Some Characteristics of SSPE Measles Virus (34718)

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The recent isolation of measles virus from patients with subacute sclerosing panencephalitis (SSPE) has established the association of this disease with a suppressed measles virus infection (1–3). The present report describes serological and tissue culture studies comparing the SSPE measles virus with vaccine and wild strains of the virus. The present study also reports an *in vitro* comparison between two SSPE measles virus isolates.

Materials and Methods. Viruses. Two strains of SSPE measles virus were isolated by the mixed culture technique using brain cells from 2 SSPE patients with HeLa cells (1, 2). At the time of biopsy, patient No. 1 was in the early stage of the disease, showing the classical clinical picture, typical EEG findings, high measles antibody titers in serum and spinal fluid, and depression of the colloidal gold curve. The biopsy material was kindly supplied by Dr. Wolfgang Zeman from the Division of Neuropathology, Indiana University Medical Center, Indianapolis, Indiana. Patient No. 2 was biopsied during a more advanced stage of the illness, which was characterized by the repetitive simultaneous myoclonic jerks of the skeletal musculature; a colloidal gold curve of the paretic type reflecting a marked increase of spinal fluid IgG; high-amplitude, low-frequency, synchronous EEG waves; and high measles antibody levels on serum and spinal fluid. This biopsy was made available through the courtesy of Dr. J. T. Jabbour from the Department of Pediatric Neurology, University of Tennessee College of Medicine, Memphis, Tennessee. The first isolate, SSPE(1), was obtained in mixed cultures of cells from a primary brain biospy tissue culture. The second strain, SSPE(2), was isolated in mixed cultures of cells from passage level 7 of a subcultured brain biopsy tissue culture. SSPE virus suspensions, obtained from infected HeLa cell monolayers frozen and thawed 3 times, were centrifuged to remove cell debris and then filtered through Millipore acetate membranes (Millipore Corporation, Bedford, Mass.). Membranes of 0.45 and 0.8 μ pore sizes were used and the virus suspensions were titered before and after filtration to establish the cell-free status of the agent.

The attenuated vaccine Edmonston strain, chick embryo tissue culture adapted virus, used in these studies, was obtained from Merck Sharp & Dohme (Lot No. 0793K, Lyovac Rubeovax). This virus, Edmonston strain 749D, was passaged 24 times in primary human embryonic kidney (HEK) tissue culture, 28 times in primary human amnion (HuA) tissue culture, 12 times in the allantoic cavity of embryonated chicken eggs, and 64 times in primary chicken embryo (CE) tissue culture.

"Wild" measles virus isolated from a child with natural disease was obtained from Miss Hope Hopps from the Division of Biological Standards, National Institutes of Health, Bethesda, Maryland, and propagated in primary human embryonic kidney tissue culture. The strain was used in this study at the fourth passage level; 3 in HEK and 1 in African green monkey kidney (AGMK) tissue cultures.

Tissue cultures. Primary canine kidney (CK), CE, and AGMK tissue cultures were purchased from Microbiological Associates. The BSC-1 continuous cell line cultures were obtained from Flow Laboratories, Inc. Tissue cultures of the established HeLa cell line have been routinely grown in our laboratory

as outlined in a previous communication (2).

Monolayers of AGMK were maintained with Eagle's basal medium containing 2% inactivated fetal bovine serum (FBS). All other tissue cultures were fed with a maintenance medium composed of Eagle's minimum essential medium (EMEM) enriched with 2% FBS. Normal human brain tissue culture was prepared with trypsinized cells from cerebral cortex obtained from a patient with cerebral palsy. Cells were grown in EMEM with 20% FBS and maintained in EMEM with 2% FBS. Primary monolayers were subcultured 3 times and then utilized in virus studies.

Virus assays. Each virus strain containing approximately 1,000 $TCID_{50}$ in 5 ml was inoculated into 8-oz bottles containing the above-mentioned host-cell systems. Cultures were then incubated for 1 hr at 37° to allow virus adsorption prior to the addition of 20 ml of maintenance medium. Infected and control cell cultures were observed daily under light microscopy for at least 10 days and cytopathic effects (CPE) were recorded as negative (—); 25% (+); 50% (++); 75% (+++); or 100% (++++). On the 11th day postinoculation, cell cultures were frozen and thawed twice and fluids were clarified by centrifugation at 1000*g* for 20 min at 4°. Harvested fluids were then assayed for infectivity and hemagglutinins.

Virus infectivity titrations were performed in AGMK tubes using serial 10-fold dilutions; 0.2 ml of each dilution was inoculated into each of 4 culture tubes. These were held in a 37° incubator for a maximum of 14 days, at which time the $TCID_{50}$/ml was calculated by the method of Reed and Muench (4).

Plaque assays were conducted in monolayers of BSC-1 cells grown in 96-well, flat-bottom, plastic plates (Falcon Plastics). Medium was removed from wells and appropriate dilutions of virus were inoculated in groups of 12 wells/dilution. A calibrated dropper (Linbro Chemical Co., Inc.) was used and the virus inoculum was 0.025 ml. Virus was allowed to adsorb for 1 hr at 37°, followed by the addition of 0.05 ml/well of Eagle's minimum essential medium containing 0.75% of methylcellulose. Cultures were then covered with a plastic cover and incubated for 7–10 days at 37° in a 5% CO_2 atmosphere. After the incubation period, medium was removed from wells and the monolayers were washed 3 times with distilled water. Cells were fixed with 15% formalin for 2 hr, washed, and stained with hematoxylin–eosin. Plaques were enumerated and measured with a microscope micrometer.

Serological tests. Hemagglutination inhibition (HI) and complement fixation (CF) tests were performed by the microtiter system. Hemagglutination tests (HA) were carried out with rhesus monkey erythrocytes in the microtiter system as described by Sever (5). The highest antigen dilution producing complete hemagglutination was considered 1 HA unit and the reciprocal of the dilution was referred to as the HA titer. Serum specimens were first treated by absorption with kaolin and then with monkey red blood cells; 4 units of antigen were used in the test. The commercial HA antigen utilized, 3-3331 from Microbiological Associates, was the Edmonston strain 84F at passage level 9 in primary human heart. All test reagents were from the same source for every assay.

For the CF antibody determinations serum specimens were inactivated at 56° for 30 min; 4 units of the antigen and 2 exact units of complement were used. The commercial CF antigen utilized, M944006 from Flow Laboratories Inc., was the Edmonston strain after 24 passages in HEK, 30 passages in HuA, 12 passages in established AV-3 cell line, and 2 passages in AGMK tissue cultures.

The SSPE measles antigen employed both in CF and HI tests was prepared in AGMK cells and concentrated 5 times by ultracentrifugation (80,000*g* for 5 hr—virus pellet resuspended in EMEM).

Neutralization tests were performed in BSC-1 cells according to the method described by Fuccillo *et al.* (6). Serial 2-fold dilutions of serum specimens were mixed with equal volumes of virus and incubated at 35° for 1 hr. Each serum dilution was assayed simultaneously against 100, 10, and 1 $TCID_{50}$ of virus. Aliquots of 0.05 ml of each mixture were added to each of 4 wells (Falcon

TABLE I. Comparative Tissue Culture Study Between SSPE(1) Vaccine, and Wild Measles Viruses.

Cell system	Virus strain: Wild HA[a] titer	Wild Infectivity[b] titer	Wild CPE[c]	SSPE(1) HA titer	SSPE(1) Infectivity titer	SSPE(1) CPE	Vaccine HA titer	Vaccine Infectivity titer	Vaccine CPE
AGMK	—[d]	4.2	++	8	6.2	++++	—	4.2	++
BSC-1	—	2.0	+	8	7.2	++++	—	2.4	+
HeLa	—	—	—	4	6.2	++++	—	4.2	++
CK	—	—	—	—	5.2	+++	—	3.2	++
CE	—	—	—	—	4.2	+++	—	3.2	++

[a] Hemagglutination.
[b] $TCID_{50}$ (log_{10}).
[c] Cytopathic effect.
[d] — = negative.

plates). The plates were then incubated at 37° and examined for typical CPE on days 7 and 14. Serum samples from 6 well-documented SSPE patients and 3 serum specimens obtained from individuals with naturally occurring measles were employed in the comparative serological assays. These specimens were coded and tested together at one time with identical lots of antigens, reagents, and cells. Antibody titrations were performed in duplicate for all serum samples to insure reproducibility of results within a 2-fold dilution. Titers of all serological determinations were recorded as the reciprocal of the highest dilution of sera showing positive reaction.

Results. The cell host range for each virus, degree of cytolysis observed, quantity of infectious particles and hemagglutinins detected in harvested materials are summarized in Table I. The very pronounced CPE induced by the SSPE (1) agent was accompanied by a very high yield of infectious virus. Both attenuated and SSPE viruses were capable of multiplying in all 5 tissue culture systems employed in this study whereas the wild virus strain did not replicate in HeLa, CE, or CK cells. Hemagglutinins were produced only by the SSPE(1) measles virus in monolayers of AGMK, BSC-1, and HeLa cells.

In general, the serum specimens from patients with SSPE and convalescent sera from children with measles reacted similarly with antigens prepared with SSPE(1) or Edmonston strains of measles virus as tested by CF and HI (Table II). The results attained with the neutralization tests, however, indicated the sera from SSPE patients 1 and 2 had 4- to 8-fold lower antibody titers with the SSPE(1) virus than with the wild strain. This difference in titers did not occur with convalescent sera from 2 children with measles tested with the same virus strains.

TABLE II. Reactivity of Sera from SSPE Patients with Natural Measles with SSPE(1) and Measles Antigens.

Antisera	Antigens: Edmonston strain[a] and wild measles CF	HI	Neut.[b]	SSPE(1) measles virus CF	HI	Neut.
SSPE						
Patient 1	2048	1024	256	1024	1024	64
2	1024	256	64	512	256	8
3	512	512	—	256	512	—
4	1024	512	—	2048	1024	—
5	1024	512	—	512	1024	—
6	512	512	—	128	1024	—
Measles						
Patient 1	128	256	128	256	256	128
3	16	16	—	16	32	—
7	32	64	—	32	64	32

[a] The Edmonston strain was utilized in HI (hemagglutination-inhibition) and CF (complement fixation) tests.
The wild measles virus was employed in the neutralization test.
[b] — = not tested.

TABLE III. Plaque Comparison Between SSPE, Wild and Attenuated Measles Virus.

Strain	Mean plaque diam (mm)	PFU/ml[a] log 10	Plaque morphology
SSPE(1)	1.0	5.4	Small, circular, irregular sharp boundaries
Vaccine	1.0	3.1	Small, circular, irregular sharp boundaries
Wild	1.6	1.7	Large, circular, irregular sharp boundaries

[a] Plaque-forming units per milliliter.

The results of plaque assays are shown in Table III. Plaques of the wild virus were large and circular with irregular sharp boundaries. Both the SSPE(1) and vaccine viruses made small circular plaques with irregular sharp boundaries. The SSPE(1) virus produced plaques within 7 days after inoculation while the other two viruses required 10 days to develop plaques.

A comparison of the 2 strains of SSPE measles virus is shown in Table IV. These viruses grew well in HeLa cells, CK, and normal human brain tissue culture. However, the SSPE(1) strain proved to be more cytolytic, producing more rapid and extensive CPE than the other isolate. This difference was further substantiated by hemagglutinin assays with rhesus monkey erythrocytes; the culture fluids from the faster growing SSPE(1) strain invariably produced HA titers 4- to 8-fold greater than those obtained with the less cytolytic virus. Infectivity titers established that virus replication was at least 100-fold greater for the SSPE(1) strain.

Both SSPE(1) and SSPE(2) viruses when filtered through 0.45 μ pore size Millipore membranes retained approximately 20% of their original infective titers. Filtration through 0.8-μ pore size membranes did not produce any reduction of infectivity. These results demonstrate the cell-free status of the retrieved SSPE measles virus as compared to its original suppressed cell-dependent condition.

Discussion. Comparative tissue culture studies (Table I) revealed that the SSPE(1) measles was more cytolytic and more readily tissue-adapted than either the vaccine strain or the wild virus. It is known that virus isolated directly from measles patients is best propagated in primary cultures of human or simian kidney cells, becoming adapted to a variety of cell systems only after a varying number of passages. Furthermore, infectivity titers of human isolates in primary cultures of human or monkey cells are usually low, ranging from 10^3 to 10^5/ml. The SSPE(1) measles virus grew in a wide spectrum of host cells producing rapid specific cell destruc-

TABLE IV. *In Vitro* Comparison of Two SSPE Measles Isolates.

Virus	Cell system	Passage level[a]	CPE[b]	HA titer	Infectivity titer[c]
SSPE(1)	HeLa	1	++++	8	6.2
		2	++++	16	6.4
	CK	1	+++	—	5.2
	Normal human brain	1	+++	2	NT[d]
SSPE(2)	HeLa	1	+++	2	3.0
		2	+++	2	2.7
	CK	1	++	—	2.7
	Normal human brain	1	+	—	NT

[a] After isolation in mixed culture.
[b] Cytopathic effect.
[c] $TCID_{50}$ (log 10).
[d] NT = not tested.

tion with unusually high virus yield and hemagglutinin levels.

The serological comparison of patients with SSPE and children with natural measles showed similar CF and HI titers using Edmonston strain virus. The neutralization titers with these patients' sera, however, were 4- to 8-fold lower with the SSPE(1) virus than with the wild measles virus, which suggests that the antibody produced by the patients is less effective against the virus of their disease than against the wild virus. These differences were confirmed in repeat titrations. If this finding is observed with other SSPE patients, it would suggest that treatment with massive doses of hyperimmune measles gamma globulin and/or specially prepared measles vaccines should be investigated.

The plaque morphology studies with the 3 strains of virus were consistent with the plaque differentiation of virulent and attenuated strains of measles virus reported by Rapp (7). Although plaque morphology was essentially the same for the strains tested, there were differences in size and time of appearance which could be used as a criteria for the study of the properties of different strains of measles. Plaque-forming units (PFU/ml) correlated with CPE endpoints obtained in tube titrations, showing a much higher titer for the SSPE(1) measles virus.

The comparison of the 2 strains of SSPE measles virus showed them to be quite similar under *in vitro* conditions. The slight differences observed could be related to the infectivity titers alone. These 2 strains of measles virus appear to resemble, more closely, attenuated measles virus than wild virus. This fact is rather interesting and suggests that either the virus is highly adapted when the SSPE patient is initially infected or passage *in vivo* in brain cells accomplishes the same result. It should be noted that SSPE(1) virus was isolated from a primary brain biopsy tissue culture while SSPE(2), the less infectious strain, was recovered from passage level 7 of a subcultured brain biopsy tissue culture. The stage of the illness, area of the brain biopsied, passage level of the brain tissue culture, and method of rescuing the virus, may prove to be important factors in affecting the characteristics of virus recovered from SSPE diseased brain tissue.

Summary. The *in vitro* comparison of two SSPE measles virus isolates and conventional measles virus demonstrated antigenic identity between these viruses. However, neutralization tests showed an avidity of the SSPE patient's neutralizing antibody towards the wild measles virus rather than to the virus isolated from diseased brain. The cytopathology and plaque formation of these measles virus strains revealed greater resemblance between the SSPE measles and the vaccine strain of the virus than between SSPE measles and wild virus. Differences were found in the growth characteristics of the two SSPE virus isolates as measured by tissue culture assays.

1. Horta-Barbosa, L., Fuccillo, D. A., Sever, J. L., and Zeman, W., Nature (London) **221**, 974 (1969).

2. Horta-Barbosa, L., Fuccillo, D. A., London, W. T., Jabbour, J. T., Zeman, W., and Sever, J. L., Proc. Soc. Exp. Biol. Med. **132**, 272 (1969).

3. Payne, F. E., Baublis, J. V., and Itakashi, H. H., N. Engl. J. Med. **281**, 585 (1969).

4. Reed, L. J., and Muench, H., Amer. J. Hyg. **28**, 493 (1938).

5. Sever, J. L., J. Immunol. **88**, 320 (1962).

6. Fuccillo, D. A., Catalano, L. W., Jr., Moder, F., Debus, D., and Sever, J. L., Appl. Microbiol. **17**, 619 (1969).

7. Rapp, F., J. Bacteriol. **88**, 1448 (1964).

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