Isolation of Measles Virus from Brain Cell Cultures of Two Patients with Subacute Sclerosing Panencephalitis (34196)

LUIZ HORTA-BARBOSA, DAVID A. FUCCILLO, W. T. LONDON, J. T. JABBOUR,[1] WOLFGANG ZEMAN,[2] AND JOHN L. SEVER

Section on Infectious Diseases, Perinatal Research Branch, National Institute of Neurological Diseases and Stroke, National Institutes of Health, Bethesda, Maryland 20014

Subacute sclerosing panencephalitis (SSPE, Dawson's encephalitis Van Bogaert's leukoencephalitis) has long been recognized as a progressive degenerative, neurological disease. Dawson described inclusions in the brains of affected individuals thereby suggesting a possible viral etiology (1, 2). Recent electron microscopic evidence of paramyxovirus-like particles and nucleocapsids (3), extremely high measles antibody titers in serum and spinal fluids of SSPE patients (4), and specific immunofluorescence with measles antibody in brain biopsies (4) provided indirect evidence that this disease was due to measles virus. In addition, trypsinized brain cell cultures from patients with SSPE also showed specific fluorescence with measles antisera (5, 6).

Several investigators attempted to isolate a virus from the brains of SSPE patients as well as to transmit the disease experimentally (7). Animals or tissue cultures inoculated with specimens from SSPE patients consistently failed to demonstrate the presence of infectious virus. The present report confirms and extends our initial observations on the successful isolation and identification of the presumed etiologic agent of SSPE (8).

Materials and Methods. The brain biopsies were obtained from two children with clinically, serologically, and pathologically documented SSPE. Patient 1 was biopsied at Indiana University Medical Center, and the specimen was shipped in Simm's balanced salt solution with 40% bovine serum, 25% bovine serum ultrafiltrate, and antibiotics to the National Institutes of Health (NIH). The biopsy from patient 2 was obtained at the University of Tennessee and similarly shipped to the NIH. This biopsy was subcultured through the seventh passage level by Mr. Monroe Vincent at Microbiological Associates Inc., Bethesda, Maryland.

Primary brain tissue cultures were prepared by mincing and trypsinizing the biopsy material according to standard procedures (9). Dispersed cells were washed three times with Hanks' balanced salt solution and then resuspended in Earle's minimum essential medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) to a final concentration of approximately 8×10^5 viable cells/ml. Two-ml aliquots of this suspension were planted in culture tubes which were sealed and placed in a 37° incubator. Growth medium was changed every 2 days until confluent monolayers developed. These contained a large population of fibroblasts with a few microglial cells scattered throughout the monolayer. Cultures were maintained in EMEM with 2% FBS. Subcultures were made by trypsinization of cell sheets which were then planted and maintained as the primary cultures.

The HeLa cultures were grown in EMEM with 10% FBS and maintained in EMEM containing 2% FBS. This cell line was originally obtained from Flow Laboratories Inc., Rockville, Maryland. Passages of this cell line were made according to standard techniques (9). The HEp II cultures were obtained from Microbiological Associates Inc. and grown and maintained in the same media mentioned above.

[1] University of Tennessee, Pediatric Neurology, Memphis, Tennessee 38103.

[2] Indiana University Medical Center, Indianapolis, Indiana 46207.

Mixed cultures of brain cells with HeLa or HEp II cells were prepared by combining 1 vol of brain cell suspension containing approximately 3×10^5 viable cells/ml with 2 vol of HeLa or HEp II suspensions with approximately 2×10^5 viable cells in each ml. These mixtures were then seeded in plastic dishes which were incubated at 37° in an atmosphere of 5% CO_2 in air.

Fluorescent-antibody staining (FA). Mixed cultures and brain cell cultures were assayed by the methods described by Smith (10). Sera employed in these assays were human paired specimens from three patients with natural measles. These were kindly provided by Dr. Albert Z. Kapikian from the National Institute of Allergy and Infectious Diseases. The acute sera were included in the tests as negative controls. Fluorescein-conjugated antihuman globulin (horse origin) produced by Progressive Laboratories, Inc., Baltimore, Maryland, was utilized for the indirect FA method.

Hemagglutination (HA) and Hemagglutination inhibition (HI). Virus hemagglutinins were detected with rhesus monkey erythrocytes using a micro-technique (11). Rubeola HI antibody determinations with the brain and mixed culture antigens were also accomplished with the micro-technique using the paired human sera from the children with natural measles. Commercial measles HA antigen was purchased from Microbiological Associates, Inc. for control titrations. Tissue culture fluid HA antigens from brain and mixed cultures were prepared by centrifuging the culture liquids at 1500 rpm for 10 min. Supernatants were used in the assays. Cell pack antigens were obtained by disrupting the cells by freezing and thawing twice.

Hemadsorption. Cultures were washed with Hanks' balanced salt solution and overlayed with rhesus monkey red cell suspension according to standard technique (12).

Infectivity assays. Titrations were performed by the end point dilution method in tube cultures of HeLa cells. Tenfold serial dilutions were prepared in maintenance media and each of four HeLa cell tubes were inoculated with 0.2 ml of each test dilution. The cultures were examined daily under light microscopy for cytopathic effect (CPE) throughout a period of 10 days. Appearance of syncytium or giant cells in the monolayers were attributed to infectious virus. This was confirmed to be measles virus by specific neutralization with paired antisera produced in monkeys.

Experimental animals. Three 1-year old rhesus monkeys, previously shown to have no detectable rubeola antibody by HI tests, were each inoculated intracerebrally with 0.6 ml of SSPE rubeola virus. The virus was originally isolated from patient 1 and had been passed twice in HeLa cells following release from mixed brain–HeLa cultures and had a titer of 5.3 $\log_{10}$/ml. Nine weanling ferrets with no detectable CF antibody to distemper and no HI rubeola antibody were each inoculated intracerebrally with 0.3 ml of the same rubeola virus preparation. The animals were observed daily for clinical signs. Daily throat swab specimens were taken from the monkeys and ferrets on days 6–14 following inoculation. Several specimens were obtained from each animal on days 21 and 28 after administration of virus.

Throat swab specimens were tested for the presence of virus by inoculation into HeLa and primary African green monkey kidney tissue cultures. All cultures were blind passed three times and observed for CPE then absorption at each passage. Monkey kidney cultures were also tested for release of hemagglutinating antigen using standard techniques (11).

Results. Primary brain tissue cultures of patient 1 were observed under light microscopy for a period of 14 days during which time no CPE appeared. However, immunofluorescent studies revealed specific intracellular viral fluorescence when monolayers were treated with convalescent but not acute sera from patients with natural measles (Figs. 1 and 2, Table I). Fluorescent particles could be seen throughout the cytoplasm but were most concentrated in the perinuclear regions. Fluorescence was seen in approximately 40% of the cells. Supernatant fluids from these cultures were inoculated into continuous cell lines known to be susceptible to measles in-

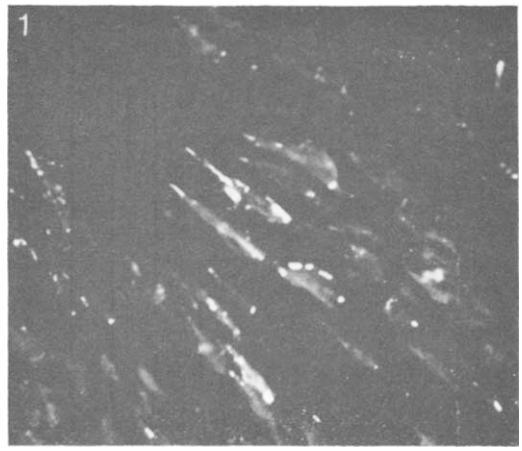

FIG. 1. Intracellular viral immunofluorescence of primary culture of brain cells from the SSPE patient; ×420.

cluding HeLa and HEp II with no evidence of a transmissible CPE producing agent. Brain cultures at the seventh passage level from patient 2 were studied by light microscopy and were found to contain syncytial formations. Intracellular measles specific immunofluorescence was observed in syncytia as well as in mononucleated cells as described above (Fig. 3 and 4, Table I).

Hemagglutination tests performed with disrupted cells from both primary and secondary brain cultures of patient 1 and seventh passage cultures of patient 2 demonstrated the presence of HA measles-specific antigen while the culture fluids alone failed to demonstrate cell-free antigen (Table I).

Mixed cultures containing the brain cells from either primary or secondary monolayers from patient 1 and 2 and HEp II cells did not develop CPE but similar mixed cultures with HeLa cells contained giant cells which became evident within 2–5 days of incubation. Fluids from these cultures were frozen, thawed, and inoculated into HeLa cell cultures and the same characteristic CPE appeared on the fourth to fifth day of incubation. The presence of measles virus in the mixed brain and HeLa cell cultures was further supported when fluids from these cultures hemagglutinated rhesus monkey red blood cells (Table I). This HA activity was inhibited by convalescent sera but not by the acute measles sera from children with natu-

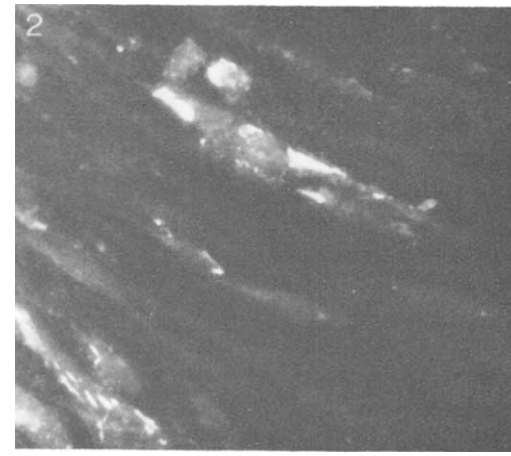

FIG. 2. Primary SSPE brain culture; indirect fluorescent antibody technique shows the intracellular rubeola antigen; ×870.

TABLE I. Rubeola Antigen and Infectious Virus in SSPE Brain Cultures and Mixed Brain–HeLa Cultures.

Type of culture	Intracellular antigen (fluorescence)	CPE	Hemagglutinating antigen (reciprocal of dilution)		Extracellular infectious virus[b]
			Culture fluids	Disrupted cells[a]	
Primary brain	+	—	<1	NT[c]	—
Secondary brain	+	+	<1	4	—
HeLa and brain cells	+	+	8	16	+[d]

[a] Cells disrupted by freezing and thawing; cell extracts were used in the tests.

[b] Based on cytopathic effects seen by inoculating culture fluids into HeLa.

[c] Not tested.

[d] Tissue culture ID_{50} (log_{10}) · titer = 5.3.

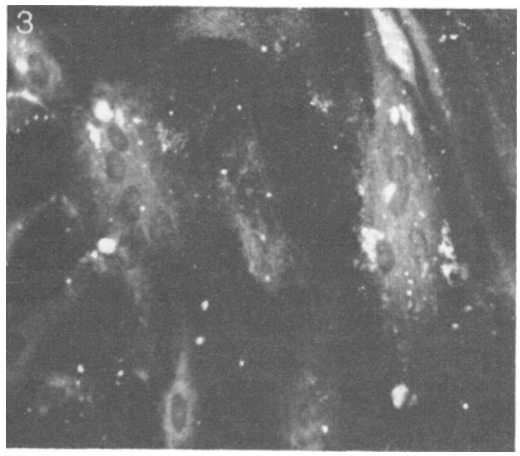

FIG. 3. Syncytial formation with rubeola immunofluorescence in a secondary culture of the SSPE brain cells; ×420.

ral measles. In addition, distemper antisera prepared in ferrets did not block the HA effect of the antigen, thus confirming the specificity of the HI reaction. Neutralization tests performed in HeLa cells showed that the infectious virus was specifically neutralized by measles antisera from monkeys.

Intracerebral inoculations of three rhesus monkeys resulted in development of HI antibody to measles. Titers ranged between 8 and 64. Similarly inoculated ferrets did not seroconvert and no clinical signs of disease were observed in any of the monkeys or ferrets through the 3 month period of isolation. Virus could not be isolated from the throat swab specimens from monkeys or ferrets.

HeLa cell monolayers infected with the SSPE rubeola virus hemadsorbed rhesus monkey erythrocytes while control cultures were nonreactive. Indirect FA tests with inoculated HeLa cell cultures were nonreactive. Indirect FA tests with inoculated HeLa cell cultures using measles antisera showed specific intracellular viral immunofluorescence (Fig. 5) whereas control monolayers did not show specific staining. HeLa cell culture fluid had an HA titer of 8. This antigen was inhibited by convalescent measles antisera but not by the acute sera.

Inoculation of susceptible cell systems such as HEp II and BSC-1 with the SSPE measles virus from the mixed brain HeLa cultures resulted in production of characteristic CPE (syncytial and giant cell formations) and replication of fully infectious rubeola virus.

Discussion. In our early attempts to recover a virus directly from SSPE brain specimens we experienced negative results. Inoculation of brain homogenates intracerebrally and parenterally into rhesus monkeys and ferrets failed to demonstrate the presence of an infectious agent. Dispersed cells obtained by trypsinization of brain biopsies also proved negative when similarly inoculated.

Recently Baublis and Payne (5) and Chen and co-workers (6), and we (8, 11) were able to grow SSPE brain cells in tissue culture and show the presence of measles antigens in the cell cultures.

Baublis and Payne (5) and Chen *et al.* (6) reported cocultivation experiments using SSPE brain cells and primate cell lines in which cytopathic effects appeared although free infectious virus was never detected. Thus while the brain cultures showed persistence of measles antigen, none of our previous studies nor those of the other investigators (5, 6) resulted in the release of virus. The release of fully transmissible rubeola virus was only accomplished when we performed a mixed culture technique with HeLa cells (8). The progressive disease which occurs in children with SSPE suggests that the suppression of the infection in the presence of antibody is incomplete. It is likely that minute amounts of complete virus are released from infected brain cells in patients with SSPE, thus pro-

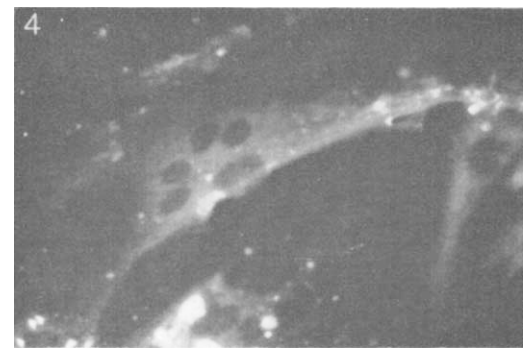

FIG. 4. Syncytium containing 5 nuclei in secondary SSPE brain culture; viral immunofluorescence is seen within cytoplasm of syncytium; ×870.

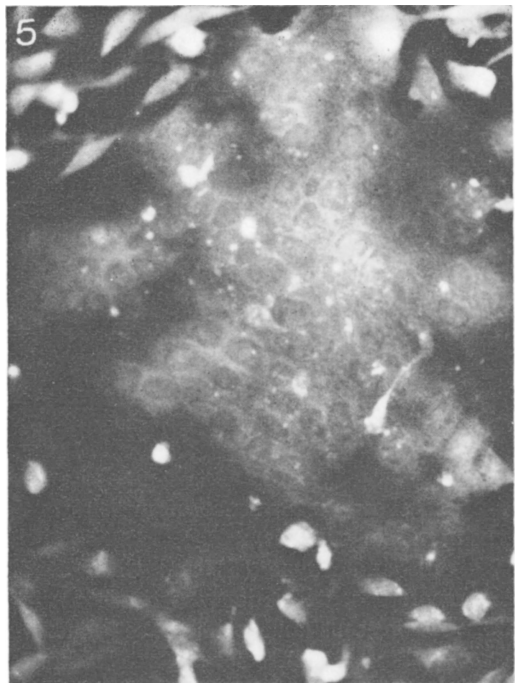

FIG. 5. Giant cell in a mixed culture of brain cells and HeLa cells; rubeola antigen is evidenced by immunofluorescence; ×420.

ducing the antigenic stimulation responsible for the high levels of antibody observed in these individuals. The infectious virus, however, would be neutralized by circulating antibody and thus infection by this route would be suppressed. A similar *in vitro* infection has been produced in HeLa cell cultures with measles virus in the presence of antibody (13). In that experimental system, the suppressed infection is maintained by transfer of viral genome to daughter cells during mitosis. In addition, direct cell to cell contact infection has been shown to occur with another myxovirus, the respiratory syncytial virus (14).

The present study established that HeLa cells function as appropriate hosts for isolating complete infectious rubeola virus when mixed with SSPE brain cells containing the suppressed virus. Just how the agent is "switched on" remains to be determined, but preliminary experiments indicate that this is accomplished by intimate cytoplasmic interaction between the brain cells harboring the suppressed virus and the adjacent susceptible HeLa cells. Once complete virus was released from the brain cells through the mixed culture it was easily cultivated and proved to be indistinguishable from measles virus. It should be mentioned that control mixed cultures, using biopsy material from two children with cerebral palsy related to birth injury were each prepared with the human brain cells and HeLa cells. These tissue cultures did not show CPE nor were hemagglutinins detected in cell extracts of these cultures. Inoculation of SSPE measles virus into monkeys resulted in antibody response but no clinical disease nor shedding of virus. These are the expected findings with natural measles virus in these animals. There was no evidence of clinical findings in ferrets inoculated, thus further indicating the presence of measles virus rather than a related virus such as distemper. Since rubeola from SSPE patients grows readily in HeLa monolayers with the production of giant cells, it appears that this virus more closely resembles laboratory-adapted or vaccine strain measles. Field strain virus requires repeated passage in primary primate or human cell cultures before propagation can be accomplished in stable cell lines.

The demonstration of suppressed measles virus infection in children with SSPE provides an opportunity to concentrate research directed towards prevention or elimination of this infection. Study of viral suppression mechanisms should give greater understanding concerning the abnormal state which occurs in these children. Furthermore, the technique of mixed cultures should be of immediate interest in the study of other diseases of unknown etiology, particularly those chronic diseases of the central nervous system and other tissues which are suspected but not yet demonstrated to be related to viral etiologies.

Summary. Measles virus was isolated from brain cell tissue cultures derived from two SSPE patients. These cultures proved to contain intracellular measles antigen which was not released in the fluid phase. Infectious, complete virus was obtained when mixed cul-

tures containing the brain cells and HeLa cells were prepared. It appears that SSPE is due to suppressed measles virus infection. Once "rescued" through the mixed culture technique, the virus recovered from SSPE patients proved indistinguishable from measles virus.

Note added in proof: At the time of galley review of this paper, confirmation of our previous isolation of complete infectious measles virus was reported by Payne, F. E., Baublis, J. V. and Itabashi, H. H. (Isolation of Measles Virus from Cell Cultures of Brain from a Patient with Subacute Sclerosing Panencephalitis, New England Journal of Medicine **281**, 585, 1969). These authors used the mixed culture technique which we reported for this purpose (Horta-Barbosa, L., Fuccillo, D. A., Sever, J. L. and Zeman, W., Subacute Sclerosing Panencephalitis: Isolation of Measles Virus from a Brain Biopsy, Nature **221**, 974, March 8, 1969) with BSC-1 cells and continued propagation of the patient's brain cells.

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