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# Temperature Sensitivity of Subacute Sclerosing Panencephalitis Virus and Its Ability to Establish Persistent Infection (40562)

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Several investigators have recently suggested that temperature-sensitive (ts) mutants play a role in the establishment and maintenance of persistent infection (PI) of animal cells by viruses (1–5). It was decided, therefore, to examine whether temperature sensitivity characterized the subacute sclerosing panencephalitis (SSPE) virus, a variant of measles virus known to be associated with persistent central nervous system infection and degenerative brain disease in man, and to determine whether this property bears any relationship to the capacity of the virus to initiate and maintain PI in a line of human cells *in vitro*.

*Materials and methods. Viruses.* The Halle strain of SSPE virus was obtained after four passages in Vero cells from Dr. L. H. Barbosa (Section on Infectious Diseases, National Institute of Neurological Communicative Diseases and Stroke, Bethesda, Md.). The strain was further passed once in HEp-2 cells and titered $1.7 \times 10^4$ plaque-forming units (PFU)/ml. Unattentuated Edmonston strain measles virus was originally obtained from the laboratory of Dr. John Enders (Harvard University) in 1962. The virus had undergone four passages in HEp-2 cells in the laboratory of Dr. R. M. Chanock (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and titered $2.4 \times 10^5$ PFU/ml.

*Cells.* HEp-2 cells were obtained from Flow Laboratories (Rockville, Md.) and grown either in roller tubes or in 24- or 96-well plastic trays (Linbro; Hamden, Conn.). Media for growth or maintenance of cell cultures consisted of Eagle's minimal essential medium (MEM) (Flow) supplemented, respectively, with either 10 or 2% fetal calf serum (Gibco; Grand Island, N.Y.). Media contained glutamine (2 m*M*), penicillin (50 units/ml), streptomycin (50 μg/ml), and amphotericin B (2.5 μg/ml). Trypsin–versene solution (Flow) was used for cell passage.

*Virus growth and plaque assays.* Viral growth at different temperatures was assayed by the methods of Gharpure *et al.* (6). Briefly, 0.1 ml of virus was inoculated onto 6 roller tube cultures of HEp-2 cells and adsorption carried out for 90 min at 35°. At the end of this time 1 ml of MEM with 2% fetal calf serum was added and duplicate tube cultures incubated at 32, 37, and 39°. Cultures at the two higher temperatures were kept in roller tubes sealed with paraffin wax and immersed in water baths kept within ± 0.05° by means of Braun water circulators (Melsungen, Germany). Cultures grown at 32° were maintained in an incubator. Infected cultures were examined daily for the presence of virus-specific cytopathic effects (CPE) and were harvested when these effects were maximal, i.e., at the point where the monolayers were 80–90% replaced by viral synctia. Virus titers were maximal at this time which was usually 6 days after infection. Virus yield was quantified by plaque assay as described by Gharpure *et al.* (6). Supernatant fluids were withdrawn from the tubes and 10-fold serial dilutions were made in Dulbecco's phosphate-buffered saline (PBS) solution (Flow). Fifty microliter portions of each dilution were inoculated onto confluent monolayers of HEp-2 cells in 96-well Linbro trays. Adsorption was for 90 min at 35°. MEM containing 10% fetal calf serum and 0.75% methyl cellulose was then added and the cultures incubated at 35° until CPE were maximal (6 days). At the end of this time the cells were fixed with 10% formalin, stained with hematoxylin and eosin, and examined under a dissecting microscope. Plaques were eosinophilic and easily enumerated.

*Fluorescent antibody techniques.* An indirect fluorescent antibody (FA) technique was



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employed according to methods already published (6). Guinea pig preimmune serum and guinea pig measles antiserum (Flow) were used in all tests along with goat fluorescein-conjugated antiguinea pig gamma globulin (Cappel Laboratories, Dowington, Pa.). Counterstain was Evans Blue (7). The specimens were examined in a Zeiss fluorescent microscope using a BG 12 exciter filter and an orange barrier filter.

*Establishment of PI.* Twelve roller tubes containing confluent monolayers of HEp-2 cells were inoculated with either measles or SSPE virus at a multiplicity of infection (m.o.i.) of 0.05 PFU/cell. After a 90-min adsorption period at 22°, 1 ml of maintenance medium was added. Cultures infected with both viruses were then incubated at 32, 37, and 39° (four tube cultures at each temperature for each virus). Temperature control was effected as described above.

At the end of 3 days, prior to the appearance of CPE, viral replication was quantitated and attempts were made to establish persistently infected cell lines. Two of the four tubes at each temperature were employed for virus quantitation. Under sterile conditions the supernatant fluid was removed and frozen at −70° for subsequent plaque assays of virus yield. These tubes were not passaged further.

The monolayers in the remaining two tubes were dispersed with trypsin–versene and the cells from each of the two tubes seeded into two new tubes. Two lines of cells, one derived from each of the original remaining tubes, were thus established.

Beginning with the first passage, growth medium (MEM containing 10% fetal calf serum) was used. This was to encourage optimal cell growth. When FA studies were required, the passages were made in Leighton tubes.

Cultures were examined daily for the presence of CPE. The virus being shed into the supernatant was assayed periodically. The medium was removed and frozen at −70° for subsequent assay. The monolayers were then washed three times with PBS to effect a $10^{-4}$–$10^{-5}$ dilution of any virus that might be present, new growth medium was added, and the incubation was continued.

When the monolayers reached confluency, passages were made as described above with half the tubes being employed for assay of the virus being shed into the supernatant and half the tubes being passed to maintain the cell line.

*Preparation of purified virus populations.* Purified populations were prepared by a single process of terminal dilution. HEp-2 cells were grown to confluency in 16-mm-diam plastic wells contained in 24-well trays. Serial half-log dilutions of the different virus strains were then made and 50-μl portions of each dilution inoculated onto 48 wells. The cultures were adsorbed at 22° for 90 min. At this point 1 ml of maintenance medium was added to each well, and the cultures were incubated at an intermediate temperature (35°) to avoid selective pressures. Trays were examined daily for the presence of CPE. Some dilutions produced CPE in five or fewer of the 48 wells inoculated. The supernatant fluids of these wells were removed and constituted the purified populations. Assays for temperature sensitivity of these populations were done without further passage.

*Results. Establishment of PI.* Measles-infected cells quickly developed typical syncytia at all temperatures and equal quantities of virus were shed into the supernatant fluid at each temperature (Fig. 1). The cultures did not regenerate after the initial appearance of CPE and could not be propagated beyond the second passage.

There were only minor variations in the amount of virus shed into the supernatant by SSPE-infected cells at the three different temperatures (Fig. 2). SSPE-infected cells at 32° were totally destroyed by the virus CPE and did not survive the second passage.

The cell monolayers infected with SSPE virus and cultured at 37° were 95–99% destroyed by the initial CPE but the remaining cells grew out and could be propagated through 22 passages over a period of more than 100 days. At no time, however, did these cells show any evidence of PI. Assays for infectious virus in the supernatant fluid and in the cell layer were repeatedly negative and FA staining consistently failed to reveal presence of virus-specific antigen. At the 13th passage some of the cultures at 37° were shifted down to 32°. Through 10 subsequent passages, assays for infectious virus were neg-

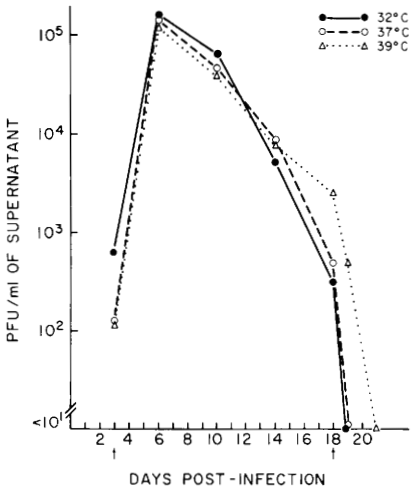


FIG. 1. Yield of Edmonston strain measles virus during attempts to establish PI at 32, 37, and 39°. Cell passages indicated by arrows.

ative. Thus, it appeared that the cells cultivated at 37° were not persistently infected with SSPE virus, but represented HEp-2 cells that had escaped infection with virus at the onset.

At 39°, SSPE-infected monolayers were also initially 95–99% destroyed, but the remaining cells grew out and could be serially passed. Initial production of infectious virus paralleled that at 37° (Fig. 2). By contrast, in further passages, there was evidence that PI had been established. FA studies, which were first done at the third passage, were consistently positive with 1–5% of cells showing evidence of virus-specific antigen production. Initially assays for infectious virus were negative, but after the sixth passage a "crisis" (8) occurred. Syncytial CPE typical of SSPE virus became apparent, the percentage of cells positive by FA increased to 20–25%, and infectious virus began to be produced. Despite this crisis, the monolayers quickly regenerated and repeated passage of the cells was possible. Crises continued on a cyclical basis through 11 subsequent passages over the next 70 days (Fig. 3). The percentage of cells positive by FA varied from 1 to 5% when infectious virus and viral CPE were undetectable to 20 to 25% during crises. Shifting cultures from 39 to 32° during periods when infectious virus and CPE were not detectable did not result in their induction.

*Temperature-dependent growth properties of the virus populations.* As is evident from the growth curve illustrated by Fig. 2, the SSPE virus gives some suggestion of being ts. However, the assays of virus growth shown in Fig. 2 represent the results of growth initiated by a potentially heterogeneous population of virions. It has now become apparent that such populations can manifest growth curves like that depicted while containing many ts subpopulations. For this reason it was decided to prepare partially purified populations from three different virus strains: (i) the SSPE strain that initiated the PI at 39°; (ii) the virus being shed into the supernatant during the

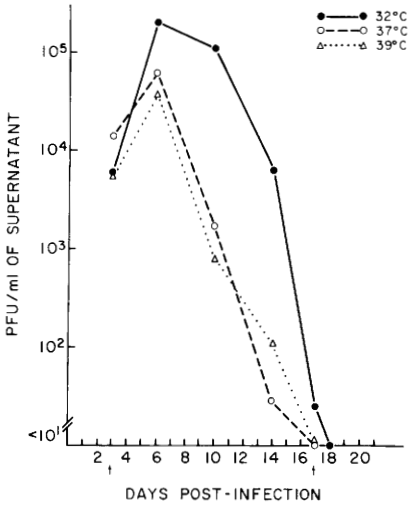


FIG. 2. Yield of Halle strain of SSPE virus during attempts to establish PI at 32, 37, and 38°. Cell passages indicated by arrows.

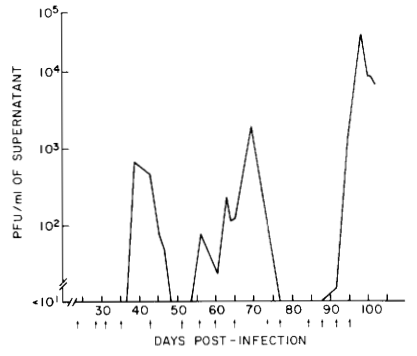


FIG. 3. Titer of virus shed into supernatant by cells persistently infected with the Halle strain of SSPE virus at 39°. Cell passages indicated by arrows.

course of the PI (virus being shed after 95 days was used); and (iii) the Edmonston strain of measles virus that failed to initiate PI.

All purified populations produced virus-specific CPE with equal facility at all temperatures. Efficiency of virus yield, however, varied greatly. Yields were assayed according to the virus growth assay procedure outlined in Methods. The yields produced by the different purified populations at different temperatures are presented in Tables I–III. Purified populations were scored as ts if the ratio of virus production at 32° compared with that at 39° was 10 or greater. All tests were done in duplicate and no significant variation between the duplicate cultures was noted. Of 16 purified populations of Edmonston measles virus, none exhibited the ts property (Table I). By contrast, 10 of 16 purified populations of the Halle strain of SSPE virus that initiated the PI exhibited this property to varying degrees as did four of 18 purified populations derived from the virus being shed into the supernatant during the course of PI (Tables II and III).

*Discussion.* These findings indicate that the Halle strain of SSPE virus has ts properties. These properties may be overlooked, however, unless attempts are made to purify potentially heterogeneous populations before temperature sensitivity testing is done or if efficiency of yield is not examined. The results are similar to those presented by Youngner *et al.* (5). The population purification procedure used by these workers was more elaborate than the one employed here, and it is possible that some of the populations reported as non-ts here might have been shown to be heterogeneous populations of both ts and non-ts virions if techniques such as triple

TABLE I. YIELD (PFU/ml) AT THREE TEMPERATURES OF PURIFIED VIRUS POPULATIONS ISOLATED FROM THE EDMONSTON STRAIN OF MEASLES VIRUS (AVERAGE OF TWO CULTIVATIONS).

| Purified population No. | $Log_{10}$ PFU/ml | | | Ratio 32/39° |
|---|---|---|---|---|
| | 32° | 37° | 39° | |
| 1 | 6.3 | 6.4 | 6.3 | 1.0 |
| 2 | 7.3 | 7.3 | 7.3 | 1.0 |
| 3 | 7.3 | 7.3 | 7.3 | 1.0 |
| 4 | 6.8 | 6.8 | 6.8 | 1.0 |
| 5 | 6.9 | 6.9 | 7.0 | 0.79 |
| 6 | 6.8 | 6.9 | 6.9 | 0.79 |
| 7 | 6.8 | 6.8 | 6.9 | 0.79 |
| 8 | 6.8 | 7.0 | 6.9 | 0.79 |
| 9 | 6.8 | 6.8 | 7.0 | 0.63 |
| 10 | 7.0 | 7.0 | 7.2 | 0.63 |
| 11 | 6.7 | 6.9 | 6.9 | 0.63 |
| 12 | 6.1 | 6.7 | 6.3 | 0.63 |
| 13 | 6.8 | 7.0 | 7.0 | 0.63 |
| 14 | 6.3 | 6.6 | 6.5 | 0.63 |
| 15 | 6.7 | 6.7 | 6.9 | 0.63 |
| 16 | 6.5 | 6.8 | 6.9 | 0.40 |

TABLE II. YIELD (PFU/ml) AT THREE TEMPERATURES OF PURIFIED VIRUS POPULATIONS ISOLATED FROM THE HALLE STRAIN OF SSPE VIRUS (AVERAGE OF TWO CULTIVATIONS).

| Purified population No. | $Log_{10}$ PFU/ml | | | Ratio 32/39° |
|---|---|---|---|---|
| | 32° | 37° | 39° | |
| 1 | 5.9 | 4.9 | 2.7 | 1600 |
| 2 | 5.0 | 4.7 | 2.4 | 400 |
| 3 | 4.9 | 4.6 | 2.6 | 200 |
| 4 | 4.8 | 4.7 | 2.9 | 79 |
| 5 | 6.4 | 6.0 | 4.8 | 50 |
| 6 | 6.3 | 6.3 | 4.7 | 40 |
| 7 | 4.8 | 4.6 | 3.4 | 25 |
| 8 | 4.8 | 4.8 | 3.5 | 20 |
| 9 | 3.8 | 4.7 | 2.5 | 20 |
| 10 | 6.7 | 6.8 | 5.6 | 13 |
| 11 | 5.9 | 6.3 | 5.4 | 3.2 |
| 12 | 4.8 | 4.3 | 4.3 | 2.5 |
| 13 | 6.7 | 6.8 | 6.5 | 1.6 |
| 14 | 6.0 | 6.3 | 5.9 | 1.3 |
| 15 | 4.8 | 6.7 | 4.8 | 1.0 |
| 16 | 5.8 | 6.7 | 6.6 | 0.16 |

TABLE III. YIELD (PFU/ml) AT THREE TEMPERATURES OF PURIFIED VIRUS POPULATIONS SHED INTO THE SUPERNATANT BY CELLS PERSISTENTLY INFECTED WITH SSPE VIRUS (AVERAGE OF TWO CULTIVATIONS).

| Purified population No. | $Log_{10}$ PFU/ml | | | Ratio 32/39° |
|---|---|---|---|---|
| | 32° | 37° | 39° | |
| 1 | 6.5 | 5.5 | 4.3 | 160 |
| 2 | 6.3 | 6.2 | 4.2 | 130 |
| 3 | 6.5 | 6.7 | 4.9 | 40 |
| 4 | 5.8 | 6.8 | 4.3 | 32 |
| 5 | 5.0 | 5.1 | 4.1 | 7.9 |
| 6 | 3.2 | 4.1 | 2.3 | 7.9 |
| 7 | 6.3 | 6.7 | 5.5 | 6.3 |
| 8 | 4.9 | 5.0 | 4.1 | 6.3 |
| 9 | 4.6 | 4.8 | 3.8 | 6.3 |
| 10 | 3.3 | 3.7 | 2.4 | 6.3 |
| 11 | 6.1 | 6.3 | 5.5 | 4.0 |
| 12 | 5.4 | 6.0 | 4.8 | 4.0 |
| 13 | 6.2 | 6.6 | 5.7 | 3.2 |
| 14 | 2.5 | 3.3 | 2.2 | 2.0 |
| 15 | 2.4 | 2.7 | 2.4 | 1.0 |
| 16 | 5.2 | 6.6 | 5.3 | 0.79 |
| 17 | 4.0 | 4.6 | 4.1 | 0.79 |
| 18 | 2.0 | 4.7 | 2.2 | 0.65 |

plaque purification were employed.

Although there is great temptation to do so, caution must be exercised before one may conclude that the Halle strain as it existed in the patient's brain contained ts elements. The virus had undergone a number of laboratory passages before these tests were done. It should be noted, furthermore, that the virus persisting in the patient's brain might have undergone considerable variation from the virus that established the infection. It should be pointed out, however, that previous cultivation of the virus had been at 37°, so that there would have been no selective pressure toward temperature sensitivity in the course of the passages.

It is of interest that the Halle strain of SSPE virus was able to initiate PI of HEp-2 cells at restrictive temperature but not at permissive temperature in duplicate cultures. The Edmonston strain of wild type measles virus, in which ts elements were not detected, was unable to establish PI at any temperature. This finding supports the hypothesis of Youngner (4, 5) that ts mutants may be of importance in the initiation of PI.

In contrast to the findings of other investigators (1, 5, 8, 9, 10), there was no indication that ts mutants were selected by the persistently infected state (Table III).

One would have expected that shifting the cultures from 39 to 32° during the intercrisis periods would have enhanced production of infectious virus. This did not occur, and the data do not provide an explanation.

Other workers have stressed the role of defective interfering (DI) particles in persistent infections caused by measles virus (11). Rima *et al.* (11) detected DI particles by examining the ratio of hemagglutination units to infectious particles in different virus populations. In this laboratory results with the hemagglutination assay were too variable to allow for firm conclusions. Others have pointed out that ts mutants are defective virions of a sort (12), even though they are not deletion mutants. The role they play in PI might be similar to the role played by DI particles.

*Summary.* The Halle strain of SSPE virus was found to consist of a heterogeneous population with regard to temperature sensitivity. Ten of 16 purified virus populations isolated from this strain were temperature sensitive (ts). The Halle strain was able to initiate persistent infection (PI) of HEp-2 cells at restrictive temperature but not at permissive temperature. The proportion of virus with ts properties did not increase with the passage of the persistently infected cells. No ts virus was detected in a strain of Edmonston measles virus, and this strain did not initiate PI at any temperature.

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