Optimal Conditions for Isolation of a Neurotropic Measles Virus from Brain Tissue (34799)

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The difficulties in isolating viral agents from the central nervous system (CNS) in postinfectious (measles, rubella, varicella, influenza) and postvaccination (rabies, vaccinia) encephalitis are well known (1–7). These very difficulties have, in fact, served in part to distinguish postinfectious and postvaccination encephalitis from the more common encephalitides caused by viruses in the arbo and entero groups (8).

Immune mechanisms have been implicated in both postinfectious and postvaccination encephalitides, and occasional successful viral isolations from brain have been interpreted as recovery of blood-borne virus present in the vascular system of the CNS (6).

It has been shown for varicella (9, 10) as well as for neurotropic measles virus (11) that transfer of the agents from one cell type to another or even transfer within the same cell type may require the presence of viable infected cells. This situation is best demonstrated by the failure to isolate measles virus from brain in acute or chronic measles encephalitis when routine isolation techniques are used (1, 2, 12–14).

Recently, several instances of successful isolation of measles virus have been reported from patients with subacute sclerosing panencephalitis—a presumed measles infection—in which isolated viable nerve cells instead of brain homogenates were used as inoculum (15, 16).

It is obviously difficult to preserve the viability of CNS elements during dissociation considering their intricate connections and vulnerability. Dissociation by micromanipulation, by a combination of mechanical and chemical methods, or by enzymatic digestion was used with variable success as regards morphological integrity, purity, and viability of individual cell types (17, 18).

The present paper quantitatively examines conditions for recovery of a hamster brain-adapted measles virus (19) using different techniques of brain dissociation. In addition, virus recovery from brain tissue at different time periods after death was investigated.

Materials and Methods. Virus. The hamster brain-adapted measles virus (19) was received from Dr. Theodore Burnstein in its 109th suckling hamster passage. It was used in the 110–112th passage throughout the present experiments. Infectious stock virus was prepared from a suspension of suckling hamster (golden Syrian; 2–3 days old) brains harvested as animals became moribund 4–5 days after intracerebral (ic) inoculation. A 10% brain homogenate in the tissue culture maintenance medium (MEME with 2% FBS) described below was stored in 1-ml aliquots at −60°. The virus titer of this suspension was $10^{5.5}$–$10^{6.0}$ suckling hamster ic LD_{50}/0.1 ml and was approximately 10-fold less in weanling hamsters.

VERO cell cultures and virus titration. The VERO African green monkey embryonic kidney cell line (20) was received from Dr. J. Rhim (Microbiological Associates, Bethesda, Md.) through Mrs. H. E. Hopps (Division of Biologics Standards, NIH, Bethesda) in its 174th passage. The cells were grown in 8-oz milk dilution bottles in medium 199 containing 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin G, 100 μg

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streptomycin sulfate, and 5 μg tetracycline HCl per ml).

For virus-titration plastic disposable microplates with 96 flat-bottomed circular wells were used (MicroTest II tissue culture plates, Falcon Plastics, Division of Bioquest; or Model IS-FB-96-TC MICRO plate, Linbro Chemical Co.) Each well was seeded with 1 drop of medium containing 25,000 cells. Several hours later 0.1 ml (2 drops) of the brain material to be titrated was added using 4–6 wells per dilution. Eagle's minimal essential medium with Earle's balanced salt solution (MEME) (21) containing 2% FBS and the above concentration of antibiotics was used for seeding the VERO cells and virus material. The plates were incubated without further change of medium in a humidified 5% CO_2 atmosphere at 37° for 10 days when the final reading was made.

Brain cell cultures. To test the viability of the brain cells obtained by different dissociation procedures 35-mm Falcon plastic petri dishes were seeded with 0.2 ml of the 10% brain cell suspension in 3.0 ml of medium. As a routine MEME with 10% FBS and the above-mentioned antibiotics were used and changed twice weekly. Cell viability was judged by the rate of outgrowth into monolayers.

Experimental animals. Weanling golden Syrian hamsters (3–4 weeks old) were obtained from the Rodent and Rabbit Production Section, Laboratory Aids Branch, NIH. After receiving an ic inoculation of 0.03 ml of the measles virus 10% brain homogenate, the animals were sacrificed 5–10 days later when clinical symptoms were evident. First neurological symptoms appeared usually on day 5 and the majority of animals died on day 7 and 8 after infection. Occasional animals survived longer or recovered.

Processing of brain tissue. Animals used in isolation studies were bled out, the brains removed, and cut into smallest possible pieces using forceps and a sharp scalpel. Teasing and pressing was avoided. The mince from several brains was pooled, washed in a petri dish three times with phosphate-buffered saline (22), and divided into the required amount of equal aliquots.

For dissociation, 0.25% trypsin (Difco), 0.02% Versene (ethylenediamine tetra-acetic acid) or 9.4 m*M* sodium tetraphenylboron at 37° were used. Mechanical disruption was achieved by pressing minced brain gently through a No. 60 wire mesh (60 threads/inch) followed by repeated pipetting in a 5-ml glass pipette. Homogenization of the brain mince was achieved using a Sorvall Omni-Mixer Micro-Attachment activated for 2 min at setting 7 (full scale divided into 10 settings) at 4°.

Brain cell suspensions resulting from enzymatic or mechanical dissociation were taken up in washing fluid, spun for 10 min at speeds varying from 400 rpm (40*g*) to 1000 rpm (250*g*) and reconstituted to a 10% cell suspension.

Assay of virus in weanling hamster brain. Serial 10-fold dilutions of the brain cell suspension or whole brain homogenate were titrated in microplate VERO cultures (0.1 ml per well) or IC in suckling hamsters (0.02 ml per animal). Endpoints based on characteristic syncytia formation were calculated by the Kärber method (23) and the titers are expressed as negative antilogarithm per 0.1 ml of a 10% cell suspension or 10% brain homogenate.

Virus recovery from brain material obtained at necropsy. Hamsters with advanced neurological symptoms of disease were sacrificed by cervical dislocation and placed in a refrigerated room (4°) for periods up to 22 hr. Thereafter the brains were removed from the skull and processed simultaneously in a way to be described.

Results. Recovery of measles virus by different procedures of brain dissociation. Six hamsters were sacrificed 10 days after infection with measles virus after showing protracted symptoms of encephalitis. The brains were minced and then divided into six equal portions which were processed as indicated in Table I.

It can be seen that tryptic digestion provided the highest virus titer when the cell suspension was assayed in VERO cells or by ic inoculation into suckling hamsters. Versene was considerably less suitable. Although mechanical disruption of the brain was very

TABLE I. Recovery of Viable Brain Cells and Measles Virus from Infected Brain Tissue by Different Dissociation Procedures.

Method of treatment of brain tissue	Volume of released cells (ml)	Viability of cells (trypan blue exclusion test)	$TCID_{50}$/0.1 ml of 10% brain cell suspension	LD_{50}/0.1 ml of 10% brain cell suspension	Primary brain cell cultures 11 days after seeding
Homogenized	—	—	0	50[a]	—
Trypsin	.05	>99%	830[a]	4150	Good outgrowth covering large part of petri dish
Trypsin + Versene	.05	>99%	830	4150	Scattered islands of outgrowth
Versene	0.1	90%	14	28	No growth
Passed through wire mesh + Versene	0.2	50%	0	ND[b]	No growth
Passed through wire mesh and pipetted	0.3	40%	0	160	No growth

[a] Negative antilogarithm of $TCID_{50}$ and LD_{50} values obtained.
[b] ND = Not done.

efficient, as judged from the volume of pelleted material, it resulted in profound damage as evidenced by the trypan blue exclusion test and failure of isolated cells to grow out in culture vessels.

Besides the techniques listed in Table I, dissociation by sodium tetraphenylboron was used in two experiments performed in collaboration with Dr. Roger A. Snyder (NINDS, Bethesda). Three and 3/4 hr of dissociation yielded sufficient amounts of morphologically well-preserved cells. In one of two attempts virus was recovered in cell cultures in the second subpassage. A marked toxic effect of tetraphenylboron on suspended cells was observed and seemed to be the main reason for unsatisfactory results.

Factors affecting the trypsinization procedure. Because of the observed sensitivity of brain cells to mechanical and chemical treatment, the effect of repeated cycles of trypsinization and centrifugation were investigated. Minced infected hamster brain was trypsinized consecutively three times for 20 min each. The harvests were collected separately in MEME containing 10% FBS and spun at 400 rpm. The supernatant fluid from each harvest was transferred into another test tube and spun again at 1000 rpm. Each cell pellet was reconstituted to give a 10% cell suspension and titrated in VERO cell cultures.

As seen in Table II, 400 rpm was sufficient to pellet most of the infectivity. Prolonged trypsinization gradually dissociated more tissue but decreased the virus titer. On mi-

TABLE II. Effect of Repeated Cycles of Trypsinization and Centrifugation on Recovery of Measles Virus from Brain Tissue.

Trypsinization cycle (20-min intervals)	A. Cell harvest spun at 400 rpm		B. Cell harvest obtained from supernate in A after spinning at 1000 rpm	
	Volume (ml)	$TCID_{50}$/0.1 ml	Volume (ml)	$TCID_{50}$/0.1 ml
First	.05	316[a]	0.1	32[a]
Second	0.1	200	.15	20
Third	0.3	50	0.2	13

[a] Negative antilogarithm of $TCID_{50}$ values obtained by the Kärber formula (23).

TABLE III. Effect of Composition of Washing Fluid on Recovery of Measles Virus from Trypsinized Brain Tissue.

Cells taken up in:	A. Cell harvest spun at 400 rpm		B. Cell harvest obtained from supernate in A. after spinning at 1000 rpm	
	Volume	$TCID_{50}$/0.1 ml	Volume	$TCID_{50}$/0.1 ml
Hanks BSS	.03	7[a]	.02	31[a]
Hanks BSS + soybean inhibitor	.05	24	.05	50
Eagle's medium w/10% FBS	.05	94	.05	5
Eagles's medium w/10% FBS + soybean inhibitor	.06	24	.02	12.5

[a] Negative antilogarithm of $TCID_{50}$ values obtained by the Kärber formula (23).

croscopic examination the cells in all six pellets showed greater than 95% viability in the trypan blue dye exclusion test. However, pellets obtained at 1000 rpm contained a considerably higher admixture of cell debris.

A brain cell suspension collected after 30 min of trypsinization was divided into four equal parts and taken up in different washing fluids as indicated in Table III. After spinning at 400 rpm the supernatant fluids were decanted and spun again at 1000 rpm. The results in Table III show that a medium containing 10% FBS was satisfactory for efficient collection and preservation of infectivity at 400 rpm. Increased recovery of infectivity in the pellet was achieved at lower speeds when 10% FBS was present in the medium. Soybean inhibitor had a protective effect when added to Hanks' BSS.

Comparison of virus recovery from trypsinized and homogenized brain tissue. Infectivity titrations of trypsin dissociated or homogenized samples derived from the same brain source were performed simultaneously in VERO cell cultures and IC inoculated suckling hamsters over a period of several months.

The comparison presented in Table IV shows that dissociated brain cells transfer infectivity more effectively than do homogenates when inoculated into hamsters and were almost a prerequisite for virus isolation in cell cultures. There was no direct relation-

TABLE IV. Comparison of Quantitative Recovery of Measles Virus from Infected Hamster Brain Processed by Trypsinization or by Homogenization.

Days after infection	Neurological symptoms	Titration in VERO cells: $TCID_{50}$/0.1 ml of: 10% trypsinized brain cell suspen.	Titration in VERO cells: $TCID_{50}$/0.1 ml of: 10% brain homogenate	Titration in suckling hamsters: LD_{50}/0.1 ml of: 10% trypsinized brain cell suspen.	Titration in suckling hamsters: LD_{50}/0.1 ml of: 10% brain homogeate	Outgrowth of brain cell cultures (days needed to achieve a 95% cell sheet)
5	Initial	17.8[a]	0	89[a]	28	8
6	Pronounced	190	0	212	18	6[b]
7	Moribund	272	13	ND	ND	ND
8	Pronounced	100	0	ND	ND	11
9	Pronounced	56	0	400	0	8
9	Moribund	32	0	158	0	8
10	Pronounced	830	0	4150	50	ND

[a] Negative antilogarithm of $TCID_{50}$ and LD_{50} values obtained by the Kärber formula (23).
[b] 0.5 ml (instead of 0.2 ml) of 10% brain cell suspension seeded per petri dish. ND = Not done.

TABLE V. Isolation of Neurotropic Measles Virus from Brain Tissue at Different Times after Sacrifice of Animals Infected 5 Days Earlier.

	Titration in VERO cells		Titration in suckling hamsters		Outgrowth of brain cell cultures	
	$TCID_{50}$/0.1 ml of:		LD_{50}/0.1 ml of:			
Brain processed (hours after sacrifice)	10% brain cell suspension	10% brain homogenate	10% brain cell suspension	10% brain homogenate	Day of reading	% area sheeted
0	18[a]	0	16[a]	0	22	50
1	6	0	9	318	22	50
3	6	0	50	88	22	60
5	18	0	88	28	8	95[b]
6	32	0	160	890	22	100
18	10	0	ND	0	22	60
22	0	0	16	0	10	80[b]

[a] Negative antilogarithm of $TCID_{50}$ and LD_{50} values obtained by the Kärber formula (23).
[b] Cells seeded in MEME with 2% FBS (see text).

ship between virus titers and the duration or severity of the disease.

Recovery of measles virus from brain tissue removed at different times after death. In order to determine to what extent autolytic changes in the CNS affected trypsinization, outgrowth of cells in monolayers, and infectivity in the two assay systems used, the following experiment was performed. From a group of hamsters infected 5 days previously two animals were sacrificed at intervals over a period of 22 hr and their bodies stored at 4°. After the last two animals were harvested the brains were removed from all animals and processed simultaneously.

Results in Table V show that measles virus could be isolated up to 18 hr after death in VERO monolayers and up to 22 hr in suckling hamsters. Unlike the titration results obtained with fresh brain samples, titers in postmortem homogenates were on several occasions higher in suckling hamsters than in cell suspensions. This difference is probably a reflection of the greater vulnerability to trypsinization of brain cells undergoing autolysis. The reduced viability of the latter was also evident from the considerably slower outgrowth in petri dishes.

The markedly accelerated growth of cell cultures from brains removed 5 and 22 hr after sacrifice warrants special attention. Unlike in the other cultures, these cells were seeded and kept for the first 18 hr in MEME with 2% FBS. We introduced this change after observing that brain cells in medium containing 10% FBS remained for the most part floating without attaching to the petri dish. After 18 hr the medium was replaced with MEME containing 10% FBS, and the latter was changed twice weekly.

Discussion. Our results are concerned primarily with techniques for recovery of CNS-grown measles virus in cell cultures. Based on tests on a variety of primary and continuous cell cultures, the VERO cell line was selected because it appeared to be the most sensitive and convenient indicator system for the neurotropic measles virus.

A method widely used in isolation of intact, metabolically active neuronal and glial elements consists in dispersion of brain tissue by sieving, following by centrifugation in sucrose and Ficoll gradients to separate the individual elements (17). As measles virus infects both glial and neuronal cells no separation of the two cell types was attempted in order to reduce cellular trauma. Our results demonstrate that neither mechanical separation nor chemical dispersion of brain cells by two effective chelating agents, Versene and sodium tetraphenylboron (18), could approach the suitability of trypsin.

The most constant finding in our experiments was the inability to transfer neurotrop-

ic measles virus into cell cultures using homogenized brain tissue. Two explanations seem probable: (a) Measles virus proliferates in weanling hamster brain in a defective cycle (24) resulting in a small quantity of infectious virus which is capable of attaching to and multiplying in brain cells but lacks the ability to infect VERO cells. The information for virus synthesis, however, is readily transmitted in intimate cell contact, *e.g.*, as provided mainly through cell fusion. (b) It is also likely that a considerable portion of infectious virus, if present, would be prevented from manifesting itself because of the presence of disrupted brain tissue. Quantitative studies in progress indicate that up to 99% of neurotropic measles virus (a high-titer suspension from suckling hamster brain) is bound by homogenates of normal or infected brain tissue. A possible interference of viral antibodies in our system could be excluded, and interferon from infected brain, if present, would probably not be effective in VERO cells.

It is conceivable that the properties of measles virus causing encephalitis in man resemble those found in the hamster model. At least in part this would explain the failure to isolate infectious virus in acute measles encephalitis (1, 2) and in a total of 31 cases of subacute sclerosing panencephalitis—a presumed chronic measles encephalitis—when standard isolation procedures were used (12–14). Exception to this line of unsuccessful attempts are instances in which brain biopsy specimens—rich in inclusion bodies—were trypsinized and grown into monolayer cultures or seeded in mixed cultures with susceptible cell lines shortly after surgery (15, 16).

Our results indicate that attempts to recover virus from patients after death may be equally successful if processing is not delayed. Furthermore, we have succeeded in preservation of virus infectivity in stored brain material provided the tissue was suspended in a cryoprotective agent and frozen at a suitable cooling gradient to —60° (Albrecht and Schumacher, to be published).

Summary. For recovery of measles virus from brain tissue dissociation by trypsinization was superior to cell isolation by mechanical and chemical treatment. Optimal techniques for dispersion of brain cells were established. When tested in cell cultures or in animals dissociated cells transferred infectivity much more effectively than did brain homogenates. Isolation of measles virus was successful up to 18 hr after the death of animals in tissue culture and up to 22 hr when assayed in suckling hamsters.

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