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Measles Virus Infection of Mouse Neuroblastoma (C 1300) Cells (40933)

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Abstract. C 1300 mouse neuroblastoma cells were permissively infected with measles virus. No cytopathic changes were observed in light microscopy although yields of infective virus comparable to those of infected Vero cells were demonstrable. Persistently infected measles virus carrier cell lines were established. The growth rate of these cells was reduced and they exhibited a higher degree of morphological differentiation than uninfected neuroblastoma cells. Infected cells demonstrated hemagglutinin and hemolysin activities on the cell surface. Electron microscopy revealed accumulation of viral nucleocapsids in the cytoplasm but no viral components in the nuclei. In association with the measles virus infection a marked reduction of acetylcholinesterase activity of C 1300 cells was detected.

Measles virus carrier cultures have been established with a variety of cells of different species (1). From these persistently infected cultures low levels of infective virus are continuously shed into the culture medium and, as a rule, cytopathic changes are present. Some of the carrier lines have been of brain cell origin (2–7) but few have been derived from neural cells. Kobune *et al.* (7) reported restricted infection without measles virus production and cytopathogenicity in an established rat glioma line while two human cells, the neuroblastoma IMR-32 line and the 118 MGC glioma line, both produced infective virus and exhibited cytopathic changes.

The established neuroblastoma cell lines are of particular interest because they offer the possibility of observing interactions of the virus with clones of neuron-like cells. No interference from non-neuronal cells present in the cultures need be considered. Neuroblastoma cell lines have maintained many of the characteristics ascribed to neurons, such as the capacity for morphological differentiation (8) and synthesis of neurotransmitters (9) and acetylcholinesterase (10). The present study describes the measles virus infection of a mouse neuroblastoma cell line (C 1300) which, upon passage of infected cultures, becomes a virus-producing carrier line. We have observed changes in the acetylcholinesterase activity of the measles virus-infected C 1300 neuroblastoma cells.

Materials and methods. Cells. Vero cells and clone 41 A_3 of C 1300 mouse neuroblastoma cells were used. A characterization of the C 1300 cell line has been given previously (11, 12). All cells were cultured as monolayers in plastic bottles (250 ml), tubes, and 5-cm dishes (Nunc, Denmark). Eagle's minimal essential medium (MEM) was used, supplemented with 10% fetal calf serum, 100 IU of penicillin, and 100 μg of streptomycin per milliliter. The C 1300 cells received in addition 4.5 g of glucose per liter of medium. For maintenance the same medium supplemented with only 2% serum was employed. Cell counting was performed according to standard methods.

Virus. The Edmonston strain of measles virus was used in all experiments. The strain (NIH research reference strain) was passaged at high dilution in monkey kidney cells.

Titrations of infectivity. To plaque the virus, Vero cells grown in 5-cm dishes were inoculated with 0.5 ml of virus suspension and after 120 min of adsorption at room temperature they were overlaid with maintenance medium containing 1% methylcellulose. The plaques were counted after the dishes were incubated for 5 days at 37°.

Fifty percent end-point titrations were carried out in tube cultures of Vero cells inoculated with 0.1 ml of tenfold serial virus dilutions. Five cultures were infected with each dilution. The infected cultures were incubated at 37° and read daily for presence of cytopathic changes. Final readings ($TCID_{50}$) were performed on Day 10.

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Hemadsorption and hemolysin activity. Infected and uninfected bottle cultures were washed four times with phosphate-buffered saline and 10 ml of a 2% suspension of fresh Cynomolgus monkey erythrocytes was added to each culture. The cultures were left at room temperature for 30 min and then carefully rinsed four times with buffer and examined by light microscopy or incubated at 37° to test the hemolysin activity. After 2 hr of incubation the overlaying buffer was cleared by low-speed centrifugation and assayed for released hemoglobin in a hemocytometer.

Electron microscopy. Infected and uninfected bottle cultures were fixed *in situ* using 1% glutaraldehyde buffered with 0.15 *M* sodium cacodylate, pH 7.4, for 15 min, followed by treatment for 1 hr with 1% OsO_4 buffered with 0.2 *M* sodium cacodylate. Dehydration was achieved by consecutive treatments with increasing concentrations of ethyl alcohol. The fixed and dehydrated cells were scraped off and pelleted by low-speed centrifugation, transferred into Epon 812 containing 50% propyleneoxid, left overnight and embedded in Epon. Sections were stained with lead citrate and uranyl acetate and observed in a Philips' 301 electron microscope.

Acetylcholinesterase activity. The acetylcholinesterase activity was assayed according to Ellman *et al.* (13) with some modifications. Infected and uninfected bottle cultures were washed three times with ice-cold Hanks' buffer, pH 7.2. The cells were scraped off into 5 ml of 0.1 *M* Na_2HPO_4 buffer adjusted to pH 8 with 0.1 *M* KH_2PO_4 containing 1 μl of Triton X-100 per 100 ml. After cell counting, the different suspensions were adjusted to contain 10^6 cells per milliliter. The suspended cells were disrupted by sonification at 1.35 A (two times for 30 sec) in a Raytheon ultrasonicator. To 100-μl samples of disrupted cells, 1.0 ml of the buffer, 20 ml of 0.75 m*M* acetylcholine iodide in buffer, and 100 μl of 0.01 *M* dithiobisnitrobenzoic acid (DTNB) in buffer were added. The reaction mixtures were left at 20° for 30 min and then immediately read for light adsorbance at 412 nm in a spectrophotometer (Abs sample). Reaction mixtures without acetylcholine iodide (Abs_1), buffer with DTNB and acetylcholine iodide (Abs_2), and buffer with DTNB only (Abs_3) were used as blanks. Protein contents were assayed according to Lowry *et al.* (14). Acetylcholinesterase activity (E) was expressed as nanomoles of thiocoline released per 30 min per milligram of protein and was calculated from the formula:

$$E = \frac{8.97}{\text{protein content of sample}} \times \text{Abs sample} - (Abs_1 + Abs_2 - Abs_3).$$

Results. Infection of C 1300 cells with measles virus. Tube cultures of C 1300 were infected with tenfold dilutions of virus, five cultures per dilution. The cultures were incubated at 37° in a roller and read daily for cytopathic changes. As controls cultures of Vero cells were infected with the same virus suspensions. No cytopathic changes were detected in inoculated C 1300 cell cultures by light microscopy, while the Vero cell controls demonstrated titers of cytopathogenicity of 4.5 to 6.6 log $TCID_{50}$.

The adsorption rate of measles virus was studied on suspended Vero and C 1300 cells prepared from monolayer cultures by trypsinization. Suspended cells were washed three times in Eagle's MEM and allowed to rest in maintenance medium for 12 hr at 37° before use in adsorption studies to avoid influence on adsorption by the trypsinization. To 0.9 ml of cell suspension containing 10^7 cells per ml, 0.1 ml of the measles virus suspension, 10^6 PFU/ml, was added. The cell–virus mixtures were kept agitated in a water bath at 37° during the adsorption. Aliquots, 100 μl each, were collected immediately after admixture of the virus and then after intervals ranging from 30 to 240 min. The samples were diluted 1:100 in Hanks' buffer, centrifuged at low speed to remove cells, and assayed by plaque titrations. The percentage nonadsorbed virus was calculated after correction for loss of infectivity in cell-free controls. The results (Fig. 1) demonstrated that measles virus adsorbed to C 1300 and Vero cells at about the same rate; 25 to 35% of the inoculated virus were recovered as nonadsorbed at 4 hr after mixing of virus and cells.

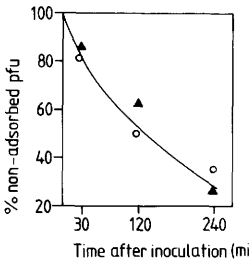

FIG. 1. Adsorption of measles virus to suspended C 1300 (open circles) and Vero (filled triangles) cells. The percentage infectivity remaining nonadsorbed in the medium, after correction for infectivity lost in cell free controls, is plotted against time.

In another set of cultures the production of measles virus in C 1300 was observed. Vero and C 1300 cell cultures were inoculated, 0.1 PFU per cell, and after 2 hr of adsorption the cultures were washed four times with Hanks' buffer and incubated with maintenance medium at 37°. At varying periods of time, three cultures of each cell type were collected. Cells and fluid phases were separately frozen, thawed, sonicated, and titrated for infective measles virus. The results (Fig. 2) revealed that the measles virus-infected neuroblastoma cells, although they did not exhibit cytopathic changes detectable by light microscopy on fixed and stained cells, yielded infective virus in concentrations comparable to those of the Vero cell cultures. Infected C 1300 cells exhibited hemagglutinin on plasma membranes as indicated by the hemadsorption of monkey erythrocytes.

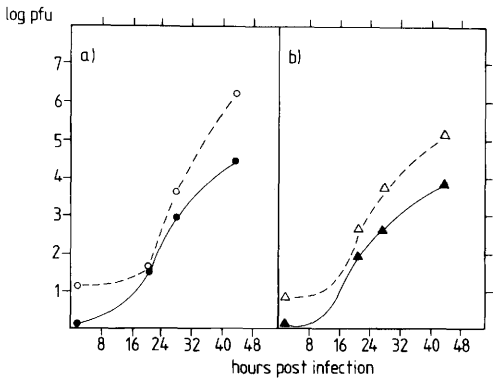

FIG. 2. Production of measles virus in C 1300 cells (a) and in Vero cells (b). The open symbols and dotted lines represent cell-associated infectivity; the filled symbols and solid lines represent virus infectivity found in the overlaying medium.

Establishment of persistent measles virus infection in C 1300 cells. Monolayer bottle cultures of C 1300 cells were infected with measles virus at a multiplicity of infection of 1 PFU per cell. After 48 hr of incubation at 37° none of the cultures showed signs of cytopathic effect (cpe) and could readily be subcultivated by dividing one culture into two every third day. Culture fluids were regularly tested for measles virus at the time of subcultivation by inoculating 0.5-ml aliquots on the Vero cell monolayer. Two milliliters of the same fluid was frozen at −70° for plaque titrations later.

A persistent state of infection was obtained with all the infected cultures. The persistently infected cells (PI cells) have since been passaged more than 100 times. They consistently gave high yields of measles virus (5×10^5 to 10^7 PFU/ml).

The PI cells showed a higher degree of morphological differentiation than uninfected C 1300 cells (Fig. 3). Both hemagglutinins and hemolysins were detected on the PI cell plasma membranes (Table I).

Persistently infected and uninfected C 1300 cells were suspended in growth medium (10,000 cells/ml), seeded into petri dishes (5 ml/dish), and incubated at 37° in CO_2 atmosphere. After 24, 48, and 72 hr of incubation five cultures of each kind were collected and the cell numbers per dish were counted. After 72 hr of incubation petri dish cultures seeded with uninfected C 1300 cells contained twice as many cells as dish cultures seeded with the same number of PI cells (passage 80, Fig. 4). Thus, the growth rate of the PI cells was regularly lower than that of uninfected C 1300 cells.

Electron microscopy. Bottle cultures of C 1300 cells infected at a multiplicity of 0.5 PFU per cell and incubated for 48 hr at 37° were examined by transmission electron microscopy. More than 60% of the cells contained nucleocapsids and tubular structures in the cytoplasm and areas with thickening of the plasma membrane, compatible with spikes and M-protein structures at sites for virus budding (Fig. 5). However, no nucleocapsids were detected in the nuclei of infected cells.

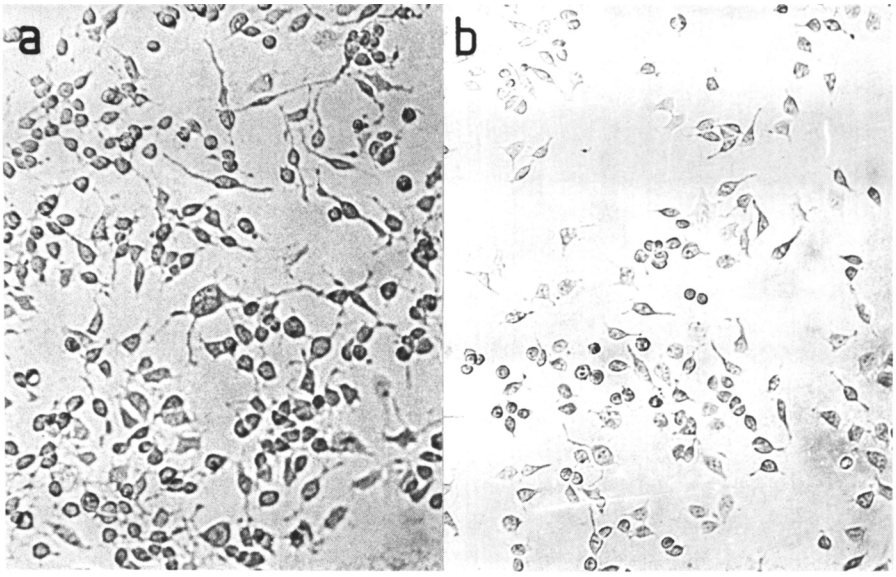

FIG. 3. Light microscopy of PI cells, passage 80 (a), and uninfected C 1300 cells (b).

Electron microscopy was also performed on PI cells (passage numbers 3, 10, 11, and 13). In the cytoplasm all cells examined contained zones lacking ordinary cytoplasmic structure surrounded by big aggregates of densely packed rough tubules of nucleocapsids with an outer diameter of 15 nm and an inner diameter of 4 nm (Fig. 6). PI cell nuclei were free from nucleocapsids. Plasma membrane alterations, i.e., thickening and surface projections as well as budding viruses, were observed.

Acetylcholinesterase activity. C 1300 cells and Vero cells infected at a multiplicity of 1 PFU per cell as well as mock-infected cells were incubated at 37° for 24 hr and assayed for acetylcholinesterase. The following controls were included in the experiments. The specificity of the enzyme activity assayed was ascertained by blocking with 10^{-6} *M* eserine. Cultures inoculated with uv-inactivated virus and virus neutralized by a measles antiserum were used as controls of specificity of the virus infection. Vero cell cultures were included to ensure cellular specificity of the enzyme activity determined. Table II shows that acute as well as persistent measles virus infection of C 1300 cells markedly reduced the apparent acetylcholinesterase activity of the cells. The thiocholine released (as nanomoles released per milligram of

TABLE I. HEMADSORPTION AND HEMOLYTIC ACTIVITY OF PI-1 CELLS

Cells	Passage number	Hemadsorption	Hemolytic activity
PI-1	1	+	NT[a]
	2	+	NT
	28	+	NT
	48	+	145[b]
	49	+	125
	50	+	100
	51	+	140
Uninfected C 1300 cells	—	—	0

[a] NT, not tested.
[b] released hemoglobin (mg/liter).

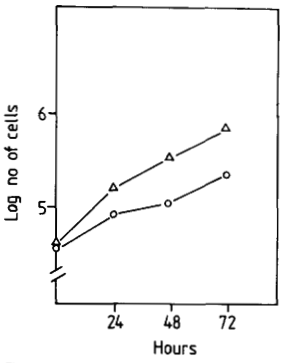

FIG. 4. Growth rate determination of PI cells (○——○) and uninfected C 1300 cells (△——△).

protein/30 min) was less than 20 to 30% of the amount released from noninfected cultures.

Discussion. Cultured neuroblastoma cells offer favorable possibilities for observing interactions of virus and neuron-like cells because neuroblastoma cells maintain many neuronal characteristics (8, 9), including the capacity to synthesize acetylcholinesterase (10). We found that mouse neuroblastoma C 1300 cells were permissively infected with measles virus. They demonstrated nucleocapsids in the cytoplasm, hemagglutinin on the plasma

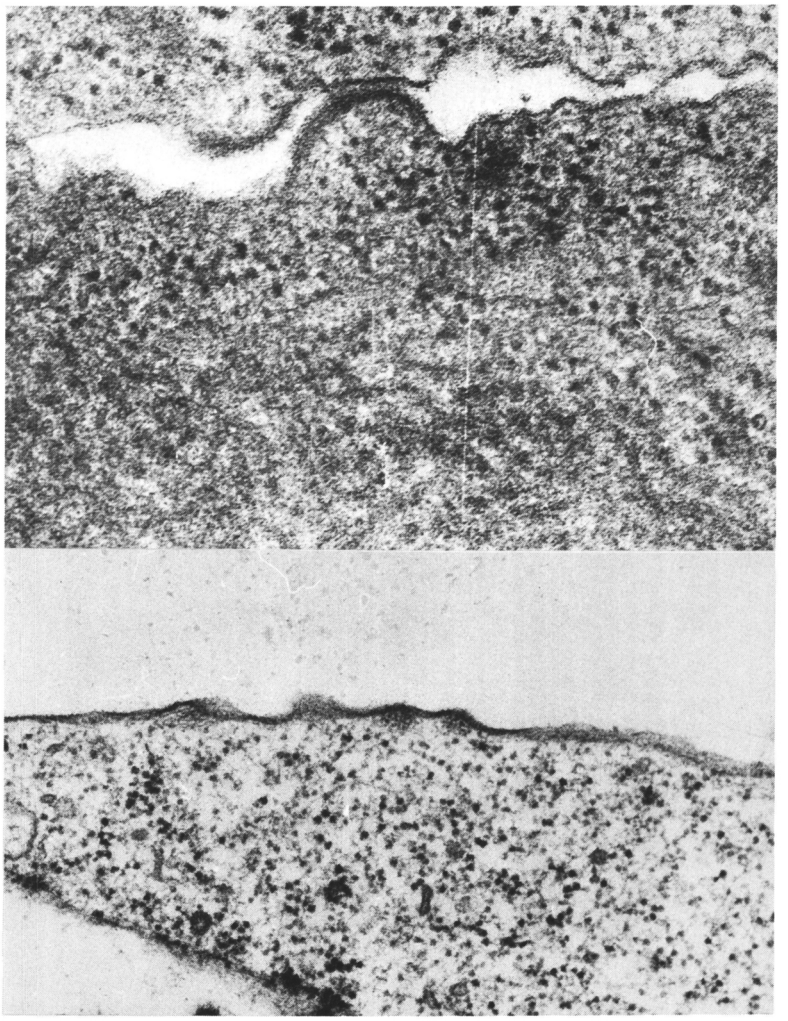

FIG. 5. Transmission electron microscopy of measles virus-infected C 1300 cells. Magnification, ×110,000 (top) and ×56,000 (bottom). Note the accumulation of nucleocapsids in the cytoplasm, and areas with thickened plasma membrane corresponding to M proteins and spikes at the sites of virus budding.

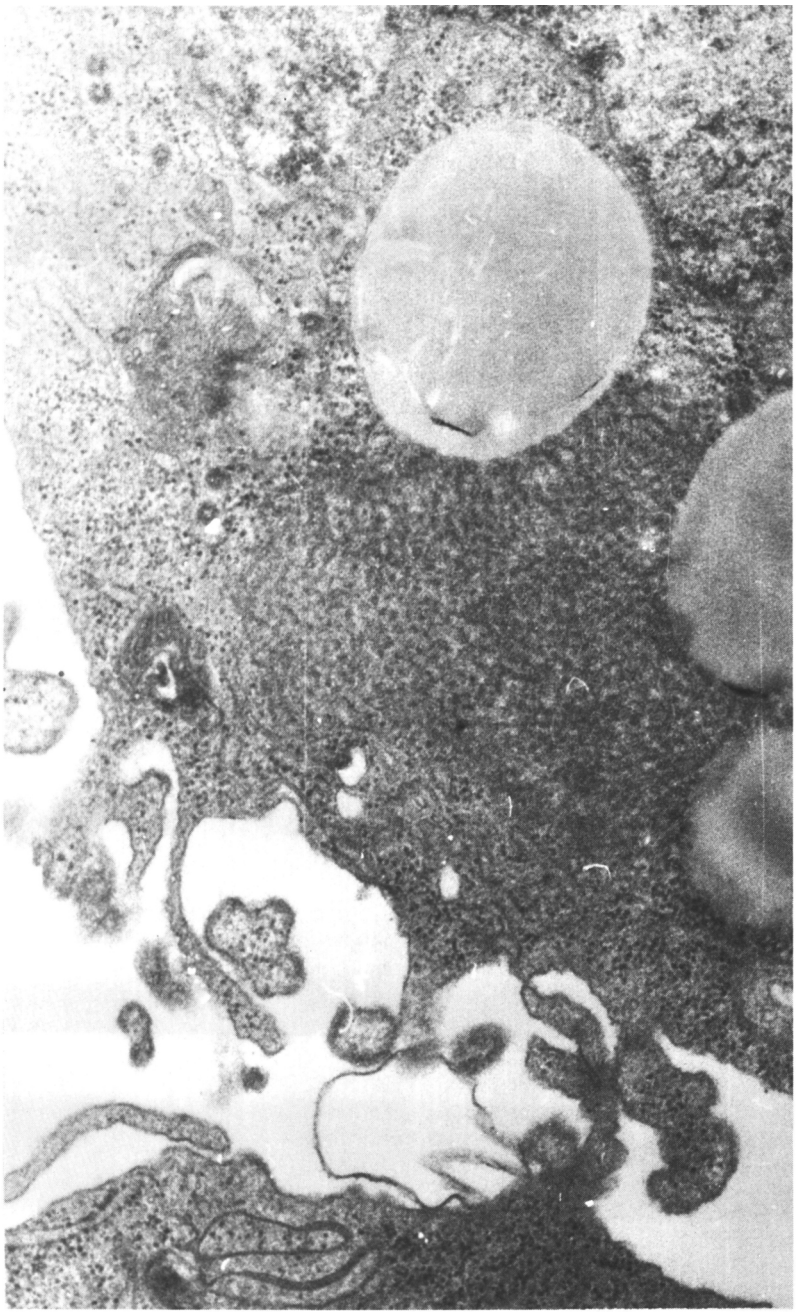

FIG. 6. Transmission electron microscopy of P-1 cells (P-8) (×35,000). Note accumulation of nucleocapsides in the cytoplasm and areas lacking ordinary cytoplastic structure.

membrane, and the morphological appearance of budding virions. No nucleocapsids were seen intranuclearly, a finding which is in accord with the observations of Kobune *et al.* (7) on the human neuroblastoma IMR-32 cells. Possibly the absence of intranuclearly located viral components is a prerequisite for the ability of the measles

TABLE II. EFFECT OF MEASLES VIRUS INFECTION ON ACETYLCHOLINESTERASE ACTIVITY OF C 1300 NEUROBLASTOMA CELLS

	Eserine						
	C 1300 cells						
	Infected[a]		Uninfected		PI[b]		Vero cells
Expt.	−	+	−	+	−	+	−
1	79 ± 9	0	260 ± 19	0	NT[c]	NT	0
2	41 ± 8	0	170 ± 25	0	NT	NT	0
3	NT	NT	190 ± 7	0	38 ± 10	0	NT
4	NT	NT	240 ± 18	0	64 ± 14	0	NT

[a] Infected at a m.o.i. of 1 PFU/cell. Tested 24 hr p.i.
[b] Persistently infected cells. Passage number.
[c] Eserine, 10^{-6} *M*, was added to block acetylcholinesterase activity.
[d] Acetylcholinesterase activity expressed as nanomoles of thiocholine released per milligram of protein/30 min. Means and ± SEM; $n = 6$.
[e] NT = not tested.

virus-infected cells to divide—as pointed out by Norrby (15—and thus for the C 1300 cells to become virus carriers.

Our most interesting finding, however, was the reduction in acetylcholinesterase activity of the measles virus-infected C 1300 cells. As the virus-infected cells, although replicating virus, maintained their morphological appearence or even exhibited a higher degree of morphological differentiation (PI cells), their reduced ability to produce acetylcholinesterases might be considered as a measles virus-induced dysfunction. A striking characteristic of measles encephalopathy in experimentally infected hamsters and mice is the specific neuronal tropism of the virus (16). In the subacute sclerosing panencephalitis (SSPE) a pronounced neuronal loss in the cerebral cortex is a characteristic feature (17), suggesting that somehow virus−neuron interactions influence SSPE pathogenesis. Such interactions do not necessarily only imply effects of virus cytopathogenic proteins. Incipient symptoms of SSPE might reflect more subtle neuronal dysfunctions. In systems of culture cells displaying complex functional properties virus infection may lead to low levels of so-called luxury functions without concomitant death of the cells (18), and in experimentally infected mice acute herpes simplex virus encephalitis stimulates a rise in the synthesis of monoaminergic neurotransmitters (19, 20).

1. Morgan, E. M., and Rapp, F., Bact. Rev. **41**, 636 (1977).
2. MacIntyre, E. H., and Armstrong, J. A., Nature (London) **263**, 232 (1976).
3. Gibson, P. E., and Bell, T. M., Arch. Ges. Virusforsch. **37**, 45 (1972).
4. Bather, R., Furecz, J., Fanok, A. G., Gill, S. D., and Yarosh, W., J. Gen. Virol. **20**, 401 (1973).
5. Fleury, H., and Pasquier, P. D., J. Neuropathol. Exp. Neurol. **36**, 842 (1977).
6. Webb, H. E., Illavia, S. J., and Lawrence, G. D., Lancet **1**, 4 (1971).
7. Kobune, K., Yamanouchi, K., Yoshikawa, Y., Hayami, M., and Shishido, A. Arch. Virol. **61**, 115 (1979).
8. Prasad, K. N., and Hsie, A. W., Nature New Biol. **233**, 141 (1971).
9. Narotzky, R., and Bondareff, W., J. Cell Biol. **63**, 64 (1974).
10. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R., and Nirenberg, M., Proc. Nat. Acad. Sci. USA **67**, 786 (1970).
11. Augusti-Tocco, G., and Sato, G., Proc. Nat. Acad. Sci. USA **64**, 311 (1969).
12. Vahlne, A., "Studies on Interactions of Herpes Simplex Virus and Neural Cells," Thesis, Göteborg (1978).
13. Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M., Biochem. Pharmacol. **1**, 88 (1961).
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
15. Norrby, E., Microbios. **5**, 31 (1972).
16. Baringer, J. R., and Griffith, J. F., Lab. Invest. **23**, 335 (1970).
17. ter Meulen, V., Katz, M., and Müller, D., Curr. Topics Microbiol. Immunol. **57**, 1 (1972).

18. Oldstone, M. B., Holmstoen, J., and Welsh, R. M., Jr., J. Cell. Physiol. **91**, (1977).

19. Lycke, E., Modigh, K., and Roos, B.-E., Brain Res. **23**, 235 (1970).

20. Lycke, E., and Roos, B.-E., Brain Res. **44**, 603 (1972).

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