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Characterization of Four Cell Lines Persistently Infected with Measles Virus

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With 1 Figure

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Summary

Persistently infected cell lines were established by infecting Vero cells with four different strains of measles virus: Edmonston "wild type", Schwarz vaccine strain passaged at high multiplicity of infection, Hallé SSPE strain, and a temperature sensitive mutant of Edmonston strain, designated *ts* 841. The four cell lines have continued to produce virus at a constant low level over a period of more than two years, although cytopathology and hemagglutinating ability have varied with cell passage. Only virus from cells originally infected with *ts* 841 appears to be temperature sensitive. In each of the cell lines a sizable population of low density, interfering virus particles was generated, indicating that this is an important mechanism for these four cell lines in maintenance of the measles virus persistent infection.

Introduction

Persistent infection with a measles virus variant is the cause of subacute sclerosing panencephalitis (SSPE), a rare chronic degenerative disease of the central nervous system in children. Measles virus has also been implicated as a possible trigger for autoimmune demyelination characteristic of multiple sclerosis (MS). Variants of standard measles virus that have reduced capacity for carrying out a productive, cytocidal infection appear to be responsible for persistent infections both *in vivo* and *in vitro* (see review by MORGAN and RAPP, 1977).

Cultured cells that survive lytic infection with measles virus can be selected and subcultured. Often these cells are found to be persistently infected and to shed low levels of infectious virus continuously into the medium (HASPEL *et al.*, 1973; NORRBY, 1967; RUSTIGIAN, 1962, 1966) without showing the normal cytopathology characteristic of paramyxovirus infection. Variants of measles virus, either temperature sensitive *(ts)* mutants or defective interfering (DI) particles, may function in the establishment and maintenance of these measles virus

persistent infections (FISHER and RAPP, 1979a; GOULD and LINTON, 1975; HALL *et al.*, 1974; JU *et al.*, 1978, 1980; RIMA *et al.*, 1977; reviews by YOUNGNER and PREBLE, 1980; and HOLLAND *et al.*, 1980). Both *ts* and DI viruses may play roles in persistence in some cells. In at least one persistently infected cell line (HASPEL *et al.*, 1973) the cells released a *ts* virus at early cell passages, but at later passages produced DI rather than *ts* virus (FISHER and RAPP, 1979a).

In addition to virus factors, host cell factors may be critical in determining whether the cells become persistently infected (FISHER and RAPP, 1979a, b; WILD and DUGRE, 1978). Cyclic AMP levels in a cell may also affect measles virus replication. MILLER and CARRIGAN (1982) and ROBBINS and RAPP (1980) have shown that addition of cAMP to infected cells inhibits virus replication and results in a selective inhibition of measles virus polypeptides. Other factors affecting the physiology of the host cell may predispose it for production of defective virus particles (BEDOWS and PAYNE, 1981).

By studying measles virus persistent infections initiated by four different variants of the virus, it may be possible to determine the relative importance of selection of *ts* or DI viruses in the establishment and maintenance of the persistent infections *in vitro*. Such studies are described here.

Materials and Methods

Persistently Infected Cell Lines

Vero cells (Flow Laboratories, McLean, VA, U.S.A.) were infected with various strains of measles virus at low multiplicity of infection (MOI$\simeq$0.01). Cells surviving the lytic infection were fed at weekly intervals with Medium 199 (K. C. Biological, Lenexa, KS, U.S.A.) supplemented with 10 percent tryptose phosphate broth and 10 percent newborn bovine serum (K. C. Biological, Lenexa, KS, U.S.A.), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. Once colonies of surviving cells reached about 3—5 mm in diameter, they were removed from the culture vessel by treatment with trypsin, placed in fresh culture bottles, and subsequently passaged once a week. The viruses used to initiate these infections were as follows: Edmonston "wild type" and temperature sensitive mutant *ts* 841 obtained from F. E. Payne (School of Public Health, Department of Epidemiology, University of Michigan, Ann Arbor, MI, U.S.A.); Hallé SSPE and Schwarz vaccine strain obtained from F. Rapp (College of Medicine, Department of Microbiology, Pennsylvania State University, Hershey, PA, U.S.A.). Each of the virus strains was grown in Vero cells. The Schwarz strain was passaged 4 times at high multiplicity of infection before use to generate defective particles.

Plaque Assay

Vero cells, grown in 60 mm dishes, were infected with virus and after 45 minutes absorption were overlayed with agar plaquing medium (Minimum Essential Medium, supplemented with 10 percent newborn bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 1 percent agar). After 5 days incubation the agar was overlayed with 0.02 percent neutral red in calcium and magnesium-free phosphate buffered saline (PBS). Plaques were counted after overnight incubation.

Immunofluorescence Screening

Cells were grown on alcohol cleaned 22×22 mm coverslips in 35 mm culture dishes until they covered 70—80 percent of the coverslip surface. The cells were fixed in 3 percent formaldehyde in PBS for 30 minutes, washed 2 times with PBS, and treated with 0° C acetone for 7 minutes. The coverslips were washed again with PBS and were stained for immunofluorescence by an indirect technique. Cells were treated at 37° C

for 1 hour with human convalescent antimeasles serum (neutralization titer approximately 2560) that had been adsorbed for 1 hour with uninfected Vero cells. The coverslips were rinsed to remove unreactive serum and the cells were incubated with fluorescein isothiocyanate conjugated *Staphylococcus aureus* protein A (Sigma Chemical Co., St. Louis, MO, U.S.A.) for 30 minutes at 37° C. The cells were rinsed with PBS, air dried, mounted, and screened for fluorescence using an Olympus BH Microscope, with mercury vapor light source.

Hemadsorption Assay

African green monkey erythrocytes (Flow Laboratories, McLean, VA, U.S.A.) were washed in PBS. A 1 percent (v/v) suspension of the cells was made in PBS. Medium was removed from infected or uninfected Vero cell monolayers and the cells were washed with PBS. They were overlayed with the suspension of erythrocytes and incubated at 37° C for 1 hour. After extensive washing with PBS the cells were scored by microscopic examination (— to + + + +) for adsorption of the monkey erythrocytes.

Virus Purification

Persistently or lytically infected Vero cells, grown in 100 mm plates, were labeled for approximately 24 hours with 20 μCi/ml [^{35}S]-L-methionine (Amersham Corp., Arlington Heights, IL, U.S.A.) in Medium 199. The labeling period began approximately 48 hours after passage for the persistently infected cells and when 70—80 percent of the cells were visibly infected for the lytic infections. The cells were lysed by freezing and thawing. Cell debris was sedimented and the virus containing medium was stirred with 2.3 percent NaCl, 7 percent polyethylene glycol at 4° C overnight. Aggregated virus was collected by centrifugation at $700 \times g$ for 30 minutes and the pellet suspended in 1 ml TE buffer (0.005 M Tris HCl, pH 7.4, 0.001 M EDTA). The virus suspension was sedimented in a 15—40 percent (w/w) potassium tartrate (in TE) gradient for 2 hours at 35,000 rpm (Beckman SW 40). The virus band was collected with a syringe, diluted in TE and sedimented to equilibrium on a 0—50 percent (w/w) potassium tartrate gradient containing 30 percent (v/v) glycerol throughout. Tubes were punctured with a needle and 12 drop fractions were collected.

Interference Assay

Virus was isolated and purified as described above. Equal volumes of the two virus density gradient fractions to be tested were mixed and 0.2 ml of the mixture was added to a 16 mm multi-well of Vero cells. When a single gradient fraction was assayed, it was mixed with an equal volume of PBS before infecting the cells. After incubation at 37° C for 72 hours the cells were scraped from the wells and medium and were assayed for virus yield by the plaque assay previously described. Percent reduction in titer was calculated as follows:

$$\frac{(\text{titer of lytic virus} + \text{titer of DI virus}) - \text{titer of mixed infection}}{(\text{titer of lytic virus} + \text{titer of DI virus})} \times 100\%$$

Results

Virus Production by Persistently Infected Cells

Once the persistently infected (PI) cells had stabilized so that they could be passaged at weekly intervals, the cells were screened periodically for cytopathology, production of infectious virus at 37° C, the ability to absorb monkey erythrocytes, and immunofluorescence. Cells infected with the Hallé strain (designated H) and cells infected with *ts* 841 (designated 841) often produced syncytia characteristic of measles virus infection. There was no apparent correlation between release of infectious virus titer and appearance of cytopathic

effect (CPE) in the cells (Table 1). Both the H and 841 cells went through numerous periods of lytic "crisis" during the first 28 months they were in culture. During these "crises", most of the cells were killed as a result of the infection. The surviving cells were fed and passaged as described in Methods. Cells infected with Edmonston strain (designated PEdm) and Schwarz strain (designated Sch) showed no CPE, though they continously produced virus (Table 1). The PEdm and Sch cell lines have undergone crisis only once in the 32 months they have been maintained in culture. Each of the four cell lines has at some time during its passage history been positive for hemadsorption. Three of them, however —PEdm, Sch, and 841—have lost the ability to hemadsorb with continued passage.

Fixed PI cells were screened for immunofluorescence and compared with uninfected Vero cells using the indirect technique described in Methods (Table 1). As might be expected by the amount of infectious virus released from the PEdm, H, and 841 cells, these cells have remained positive for immunofluorescence throughout their culture history. The Sch cells, however, have had very little immunofluorescence at any time, even though they were releasing comparable

Table 1. *Biological characteristics of persistently infected cells*

Cell line	Passage number	CPE	HAd[a]	IF[b]	PFU/ ml
PEdm	19	−	++	++ (55%)	2×10^3
	27	−	−	+++ (65%)	2×10^3
	45	−	−	++ (60%)	5×10^2
	52	−	−	++ (40%)	2.3×10^3
Sch	11	−	++++	+ (<10%)	3×10^3
	22	−	+	−	1×10^3
	32	−	−	−	3×10^3
	33	−	−	−	<10
	45	−	N.D.[c]	N.D.	6×10^1
H	14	+	++++	N.D.	9×10^3
	26	+	++++	± (<10%)	9×10^3
	35	−	+	++ (20%)	1×10^3
	39	−	++	++++ (85%)	2×10^4
841	17	+	++	+ (20%)	2×10^2
	25	−	−	± (<10%)	1×10^3
	28	−	−	++ (45%)	3×10^2

[a] Hemadsorption
[b] Immunofluorescence
[c] Not done

Approximately 4 days after cell passage, culture fluids were removed from persistently infected cells, cleared of cell debris by centrifugation at $100\times g$, 10 minutes, and assayed at 37° C for virus yield by plaque titration in Vero cells. The infected cell monolayers were used for the hemadsorption assay as described in Methods. Fixed cells were screened for immunofluorescence after being prepared as described in Methods. Relative brightness of fluorescence was rated — to ++++. The percentage of fluorescent cells in the specimens is indicated in parentheses

amounts of virus to the other PI cells. No precise quantitative correlation was observed between infectivity titer or hemadsorption activity and immunofluorescence.

Presence of Temperature Sensitive Viruses in Persistently Infected Cells

One of the mechanisms often cited for maintenance of virus persistence in cell culture is the selection of temperature sensitive *(ts)* viruses in the virus population (see review by YOUNGNER and PREBLE, 1980). The mutations in these viruses are such that the viruses cause subacute infections that are characteristic of virus persistence *in vitro*. In order to determine whether virus produced by these cell lines is *ts*, the cells were grown at 37° C, 72 hours after cell passage the cells were scraped into the culture medium with a teflon policeman and were dispersed into a single cell suspension. The cells and culture medium were analyzed for infectious virus using the plaque assay technique described in Methods. The plaque assays were performed in duplicate at 33.5°, 37°, and 39.5° C. At the earliest cell passages tested, only 841 cells, those originally infected with a *ts* measles virus, produce virus with decreased plaquing ability at 39.5° C (Table 2). At later cell passages (49 through 55), however, virus produced in PEdm cells is increasingly more temperature sensitive.

Effect of Culture Temperature on Maturation of Viruses in Persistently Infected Cells

Production of virus in persistently infected cells may be temperature dependent (FISHER and RAPP, 1979b). The maturation and release of infectious virus may not occur efficiently at elevated temperatures even though the virus itself is not *ts*. Production of virus in the persistently infected cells grown at 33.5°, 37°, or 39.5° C was tested by assaying them for virus yield by plaque

Table 2. *Temperature sensitivity of virus produced by persistently infected cells*

Cell line	Passage number	33.5° C	37° C	39.5° C
841	27	2×10^4 [a]	1×10^1	1×10^1
	42	7×10^1	N.D.[b]	<10
H	26	2×10^2	8×10^2	3×10^2
	41	1.1×10^3	N.D.	1×10^3
Sch	10	7×10^3	8×10^3	7×10^3
	34	1.2×10^3	N.D.	1×10^4
PEdm	31	6×10^3	7×10^3	6×10^3
	49	2×10^5	N.D.	1.4×10^4
	55	1.6×10^5	N.D.	5×10^3

[a] PFU/ml
[b] Not done

Persistently infected cells were grown at 37° C. Seventy-two hours after cell passage the cells and culture fluids were assayed by plaque assay in Vero cells at the temperatures indicated

assay at 37° C. The temperature dependence of virus production seems to decrease with the amount of time the cells have been in culture. In each of the 4 cell lines, cells that have been passaged relatively few times produce virus in a temperature dependent fashion. One to two $\log_{10}$ less virus is produced at 39.5° C than at 33.5° C (Table 3) at those early cell passages. At later cell passages, however, virus is synthesized in the cells regardless of the temperature of culture.

Defective Interfering Virus Particles from Persistently Infected Cells

Although some degree of temperature sensitivity was evident early in the passage history of the cells, it did not seem to play a clear role in the maintenance of the persistent infections (except for the cell line originally infected with a *ts* virus). The cells were analyzed for the presence of defective interfering (DI) virus particles. Virus was purified from [^{35}S]-L-methionine labeled persistently infected cells as described in Methods. In addition, Vero cells were infected with the Edmonston strain of measles virus as a lytic control. After the virus was sedimented to equilibrium, the gradients were fractionated and 100 μl fractions weighed to determine buoyant density. Virus produced in a lytic infection has an average buoyant density of 1.26 g/cm^3 (Fig. 1a). PEdm (Fig. 1b), H (Fig. 1e), and 841 (Fig. 1d) cells produce 2 virus populations. PEdm and H cell viruses each contain a major peak with a density characteristic of the wild type virus and one with low density (1.15 and 1.13 g/cm^3, respectively) characteristic of a defective interfering virus particle. The larger population in 841 cells has a peak buoyant density of 1.25 g/cm^3 and forms a broad band, indicating some degree of heterogeneity in density. The peak density of the smaller population is 1.14 g/cm^3. The Sch cells produce one population of virus with an average buoyant density of 1.17 g/cm^3 (Fig. 1c). Very little virus is detected at the standard virus density of 1.26 g/cm^3. It should be noted that the virus used to initiate the persistent infection in Sch cells contained a high proportion of defective particles.

Table 3. *Temperature dependence of virus production in persistently infected cells*

Cell line	Passage number	33.5° C	37° C	39.5° C
841	27	3×10^2 [a]	4×10^2	$< 1.0 \times 10^1$
	45	1.4×10^2	2.4×10^1	1.4×10^1
H	26	1.3×10^2	1.9×10^2	6×10^1
	41	1.6×10^3	N.D.[b]	1.6×10^3
Sch	10	2.1×10^2	3.2×10^2	$< 1.0 \times 10^1$
	34	1×10^3	N.D.	1×10^4
Edm	31	1×10^1	2×10^1	$< 1.0 \times 10^1$
	49	3×10^2	N.D.	1×10^3
	55	7×10^3	N.D.	4×10^3

[a] PFU/ml
[b] Not done

Persistently infected cells were grown at the temperatures indicated. At 72 hours after cell passage the cells and culture fluids were assayed at 37° C for virus yield by plaque titration in Vero cells

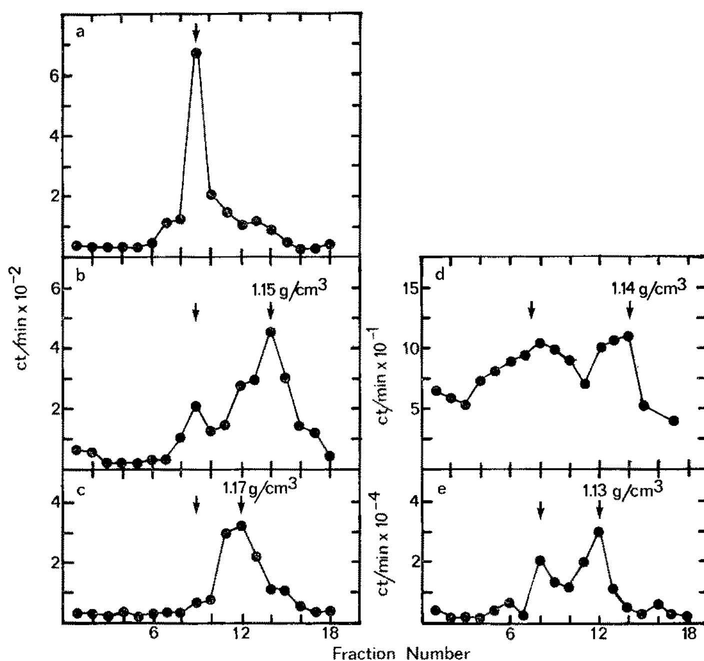

Fig. 1. Equilibrium density gradient profiles of virus isolated from infected cells. [35S]-methionine labeled virus was purified from infected cells as described in Methods. The position of standard density virus (1.26 g/cm³) is indicated by arrows. *a* Edmonston "standard virus" isolated from Vero cells following lytic infection; *b*—*e* virus isolated from persistently infected cells: *b* PEdm, *c* Sch, *d* 841, *e* H

Table 4. *Interference with replication of standard density virus by low density virus*

Virus	Gradient fraction number	Titer (PFU/ml)
Edm (lytic)	9	1.8×10^2
Hallé (lytic)	10	1.6×10^1
H	12	1.2×10^2
PEdm	14	1.2×10^1
Sch	12	1.6×10^1
841	13	1.0×10^2

Virus mixture	Titer (PFU/ml)	% reduction from expected
Edm (lytic) + Edm (lytic)	1.4×10^3	0
Edm (lytic) + PEdm	<10	>94.8
Edm (lytic) + Sch	1.4×10^1	92.9
Edm (lytic) + 841	4.0×10^1	85.7
Hallé (lytic) + H	1.0×10^1	92.6

Selected fractions from the density gradients (see Fig. 1) were assayed for infectivity either singly or in pairs and virus replication interference was determined from the double infections as described in Methods

The presence of large numbers of low density particles as compared to standard density particles in the population of viruses produced by these four persistently infected cells is indicative of DI particle production in the cells. If these viruses were DI particles, they should interfere with measles virus replication. In order to determine whether interference occurred, aliquots of density gradient fractions were mixed and used as inoculum for Vero cells as described in Methods. Peak low density virus fractions from the persistently infected cells were mixed with peak standard density virus fractions to assay for interference with virus replication as described in Methods. Reductions of 86 to 94 percent were observed in virus titers as compared to titers expected if no interference occurred (Table 4).

Discussion

Establishment of viral persistent infections may occur in a variety of situations where virus replication and production of infectious particles is limited. Any of a number of replication defective virus variants, including temperature sensitive mutants, defective interfering virus particles, and "cell-associated" viruses may be involved. In addition, some cells may allow only limited replication and maturation of specific viruses.

The four persistently infected cell lines analyzed in these studies have continued to produce infectious virus throughout their passage history. At some passages, the level of virus production has dropped significantly (to $\simeq 10$ PFU/ml), only to increase on subsequent passages (data not shown). In the two cell lines (H and 841) which have shown some cytopathology, the tendency for production of syncytia has decreased with continued cell passage. As a general rule, the ability of the persistently infected cells to hemadsorb has also decreased with continued passage. Only the virus isolated from 841 cells, originally a *ts* virus, is significantly temperature sensitive ($10^{3.5}$ less virus produced when assayed at 39.5° C than when assayed at 33.5° C). The 841 cells themselves also produce virus more efficiently at 33.5° C than at 39.5° C. Each of the four cell lines was found to generate a large population of virus particles with average buoyant densities ranging from 1.13—1.17 g/cm^3. Standard virus density is 1.26 g/cm^3. These low density virus particles interfered with the replication of virus of standard density. Only the Sch cells were initiated with inoculum rich in defective particles.

It appears that in each of the four cell lines, generation of low density, interfering particles plays an important role in maintaining persistence. It might be noted, however, that the high proportion of interfering particles in the inoculum used to establish Sch cells did not hasten the induction of the initial persistent infection (data not shown). McKIMM-BRESCHKIN *et al.* (1982) recently reported that Hallé SSPE measles virus isolates grown in their laboratory were composed of both syncytia (syn)$^+$ and syn$^-$ variants. The syn$^-$ virus produced low density interfering particles under some culture conditions. Since the Hallé strain used to establish H cells was originally grown in the same laboratory from the same parental virus as used in the McKimm-Breschkin studies, it is quite likely that such syn$^-$ variants pre-existed in the Hallé strain inoculum.

It is still unclear what selective pressures operate in the initial establishment of a persistent infection. Possibly host cell factors prevent efficient virus replication and maturation. This could result in a subacute infection where only limited amounts of infectious virus are produced. It does appear, though, that after the persistent infection is established, the generation of a class of virus particles that interferes with the replication of standard virus is important in maintenance of the persistent state. Preliminary analysis of virus nucleocapsids from Sch cells indicates the presence of a predominance of [^{3}H]-uridine labeled structures that sediment more slowly than nucleocapsids isolated from cells lytically infected with the Schwarz strain of measles virus. This observation is consistent with the presence of low density, interfering particles described in Fig. 1 and Table 4.

Further study of these four measles virus persistent infections should help to determine whether defects exist in virus maturation pathways in the cells. In addition, a look at the nature of virus-specific genomic and messenger RNAs in the persistently infected cells as compared to lytically infected ones will provide information about the mechanisms involved in maintenance of measles virus persistence in cell culture.

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