Arch. Inst. Razi, 1978,30,3-15

# Isolation and Characterization of a Defective Measles Virus from Brain Biopsies of Three Patients in Iran with Subacute Sclerosing Panencephalitis [1]

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*Key Words.* SSPE. Subacute sclerosing panencephalitis. Measles-like defective SSPE virus. Defective virus, SSPE

*Summary.* Three cytopathic strains of subacute sclerosing panencephalitis (SSPE) virus were isolated from brain biopsies of three patients. These strains were isolated and maintained by cocultivation of infected brain cells with fresh Vero cells. The biological characteristics of two strains were studied. It was found tha these strains remain cell-associated after repeated cocultivations with Vero cells and produce plaques under fluid medium or tragacanth overlay. The correlation with measles virus was demonstrated by the plaque reduction test as well as by the immunofluorescence test. Large numbers of nucleocapsids were observed in the cytoplasm of infected cells but none in nuclei. Intracerebral inoculation of monkeys, adult guinea pigs, newborn and adult hamsters or mice was followed by acute encephalitis and death.

[1] The authors wish to express their thanks to Dr. M. Kaveh, Director General, Razi Institute, for his support and encouragement.

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(1) Reprinted from Intervirology 9: 106–118 (1978)

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The association of measles virus with subacute sclerosing panencephalitis (SSPE) and the isolation of measles-like virus from the brains or lymph nodes of patients with SSPE have been reported by several groups of workers (1–17). In this paper we report isolation of a cell-associated measles-like virus in Iran

*Table I.* Clinical information on SSPE patients diagnosed over a 12-month period at Dariush-Kabir Hospital[1]

| Patient (code) | Age years | Sex | Duration (months) of disease before diagnosis | History of | | EEG[2] | Diagnosis confirmed by measles HI titer[3] | | | Brain biopsy | Measles-like virus isolated |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | natural measles | measles vaccination | | serum | CSF | saliva | | |
| EB | 8 | M | unknown | unknown | unknown | + | 2,048 | | | + | |
| SI | 8 | M | 4 | + (4) | − | + | 4,096 | 64 | 24 | + | + |
| ZH | 8 | F | 12 | + | unknown | − | 2,048 | 24 | | + | + |
| MB | 11 | F | 8 | + (2–3) | − | + | 4,096 | 64 | 16 | ND | |
| AY | 12 | M | 6 | + | − | − | 1,024 | | 8 | ND | |
| SK | 12 | F | 3 | + (1) | − | + | 2,048 | 96 | | + | |
| RP | 16 | M | 2 | unknown | − | + | 2,048 | 64 | 8 | + | |
| MA | 18 | M | 1 | unknown | − | + | 2,048 | | | ND | |
| MA | 11 | M | 9 | unknown | − | + | 2,048 | | | ND | |
| GN | 4 | M | 2 | + (3) | − | + | 1,024 | | | ND | |
| AM | 12 | F | 8 | + (1.5) | − | + | 1,024 | 12 | 0 | ND | |
| SA | 14 | F | 4 | + | − | + | 2,048 | | 6 | + | + |
| RA | 16 | M | 60 | + (1.5) | − | − | 4,096 | | | ND | |
| RB | 10 | M | 12 | + | − | + | 1,024 | | | ND | |
| NS | 24 | F | 8 | unknown | − | − | 2,048 | 16 | | + | |

[1] + = History, sign, or test result positive; with history of natural measles, age in years at time of illness is given in parentheses; − = history absent, sign or test result negative; ND = not done.

[2] Characteristic electroencephalogram pattern for SSPE.

[3] Hemagglutination inhibition test. Titer is shown as the reciprocal of the highest dilution of serum or other fluids which inhibits hemagglutination of monkey red blood cells.

from the brain biopsies of three ptients. Biological characteristies of two isolates are discussed brifly.

## *Materials and Methods*

*Patients.* During a period of 12 months in 1976, 15 cases of SSPE were diagnosed at the Dariush-Kabir Hospital of Tehran. The diagnosis was based on clinical and serological findings. Age, sex, history of infection with natural measles, and other data are reflected in table. I. In 7 cases, diagnosis was confirmed by histopathological examination of the brain tissue after biopsy. 7 specimens of the brain tissues were subjected to virus isolation attempts; from 3 of these (patients ZH, SI, and SA), a cell-associated virus was isolated.

*Cultivation of the brain cells.* Virus isolation attempts were made with portions of brain cortex (2–3 cm3). The tissues were kept in BME (basal medium, Eagle's) without serum, and were transferred to the laboratory within 1 h. They

were trypsinized with 0.25% trypsin solution (Difco) for 30 min at 37°, with gentle shaking at intervals. The isolated cells were sedimented by low-speed centrifugation and washed once with BME. These cells were cocultivated with fresh suspensions of Vero cells (1:3 to 1:5) in Roux bottles. The growth medium was BME supplemented with 4% fetal calf serum, 100 ug/ml streptomycin and 50 ug/ml neomycin. After 3 days a confluent monolayer was formed. The growth medium was then changed to maintenance medium (similar to growth medium but containing 2% fetal calf serum). This medium was changed twice a week. After 2 weeks the monolayer was treated with trypsin solution and was again cocultivated with a fresh suspension of Vero cells. After 3–5 such passages, cytopathic effects of the virus were observed. In the present paper some biological characteristics of two of the strains, ZH and SI, will be presented.

*Plaquing and plaque counting.* For plaquing, cultures at the 7th passage were selected. 4-oz bottles were seeded and after 2–3 days, when plaques were macroscopically observable, the bottles were decanted and the monolayers were stained with crystal violet (0.2% in ethanol). The syncytial plaques also appeared under fluid tragacanth (18) which was added 1 day after the start of cocultivation. For staining, the overlay was removed on the 3rd day and plaques were stained as mentioned above.

*Antiserum.* The antiserum used for plaque reduction was a pool of sera obtained from children 8 weeks after immunization against measles. The HI antibody titer of this pool against measles virus was 360. For immunofluorescence, equal portions of measles virus (virulent Edmonston strain at a concentration of $2 \times 10^5$ $TCID_{50}$/ml) and complete Freund's adjuvant were homogenized, and each of the 2 vervet monkeys was given 4 injections of 4.0 ml of virus-adjuvant mixture (2.0 ml in each side) at 2-week intervals. The animals were bled 14 days after the last injection, and the sera were stored at – 20.

*Immunofluorescence.* Coverslip cultures of the cocultivated cells at passage 7 were fixed in cold acetone for 10 min. The monkey immunoglobulins were separated from the whole serum and conjugated with fluorescein isothiocyanate according to the method described by Schieble *et al.* (19). Coverslips were stained by the direct method and examined with a Zeiss fluorescence microscope, illuminated with an Osram HBO 200 lamp. A UG5 exciter filter and a GG4 barrier filter were used. For microscopic observation and microphotography, 25 × and 40 × objective lenses were selected.

*Hematoxylin and eosin staining.* Coverslips of the same cultures were fixed with Bouin's solution for 15 min and stained with HE by routine histopathological techniques.

*Electron microscopy.* Cells infected with virus strain ZH and suspended in phosphate buffer (pH 7.0) were homogenized in a homogenizer for 2 min. The homogenate was then centrifuged at 6,000 rpm for 10 min and the supernatant

was saved and used for electron microscopic (EM) examination. Samples were negatively stained with sodium silicotungstate.

Monolayer cultures of ZH strain were gently harvested with a rubber policeman, washed once with phosphate buffer (pH 7,0), fixed with 3% glutaraldehyde, washed again with phosphate buffer (pH 7.0), post-fixed in 2% osmium tetroxide for 2 h, then embedded in Epon 812. Sections were cut and stained with uranyl acetate followed by lead citrate.

*Animal hosts.* Mice (NIH strain), Syrian hamsters and guinea pigs were received from our local stocks. These animals were healthy and free from known contaminants. African green monkeys (vervet) were imported from Tchad. They were free from measles antibodies.

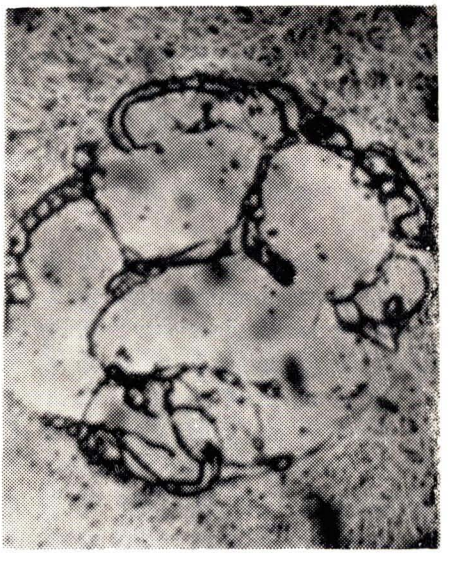

*Fig.*1. A syncytium in Vero cell culture of SSPE virus, strain ZH.

*Storage of SSPE.infected cells.* At passages 7 and 14, infected monolayers were trypsinized and cells were suspended in medium containing 10% dimethylsulfoxide (DMSO), 10% sucrose, 10% bovine albumin, 30% fetal calf serum and 40% BME. About 3 million cells/ml were suspended in this medium; the suspension was distributed in 1.0-ml amounts and kept frozen at – 70°.

*Hemadsorption and hemagglutination.* 0.3 ml of a 0.5% suspension of African green monkey erythrocytes were added to the SSPE cultures ( strain ZH at subculture 11 and strain SI at subculture 9) in 4–oz bottles. After 2 h incubation at 37°, cultures were washed 4 times with phosphate-buffered saline (PBS) and were examined microscopically.

Cultures of both strains at the same passage level were disrupted by three cycles of freezing-thawing in dry ice/acetone. The supernatants were centrifuged 15 min at 3,000 rpm at 4° and were tested for hemagglutination.

## *Results*

*Development of cytopathic effect of virus.* The CPE of the agent appeared after 3–5 subcultures by cocultivation of the brain cells with Vero cells. The first CPE was a circular syncytial giant cell (fig. 1) which was observed macroscopically on the 2nd to 3rd day. This CPE was not progressive and remained as rounded plaques, 1.5–3 mm in diameter (fig. 2). Later, the giant cell was destroyed and became detached from the glass. After the infected cells were stained with HE, inclusion bodies, similar to those of measles virus, were seen only in cytoplasm of syncytial giant cells. Round plaques were formed under fluid medium. The plaques had the same size under tragacanth overlay and fluid medium (fig. 2), but in several experiments the number of plaques increased by 25–30% when tragacanth overlay was used.

*Absence of cell-free virus.* Both strains were cocultivated with Vero cells up to 14 passages. At several occasions cells were frozen-thawed or sonicated and centrifuged at 3,000 rpm at 4° for 30 min. In all trials Vero cells, HeLa cells, and human diploid cells (MRC-5) remained negative when inoculated with cell-free supernatant of the lysed cells.

*Plaque reduction test.* Measles antiserum dilutions 1:10 and 1:25 in PBS were added to the growth medium for cocultivation of SSPE virus (strain ZH). After 3 days the growth medium was discarded and the bottles were stained with crystal violet. 1:10 dilution of serum prevented the development of 95% of plaques (i.e., in these bottles only 5% of the plaques developed, as compared with controls). In the presence of 1:25 dilution of serum, 12% of the plaques developed, i.e., 88% were inhibited (fig. 3).

*Immunofluorescence staining.* Syncytial giant cells, fixed with cold acetone and stained with fluorescein isothiocyanate-conjugated monkey immunoglobulins, showed intracellular fluorescence (fig. 4). The fluorescence was observed as fine grains distributed in the cytoplasm and around the nuclei of giant cells. In old cultures, fluorescence was weak.

*Freezing storage of SSPE cells.* Ampules of frozen SSPE cells of both strains kept at – 70° were thawed 3 months later and cocultivated with fresh Vero cells. A monolayer was formed in 36h, and multinucleated giant cells were observed after 2–3 days of cultivation.

*Hemadsorption and hemagglutination.* Although our routine cultures of measles vaccine strains in MRC-5 cells show hemadsorption with green monkey erythrocytes and produce hemagglutinin at various titers, the syncytial giant


*Fig.2.* Plaques of SSPE virus, strain SI. T.O.=Tragacanth overlay; F.M.=fluid medium.

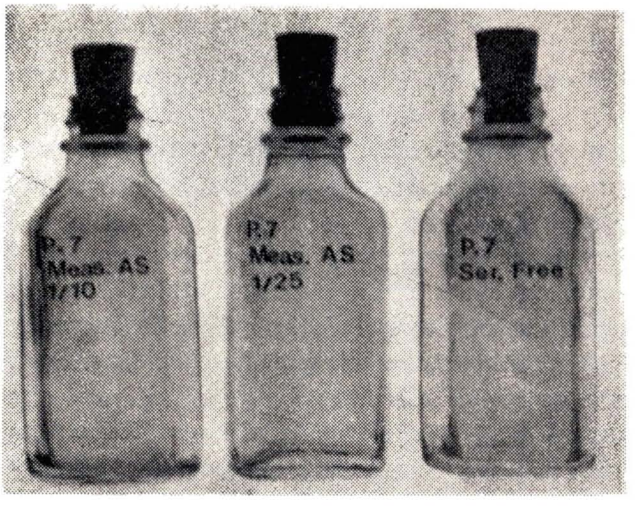


*Fig.3.* Reduction of plaque counts by measles antiserum. Left to right: with antiserum dilution 1:10; with antiserum dilution of 1:25; without antiserum.

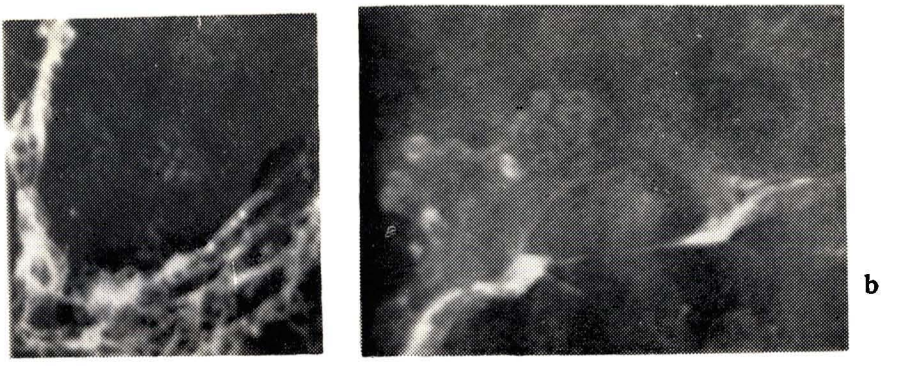


*Fig.4.* Direct staining with FITC-labelled monkey anti-measles globulin. a Syncytial giant cell in culture infected witk SSPE, strain ZH; diffuse intracytoplasmic immunofluorescence is shown. b Syncytial giant cell in culture infected with SSPE, strain SI; intracytoplasmic immunofluorescence is shown.

cells of SSPE, strains ZH and SI, failed either to adsorb monkey red cells or to produce hemagglutinin.

*Electron microscopic findings.* The negative staining of the infected cells revealed large numbers of nucleocapsid-like structures (fig. 5). In the fixed preparations, aggregates of nucleocapsids were only seen in the cytopalsm of cells. Particles suggesting a paramyxovirus were rarely detected in the fluid from frozen-thawed or ultrasonicated cells.

*Intracerebral inoculation of laboratory animals.* Palques of strain ZH, subculture 7, developed in a Roux bottle, were counted; cells were gently scraped off the glass and resuspended in PBS at a concentration of $3 \times 10^3$ PFU/ml. Newborn and adult mice, hamsters, and adult guinea pigs were injected intracerebrally with this suspension in doses of 0.03, 0.05 and 0.25 ml, respectively.

The results are shown in table II. Most of the inoculated animals showed neurologic disorders 3–6 days after inoculation, and all of them died. No symptoms were recorded in controls inoculated with normal Vero cells. This experiment was repeated with SI strain, and similar results were recorded. In agreement with Doi *et al.* (15), we found that mice and hamsters were more susceptible to SSPE agent than guinea pigs, which showed a longer period of incubation.

The cell-associated virus was easily isolated from the brains of these animals when brain homogenates were cocultivated with Vero cells. Pathological changes included perivascular cuffing, diffuse inflammatory infiltration, absence of inclusion bodies and astrocytic hyperplasia, and to some extent loss of neurons, showing acute encephalitis in these animals.

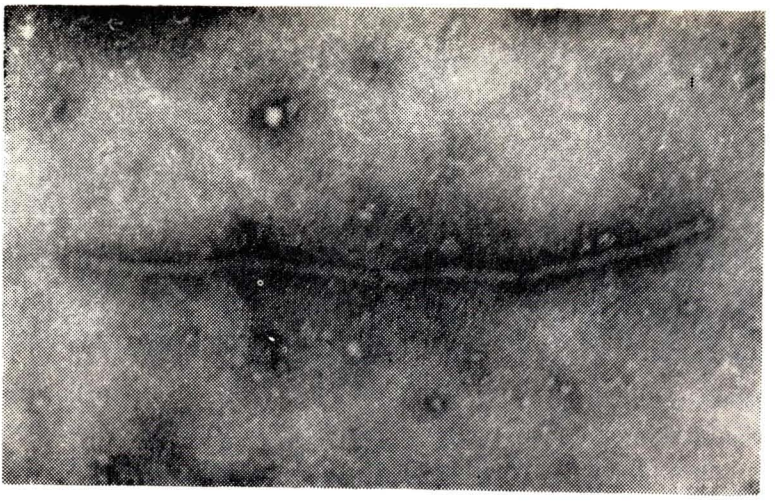

*Fig.*5. A segment of nucleocapsid of measles-like virus stained with sodium silicotungstate, showing helical structure of the nucleocapsid with serrations of regular periodicity and a central hole about 40 A in diameter. × 110,000,

*Table II.* Results of intracerebral inoculation of experimental animals

| Experimental animals | Inoculum: ZH strain of SSPE virus | | | Inoculum: control Vero cells | |
|---|---|---|---|---|---|
| | number of PFU inoculated | mortality[1] | incubation days | number of cells inoculated | mortality |
| Mice | | | | | |
| Newborn | 45 | 12/12 | 5–8 | 90 | 0/8 |
| Adult | 90 | 12/12 | 6–8 | 180 | 0/8 |
| Hamsters | | | | | |
| Newborn | 45 | 10/10 | 4–6 | 90 | 0/6 |
| Adult | 150 | 10/10 | 4–7 | 300 | 0/7 |
| Guinea pigs | | | | | |
| Adult | 750 | 10/10 | 7–10 | 1,500 | 0/5 |

[1] Number of encephalitis deaths/number of animals inoculated.

*Transmission of the SSPE virus to monkeys.* ZH strain at the 14th subculture was used for inoculation of monkeys. Six vervets (*Cercopithecus aethiops*) of 3–4 kg weight were inoculated with a suspension of infected cells at $3 \times 10^3$ PFU/ml in PBS as follows: each of 2 monkeys was inoculated intracerebrally with 0.5 ml of cell suspension in each side; 2 monkeys were inoculated subcutaneously with 1 ml each; and each of the last 2 animals received 1 ml of suspension intramuscularly.

*Clinical observations.* Only the first 2 monkeys soon developed symptoms of CNS involvement, whereas the other 4 did not show any symptoms during 5 months following inoculation; they are still under close observation for the possible later involvement. The following symptoms were recorded for the 2 primates inoculated intracerebrally.

*Monkey No.* 1: 3 days after inoculation, stiffness of the neck was observed. Lack of appetite and incoordination in movements were also noticed. The animal died 9 days after inoculation. At autopsy, subdural hemorrhages were observed. Scattered hemorrhages and monocytic cuffing were found in histologic slides prepared from CNS and stained with HE.

*Monkey No.* 2: This animal developed restlessness and tremor 7 days post-inoculation. 10 days after inoculation the animal was excited and from time to time hit his head against the cage's wall. He became moribund 12 days after inoculation and died on the following day. At necropsy no macroscopic change was seen in the organs. In histologic slides of the CNS, the following changes were recorded: vessels in the vicinity of inoculation trauma showed prominent monocytic cuffings, and the endothelial cells of the vessels were swollen. Scattered pin-point hemorrhages were noted throughout. Very scarcely scattered vacuolated or pyknotic neurons were observed. These changes were confined to the regions of inoculation trauma.

The serological responses of monkeys are reported in table III.

## *Discussion*

SSPE is known as a relatively rare disease with an estimated morbidity of 1:1,000,000 (20). It is, however, not so rare in Iran, where measles morbidity and mortality were high until 1970 when mass vaccination campaigns were initiated. To estimate the relative prevalence of the disease, it is noteworthy that 15 cases were diagnosed during the period of 1 year, in only one of the hospitals in Tehran (table I).

Out of 7 specimens of brain tissues, 3 cell-associated strains of SSPE virus were isolated. The histopathological changes in the other 4 samples, as well as the clinical and serological data on the patients, confirmed the diagnosis of the disease despite the fact that an SSPE agent could not be isolated from them. Katz and Koprowski (21) previously isolated two strains of SSPE from eight

*Table III.* Serological response of monkeys after SSPE virus inoculation

| Monkey No. | Route of inoculation[1] | Serological response | |
|---|---|---|---|
| | | days post-inoculation | HI titer |
| 1 | IC | 9 | 4 |
| 2 | | 13 | 4 |
| 3 | SC | 60 | 12 |
| 4 | | 60 | 6 |
| 5 | IM | 60 | 24 |
| 6 | | 60 | 4 |

[1] IC = Intracerebral; SC = subcutaneous; IM = intramuscular.

specimens of human brain, by cocultivation of brain cells with cells of human or simian origin in the presence of the cell-fusing Sendai virus. A low concentration of defective virus may be limiting the number of isolations from human brain. We had no problem in isolating the SSPE agent from the brain homogenates of baby and adult mice, hamsters and guinea pigs. These rodents had a high titer of defective SSPE virus in their brains, and in more han 20 specimens we had complete success in isolating the agent by cocultivation with Vero cells.

Cell-free virus has been isolated from the brain of patients with SSPE upon cocultivation of brain cells with HeLa cells (7,8). Our two strains remained cell-associated after repeated subcultures. A similar finding has been reported by Doi (personal commun., 1976), who has already subcultured the Niigata-1 strain over 200 times.

Because of the isolation of suppressed measles virus from the lymph nodes of patients with SSPE (9) and the demonstration of measles antigen in kidney, spleen and lung cells, it is suggested that SSPE infection is not restricted to the CNS, but rather is disseminated throughout the body. The presence of measles HI antibody in saliva of some of our patients suggests that it might be worthwhile to search for the SSPE agent in biopsies of salivary glands.

Plaques of both strains characterized in our study were smaller than those described by other workers (15), and the number of plaques was persistently 25–30% higher under tragacanth than under fluid medium. Under tragacanth, higher yields of plaques of other viruses also have been obtained (18,22). It is also worth mentioning that 'Comet plaques' (23) were not produced in our cell system by either of the strains under study. Comet plaques were observed (24) when an SSPE strain was obtained in a mixed culture of brain cells and BSC-1 cells.

In the present study, tubular filaments resembling nucleocapsids of paramyxovirus were observed only in the cytoplasm of SSPE cell cultures. Tubular

filaments as detected by other investigators (25–27) in the nucleus of cultured SSPE cells were not found.

Another point of interest in our EM study was the presence, although rare, of undefined particles similar in size and aspect to complete and mature measles virus. This finding has also been reported by other investigators (28). Other investigators also have reported an intracytoplasmic particle without a membrane, resembling members of the papovavirus group (29,30), as a second viral agent, perhaps playing a role in the development of SSPE. In our study no such particles were observed.

Both strains under study induced acute encephalitis in baby and adult hamsters and mice as well as in guinea pigs; similar findings have been reported by other workers (15,31). Suckling hamsters have been particularly used for the study of SSPE replication in CNS. By intracerebral inoculation of SSPE agent, animals progress rapidly through a phase of lethargy to death in approximately 6 days (32). In monkeys also, a model of the long-term course of human SSPE has not yet been obtained: intracerebrally inoculated animals developed acute encephalitis; and animals inoculated by other routes thus far have evidenced no disease. Perhaps a latent infection might have been established if the monkeys had been inoculated with extremely low doses of the SSPE agent.

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