ISOLATION OF A HETEROGENEOUS POPULATION OF TEMPERATURE-SENSITIVE MUTANTS OF MEASLES VIRUS FROM PERSISTENTLY INFECTED HUMAN LYMPHOBLASTOID CELL LINES*

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A number of slow neurological diseases occurring in animals and in man appear to be associated with a long-term, chronic infection of the host by a virus or viral-like agent. Measles virus, or a variant of measles, has been shown to be the etiologic agent of the chronic and invariably fatal human disease, subacute sclerosing panencephalitis (SSPE).[1] In SSPE, the virus appears to produce a persistent infection of both the central nervous system and lymphoid cells (1–3). There is also increasing evidence that measles virus might be associated with multiple sclerosis (MS), and possibly with other chronic human diseases as well (4).

Several observations suggest that lymphoid cells might serve as a reservoir for measles virus during the long incubation period in a chronic infection before the onset of clinical manifestations. One of the first isolations of the measles variant virus associated with SSPE was from lymph nodes of SSPE patients (3). Recently, detection of measles viral antigens in the mononuclear cells of the jejunal mucosa of patients with MS has been reported (5), and measles-like virus has been isolated from jejunal biopsies (6).

Persistently infected cultured cells have potential usefulness as models for the study of chronic diseases of viral etiology. Several mechanisms have been suggested for establishing or maintaining the carrier state. Factors such as interferon (7), conditional lethal mutants (8), defective interfering (DI) particles (9), and conceivably the production of DNA provirus copies (10, 11) might be involved. This report deals with the study in vitro of a persistent infection of human lymphoblastoid cell lines (LCL) by measles virus. Establishment and

the characteristics of the persistently infected cells are described, and in addition, we report the isolation and preliminary characterization of a highly heterogeneous population of temperature-sensitive (ts) mutants produced by the carrier cultures.

Materials and Methods

Cells. The human LCL WI-L2 and 8866 were propagated as suspension cultures in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.), supplemented with 17% fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, Md.), and glutamine. Both WI-L2 and 8866 are presumably transformed by Epstein-Barr virus, and carry markers characteristic of human B lymphocytes (12).

Monolayer cultures of Vero cells, derived from green monkey kidney cells, were used for all viral isolations, titrations, and characterization assays. They were the gift of Dr. Philip Marcus, University of Connecticut, Storrs, Conn. Cultures were grown in Dulbecco's modified Eagle's medium (DME; Grand Island Biological Co.) supplemented with 10% FCS and glutamine. All cultures were incubated in a humidified atmosphere of 5% CO_2 in air, at 37°C unless otherwise noted.

Viruses. The Edmonston strain of measles virus was originally obtained from Dr. Bernard Fields. Parental virus was doubly plaque-purified on Vero cells, and stock supernatant virus was produced by growth in Vero cells at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU)/ml. Supernatant virus was then separated from cell debris by sonication, followed by clarification of the supernate by low-speed centrifugation. The stock virus was stored in 10% dimethylsulfoxide at -60°C, and contained 5×10^6 PFU/ml by plaque assay on Vero cell monolayers at 37°C. The Indiana (HRC) strain of vesicular stomatitis virus (VSV) was the gift of Dr. Philip Marcus.

Virus Titrations. Virus preparations were titered by a semiquantitative plaque assay (13). Vero cell monolayers were trypsinized and plated in 96-well plastic trays (Linbro Scientific Co., Hamden, Conn.). Approximately 1.3×10^5 cells were plated in each 16-mm well and incubated at 37°C overnight. 10-fold dilutions of virus samples were made in DME with 2% FCS. The 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 31°C, 1 ml of growth medium containing 0.75% carboxymethylcellulose was added. The monolayers were then incubated at the appropriate temperature for 5 days, then fixed with 10% formyl saline and stained with crystal violet.

Persistent Infection of LCL. The persistent infection of the LCL was established using a modification of the method of Barry et al. (14). Briefly, 2.5×10^6 lymphoblastoid cells were infected at an MOI of $1-4 \times 10^{-6}$ PFU/cell. The inoculum used was a 10% hamster-brain suspension, derived from neonatal hamsters injected intracerebrally with parental virus and containing 10^6 PFU/g of tissue. Medium from infected cultures grown at 37°C was changed weekly, and the cultures were able to be passaged $\cong 5$ wk after infection.

Immunofluorescence Staining. Measles viral membrane antigens were detected by the indirect staining technique. Cells in suspension were stained with a rabbit anti-measles serum produced in this laboratory, followed by staining with rhodamine-conjugated goat anti-rabbit serum (N. L. Cappel Laboratories Inc., Cochranville, Pa.). The cells were washed, dried on glass slides, post-fixed in absolute ethanol, mounted in buffered glycerol, and examined microscopically with ultraviolet illumination. Measles viral cytoplasmic antigens were detected by direct staining of cells dried onto glass slides and prefixed with absolute ethanol. The fixed cells were then stained with fluorescein-conjugated rabbit anti-measles serum diluted in phosphate-buffered saline containing 80% FCS and 0.5% Evans blue counterstain. Cells were washed extensively, mounted in buffered glycerol, and examined by ultraviolet illumination.

Electron Microscopy. Cells were pelleted and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. The cells were then post-fixed in 1% OsO_4, dehydrated in a graded series of ethanol, and embedded in Araldite-Epon. Sections were cut, stained with lead citrate-uranyl acetate, and examined with a Siemens 102 electron microscope (Siemens Corp., Cherry Hill, N. J.).

Superinfection of Persistently Infected (PI) LCL. WI-L2 and WI-L2$_n$ I were tested for resistance to infection by VSV(HRC) or by parental measles virus. Each cell line was either

infected with VSV at an MOI of 1 or 10, or with measles virus at an MOI of 0.01. VSV-infected and uninfected cultures were incubated at 37°C for 40 h, and samples were removed for viable cell count by trypan blue exclusion, and for assessing the amount of VSV produced by titration on Vero cells. The amount of DNA synthesis was also measured in surviving cells by uptake of [³H]thymidine (New England Nuclear, Boston, Mass.) in a microtiter assay for 18 h. Quadruplicate samples were labeled and collected on a multiple cell culture harvester. Measles-infected and uninfected control cultures were incubated at 37°C for 6 days before samples were removed for viable cell counts and assessing yields of measles virus.

Transfection Studies with DNA from PI LCL.   DNA was isolated from $\cong 10^8$ WI-L2$_{PI}$ I cells by the method of Marmur (15). Transfection of Vero cell monolayers was performed by the calcium precipitation technique of Graham and Van der Eb (16). Treated cells were incubated at 33°C for 14 days and examined for evidence of cytopathology.

Interferon Assay.   Supernates of PI and control cultures were assayed for interferon production by the interference assay of VSV replication on human foreskin cultures (17). Any measles virus present was removed by ultracentrifugation or by adjusting the supernates to pH 2-3.

Production and Quantitation of DI Particles.   Measles DI particles were produced and amplified by multiple undiluted passages of parental virus in Vero cell monolayers. Samples were assayed for interference by mixing 0.1 ml of serial twofold dilutions of a DI preparation with 0.1 ml of a preparation containing parental measles virus. The mixtures were inoculated in duplicate onto Vero cell monolayers in plastic trays (Linbro Scientific Co.) containing 24 wells of 16-mm diameter. Control wells received 0.1 ml of each twofold dilution of the DI preparation, or 0.1 ml of the parental virus. Samples were incubated and scored as in the virus titration assays.

Plaque Purification of Viral Isolates from PI LCL and Screening for ts Mutants.   PI cell cultures were grown at 31°C for 24-48 h. The supernates 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 (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). An appropriate dilution of the filtered supernates was used to give 25-50 plaques per dish. After a 2-h adsorption period at 31°C, the monolayers were overlaid with 4 ml of modified Eagle's medium containing 10% FCS and 0.75% agarose. Plates were incubated at 31°C for 7-10 days, then overlaid again with 2 ml of agarose medium containing 0.00125% neutral red, and incubated for an additional 18 h at 31°C. Well-isolated plaques were selected and inoculated onto individual Vero monolayers in 16-mm Linbro tray wells, with the addition of 1 ml of growth medium. The plaque-purified isolates were incubated at 31°C and harvested when 90% of the monolayer showed cytopathology (CPE). The entire culture fluid (with cell debris) was then inoculated onto Vero monolayers in 25 cm² Falcon flasks (Falcon Plastics), incubated at 31°C, and harvested when 90% of the monolayer showed CPE. Each isolate was then screened for temperature sensitivity by plaque assay at 31 and 39°C. A plating efficiency (P.E.) ratio for each isolate was calculated as follows:

$$\text{P.E. ratio} = 39°\text{C titer}/31°\text{C titer}.$$

Thermolability of ts Mutant Isolates.   Thermolability was determined by measuring the rate of reduction in PFU/ml at 31°C after incubation of virus samples at 45°C. Undiluted virus stocks were incubated in 12-ml glass conical centrifuge tubes in a 45°C water bath. Samples (0.2 ml) were withdrawn into 1.8 ml of ice-cold DME containing 2% FCS at 0, 15, 30, and 45 min. Residual infectivity was determined by plaque assay at 31°C.

Results

Establishment of PI Cell Cultures.   Persistent infection of LCL was initiated by a modification of the method of Barry et al. (14) by infection of cultures at an MOI of 10⁻⁶. Even at this low input MOI, a crisis ensued, and the majority of the cells in the culture lysed as in an acute infection. The surviving cells were passaged $\cong 5$ wk after infection. The three PI LCL to be described here were independently established: WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, and 8866$_{PI}$. In the 2 yr since establishment of the PI cultures, the LCL have remained stable. No cycles of crisis and recovery have been observed.

TABLE I

Characteristics of Measles PI Lymphoblastoid Cell Lines

Characteristic	Cell lines		
	WI-L2$_{PI}$ I	WI-L2$_{PI}$ II	8866$_{PI}$
Immunofluorescent staining*	70–80%	70–80%	90%
Electron microscopy	many cells with virus budding, nucleocapsid matrices in cytoplasm	ND‡	ND
Infectious centers§	1–2%	2–3%	1–2%
Supernate viral yield (PFU/ml)‖	1	1–2	1–2
Intracellular viral yield (PFU/ml)	1–2	1–2	1–2
Interferon (U/ml)¶	291	57	49

* Percent of cells in culture containing membrane viral antigen by indirect immunofluorescent staining.
‡ ND, not done.
§ Percent of cells in culture producing infectious virus.
‖ PFU/infectious center.
¶ Uninfected control WI-L2 had less than 10 U/ml. Uninfected control 8866 had 88 U/ml.

Properties of PI LCL. The PI lines appear morphologically normal, with fewer than 1% giant cells in the cultures. The LCL were examined soon after establishment for the presence of measles virus. The results (Table I) confirm previous reports from other laboratories (14, 18) that the cultures were PI. Immunofluorescent staining (Fig. 1) and electron microscopy (Fig. 2) indicated that the vast majority of the cells in each infected culture were producing measles viral antigens and virus-like particles. However, an infectious centers assay (19) showed that only 1–2% of the cells were producing infectious virus at 37°C. In addition, very few infectious viral particles could be detected by plaque assay on Vero cells. Only 1–2 PFU were released into the supernate from each infectious center ($\cong 10^4$ PFU/ml). When cells were disrupted by freezing and thawing, each productive cell again released only 1–2 infectious particles.

By immunofluorescence, the PI LCL cultures appeared heterogeneous in the intensity of staining for measles cytoplasmic antigens (Fig. 1). One line, WI-L2$_{PI}$ I, was cloned by the soft agarose technique (20). 23 cell clones were grown up and screened for membrane antigen by the indirect or direct staining method. 2 of the 23 clones were negative for viral antigens. The remaining 21 clones exhibited a homogeneous staining pattern in the cells within each clone. But a range of intensity of staining among the clones was seen, from faintly positive clones to clones that were highly fluorescent. One intensely fluorescent cell line, cell clone 16, was chosen to be doubly cloned for further study.

Possible Factors Involved in Maintenance of the Carrier State

INTERFERON. Some LCL have been shown to secrete continuously low levels of interferon (21), although they appear to be relatively resistant to the antiviral effects of interferon (22). A rise in interferon levels was seen in the supernates of both PI lines derived from WI-L2 (Table I), from a base level of <10 U/ml in the control culture. In contrast, the level of interferon declined
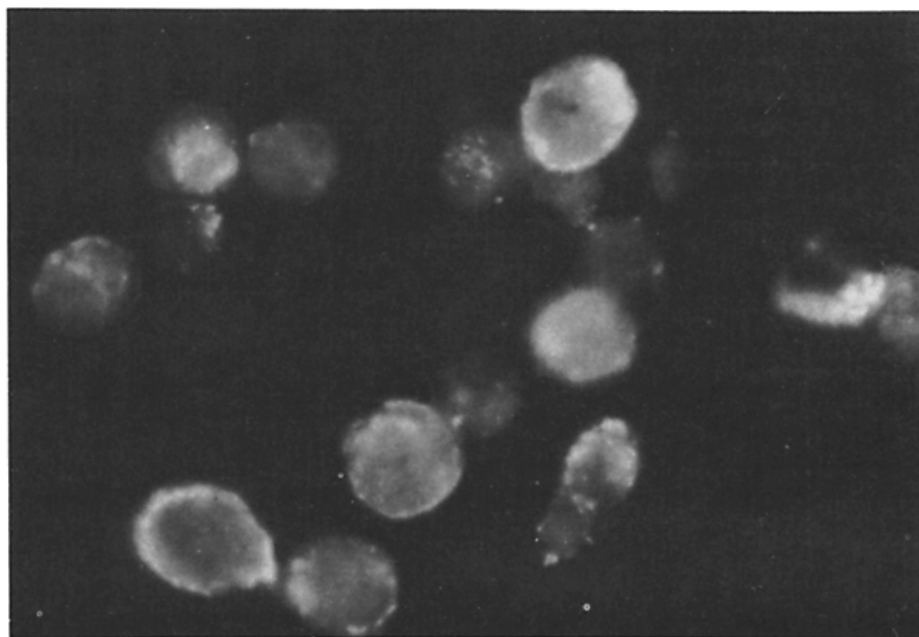
FIG. 1. Measles viral antigens in the cytoplasm of WI-L2$_{PI}$ I cells, by direct immunofluorescent staining of alcohol-fixed cells with fluorescein-conjugated rabbit anti-measles serum in Evans blue counterstain. Magnification, 100 ×.

when 8866 became PI (from 88 to 49 U/ml). In these experiments, although an increased production of interferon was not always associated with a persistent measles infection in the LCL, we cannot exclude the possibility that the low levels of interferon produced by uninfected cells were sufficient to establish the carrier state.

If interferon were a major factor in maintaining the carrier state, one might expect the PI LCL to be resistant to infection by other viruses. The resistance of WI-L2$_{PI}$ I to challenge with VSV(HRC) or measles virus was determined (Tables II and III). Both control WI-L2 and WI-L2$_{PI}$ I were susceptible to infection by VSV, as indicated by the viral yields and by CPE as assayed by trypan blue exclusion. Cell clone 16 of WI-L2$_{PI}$ I was also as susceptible as control WI-L2 cells to VSV, as measured by reduced uptake of [^{3}H]thymidine in infected cultures (Table III).

When challenged with the parental measles virus, the PI LCL exhibited homologous interference (Table II). Yields of measles virus from unchallenged and superinfected WI-L2$_{PI}$ I were comparable, and were lower than the yields obtained from control WI-L2 cells infected with measles. The control cells also showed extensive cell destruction (>90% nonviable by trypan blue exclusion), whereas a much lower amount of CPE was seen with the superinfected WI-L2$_{PI}$ I culture (30% nonviable cells).

DNA PROVIRUS COPIES. It has been suggested by Simpson and Iinuma (10) and Zhdanov (11) that enveloped RNA viruses could maintain a persistent viral infection by the production and integration into host cell DNA of a DNA
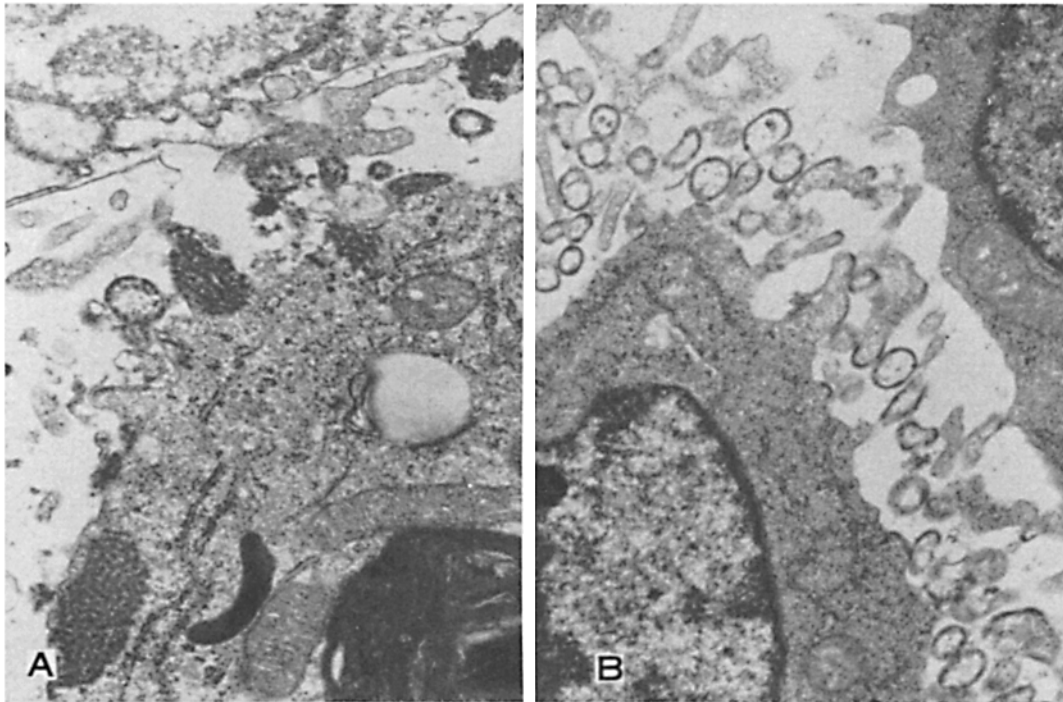
FIG. 2. Electron photomicrographs of lymphoblastoid cells PI with measles virus. (A) WI-L2$_{PI}$ I cells with aggregates of viral nucleocapsids aligned along the cell membrane. Magnification, 24,000 ×. (B) Measles virus-like particles released from WI-L2$_{PI}$ I cells. Magnification, 30,000 ×.

TABLE II

Superinfection of WI-L2 and WI-L2$_{PI}$ I with VSV and Measles Virus

Cell line	Virus*	Time after infection	Viable cell count	CPE‡	Supernatant virus yields§	
					PFU/ml	PFU/10^6 viable cells
			cells/ml	%		
WI-L2	VSV	40 h	4.7 × 10^5	50	2.5 × 10^7	5 × 10^7
WI-L2$_{PI}$ I	VSV	40 h	6 × 10^4	80	1 × 10^7	1.6 × 10^8
WI-L2	measles	6 days	1.6 × 10^5	>90	6.2 × 10^3	4 × 10^4
WI-L2$_{PI}$ I	measles	6 days	4.8 × 10^5	30	2 × 10^3	6.5 × 10^3
WI-L2$_{PI}$ I	none	6 days	8 × 10^5	<1	5 × 10^3	4.2 × 10^3

* VSV: MOI = 1; measles: MOI = 0.01.
‡ Percent of cells that did not exclude trypan blue.
§ Assayed by plaque titration on Vero cells at 37°C.

provirus. Transfection studies using DNA extracted from WI-L2$_{PI}$ I cells were negative for production of measles viral CPE on Vero cells at 33°C. Similar negative results have been reported by Holland et al. (23) for cells persistently infected with VSV, measles, rabies, or mumps (23).

DI PARTICLES. Since persistent infection by several other RNA viruses has

TABLE III

Superinfection with VSV

Cell line	MOI	[³H]Thymidine incorporation*		Viable cell count
		cpm	Uninfected	
			%	cells/ml
WI-L2	0	265,469	100.00	1.9×10^6
	1	338	0.13	$<10^4$
	10	309	0.12	$<10^4$
WI-L2$_{PI}$ I, clone 16	0	159,543	100.00	1×10^6
	1	373	0.23	$<10^4$
	10	333	0.21	$<10^4$

* 0.2 ml of cell cultures was incubated with 1 μCi of [³H]thymidine per well in microtiter plates for 18 h at 37°C, on day 3 post-infection. Each value is an average of four wells.

been shown to be mediated by DI particles (9, 23, 24), the PI LCL were examined for the production of particles with interfering activity. A stock DI-enriched preparation of measles was produced by seven undiluted passages of parental virus. This stock contained no PFU when assayed on Vero cells at 37°C, and 0.1 ml of this sample reduced the yield of parental measles virus by at least one log of infectivity. Attempts were made to demonstrate the presence of DI particles in cell supernates. No evidence was obtained for the presence of significant numbers of DI particles in fluids harvested from PI LCL. Undiluted PI supernates harvested from WI-L2$_{PI}$ I or from 8866$_{PI}$ (grown at 31°C or at 37°C) failed to produce any interference in the replication of the stock measles virus at 37°C. The interference assay was also performed with undiluted supernates passaged one, two, or three times on Vero cell monolayers at 31°C. These cycles of amplification for DI particles did not result in any detectable interference with the parental measles replication.

TS VIRAL MUTANTS. Persistent infection of a variety of mammalian cell lines has been reported to be associated with the appearance of ts mutants (8). Recent studies have demonstrated a similar finding in several cell lines persistently infected with measles (25-28). Therefore, the LCL cultures were examined for the appearance of ts mutants. The P.E. ratio of total progeny virus from the PI cells did not differ significantly when titrated at 31, 37, or 39°C (data not shown). The possibility remained that ts mutants might exist as a fraction of the total viral population. This was evaluated by plaque purification of virus from filtered supernates of PI cultures grown at 31°C for 24-48 h. Individual isolates were propagated on Vero cells, and titered by the plaque assay at 31 and 39°C. The P.E. ratios (39°C titer/31°C titer) were determined for viral plaque-purified isolates from the three parental cell lines (Table IV). Each of the parental cell lines, WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, and 8866$_{PI}$, produced a population of viral isolates with a range of P.E. ratios from $>10^{-1}$ to $<10^{-4}$.

The appearance of a heterogeneous population of ts mutants could have been attributed to the heterogeneity of the cell population, with different cells producing a particular ts mutant. Therefore, cell clone 16, a doubly cloned cell line of WI-L2$_{PI}$ I, was also examined for the production of ts mutants by plaque purification. Again, isolates with a range of P.E. ratios were obtained, suggest-

TABLE IV

Ratios of P.E. at 39 and 31°C of Viral Isolates from Supernates of PI LCL and of Parental Stock Virus

	Cell Line					
	WI-L2$_{PI}$ I	WI-L2$_{PI}$ II	8866$_{PI}$	Clone 16	Total	Parental stock
Total number of isolates	17	15	17	21	70	21
Number of isolates with P.E. ratios*						
$1-10^{-1}$	3(18%)‡	8(53%)	7(41%)	1(5%)	19(27%)	21(100%)
$10^{-1}-10^{-2}$	2(12%)	4(27%)	7(41%)	5(24%)	18(26%)	0
$10^{-2}-10^{-3}$	2(12%)	2(13%)	0	6(29%)	10(14%)	0
$10^{-3}-10^{-4}$	7(41%)	1(7%)	0	7(33%)	15(21%)	0
$10^{-4}-10^{-5}$	3(18%)	0	3(18%)	2(9%)	8(11%)	0

* P.E. = ratio = 39°C titer/31°C titer.

‡ (%) = number of isolates with a given P.E. ratio/total number of isolates × 100.

ing that even a clone of lymphoblastoid cells could produce a heterogeneous virus population. In Table IV are presented the P.E. ratios of 70 viral isolates from the 4 PI LCL. P.E. ratios greater than 10^{-1} were found with 19 of the 70 isolates, but the majority of the plaque-purified isolates (51/70) showed at least some degree of temperature sensitivity. It was also observed during the plaque purification procedure that isolates differed in plaque size and morphology. However, this phenotypic marker did not breed true, and plaque size did not correlate with P.E. ratios (i.e., virus from an initially small plaque did not always exhibit a low P.E. ratio).

To discount the possibility that viral mutants were present in the original inoculum, parental measles virus was grown at 31°C and plaque-purified by the same method as used with the PI LCL supernates. When 21 viral isolates were examined, all 21 had a P.E. ratio of 1 (Table IV). This suggests that the appearance of ts mutants in the stock virus is not a high frequency event, and that the population of ts mutant virus was induced during the establishment of the persistent infection, not selected from preexisting mutants.

To examine the question of whether or not the plaque-purified isolates were stable viral mutants, isolates from cell clone 16 were tested by a second plaque purification (Table V). Two viral isolates with initial P.E. ratios of $<10^{-4}$ and two isolates which appeared to be leaky mutants (P.E. ratios $>10^{-1}$) were selected. All plaque-purified isolates of 4M and 9S retained the ts phenotype, exhibiting P.E. ratios comparable to those of the original viral stocks. However, the viral isolates 1S and 7M generated plaques with a range of P.E. ratios from 10^{-1} to 10^{-5}. These two initially leaky isolates were either highly unstable, or consisted of a mixed population of virions. Isolates from 1S are being examined after a third plaque purification for the ability to retain their initial P.E. ratios.

Thermolability of the ts Mutants. A ts mutation affecting a structural component of the virion might in some cases result in altered thermolability of the mutant virus. Therefore, the kinetics of heat inactivation of several of the ts isolates were compared to those of the parental virus. The parental measles virus was completely inactivated after 60 min at 45°C (Fig. 3). Seven viral

TABLE V

Ratios of Plating Efficiencies of Viral Isolates after a Second Plaque Purification

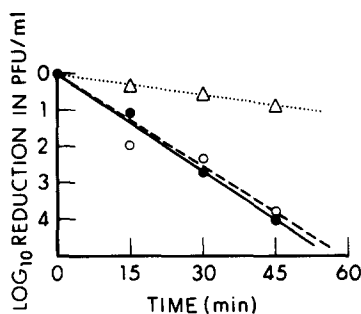|	Viral isolate			
	1S	7M	4M	9S
Initial P.E. ratio	10^{-1}	10^{-1}	10^{-4}	10^{-4}
Total no. of isolates	7	11	11	12
Number of isolates with P.E. ratio				
10^{-1}–10^{-2}	1(14%)	0	0	0
10^{-2}–10^{-3}	1(14%)	1(9%)	6(55%)	0
10^{-3}–10^{-4}	3(43%)	9(82%)	5(45%)	11(92%)
10^{-4}–10^{-5}	2(29%)	1(9%)	0	1(8%)

FIG. 3. Thermolability of parental and ts isolates of measles virus at 45°C. Undiluted virus stocks were incubated in 12-ml glass conical centrifuge tubes in a 45°C water bath. Samples (0.2 ml) were withdrawn into 1.8 ml of ice-cold medium containing 2% FCS at 0, 15, 30, and 45 min. Residual infectivity was determined by plaque assay at 31°C. Parental virus (●), ts isolate 12 (○), and ts isolate 9S (△).

isolates from two of the PI LCL (representing a range of P.E. ratios from 10^{-1} to 10^{-5}) were tested and could be divided into two groups. One group, which included five of the seven isolates tested, showed a rate of inactivation similar to that of the parental virus. The thermolability curve of isolate 12 (from cell line 8866_{PI}) is representative of this first group. Two other viral isolates appeared to be more thermostabile than the parental virus, as has been reported with virus from another measles PI cell line (26). The inactivation curve of one of these isolates, 9S from cell clone 16, is also shown. The thermolability of the isolates did not correlate with the P.E. ratios, since both isolates 12 and 9S had P.E. ratios $<10^{-4}$. The data on kinetics of heat inactivation suggest that there exist at least two groups of ts mutants that can be distinguished by comparison of their thermolability at 45°C, again confirming the heterogeneity of mutants produced by the PI lines.

Measles Antigen Production by ts Mutants at 39°C. The amount of measles-specific antigens synthesized by several ts isolates at the nonpermissive temperature was assayed by direct immunofluorescence on alcohol-fixed infected cells. When parental virus was used to infect Vero cells at 39°C for 12 h, extensive cell destruction could be seen in the monolayer. Immunofluorescent staining showed intense fluorescence in the cytoplasm, with some antigen
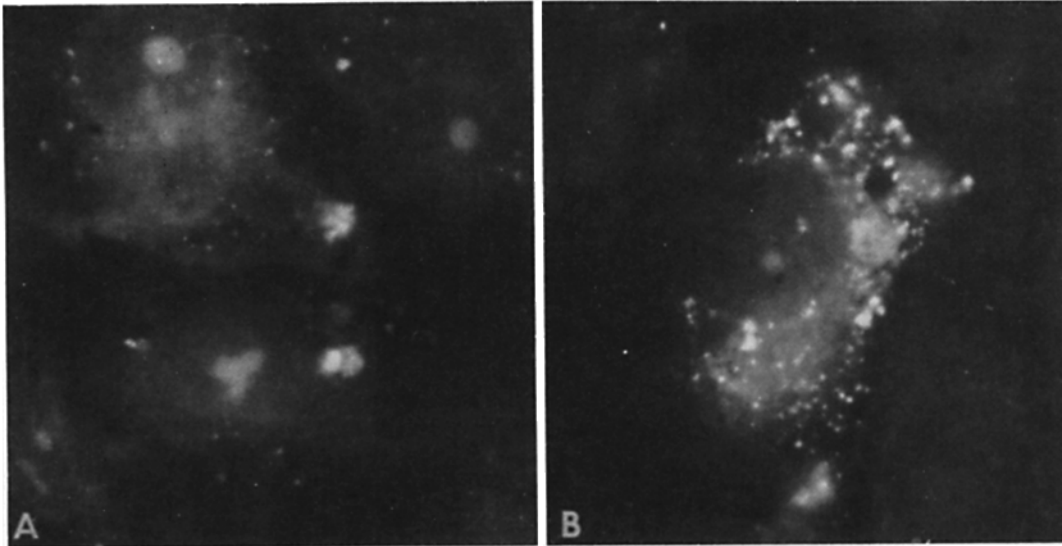
FIG. 4. Differences in antigen production at the nonpermissive temperature (39°C) by ts isolates of measles virus. Vero cell monolayers were infected at an MOI of 1-2 with individual mutants, and incubated at 39°C for 3 days. Monolayers were washed, alcohol-fixed, and stained with fluorescein-conjugated rabbit anti-measles serum in Evans blue counterstain. (A) Vero cells infected with isolate 8M from WI-L2$_{PI}$ II, showing faint cytoplasmic fluorescence. Magnification, 100 ×. (B) A single Vero cell infected with isolate 15M from 8866$_{PI}$, showing intense cytoplasmic fluorescence. Magnification, 100 ×.

localized in the nucleus. Control uninfected Vero cells exhibited no specific measles fluorescence. Four ts isolates, one each from WI-L2$_{PI}$ I, WI-L2$_{PI}$ II, clone 16, and 8866$_{PI}$, were selected for study. All four isolates had P.E. ratios <10^{-5}. Infection of Vero cells was initiated at an MOI of 1-2 by a 2-h adsorption period at 31°C. Growth medium was then added and the cultures were incubated for an additional 4 h at 31°C to allow penetration. The infected monolayers were then shifted up to 39°C and incubated for an additional 3 days.

None of the monolayers infected with ts mutants showed any visible signs of CPE. With three of the four mutants, the pattern of antigen production indicated that these isolates were early mutants, synthesizing only a very small amount of measles proteins (Fig. 4 A). Monolayers infected with the fourth mutant (15 M from 8866$_{PI}$) showed a staining pattern resembling that of the parental virus, with intense cytoplasmic fluorescence and a faint nuclear staining (Fig. 4 B).

The range in P.E. ratios of the ts isolates has been accompanied by differences in thermolability and in antigen production at the nonpermissive temperature. It is probable, therefore, that the mutants that exhibit phenotypic differences also represent a genotypically mixed population. By genetic complementation analysis, we are examining whether or not the ts isolates from different cell lines have lesions in different virus genes.

Attempts to Cure the PI LCL.   The inclusion of rabbit anti-measles serum to the culture medium was examined for its effect on the persistent infection of WI-L2$_{PI}$ I. Antibody (0.5 ml of an undiluted serum), capable of neutralizing 10^8

PFU, was added to 10 ml of medium at each cell passage. After 3 mo of culture in anti-measles serum, the cells were grown for 1 wk without antibody and then assayed by indirect immunofluorescence staining for measles membrane antigens. No difference in fluorescence pattern was observed from the untreated PI cells; >80% of the cells remained positive for viral membrane antigens. The failure to cure the PI LCL with antibody to measles implies that the role of extracellular virus is not an important one in maintaining the carrier state.

To study the effects of temperature shifts on antigen expression, cultures of WI-L2$_{PI}$ I were shifted from 37°C, the usual incubation temperature, to 39°C, and grown at this temperature for 1 mo. No CPE was seen in the shifted cultures, and indirect immunofluorescence revealed that the vast majority of the cells still expressed measles membrane antigens. When cultures of the PI LCL were incubated at 31°C, the cells showed extensive CPE and were usually destroyed. Cultures that did survive the crisis at 31°C continued to express measles membrane antigens. Preliminary experiments suggest that some ts mutant viruses can interfere with replication of both wild-type and other mutant viruses.

Discussion

PI cultured cells have potential usefulness as models for the study of chronic diseases of viral etiology (29). Measles virus has been associated with at least two slow neurological diseases in man characterized by demyelination, SSPE and MS (1–4). Interest in measles virus latent infection has resulted in the generation of a variety of mammalian cell lines persistently infected with measles, including HeLa (27, 30, 31), HEp-2 (26), the human lung line Lu 106 (32), monkey kidney cell lines Vero (33) and CV$_1$ (34), and human lymphoblastoid cell lines (14, 18, 28).

The study of PI LCL is of particular interest because of the characteristics of lymphocytes with respect to virus infection. First, resting lymphocytes are restrictive for virus growth and readily harbor virus in a latent state (35). But lymphocytes activated by mitogens or specific antigens are then able to support the replication of several viruses, including VSV (36), measles (37), mumps (38), vaccinia (39), and Herpes simplex virus (40). Second, infection by a number of viruses including measles and mumps causes transient immunosuppression in the host (41). This temporary period of immunologic incompetence could provide the virus with an escape from the host's immune defenses and allow a persistent infection to be established in lymphoid or other cells. Third, measles virus has been isolated from lymphocytes obtained from patients months and sometimes years after an acute measles infection. Recovery of measles virus has been obtained by cocultivation on susceptible cell monolayers without mitogenic stimulation (42). Lymph node biopsies provided some of the first isolates of the measles variant implicated in SSPE (3). In addition, localization of measles antigen in mononuclear cells of jejunal biopsies of MS patients has been reported recently (6). Since some lymphocytes are known to be extremely long-lived (43), these observations suggest that lymphoid cells might serve as a reservoir for harboring the viral agents in chronic infections before the appearance of clinical symptoms and disease (35).

The PI LCL differ from other measles infected cell lines in several important characteristics. The carrier state in LCL is more readily and frequently established than in other cells, with the LCL cell culture stabilized 5 wk after infection. In contrast, a PI CV_1 line was first passaged 260 days after infection (34). Antibody to measles in the culture medium is not required, as in the case of the HEp-2 (26) measles PI cultures, nor was the addition of DI particles necessary (33). In some of the measles PI lines, no infectious virus can be detected (34). When infectious virus has been previously isolated from other cell lines, some degree of temperature sensitivity has been noted (25–27, 32), but the progeny virus were assumed to be homogeneous, and no clonal analysis was attempted. Previous reports of the PI LCL did not mention any ts characteristics of the virus being produced by the PI cultures (14, 18), and although Minagawa et al. (28) reported a slight temperature sensitivity (P.E. ratio $>10^{-2}$) when total supernatant virus was titered, this P.E. difference was not considered significant.

It is important to note that a plating efficiency ratio for total supernatant virus may be misleading in determining the presence or absence of ts mutants in a given virus population. Heterogeneous populations of virions with different P.E. ratios can become aggregated and possibly self-complementary, masking the appearance of temperature sensitivity. Clonal analysis is necessary to reveal the fraction of ts mutants in a total viral population. We have shown that a clonal analysis of progeny virus from measles-infected LCL reveals that the majority of virus produced was ts. The population of ts mutants was strikingly heterogeneous in P.E. ratios, as well as in biological assays for thermolability and antigen production at the nonpermissive temperature.

Clonal analysis has been carried out for progeny virus from a VSV-PI L cell line (44). The 34 VSV clones from this mouse cell line also showed a range of P.E. ratios from 10^{-3} to $<4 \times 10^{-7}$. However, all 34 ts clones were defective in RNA synthesis at the nonpermissive temperature. Complementation analysis indicated that the eight mutants tested belonged to complementation group I, the most common mutant class that appears among spontaneous mutants in wild-type VSV populations. Complementation analysis of the ts isolates from the PI LCL is in progress, and analysis of RNA synthesis at 39°C indicated that both RNA-positive and RNA-deficient mutants are present in the ts viral population. Therefore, preliminary results suggest that these ts mutants will represent genetic differences, and are the result of lesions in different viral genes.

Although ts mutant viruses are the most outstanding characteristics of the LCL carrier state, other factors involved in persistent infection, such as interferon and DI particles, cannot yet be excluded. Interferon and DI particles may be more important in the period before establishment of a stable carrier culture, rather than in the maintenance of persistence, as has been suggested (44).

The origin of the heterogeneous population of ts mutants is an intriguing question. It seems unlikely that multiple ts mutants could be present in the initial inoculum, because of the low MOI used (1 PFU/10^6 cells) and the failure to detect a high frequency of ts mutants in the parental virus stock used for infection. Therefore, it is probable that mutations were generated in the

multiple rounds of replication during the period of 5 wk before the cultures stabilized. The phenotypic differences in the ts isolates suggest that no single class of mutants dominated, and that infection of LCL can induce a variety of mutations. Whether or not a single cell can produce a heterogeneous population of mutants is under investigation; but the likelihood of this is suggested by the clonal analysis of progeny virus from the doubly cloned culture of cell clone 16.

It is our hope that the LCL may prove useful in the generation of ts mutants, probably of different complementation classes. Persistent infection of LCL might be a close reflection of what can occur in vivo, and the study not only of ts mutants derived by chemical mutagenesis (45, 46), but also of those which arise spontaneously by persistent infection of lymphoid cells may be of importance.

Temperature-sensitive viral mutants have been useful in the study of chronic diseases. While mice infected with wild-type reovirus Type 3 develop an acute, lethal encephalitis, animals infected with some ts mutants develop a slow neurological disease (47). Inoculation of ts mutants of measles virus intracerebrally into neonatal hamsters can result in hydrocephalic changes of the brain (48). Several mechanisms based on persistence of virus in lymphoid cells could explain the development of a slow neurological disease. There could be escape of mutant virus or of wild-type revertants from the reservoir of persistence to a susceptible neurological site. It is also possible that PI lymphocytes might migrate into the central nervous system, with initiation of an inflammatory immune response against the viral antigens or infected cells, leading to demyelination or other pathological lesions as a bystander effect (49).

Whereas the viral etiology of MS is by no means established, and its association with a measles-like virus remains even more tenuous, the study of viral mutants could provide insight into one of the most puzzling aspects of this disease, namely the unpredictable exacerbations and remissions. Based on an analogy with perhaps the most classical relapsing infectious disease, African trypanosomiasis (50), one mechanism explaining exacerbations would be the emergence of variants with altered antigens. If a slow neurological disease like MS were produced by a measles-like agent, it is possible that the initial persistence of virus would involve the generation and establishment of mutant viruses. We would suggest that subsequent exacerbations might be precipitated by the emergence of antigenically altered secondary or tertiary mutants which could flourish for short periods until an appropriate primary immune response developed to suppress them and induce remission. The feasibilty of such a model for a slow neurological disease has been elegantly demonstrated recently for chronic demyelination produced in sheep by successive, antigenically variant mutants of visna virus presumably selected for by the immune response to the initial antigenic type (51).

Summary

Two human lymphoblastoid B-cell lines, WI-L2 and 8866, were infected with the Edmonston strain of measles virus at a multiplicity of infection of 10^{-6}, and stable persistent infections were established. By immunofluorescence and electron microscopy, the vast majority of cells from both cell lines were expressing viral antigens and releasing virion-like particles. However, very little infectious virus could be detected at 37°C, either by an infectious centers

assay or by titration of supernates from persistently infected cultures. When cultures were shifted to 31°C, the cells released a population of virus that was temperature-sensitive. Clonal analysis of supernatant virus at 31°C revealed a highly heterogeneous population of temperature-sensitive mutants, differing in plating efficiency ratios, thermolability, and antigen production at the nonpermissive temperature. Factors such as interferon, defective interfering particles, and extracellular virus do not appear to be important in maintaining the persistent carrier state. These studies have important implications for persistent infections of lymphoid cells in vivo, and the slow neurological diseases associated with measles, subacute sclerosing panencephalitis, and multiple sclerosis.

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