Presence of Two Different Viral Agents in Brain Cells of Patients with Subacute Sclerosing Panencephalitis[1] (34765)

G. BARBANTI-BRODANO,[2] S. OYANAGI, M. KATZ, AND H. KOPROWSKI
The Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

Subacute sclerosing panencephalitis (SSPE) is a progressive neurological disease of children, characterized by the presence of intranuclear inclusions in the neurons and glial cells, first described by Dawson (1). The relationship of this disease to a paramyxovirus was suggested by the finding of structures resembling nucleocapsids in the brains of patients with SSPE (2). Subsequently, elevated titers of measles antibodies were observed in the sera and cerebrospinal fluids of most patients with this disease (3). Immunofluorescence revealed an antigen reacting with measles antibody in the brain tissue of some of these patients (4, 5). Presence of a transmissible agent was later demonstrated when intracerebral inoculation of ferrets with homogenates of brain tissue from SSPE patients produced encephalitis whose histologic characteristics resembled those of SSPE (6). An infectious agent which shared antigenic determinants with measles virus was isolated from cultured brain cells of patients with this disease (7, 8). It was encephalitogenic for ferrets (9).

In this report, we present observations related to the presence of two different viral agents within the same brain cells in cultures derived from three patients with SSPE.

Materials and Methods. Cell cultures were derived from brain biopsies of three patients with SSPE (ROB, JAC, LEC) and one patient with Schilder's disease (BOG III) by placing minced fresh brain tissue explants in plastic bottles of 25-cm^2 surface area in 10 ml of Eagle's basal medium (BME) with 10% fetal calf serum (FCS). The explants were left undisturbed for 2 weeks, by which time cells migrating from the explants had formed a monolayer. The cells were then dispersed with 0.25% trypsin solution made in 0.1% versene and transferred to another culture. They grew uniformly and were split at weekly intervals in a ratio of 1:2.

The brain tissue cultures consist of morphologically heterogeneous cells which do not stain with silver and do not contain reticulin or collagen (10); therefore, they probably are of histiocytic or endothelial origin. At the time of our investigation, which was carried out between the thirteenth and nineteenth passage levels, the SSPE cultures contained a large proportion of giant multinucleated cells (11).

Indicator cells. The following cell types were employed for isolation of the agents: (i) primary African green monkey kidney (AGMK) cells; (ii) CV-1, a continuous line of cells derived from AGMK (12); (iii) HeLa cells; (iv) WI38, human diploid cell strain (13); and (v) W18Va2, a line of human cells transformed by SV40 virus (14). All of these cells were grown in BME, supplemented with 10% FCS and a double concentration of amino acids and vitamins, and maintained in the same medium supplemented with 2% FCS.

Cell fusion. One million cultured brain cells and an equal number of indicator cells were dispersed with trypsin and mixed together in the presence of β-propiolactone-

[1] Supported, in part, by U.S. Public Health Service Research Grants RO1-NS 06859 from the National Institute of Neurological Diseases and Stroke, SO1-FR 05540 from the General Research Support Branch, and RO1-CA 10028 from the National Cancer Institute, and by a Grant (596-A-2) from the National Multiple Sclerosis Society.

[2] Fellow at the Wistar Institute from the Institute of Microbiology, University of Bologna, Bologna, Italy.

inactivated Sendai virus at a concentration of 400 hemagglutinating units (HAU). In addition, fusion was effected within the brain cell and indicator cell cultures themselves. Fusion of cells was carried out according to a previously described technique (15). After fusion, the mixed cultures were seeded in milk dilution bottles and examined for 15 days to detect the appearance of a cytopathic effect (CPE).

The Sendai virus used to produce fusion was grown in the allantoic cavity of hens' eggs. The allantoic fluid was harvested at 72 hr after infection, concentrated by high-speed centrifugation and inactivated by exposure to β-propiolactone at the final concentration of 0.03% for 24 hr at 4°. Inactivated Sendai virus thus obtained agglutinated guinea pig red blood cells at a titer of 32,000–40,000 HAU/ml.

Absence of infectious Sendai virus (16, 17) was determined in several controls established with indicator cells and with embryonated chicken eggs.

Detection of viral agents. The medium and cells of the heterokaryon cultures were harvested every 5 days during the 15-day observation period. The cells were disrupted by sonication (Branson W-185C; total input, 1200 W) and the infectivity of the cell extracts and medium was determined separately by inoculation into cultures of the same indicator cells used in fusion. The isolated viral agents were then propagated in the same cell substrate.

Immune sera. The following sera were used in neutralization tests: rhesus monkey serum, containing antibodies against measles virus; convalescent dog serum, containing antibodies against distemper virus; rabbit serum, containing antibodies against rinderpest virus; mouse serum, containing antibodies against Sendai virus; and serum from SSPE patient LEC, obtained late in the disease.

The first three sera listed above were also used in an indirect immunofluorescence test, together with fluorescein conjugated specific (antispecies) gamma globulins. Immunofluorescence tests were carried out on indicator cells seeded on coverslips and infected with the isolated agents. Parallel coverslips were stained with hematoxylin and eosin.

Electron microscopy. Cells in monolayers grown in plastic petri dishes were fixed with 2% glutaraldehyde, postfixed with 1% osmium tetroxide, and embedded *in situ* in epoxy resin. The sections were stained with lead citrate and uranyl acetate and examined under a Siemens Elmiskop I electron microscope.

Hemagglutination. The hemagglutination tests were performed with rhesus, guinea pig and chicken red blood cells (RBC) according to the standard technique (18).

Neutralization. Neutralization tests were carried out according to the standard technique (18). Tubes of AGMK cells were infected with 100 plaque-forming units (PFU) of the isolated viral agents in 0.1-ml amounts previously mixed with an equal volume of specific serum in serial twofold dilutions. The virus–antibody mixtures were incubated for 1 hr at room temperature. The highest dilution of serum which completely inhibited viral CPE in 50% of the inoculated tubes was taken as the neutralization titer.

Results. Activation of syncytiogenic agents from brain cells in culture. No infectious agents were isolated by direct inoculation of the medium or cell extracts from SSPE brain cell cultures on indicator cells.

Heterokaryon cultures obtained by fusion of JAC and LEC cells, respectively, with AGMK and CV-1 cells developed a CPE characterized by the formation of large syncytia on the third to fifth days following fusion. These syncytia increased in size and number, leading, in some instances, to the destruction of the entire culture on the 10th to 12th days after fusion.

Syncytia were also observed in heterokaryon cultures obtained from fusion of JAC or LEC cells with WI38 cells, but the CPE was minimal.

When JAC cells were fused with HeLa cells, numerous syncytia appeared and a pronounced cytopathic effect was noted. When LEC cells were fused with W18Va2 cells the formation of syncytia did not occur.

Syncytia did not develop after fusion of cells from a third SSPE patient (ROB) with

TABLE I. Isolation of Infectious Agents from SSPE Brain Cells in Culture.

Case		Nucleocapsids seen in cells of brain cultures (11)	Infectious agents isolated after fusion of human brain cells with cells of the following cultures:[a]				
SSPE	Schilder's disease		AGMK	CV-1	HeLa	WI38	W18Va2
ROB		Yes	—	—	ND	—	—
LEC		Yes	++++	+++	ND	±	—
JAC		Yes	++++	+++	++	±	ND
	BOG III	No	—	—	—	—	ND

[a] + and — refer to presence and degree of CPE in different indicator cells infected with the isolated agents; ND = not done.

the indicator cells. Fusion of BOG III cells, derived from a patient with Schilder's disease, with indicator cells, as well as fusion of indicator cells with each other, also failed to produce a CPE. These cultures showed only the small, stable polykaryocytes produced during fusion by β-propiolactone-inactivated Sendai virus.

Isolation of viral agents. As shown in Table I, a CPE was observed in AGMK, CV-1 and HeLa cells that were exposed to either medium or cell extracts obtained from heterokaryon cultures originating from fusion of JAC and LEC cells with the same indicator cells. Medium or cell extracts of heterokaryon cultures produced by fusion of ROB and BOG III cells, respectively, with indicator cells, failed to produce a CPE in the indicator cells.

A slight CPE was also observed after exposure of WI38 cells to JAC and LEC virus, but the W18Va2 cells showed no CPE when exposed to the LEC virus.

Fusion of LEC cells with each other also resulted in the isolation of a virus infectious for green monkey kidney cells (AGMK and CV-1).

The isolated infectious agents propagated in AGMK and CV-1 cells produced a cytopathic effect first observed on the third to fourth days after infection. CPE was characterized by formation of giant multinucleated cells, which then coalesced to produce syncytia, and these increased progressively in size until the cells forming the syncytium became detached from the glass surface.

The LEC and JAC viruses have been serially propagated in green monkey kidney cells for 10 consecutive passages. Titration was performed in the same cells. Infectivity titer in PFU/ml of the late virus pools was $10^{4.3}$ for the LEC virus and $10^{3.7}$ for the JAC virus.

Characteristics of the isolated agents. Neutralization and immunofluorescence tests were carried out using antimeasles, antirinderpest and antidistemper sera. Serum from the LEC patient was also tested in a neutralization test with the virus isolated from LEC cells.

As shown in Table II, antimeasles serum

TABLE II. Neutralization of the Isolated Viruses by Immune Sera.

	Immune sera[a]						
	Antimeasles		Antirinderpest		Antidistemper		LEC
Viruses	NT	I	NT	I	NT	I	NT
LEC	1:512	+	1:64	+	<1:4	—	1:1024
JAC	1:256	+	1:32	+	<1:4	—	ND

[a] NT = neutralization titer; I = immunofluorescence: first serum was used at 1:4 dilution; fluorescein-conjugated gamma globulins were employed at 1:10 dilution. Cells seeded on coverslips were stained 3, 4, and 5 days after infection. ND = not done.

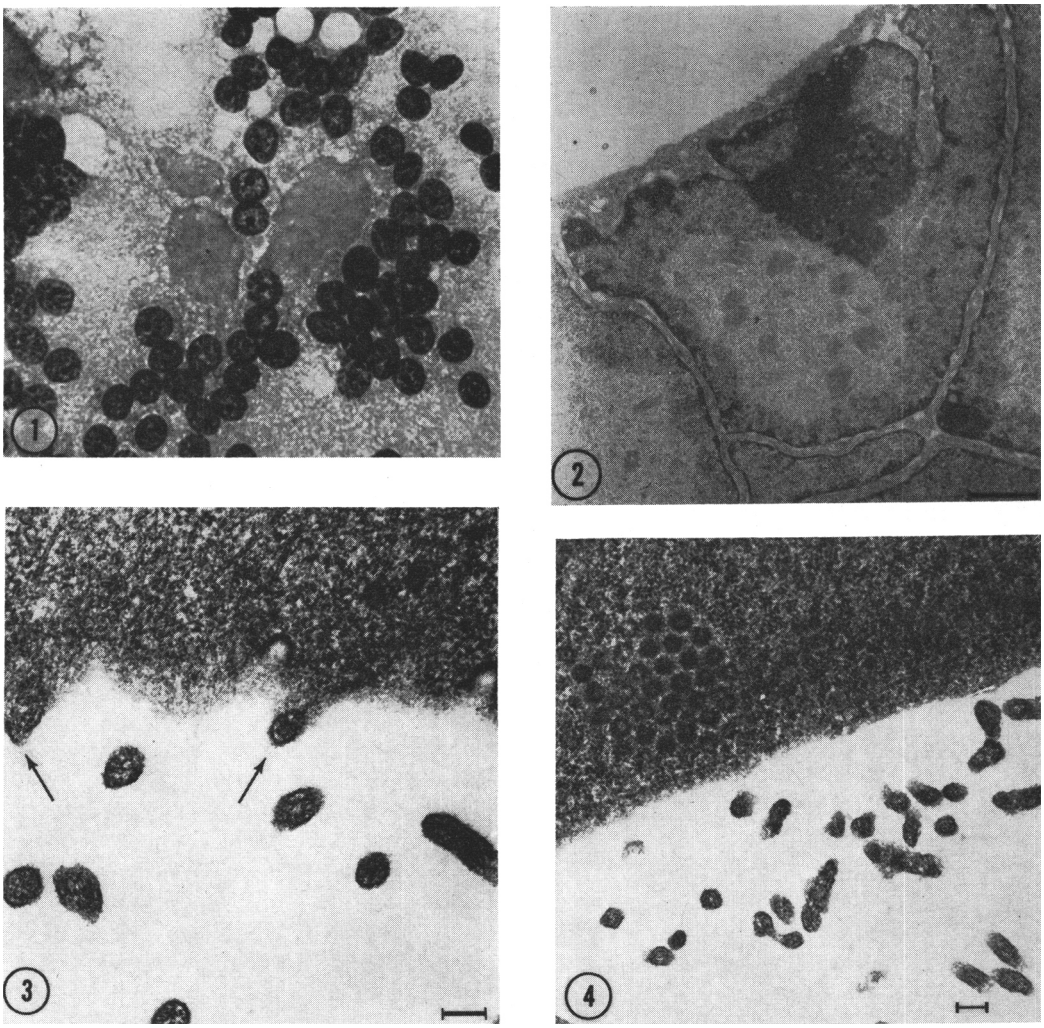

FIG. 1. CV-1 cells infected with LEC virus. Inclusion bodies occupy the cytoplasm of a syncytium; 5 days postinfection; (H and E) 400×.

FIG. 2. CV-1 cells infected with LEC virus. Inclusions formed by viral nucleocapsids are evident in the nuclei of a syncytium; 5 days postinfection; electron micrograph; scale= 1 μ.

FIG. 3. AGMK cells infected with JAC virus. Budding of viral particles (90 to 120 mμ in diameter) from the cell surface (arrows); 4 days postinfection; electron micrograph; scale = 0.1 μ.

FIG. 4. AGMK cells infected with JAC virus, showing a cluster of virions in the cytoplasm. Note spherical and filamentous forms released at the cell surface; 5 days postinfection; electron micrograph; scale = 0.1 μ.

neutralized the LEC virus in a dilution of 1:512 and the JAC virus in a dilution of 1:256. The neutralizing titer of the antirinderpest serum was 1:64 against LEC virus and 1:32 against JAC virus. Neither virus was neutralized by antidistemper serum. LEC serum neutralized LEC agent in dilution of 1:1024. No further tests could be carried out with this serum because of its limited availability. No JAC serum was available.

Immunofluorescence tests with antimeasles and antirinderpest sera revealed virus antigen only in the cytoplasm of syncytia; no nuclear fluorescence was detected. Antidistemper serum did not reveal any antigen.

Green monkey kidney cells grown on cov-

erslips and infected with the isolated viruses showed eosinophilic inclusions within the cytoplasm and the nuclei in the syncytia (Fig. 1). Electron microscopy of the green monkey kidney cells infected with the JAC and LEC viruses showed intracytoplasmic and intranuclear inclusions formed by viral nucleocapsids (Fig. 2) and viral particles budding from the cell surface (Fig. 3). Both agents agglutinated rhesus RBC at the 1:4 dilution.

The second virus. Electron microscopy disclosed spherical particles without envelope in the cytoplasm (Fig. 4). They were seen in the ROB cells examined at the 18th passage level. However, there was no evidence of transmission of these particles from the ROB cells to indicator cells by fusion, or to animals by intracerebral inoculation. They were also seen in green monkey kidney cells infected with the LEC and JAC viruses at various passage levels. In some cells these particles were seen alone and in others in association with the budding paramyxoviruses. Identical particles were seen in a ferret brain inoculated with LEC virus at the second passage level in green monkey kidney cells. Examination of approximately 100 uninfected green monkey kidney cells per each lot failed to reveal any similar structures.

Tests of inactivation of Sendai virus. (i) No CPE was observed in indicator cells inoculated with supernatant or cell extract of cultures obtained by fusion of indicator cells with each other in the presence of β-propiolactone-inactivated Sendai virus; (ii) allantoic fluids harvested 72 hr after infection of chick embryos with the LEC and JAC viruses did not agglutinate chicken and guinea pig RBC, while the allantoic fluids of embryonated eggs inoculated with comparable amounts of infectious Sendai virus had an hemagglutinating titer of 320 HAU/ml; (iii) the two isolated viruses were not neutralized by mouse anti-Sendai serum.

Discussion. Although nucleocapsids structurally resembling paramyxoviruses were observed in the three SSPE brain cell cultures (11), infectious syncytiogenic agents could be isolated only from JAC and LEC cultures. In contrast to JAC and LEC cells, no viral antigen could be detected in ROB cells (Ter Meulen *et al.*, unpublished data) although they formed syncytia in culture. It is thus probable that the ROB cells contained a defective form of viral genome whose only detectable function was the formation of syncytia. These data *per se* do not help in the identification of the agent in the ROB cells, but they suggest that it may belong to the paramyxovirus group.

Unlike its form in ROB cells, the viral genome in cells of JAC and LEC cultures was able to produce an antigen detectable by immunofluorescence in the presence of measles-immune serum (11) and to synthesize infectious virus after fusion with susceptible indicator cells. It is noteworthy that a small amount of the infectious agent was isolated from LEC cells fused with each other, but not from JAC cells in three separate attempts under the same conditions. This may lead to a speculation that the agent in LEC cells is in a more complete form than in the JAC cells.

LEC and JAC viruses could be propagated in green monkey kidney cells by inoculation with infectious media or cell-free extracts from heterokaryon cultures but in contrast it was impossible to isolate the same agents directly from media or cell-free extracts of human brain cells. It is likely that only a few of the JAC and LEC brain cells contained viral genomes sufficiently complete for their activation. These cells then, when fused with susceptible indicator cells, gave rise to the infectious virus.

The JAC and LEC viruses propagated in green monkey kidney cultures share antigenic determinants with measles virus, and, to a slightly less degree, with rinderpest virus, but not with distemper virus. This serologic pattern is characteristic for measles virus (19, 20). In addition, the LEC and JAC viruses share with measles virus the property of agglutinating rhesus RBC.

Production of syncytia, presence of inclusion bodies, and electron microscopic demonstration of viral particles budding at the cell surface are observations not inconsistent with those of measles virus (21, 22), but are insufficient as criteria for the identification of the LEC and JAC viruses as measles virus.

The host range of SSPE viruses was found to be different from that of measles virus (Edmonston and "wild" strains) since in preliminary experiments neither the LEC nor JAC virus was capable of infecting ROB and BOG III human brain cells; whereas, measles virus did infect these cells. In addition, LEC virus did not infect SV40-transformed human and simian cell cultures; whereas, the Edmonston strain and wild measles virus did.

Therefore, it would be premature to classify the two SSPE agents as measles virus before their properties have been more completely characterized.

The role played by the syncytiogenic viruses in the etiology of SSPE is uncertain because of their association with particles representing a different virus. These particles were seen in the ROB brain cell cultures and were apparently transferred, together with the paramyxovirus, from LEC and JAC cells to the green monkey kidney cells. Their identification is the subject of current investigation. Further characterization of these particles as to their structure and biological and serological properties may permit clarification of their role in SSPE. At this point it would be a mere hypothesis to consider, for instance, that they represent either defective or complete virus particles and that they may contribute either primarily or secondarily to the pathogenesis of the disease. Within the context of such an hypothesis, however, interaction of the two types of agents resulting in the disease may be postulated. The two agents may either interact on the molecular level or the paramyxovirus may facilitate the spread of the second agent by cell fusion and formation of syncytia. It is also possible, judging by the form in which the two agents seem to be present in ROB cells, that neither of them represents a complete virus in brain cells of SSPE patients. If a method for the separation of the two agents can be devised so that each can be tested *in vitro* and *in vivo*, it will be possible to assess their respective contributions to the pathogenesis of SSPE.

Summary. A measles-related virus was isolated from two out of three brain biopsies of patients with SSPE by the fusion of brain cells with susceptible cells. β-propiolactone-inactivated Sendai virus was used in the fusion experiments. The mechanism of activation and the characterization of the isolated agents are discussed. Their association with another virus is reported in light of the etiology of SSPE.

We thank Dr. A. H. Dardiri of the United States Department of Agriculture for providing antirinderpest serum and Dr. J. D. Todd of the Veterinary School, University of Pennsylvania, for providing antidistemper serum.

1. Dawson, J. R., Jr., Amer. J. Pathol. **9**, 7 (1933).

2. Bouteille, M., Fontaine, C., Vendrenne, C. L., and Delarue, J., Rev. Neurol. **118**, 454 (1965).

3. Connolly, J. H., Allen, I. V., Hurwitz, L. J., and Millar, J. H. D., Lancet **1**, 542 (1967).

4. Lennette, E. H., Magoffin, R. L., and Freeman, J., Neurology **18**, (Pt. 2), 21 (1968).

5. Ter Meulen, V., and Müller, D., Acta Neuropathol. **10**, 74 (1968).

6. Katz, M., Rorke, L. B., Masland, W. S., Koprowski, H., and Tucker, S. H., N. Engl. J. Med. **279**, 793 (1968).

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

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

9. Katz, M., Rorke, L. B., Masland, W. S., Barbanti-Brodano, G., and Koprowski, H., J. Infec. Dis. **121**, 188 (1970).

10. Ter Meulen, V., Müller, D., Katz, M., Käckell, Y. M., and Joppich, G., Acta Neuropathol. **15**, 1 (1970).

11. Katz, M., Oyanagi, S., and Koprowski, H., Nature (London) **222**, 888 (1969).

12. Jensen, F. C., Girardi, A. J., Gilden, R. V., and Koprowski, H., Proc. Nat. Acad. Sci. U.S., **52**, 53 (1964).

13. Hayflick, L., and Moorhead, P. S., Exp. Cell Res. **25**, 585 (1961).

14. Koprowski, H., Pontén, J. A., Jensen, F. C., Ravdin, R. G., Moorhead, P. S., and Saksela, E., J. Cell. Comp. Physiol. **59**, 281 (1962).

15. Knowles, B. B., Jensen, F. C., Steplewski, Z., and Koprowski, H., Proc. Nat. Acad. Sci. U.S. **61**, 42 (1968).

16. Lo Grippo, S. A., Ann. N. Y. Acad. Sci. **83**, 578 (1960).

17. Neff, J. M., and Enders, J. F., Proc. Soc. Exp. Biol. Med. **127**, 260 (1968).

18. Lennette, E. H., and Schmidt, N. J., "Diagnostic Procedures for Viral and Rickettsial Diseases," 4th ed. Amer. Pub. Health Ass., New York (1969).

19. Cabasso, V. J., Avampato, J. E., Kiser, K. H., and Stebbins, M. R., Proc. Soc. Exp. Biol. Med. **104**, 526 (1960).

20. Bussell, R. H., and Karzon, D. T., *in* "Basic Medical Virology" (J. E. Prier ed.). Williams & Wilkins, Baltimore (1966).

21. Matsumoto, N., Bull. Yamaguchi Med. Sch. **13**, 167 (1966).

22. Nakai, T., Shand, F. L., and Howatson, A. F., Virology **38**, 50, (1969).

Received Nov. 10, 1969. P.S.E.B.M., 1970, Vol. 134.