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## Mumps Virus-induced Alterations in Cellular Excitability During Persistent Infections

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### SUMMARY

Persistent mumps virus infections were established in rat pheochromocytoma (PC-12) and human medulloblastoma (TE-671) continuous cell lines. Significant amounts of infectious virus were produced by the PC-12 cells; infectious virus production by the TE-671 cells was limited. This restricted replication may be due to decreased production of viral envelope glycoproteins by TE-671 cells. The presence of virus changed the distribution of stimulus-evoked electrical responsiveness of both cell lines from responsiveness composed primarily of normal, rapidly rising, all-or-nothing action potentials to one dominated by abnormal, slowly rising, graded responses or by no response at all. Such changes have the potential to disrupt neural integration within the nervous system, and suggest a new mechanism by which persistent virus infections might play a role in chronic neurological and/or mental disease.

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Mumps virus infections commonly involve the central nervous system and, indeed, this virus is the single most common cause of an acute aseptic meningitis and a mild encephalitis (Johnson, 1982; Wolinsky & Sever, 1985). However, various neurological symptoms (including ataxia and psychological disorders) can follow acute infections and have been reported to be associated with elevated mumps virus antibody titres in the cerebrospinal fluid years after the infection (Koskiniemi *et al.*, 1981, 1983; Vaheri *et al.*, 1982; Julkunen *et al.*, 1985). These long term effects of mumps virus infection could be due to irreversible brain damage following the acute infection or to a chronic infectious process.

Other members of the paramyxovirus family cause persistent infections of the brain. Canine distemper virus and a measles virus variant which causes subacute sclerosing panencephalitis are prominent examples. Both of these viruses persistently infect neural cell lines and alter membrane receptor-associated neural function in these cells (Halbach & Koschel, 1979; Koschel & Muenzel, 1980; Muenzel & Koschel, 1982).

These modifications of cellular 'luxury functions' [functions not necessary for cell survival (Oldstone *et al.*, 1977)] prompted us to examine the possibility that persistent mumps virus infection could alter another membrane-associated luxury function, excitability. This type of alteration has been demonstrated in acute, and in reactivating latent herpesvirus infections (Fukuda & Kurata 1981; Oakes *et al.*, 1981; Kiraly & Dolivo, 1982; Fukuda *et al.*, 1983; Mayer *et al.*, 1985, 1986), but has not been identified as a component of persistent virus pathogenesis. This report describes mumps infectious virus and antigen synthesis and virus-induced alterations of stimulus-evoked action potentials (SEAPs) in rat PC-12 pheochromocytoma and human TE-671 medulloblastoma cells persistently infected with mumps virus. A brief preliminary report of these data has been presented elsewhere (Ziegler & Stauffer, 1986).

Cultures of mock-infected (MI) and persistent mumps virus-infected (PI) PC-12 and TE-671 cells were established in the following manner. Five × 10<sup>4</sup> PC-12 and TE-671 cells were placed in separate wells of a 24-well plastic tissue culture plate and cultured 48 to 72 h, until 60% to 70%

Table 1. *Viral antigen and infectious virus production in persistently infected cultures*

Cells	Supernatant virus titre*	Antigen-positive cells (%)†			
		NP	HN	M	F
PC-12 (P <sub>4</sub> )‡	1.4 × 10 <sup>6</sup>	95	95	95	95
PC-12 (P <sub>8</sub> )	3.0 × 10 <sup>4</sup>	80	ND§	ND	ND
PC-12 (P <sub>12</sub> )	8.3 × 10 <sup>3</sup>	60	60	60	60
TE-671 (P <sub>4</sub> )	4 × 10 <sup>2</sup>	75	ND	ND	ND
TE-671 (P <sub>8</sub> )	1 × 10 <sup>1</sup>	80	18	11	0.02
TE-671 (P <sub>24</sub> )	3 × 10 <sup>1</sup>	75	14	21	0.08

\* Released into growth medium during a 72 h period by 10<sup>6</sup> cells. Approximately equal amounts of virus were cell-associated. Standard plaque assays utilizing Vero cells were employed.

† 10<sup>5</sup> cells in 24-well tissue culture plates were fixed for 10 min in cold (-20 °C) methanol and stained by indirect immunofluorescence methods. Mouse ascites fluid containing monoclonal antibodies [HNOT 1, HNOT 6, HN 10, HN 12, M 1, F 1, NP 3 (Wolinsky *et al.*, 1985) to the mumps virus HN, M, F and NP antigens] were obtained from Dr J. Wolinsky, University of Texas Health Science Center, Houston, Tx., U.S.A. Fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin was the secondary antibody. At least 10<sup>3</sup> cells were examined for each antigen. Percentages represent average of two duplicate well counts by two observers. Counts did not vary more than 10% between observers for fluorescent positive cells. Abbreviations: NP, nucleocapsid protein; HN, haemagglutinin-neuraminidase protein; M, matrix protein; F, fusion protein.

‡ Passage number.

§ ND, not determined.

confluent. PC-12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 5% heat-inactivated horse serum (HS) containing 20 µg/ml gentamicin. TE-671 cells were cultured in the same medium without the HS. The cells were then exposed at an m.o.i. of 0.05 to 0.1 p.f.u./cell to a Vero cell frozen and thawed lysate containing the Kilham strain of mumps virus or to a similar appropriate dilution of uninfected Vero cell lysate. Both types of lysates were clarified by centrifugation at 800 *g* for 5 min to remove cellular debris, diluted in Hanks' balanced salt solution, and applied to the cell monolayers in 0.25 ml volumes. After 1 h incubation at 37 °C, the inoculum was removed and replaced with 0.5 ml of growth medium. Surviving cells were grown to confluence (3 to 4 days) and passaged once per week.

Cultures of MI and PI cells at various passage levels were used for studies of viral replication and antigen synthesis, or cellular excitability, or both. The viral replication and antigen studies were performed on cells which had been cultured under conditions that maximize cellular excitability. In the case of PC-12 cells, these conditions involved plating 10<sup>5</sup> cells onto collagen-coated 35 mm tissue culture dishes, and a 14 to 17 day incubation period in DMEM with 10% FBS and 5% HS containing 20 µg/ml of 7s nerve growth factor (NGF) (Dichter *et al.*, 1977). For TE-671 cells these conditions involved identical plating procedures, but the incubation time was 10 to 12 days and the growth medium was DMEM with 1% FBS.

Data relevant to the pattern of mumps virus replication in the PI PC-12 and TE-671 cells cultured under these conditions are presented in Table 1. Several conclusions are obvious. First, significant amounts of infectious virus were produced by PI PC-12 cells, whereas infectious virus production by PI TE-671 cells was limited. This difference emphasizes the role that host cell factors can play in persistent infections. Second, infectious virus production in both cell types decreased with cell passage. The decreased production in PC-12 cells seemed to be correlated with a spontaneous 'curing' of the culture of virus-infected cells since the number of mumps virus antigen-containing cells also decreased with cell passage (95% at passage 4 to 60% at passage 12). The same did not occur with TE-671 cells because the number of virus antigen-positive cells remained relatively constant (75% to 80% for nucleocapsid protein) during cell passage. Finally, in contrast to PI PC-12 cells, not all mumps virus antigens were present in the same proportion in PI TE-671 cells. The F glycoprotein was, in fact, present in very few (0.02 to 0.08%) of the PI cells. The limited production of this glycoprotein may be partly responsible for the low level of infectious virus production by these cells. Restricted expression of the matrix

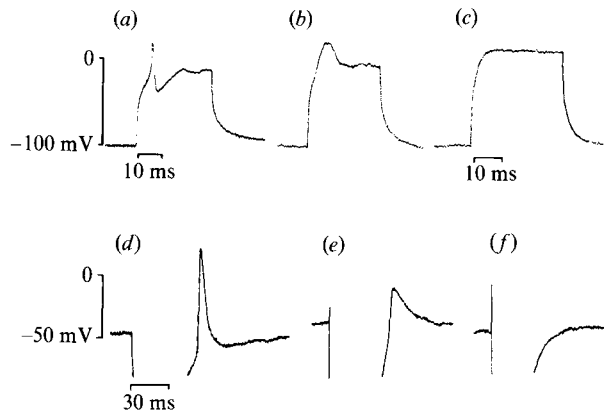


Fig. 1. Intracellular potentials of PC-12 and TE-671 cells (stimulus intensities listed in parentheses). (a to c) PC-12 responses to 30 ms depolarizing currents, 15 days post-differentiation, recorded in Earle's salts medium (a) Normal response (0.9 nA); (b) abnormal response (1.0 nA); (c) no response (2.0 nA). Cells were hyperpolarized to  $-100$  mV. Voltage scale applies to (a) to (c); same time scale in (a) and (b). (d to f) TE-671 responses to 30 ms anode-break currents, 14 days post-plating, recorded in DMEM supplemented with 1% FBS, buffered at pH 7.4 with 0.01 M-HEPES. (d) Normal response (0.9 nA); (e) abnormal response (1.6 nA); (f) no response (2.8 nA). In (d) and (e) the response is erupting from the electrotonic potential of the anode-break stimulus (off scale in the negative direction). The sharp upward deflection in (e) is an artefact. Voltage and time scales apply to (d) to (f).

(M) protein has been observed with subacute sclerosing panencephalitis virus persistent infections (Hall & Chopin, 1981*a, b*; Johnson *et al.*, 1981; Baczko *et al.*, 1984).

NGF induces PC-12 cells to extend neurites from their cell bodies (Dichter *et al.*, 1977). This ability was not affected by the presence of the mumps virus in the PI PC-12 cells: the neuritic network content of MI and PI PC-12 cell cultures was similar. This finding was consistent with our general observation of no effect of the virus on the cellular morphology of both PC-12 and TE-671 cells.

Records on transmembrane voltage changes were obtained by methods similar to those of Oakes *et al.* (1981). Intracellular recordings were made from cells impaled with fine-tipped glass micropipettes (35 to 80 m $\Omega$ ; 4 M-potassium acetate, pH 7.0) connected to an amplifier containing a bridge circuit for injecting stimulating currents. All signals were monitored on an oscilloscope and simultaneously recorded on an FM tape recorder (d.c., 1250 Hz) for later analysis. Recording time was restricted to 30 to 45 min for each culture (36 to 37 °C). Three SEAP parameters (amplitude, measured from the point of inflection where a SEAP erupted from the electrotonic response; rate of rise, measured as the slope of the tangent drawn on the steepest portion of the SEAP depolarization; and SEAP duration at half maximum amplitude) were collected from permanent records obtained with an electrostatic chart recorder. Data were averaged and compared using standard statistical software. Average values of the data are reported as the mean  $\pm$  S.D.

The effect of mumps virus on the excitability of the cells was determined by comparing the responsiveness of MI cultures with paired, passage level- and age-matched PI cultures. Responsiveness was assessed as the cells' capacity to generate SEAPs. PC-12 cells were hyperpolarized to  $-80$  to  $-100$  mV (Dichter *et al.*, 1977). TE-671 cells respond most consistently to anode-break stimulation, and only cells with resting membrane potentials (RMP)  $> 15$  mV were accepted for analysis (Syapin *et al.*, 1982). In both cell lines (whether MI or PI), three qualitative types of responsiveness were observed: normal, abnormal and no response (Fig. 1). Occasionally, it was difficult to determine by visual inspection whether or not a cell's response was normal or abnormal. The final differentiation between the well developed, all-or-nothing, normal SEAPs and the poorly developed, graded, abnormal SEAPs of all data,

Table 2. *Effects of mumps virus on the qualitative nature of SEAPs of MI and PI PC-12 and TE-671 cells\**

Expt.	Culture	Type of action potential			
		Normal	Abnormal	No response	
A†	PC-12 MI	30	12	9	$P < 0.001$
	PC-12 PI	7	21	24	
B‡	PC-12 MI	8	3	1	$P < 0.01$
	PC-12 AI	0	10	6	
C§	TE-671 MI	41	18	7	$P < 0.001$
	TE-671 PI	7	22	31	

\* For PC-12 and TE-671 cells respectively, normal as in Fig. 1(a) and (d), abnormal as in Fig. 1(b) and (e), no response as in Fig. 1(c) and (f).

† Data refer to number of cells that exhibited the type of action potential in response to a 30 ms depolarizing stimulus;  $\chi^2 = 23.56$ .

‡ Data refer to number of mature cells (i.e. cells with already developed membrane channels) that exhibited the type of action potential in response to a 30 ms depolarizing stimulus; AI, acutely infected;  $\chi^2 = 12.27$ .

§ Data refer to number of cells that exhibited the type of action potential in response to a 30 ms anode-break stimulus;  $\chi^2 = 39.44$ .

therefore, was done by a computer using a K-means clustering algorithm (Engleman & Hartigan, 1985; Hartigan, 1975) with the three SEAP parameters as the basis of partitioning.

PC-12 cells from MI cultures exhibited a variety of transmembrane voltage profiles (Fig. 1a to c). The greater proportion of the MI cells (58.8%; Table 2, expt. A) responded with relatively large, rapidly rising and brief SEAPs (Fig. 1a). These responses were identical to those reported by other workers as being the normal or typical response of PC-12 cells (Brandt *et al.*, 1976; Dichter *et al.*, 1977). A lower number (23.5%) of PC-12 MI cells produced abnormally small, slowly rising and long lasting SEAPs (Fig. 1b), or no response at all (i.e. only electrotonic voltages) (17.6%; Fig. 1c, Table 2, expt. A). These smaller responses were graded in nature and never erupted into fully developed, all-or-nothing SEAPs even when intracellular stimulating currents were clearly suprathreshold for the cell in question. The abnormal responses were significantly smaller in amplitude, and slower and longer in time when compared to the all-or-nothing normal SEAPs (Table 3). The abnormal responses were similar to those described in earlier reports (Dichter *et al.*, 1977; O'Lague & Huttner, 1980) as typical of undifferentiated PC-12 cells and judged by us, therefore, as indicative of a relatively small proportion of the normal population with un- and/or underdeveloped spike-generating mechanisms.

In contrast to the MI cells, the overall responsiveness of the PI cultures was dominated (86.5%) by the smaller, abnormal SEAPs (40.4%; Fig. 1b), or no response at all (46.2%; Fig. 1b, Table 2, expt. A). Only 13.4% of the PI cells produced normal, all-or-nothing SEAPs. There was a highly significant difference between the two conditions. Thus, the distribution of PC-12 responsiveness (i.e. a higher incidence of abnormal and/or no responses) was dependent on the presence of the mumps virus in the culture plates. Mean RMP for the infected cultures was not significantly different from the control plates (Table 3).

The observed effects of the virus on PC-12 cells could have arisen from interference with (i) the NGF-dependent development of the ionic channels which occurs during the differentiation period (Dichter *et al.*, 1977), or (ii) the functioning of already developed ionic channels, or (iii) both. To gain further insight into which of these possibilities might be the actual mechanism, data were collected from MI cells and from acutely-infected (72 h post-infection) differentiated (14 days post-NGF treatment) cells (Table 2, expt. B). In the latter culture, the ionic channels were already developed (Dichter *et al.*, 1977) and any observed viral effects would have to arise from direct interference with channel operation rather than channel development. Comparison of Table 2 expt. A with expt. B showed that there was no difference between the two distributions of PI cells (i.e. MI of Table 2, expt. B compared with expt. A:  $\chi^2 = 0.64$ ,  $P > 0.8$ ; PI of Table 2, expt. A compared with expt. B:  $\chi^2 = 2.38$ ,  $P > 0.05$ .) Note, however, that the

Table 3. *Electrophysiological properties of normal and abnormal SEAPs recorded from mock-infected and virus-infected cells in tissue culture\**

	PC-12	TE-671
Resting membrane potential† (mV)	43.7 ± 5.3 (51) 42.3 ± 5.6 (52)	29.2 ± 9.8 (66) 29.1 ± 9.2 (52)
SEAP amplitude‡ (mV)	39.7 ± 8.1 (37)§ 24.2 ± 7.0 (33)	70.9 ± 16.3 (48)§ 46.8 ± 13.0 (40)
SEAP rate of rise‡ (V/sec)	48.1 ± 12.7 (37)§ 20.0 ± 10.3 (33)	40.8 ± 20.8 (48)§ 28.1 ± 14.7 (40)
SEAP duration‡ (ms)	1.4 ± 0.5 (37)§ 2.7 ± 1.3 (33)	5.4 ± 2.1 (48)§ 8.7 ± 4.4 (40)

\* Mean ± S.D. respectively. Parentheses indicate number of cells examined.

† Upper value is from mock-infected cells; lower value is from virus-infected cells.

‡ Upper value is for normal SEAPs (Fig. 1*a, d*); lower value is from abnormal SEAPs (Fig. 1*b, e*).

§  $P < 0.001$ , *t*-test for null hypothesis: unequal means between normal SEAPs and abnormal SEAPs.

mumps virus still altered the distribution of responsiveness whether PI or acutely infected. These data indicate, therefore, that channel operation is altered by the presence of mumps virus, not channel development.

Although SEAPs of TE-671 cells have qualitatively different profiles from those of PC-12 cells, the TE-671 distribution of responsiveness was still dependent upon the presence or absence of the mumps virus (Table 2, expt. C). The most common (62.1%) type of SEAP recorded from MI cells is shown in Fig. 1(*d*) and was similar to the normal response of a previous study (Syapin *et al.*, 1982). A lower proportion (27.3%) of MI cells generated a small abnormal type of SEAP (Fig. 1*e*) or no response at all (10.6%; Fig. 1*f*). Like the PC-12 data, the abnormal type of response was significantly smaller, slower and longer than the normal type of response (Table 3). In contrast to the MI cells, the overall distribution (88.3%) of TE-671 responsiveness was significantly skewed towards the abnormal (36.7%) or no response (51.7%) category when the mumps virus was present in the cultures. PI cultures had a mean RMP that was not significantly different from that of the MI cells. [Since TE-671 cells are capable of supporting SEAPs at 1 day post-plating (Syapin *et al.*, 1982), the question of channel development is still unclear with regard to these cells.]

Our findings of a virus-induced decrease of excitability during persistent infection are consistent with those from some previous studies of excitable cells acutely infected with herpes simplex virus (Fukuda & Kurata, 1981; Oakes *et al.*, 1981; Fukuda *et al.*, 1983). However, they contrast with other studies of acute herpes virus infections which show an increased excitability as reflected by the appearance of spontaneous electrical activity in infected cells (Kiralý & Dolivo, 1982; Mayer *et al.*, 1985, 1986). These opposite effects are probably the result of different virus-specific proteins interacting in various ways with the host cells. Our data would support the view that increased or decreased excitability is determined by the infecting virus rather than the host cell because our results show a strain of mumps virus to have the same qualitative effect on two excitable cell lines derived from different organs (PC-12 from the adrenal medulla and TE-671 from the cerebellum).

Although the actual mechanism involved in the mumps virus-induced redistribution of responsiveness is beyond the scope of this report, it seems appropriate to consider one possibility: mumps virus envelope proteins could alter voltage-gated channel operation, either by a direct binding to the active channel proteins or indirectly by modifying the physicochemical (biophysical) environment around the ionic channel. In the latter case, for example, insertion of viral proteins into the membrane near the active ionic channels could alter the electromagnetic field situated around the channel such that gating and/or other channel kinetics are compromised. In either case (i.e. directly or indirectly), the net effect is an altered ionic current flow through the channels and this, in turn, would result in different voltage profiles for any propagated action potential. Since the amount of transmitter released from presynaptic

terminals is a function of the ionic current flow brought on by the voltage changes of the arriving action potential, these virus-altered action potentials would lead to a disruption of normal synaptic transmission in any neural pathway involving the infected neuron. This, in turn, would alter the normal integrative processes associated with that particular pathway and, therefore, might lead to serious neurophysiological dysfunction. In the present instance, both of the findings (i) that RMP values were not different between the control and test conditions [indicating the lack of viral interference with the transmembrane ion gradients or with the 'electrogenically inert' channels/conductances associated with the resting state (Grundfest, 1966)], and (ii) that the infected cultures are dominated by SEAPs with reduced amplitudes, reduced rates of rise and increased durations (indicating that the initial rapidly rising depolarization phase of the SEAP has been compromised), are consistent with an hypothesis that the voltage-gated Na<sup>+</sup> channel may be the primary site of the viral action (Oakes *et al.*, 1981; Fukuda *et al.*, 1983).

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