Isolation and Biological Characterization of a Measles Virus-Like Agent from the Brain of an Autopsied Case of Subacute Sclerosing Panencephalitis (SSPE)

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Abstract Isolation of a cytopathic agent causing formation of syncytial giant cells in co-cultivated Vero cells from the brain of an autopsied case of subacute sclerosing panencephalitis (SSPE) is reported. The syncytia usually autolysed from the center after growing to 1 to 2 mm in diameter and then detached from the culture vessels, and finally made macroscopically recognizable round plaques on the monolayer under liquid overlay. The agent was identified serologically as an agent related to measles virus, by both immunofluorescent tests and plaque reduction tests using anti-measles sera. However, the infected cells did not produce either virions or hemagglutinin, and failed to show hemadsorption and hemolysis of African green monkey red cells even after the 55th passage through Vero cells. Newborn mice, adult mice and hamsters showed neurologic signs after intracerebral inoculation of the infected cells, and most of them died from acute encephalitis. Guinea pigs were unsusceptible. From the brain of the animals with neurologic signs, a similar agent to the inoculated one was recovered.

The new isolate appears to be a strain closely related to measles virus on the basis of serology, and was designated as SSPE-"Kitaken-1" strain.

Subacute sclerosing panencephalitis (SSPE) is a progressive, degenerative, neurologic disease which includes Dawson's inclusion body encephalitis, Van Bogaert's subacute sclerosing leukoencephalitis and nodular panencephalitis of Pette and Döring (1).

The viral etiology of SSPE was first proposed by Dawson (2) in 1933 because he observed inclusion bodies in the brain tissue obtained at autopsy. Subsequently, Bouteille et al (3) observed electron microscopically nucleocapsids similar to those characteristic for paramyxoviruses in the affected brain in 1965. Later, detection of extremely high titers of measles antibody in the serum and cerebrospinal fluid (CSF) of SSPE patients in 1967 (4), and the close relation of isolates from biopsied
brain with measles virus found in 1968 (5) and 1969 (6, 7) led to a hypothesis that SSPE might be a consequence of measles. The isolation of measles virus-like agent from the brain or lymph nodes of patients with SSPE has already been reported by several investigators (8–16). In Japan, Doi et al (13) reported the first case of isolation of an SSPE-agent from a brain biopsy specimen in 1972, and later Ueda et al (16) described the second case from an autopsied brain in 1975.

Since there are some discrepancies in biological characteristics among the SSPE-agents isolated, a new investigation will be of some help to further understand the significance of these isolated viruses in the chronic illness of the central nervous system.

The purpose of this presentation is to report the third case of isolation of the SSPE-agent in Japan and to discuss some biological characteristics of this strain.

MATERIALS AND METHODS

Case report. The patient was a 14-year-old boy. He began to show disorientation and memory disturbance insidiously around the end of 1973. He noted myoclonic jerks on both extremities in February 1974 and experienced generalized convulsion in April which brought him to Keio University Hospital. The CSF was aseptic and the gamma globulin level was 24.1% with 10 mg/dl of IgG. He showed enormously high titers of measles antibodies in serum and in CSF (Fig. 1). An electroencephalogram (EEG) showed typical periodic synchronous discharge. Clinical symptoms, CSF findings, titers of measles antibodies and EEG findings suggested a clinical diagnosis of SSPE. Details of clinical findings have been presented elsewhere (18). His condition gradually deteriorated and he died about 17 months after the onset of illness. Autopsy was performed 4 hr after death. The specimens were immediately sent under chilled condition within 1 hr to Virology Department, The Kitasato Institute.

Isolation of virus from specimens obtained at autopsy. Brain cells and lymphoid cells from the patient were co-cultivated with Vero cells (17) for isolation of virus.

Each piece of the frontal, parietal and occipital lobe tissues was minced into small fragments, and trypsinized by the technique described by Kettyls et al (10). The dispersed cells from each lobe tissue were then suspended in growth medium consisting of Eagle's minimum essential medium (EMEM) supplemented with 10% heat inactivated calf serum, 100 µg/ml of streptomycin and 100 units/ml of penicillin to a final concentration of approximately $1 \times 10^5$ viable cells/ml. The cell suspension of each lobe tissue was mixed with an equal volume of a Vero cell suspension with a concentration of approximately $1 \times 10^5$ viable cells/ml in the same medium. These mixtures were then seeded into bottles (2-oz), which were then incubated at 37 C.

For collection of lymphoid cells, after cutting capsule, the lymph node was pressed in a Petri dish containing the above growth medium. Co-cultivation of the lymphoid cells with Vero cells was performed in a similar manner as that of brain cells.
When a confluent monolayer developed in the co-culture, the growth medium was changed to a maintenance medium consisting of EMEM with 2% calf serum, and this medium was renewed twice a week. When the original culture did not show cytopathic effect (CPE) within 21 days of incubation, it was treated with a mixture of 0.25% trypsin and an equal volume of 0.02% EDTA, and subcultured using a growth medium consisting of EMEM and 5% calf serum. When the culture showed distinct CPE within 21 days, the CPE-agent was subjected to the following procedures.

**Passage of the CPE-agent.** As described in RESULTS, infectious cell-free virus was never detected in the CPE-positive culture. However, the agent was successfully passaged by "cell-to-cell" infection.

An infected monolayer which was incubated for less than 5 days at 37°C, was dispersed by treating with a mixture of trypsin and EDTA as described above. The cells were then suspended in EMEM with 5% calf serum at a concentration of approximately $1 \times 10^5$ cells/ml. One volume of this cell suspension was mixed with 1 to 100 volumes of a fresh Vero cell suspension prepared in the same medium at the same cell concentration as the infected cells, and then the mixture was seeded into
culture-bottles and incubated at 37 C. When Petri dishes were used, they were kept at 37 C in an atmosphere of 5% CO2.

Serological examinations. Measles-neutralizing (NT) antibody titers were measured by a Vero cell tube method (22) using Edmonston strain as the challenge virus. The passage history of Edmonston strain used in this test was as follows: it had been passaged in primary human tissue cultures as described previously (20) before we received it from Dr. J.F. Enders, and was passaged additionally 3 times in Vero cell cultures. The hemagglutination inhibition (HI) test was carried out by a microplate method (22). Sera and CSF were inactivated at 56 C for 30 min and stored at -20 C until tests. For the HI test of the CSF, kaolin treatment was omitted.

Fluorescent-antibody (FA) staining. Smears of each lobe tissue of the autopsied brain, coverslips in the cultures of infected or normal Vero cells, and smears of these cells were fixed in acetone for 10 min at 4 C, and examined by means of the direct and indirect FA-staining. Surface antigen(s) of the infected cells was examined by staining of unfixed cells.

Sera employed in the indirect FA-test were acute and convalescent sera from 2 measles patients. Measles NT-antibody titers of these sera were as follows: (case No. 1): <2× in the acute serum and 512× in the convalescent serum; (case No. 2): acute serum, <2× and convalescent serum, 1,024×. The acute sera were used as negative control. The serum and CSF obtained from this SSPE patient were also used in the test. Their measles NT-antibody titers were 8,192× and 1,024×, respectively. Fluorescein-conjugated anti-human gamma-globulin (rabbit origin, Eiken Chemical Co., Ltd., Tokyo) and fluorescein-labeled rabbit antiserum against measles virus (Toshiba Institute of Biological Science, Niigata) were procured commercially.

Plaque reduction test. The test was performed using the heat-inactivated sera from the above-described 2 measles patients and hyperimmune rabbit serum against Edmonston strain of measles virus adapted to HeLa cells.

Volumes of 0.5 ml of Vero cells infected with the CPE-agent suspended in maintenance medium were mixed with an equal volume of a diluted test-serum specimen. After 1-hr incubation at 37 C, 0.2 ml of the mixture was added into 5 ml of a normal Vero cell suspension in EMEM with 5% calf serum at a concentration of about 1×10⁵ cells/ml in 4 bottles (2-oz). They were incubated for 3 days at 37 C without medium change, and then stained with 0.1% crystal violet in 20% ethanol after fixation with methanol. Plaques were counted macroscopically.

Detection of infectious cell-free virus. Nine-tenths volume of the medium was removed from the infected cultures which were incubated for 3–5 days at 37 C. The cells were scraped with a rubber policeman, collected into the remaining medium, and then disrupted by 3 cycles of freezing-thawing in a dry ice-acetone bath. Then they were centrifuged at 3,000 rpm for 30 min at 4 C, and 0.5 ml of the supernatant fluid was inoculated into Vero cell tubes. After 1 hr of incubation at 37 C, 1 ml of maintenance medium was added. The tubes were kept at 37 C for 2 weeks. The medium was renewed at a few days interval. During this period, the cells were examined microscopically for characteristic CPE and appearance of measles antigen(s) by a direct FA-staining of their smears.
ISOLATION AND BIOLOGICAL CHARACTERIZATION OF SSPE-AGENT

Hemagglutination, hemadsorption and hemolysis tests. Hemagglutination and hemadsorption tests were carried out by the standard techniques (19) using African green monkey red cells. Cultures for the hemadsorption test were simultaneously employed for the hemolysis test as follows: after hemadsorption was examined, the cultures were kept further for 2 hr at 37°C with red cells, and then added 1 ml of saline and agitated. The fluids decanted from the cultures were centrifuged at a low speed, and then the supernatant fluids were examined for red color. As a positive control, Edmonston strain (Vero cell-passaged line described above) was inoculated into Vero cells, and similarly examined.

Hematoxylin and eosin staining. Coverslip cultures of Vero cells infected with the CPE-agent were fixed with 12% formol phosphate-buffered saline containing 1% acetic acid and stained with hematoxylin and eosin for cytological examination.

RESULTS

Isolation and Identification of Viral Agent

The original co-culture of the frontal lobe cells with Vero cells showed syncytial foci after 14 days of incubation. The agent inducing syncytia was successfully transmitted by “cell-to-cell” infection and has been passaged 55 times to date by co-cultivation with Vero cells. After the second passage, the agent induced syncytial giant cells within one day of incubation. The syncytia formation grew to 1 to 2 mm in diameter and began to autolysed from the center and then detached from the culture vessels, and finally developed macroscopically recognizable round plaques. However, the site of giant cell detachment was covered by regrowth of surrounding cells, and additional plaques appeared apart from the original site of the monolayer. Neither infectious cell-free virus nor hemagglutinin was detected in the Vero cells infected with the isolate at the 3rd, 4th, 12th, 20th and 55th cell passages (Table 1).

As shown in Table 1, the fixed syncytial giant cells which were induced by the isolate showed only intracellular fluorescence by the indirect FA-tests using sera containing measles antibody, such as convalescent sera of measles patients, and the serum and CSF of an SSPE patient. The acute sera of measles patients failed to give a specific fluorescence and did not contain measles antibody. The syncytia also showed intracellular measles-specific immunofluorescence by the direct FA-tests, while normal Vero cells revealed negative results. The fluorescence was observed as fine grains diffusely distributed mainly in the cytoplasm of syncytial giant cells (Fig. 2). On FA-staining of unfixed syncytial giant cells, ring-shaped and speckled fluorescence was observed around and on the cell surface when the cells were treated with measles antibody (Fig. 3).

In the direct FA-test on smears of the frontal lobe tissue of the autopsied brain, intracellular measles antigen(s) was similarly observed (Fig. 4).

The above data suggested that the isolate appeared to be an agent closely related to measles virus.

Efforts were made to isolate a similar agent from the parietal and occipital lobe cells and lymphoid cells of the patient, but 3 consecutive subcultures failed to detect an agent which induced CPE or immunofluorescence specific for measles.
Table 1. Summary of findings in Vero cells infected with the SSPE-agent (Kitaken-1 strain)

<table>
<thead>
<tr>
<th>Co-culture, CPE passage No. (syncytia)</th>
<th>Time of CPE appearance (days)</th>
<th>Hemadsorption&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hemolysis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hemagglutinin&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cell-free virus</th>
<th>Intracellular immunofluorescence&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Serum of measles-patient</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Case 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>14</td>
<td>N.T.&lt;sup&gt;g&lt;/sup&gt;</td>
<td>N.T.</td>
<td>N.T.</td>
<td>N.T.</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>1</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>1</td>
<td>N.T.</td>
<td>N.T.</td>
<td>-</td>
<td>N.T.</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>+</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> These tests were done using African green monkey red cells.
<sup>b</sup> The syncytial giant cells were examined by an indirect FA-staining after acetone-fixation.
<sup>c</sup> Convalescent serum.
<sup>d</sup> Cerebrospinal fluid.
<sup>e</sup> Not tested.
Plaque Formation and Plaque Reduction

The plaques stated above, developed not only in co-cultures with liquid medium, but also in those overlaid after one day of incubation with an agar medium which was previously reported by us (20). Plaques were also observed in the ordinary cultivation. Cell monolayers were inoculated with the infected cells and subsequently overlaid with liquid or agar medium after standing at 37°C for 1 hr. Linearity between the plaque counts and the number of infected culture cells was confirmed in the culture system stated above. In Fig. 5, the plaque counts in each
Fig. 4. Intracellular measles immunofluorescence of a brain cell in smear of frontal lobe tissue from the SSPE patient. Direct FA-staining.

Fig. 5. Plaque counts in various culture systems during incubation at 37°C. ○, co-culture overlaid with liquid medium; ●, co-culture overlaid with agar medium; △, ordinary culture overlaid with liquid medium; ▲, ordinary culture overlaid with agar medium.
culture system during incubation at 37°C are graphically presented. This figure shows how the number of plaques increased and declined. Decrease of the plaque number was due to regrowth of surrounding cells at the site of giant cell detachment. As shown in this figure, the co-cultures with liquid medium gave high plaque counts on the shortest incubation. Therefore, this system was used for routine counting which was carried out on the 5th day of incubation.

The plaques decreased in number and size when the infected cells were treated with measles antibody as shown in Table 2 and in Fig. 6.

Table 2. Plaque reduction in Vero cells infected with Kitaken-1 strain by treatment with measles antibody

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Seraa)</th>
<th>Measles NT-titer</th>
<th>Plaque countb)</th>
<th>Percent reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rabbit antiserum against measles virusc)</td>
<td>128 ×</td>
<td>104.5</td>
<td>Not estimated</td>
</tr>
<tr>
<td></td>
<td>Normal rabbit serum</td>
<td>&lt;2 ×</td>
<td>Confluence</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Serum from measles-patient (Case No. 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>64 ×</td>
<td>24.2</td>
<td>64.5</td>
</tr>
<tr>
<td></td>
<td>Acute</td>
<td>&lt;2 ×</td>
<td>68.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serum from measles-patient (Case No. 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Convalescent</td>
<td>256 ×</td>
<td>4.1</td>
<td>94.5</td>
</tr>
<tr>
<td></td>
<td>Acute</td>
<td>&lt;2 ×</td>
<td>74.7</td>
<td></td>
</tr>
</tbody>
</table>

a) Serum which had been diluted to a measles-neutralizing (NT) antibody titer shown in table was mixed with an equal volume of an infected Vero cell suspension.

b) Mean of plaque numbers in 4 cultures.

c) Edmonston strain of measles virus, HeLa cell-passaged line.

Fig. 6. Reduction of the plaque count in Vero cell culture (overlaid with liquid medium) infected with Kitaken-1 strain by treatment with measles antiserum.
**Histology of Syncytial Giant Cells Produced by the Isolate**

Syncytial giant cells induced by the isolate were morphologically indistinguishable from those of Edmonston strain of measles virus (20). When stained with hematoxylin and eosin, eosinophilic inclusion bodies were observed in the cytoplasm and nuclei of the syncytial giant cells (Fig. 7).

![Fig. 7. Syncytia formation in Vero cells infected with Kitaken-1 strain; H&E strain; (A) Cytoplasmic inclusion (ci) bodies are observed. (B) Higher magnification of (A), intranuclear inclusion (ni) bodies are observed.](image)

**Hemadsorption and Hemolysis**

Vero cells infected with Edmonston strain hemadsorbed strongly and also caused hemolysis of African green monkey red cells. On the other hand, the Vero cells infected with the present CPE-agent or left uninfected, failed to show these reactions (Table 1).

**Neuropathogenicity of the Isolate in Animals**

Newborn mice, adult mice, hamsters and guinea pigs were inoculated intracerebrally with the cells infected with the CPE-agent. Infectivity titers of the inoculum varied in plaque-forming units (PFU), as shown in Table 3. As presented in this table, all animals, except for guinea pigs, showed neurologic signs after various incubation periods and most of them died. The susceptibility of animal species to the CPE-agent was in variety, and LD$_{50}$ of the agent for each animal species was as follows: $\geq$3.0 PFU in newborn mice, 15.2 PFU in adult mice, and $\geq$15.1 PFU in adult hamsters. None of the guinea pigs inoculated with 0.14 PFU to 500,000 PFU developed any clinical signs for 3 months.

Virus-recovery from the brain of the animals with neurologic signs in each species were tested in a similar manner as stated in MATERIALS AND METHODS (isolation of virus from human brain). They revealed positive results as shown in Table 3. The recovered agents produced syncytia containing measles-specific immunofluorescent antigen, but virions. They were transmitted only by “cell-to-cell” infection as the inoculated agent.
DISCUSSION

We have described herein the isolation of a transmissible CPE-agent from the brain of a patient with SSPE. This patient showed elevated titers of measles antibody in the CSF, and specific measles antigen was found in the brain cells by the direct FA-staining test. The isolation of a viral agent which produces syncytia containing measles antigen(s) from such a patient, confirm the previous supposition (5-16) that this agent was responsible for the development of this disease. This newly isolated agent was designated as SSPE-“Kitaken-1” strain.

B.R. strain of SSPE-agent which was isolated by Felici et al (15) did not show any neuropathogenicity for newborn mice, rats or hamsters, but our strain and

Table 3. Intracerebral inoculation of animals with Vero cells infected with Kitaken-1 strain

<table>
<thead>
<tr>
<th>Animal</th>
<th>Inoculated PFU</th>
<th>Mortality</th>
<th>Incubation period (days)</th>
<th>Re-isolation and characterization of virus from the brain of animals with neurologic signs</th>
<th>Virus recovery</th>
<th>Inducing syncytia</th>
<th>Producing cell-free virus</th>
<th>Measles fluorescence in infected cell(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn mouse</td>
<td>1.4 (1/6) 0/6</td>
<td>3.0</td>
<td>4</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adult mouse</td>
<td>4.2 1/9</td>
<td>7-8</td>
<td>12</td>
<td></td>
<td>+</td>
<td>N.T.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adult hamster</td>
<td>7.0 2/4 0/4</td>
<td>15.1</td>
<td>11-18</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Adult guinea pig</td>
<td>500,000 0/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult pig</td>
<td>5,000 0/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 0/4</td>
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</tbody>
</table>

(a) Stained by the direct FA-test.

(b) Denominator is the number of inoculated animals. Numerator is that of animals died after appearance of neurologic signs. ( ) : Number of sacrificed animals with neurologic signs for virus-recovery tests.

(c) Not tested.
Niigata-1 strain (13) gave positive results on these animals. Guinea pigs were susceptible to Niigata-1 strain (13), but not to Kitaken-1 strain. The reason why neuropathogenicity in the animals appears to be different among the strains previously reported is uncertain.

Kitaken-1 strain produced cell surface viral antigen(s) detected by the FA-staining and plaque reduction tests using measles antiserum. On the other hand, as described above, neither virions nor hemagglutinin was produced by this strain. Nevertheless, high titers of measles-neutralizing antibody and lower titers of hemagglutination-inhibition antibody were detected in sera and CSF of this patient as shown in Fig. 1. The relationship between the antigen(s) against these humoral antibodies and the cell surface antigen(s) which is produced by Kitaken-1 strain is under study. The discrepancy between the lack of production of hemagglutinin by this strain and the existence of hemagglutination-inhibition antibodies in the CSF of the patient, is still uncertain.

REFERENCES


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