. Iida.** 1976. Characterization of measles virus in establishment of persistent infections in human lymphoid cell line. J. Gen. Virol. **33**:361–379.
24. **Norrby, E., S. N. Chen, T. Togashi, H. Shesbaradaran, and K. P. Johnson.** 1982. Five measles virus antigens demonstrated by use of mouse hybridoma antibodies in productively infected tissue culture cells. Arch. Virol. **71**:1–11.
25. **Nowakowski, M., J. D. Feldman, S. Kano, and B. R. Bloom.** 1973. The production of vesicular stomatitis virus by antigen or mitogen stimulated lymphocytes and continuous lymphoblastoid lines. J. Exp. Med. **137**:1042–1059.
26. **Payne, F. E., J. V. Baublis, and H. H. Itabashi.** 1969. Isolation of measles virus from cell cultures of brain from a patient with subacute sclerosis panencephalitis. N. Engl. J. Med. **281**:585–590.
27. **Rager-Zisman, B., and T. C. Merigan.** 1973. A useful semiquantitative method for viral plaques assay. Proc. Soc. Exp. Biol. Med. **142**:1174.
28. **Rammohan, K. W., H. F. McFarland, and D. E. McFarlin.** 1981. Induction of subacute murine measles encephalitis by monoclonal antibody to virus hemagglutinin. Nature (London) **290**:588–589.
29. **Rentier, B., A. Claysmith, W. J. Bellini, and M. Dubois-Dalq.** Chronic measles virus infection of mouse nerve cells in vitro. 1981. *In* D. H. L. Bishop and R. W. Compans (ed.), The replication of negative strand viruses. Elsevier/North-Holland Publishing Co., Amsterdam.
30. **Rima, B. K., W. B. Davidson, and S. J. Martin.** 1977. The role of defective interfering particles in persistent infection of Vero cells by measles virus. J. Gen. Virol. **35**:89–97.
31. **Rustigian, R.** 1966. Persistent infection of cells in culture by measles virus. I. Development and characteristics of HeLa sublines persistently infected with complete virus. J. Bacteriol. **92**:1792–1804.
32. **Schubert D., S. Humphreys, C. Baromi, and M. Cohn.** 1969. *In vitro* differentiation of a mouse neuroblastoma. Proc. Natl. Acad. Sci. U.S.A. **64**:316–323.
33. **Sekellick, M. J., and P. E. Marcus.** 1978. Persistent infection. I. Interferon-inducing defective-interfering particles as mediators of cell sparing: possible role in persistent infection by vesicular stomatitis virus. Virology **85**:175–186.
34. **Stohlman, S. A., A. Y. Sakaguchi, and L. P. Weiner.** 1979. Characterization of the cold sensitive murine hepatitis virus mutants rescued from latently infected cells by cell fusion. Virology **98**:448–455.
35. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
36. **Wear, D. J., and F. Rapp.** 1978. Latent measles virus infection of hamster central nervous system. J. Immunol. **107**:1593–1598.
37. **Wright, P. J., and P. D. Cooper.** 1974. Isolation of cold-sensitive mutants of poliovirus. Intervirology **2**:20–24.
38. **Youngner, J. S., and O. T. Preble.** 1980. Viral persistence: evolution of viral populations, p. 73–165. *In* H. Fraenkel-Conrat and R. Wagner (ed.), Comprehensive virology (vol. 16). Plenum Publishing Corp., New York.