Subacute Sclerosing Panencephalitis Measles Virus: Study of Biological Markers

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Comparative studies between two measles virus strains isolated from patients with subacute sclerosing panencephalitis (SSPE) and a prototype low tissue culture passage Edmonston measles virus are described. Differences were noted in several properties. The findings described in this report suggest that strains of measles virus associated with SSPE have different biological properties and apparently cannot be distinguished from laboratory and field strains of the virus.

Subacute sclerosing panencephalitis (SSPE) is a progressive, uniformly fatal disease of childhood and adolescence characterized by dementia, myoclonus, seizures, and coma. Symptoms generally begin 4 to 8 years after clinical rubeola, and the brain degeneration is known to result from chronic intracellular measles virus infection (7-9).

Since the isolation of the SSPE measles virus from brain and lymph nodes of patients (2, 3, 5, 16), there has been a considerable effort to compare the SSPE isolates with prototype measles virus (1, 4, 7, 14). In an earlier report we described the susceptibility of different tissue cultures to two SSPE measles isolates, a vaccine strain, and a strain of virulent measles virus. We compared the cytopathology and plaque morphology as well as the serological properties of each of these viruses (4). Differences were found in the growth characteristics of the SSPE virus isolates as measured by *in vitro* assays, and it was suggested that the stage of the illness, area of the brain biopsied, passage level of the chronically infected brain tissue culture, and the method of rescuing the virus would be important factors affecting the characteristics of the virus.

The present study was designed to examine further strain differences of SSPE isolates by utilizing a standard virulent Edmonston measles virus for comparison.

MATERIALS AND METHODS

Viruses. The virulent Edmonston strain was obtained from Paul Albrecht from the Division of Biologics Standards. This virus had undergone four passages in primary human embryonic kidney cell cultures and six passages in Vero cell line and had retained its pathogenicity for rhesus monkeys.

One of the SSPE strains included in the study, the Halle virus, was isolated from the lymph node of a patient as described elsewhere (5). The isolation was obtained by cocultivation of the primary lymph node cells with HeLa cells. For this study the Halle virus was utilized after two additional passages in HeLa monolayers. The other SSPE virus, the Dean strain, was isolated from brain tissue as reported earlier (3). This virus was found to be in a suppressed state throughout seven serial passages of the SSPE brain tissue. Cocultivation of the seventh *in vitro* brain passage with HeLa cells resulted in the release of infectious measles virus. After two sequential passages in HeLa cells the Dean virus was included in this investigation.

Cell cultures. Vero cell cultures were selected as the host system since the Edmonston strain and the SSPE viruses propagate equally as well in this continuous cell line. Vero cells were obtained commercially (Flow Laboratories, Inc., Rockville, Md.) in the form of a suspension containing $10^{5.0}$ cells per ml of the Vero cell suspension. The growth medium was Eagle minimal essential medium with Earle balanced salt solution (EMEM) containing 10% fetal bovine serum, 100 U of penicillin G per ml, and 100 μ g of streptomycin sulfate per ml. Cell monolayers were confluent after 3 to 5 days when they were suitable for use. Maintenance medium consisted of EMEM with 2% fetal bovine serum and antibiotics.

Primary African green monkey kidney (AGMK) cells were utilized for infectivity assays. Roller tubes with AGMK monolayers were purchased from Flow Laboratories, Inc. Tissue maintenance medium for virus titrations consisted of Eagle basal medium (EBME) supplemented with 2% fetal bovine serum, 100 U of penicillin G per ml, and 100 μ g of streptomycin sulfate per ml in addition to 0.5% rabbit SV5 antisera.

Infectivity assays. Virus infectivity titrations were performed in AGMK tubes by using serial 10-fold dilutions in EBME; 0.2 ml of each dilution was inoculated into each of four culture tubes. These were incubated at 37 C for 14 days, during which time they

were examined daily under a light microscope for evidence of cytopathic effect (CPE). The end point was calculated by the method of Reed and Muench (20) and expressed in terms of mean tissue culture infective dose (TCID₅₀).

Hemagglutinin titrations. Hemagglutination (HA) tests were carried out with rhesus monkey erythrocytes by the method described by Katz and Enders (10).

Immunofluorescent tests. Cell cultures were assayed for measles antigen by the indirect fluorescent-antibody (FA) technique (21). The serum employed in these tests was a human anti-measles specimen obtained from a patient with natural rubella. Fluorescein-conjugated antihuman globulin (horse origin) produced by Progressive Laboratories, Inc., Baltimore, Md., was utilized for the FA staining. The number of fluorescent cells on each culture assayed was determined by the immunofluorescent-cell counting technique (6).

Serological tests. Hemagglutination inhibition (HI) and complement fixation (CF) tests were performed by the microtiter method (22). For the HI assays, serum specimens were first treated by adsorption with kaolin and then with rhesus monkey red blood cells; 4 U of antigen were used in the test. Measles antigen was obtained from Microbiological Associates, Bethesda, Md., and was prepared with the Edmonston strain 84F at passage level 9 in primary human heart tissue culture. All test reagents were from the same source for every assay. For the CF antibody determinations, serum specimens were inactivated at 56 C for 30 min; 4 U of the antigen and 2 exact U of complements were used. The CF antigen used was obtained from Flow Laboratories, Inc., and was prepared from AGMK cultures infected with the Edmonston strain. Titers of all serological determinations were recorded as reciprocals of the highest dilution of sera showing positive reaction.

Electron microscopy. For electron microscopy (EM), pellets of virus-infected Vero cells showing 3+ CPE were fixed in phosphate-buffered 2.5% glutaraldehyde, postfixed in osmium, and embedded in Epon. Ultrathin sections, double-stained with uranyl acetate and lead citrate, were examined with the use of a Hitachi 12 microscope.

Preparation of rat antisera. Antisera to each of the strain of measles virus were prepared in adult

Lewis rats. These samples were tested by CF and HI assays. Ten-thousand TCID₅₀ of each virus under study, contained in 1 ml, were UV inactivated and mixed with equal volumes of complete Freund adjuvant prior to inoculation. UV inactivation was done by placing 1 ml of the virus suspension in a 60-mm petri dish and irradiating with a germicidal lamp (Sterilamp type G36T6, General Electric). The maximum output of the lamp was at 25,370 nm, and the dish was kept at 12 inches from the lamp for 6 h. Five rats were inoculated subcutaneously with a single inoculation of 0.1 ml in each rear footpad. Three weeks after inoculation all animals were bled by heart puncture and sera were pooled according to group.

Virus propagation. Each virus strain diluted in EMEM to contain 10,000 TCID₅₀ in 1 ml was inoculated into each of 30 plastic petri dishes (35 mm) with confluent Vero cell monolayers. Cultures were then incubated for 1 h at 37 C to allow virus absorption prior to the addition of 2 ml of maintenance medium. Infected and control cultures were placed in 37 C incubator with 5% CO₂ atmosphere and observed daily under phase microscopy. At 24-h intervals, for at least 12 days, two cultures of each virus were fixed for FA. At the same time tissue culture fluids of these cultures were collected, clarified by light centrifugation (5,000 × g), and frozen at -70 C for infectivity assays and hemagglutinin determinations. Electron microscope examinations were conducted upon appearance of early CPE and thereafter.

RESULTS

Cytopathic changes in Vero cells substrate. Table 1 summarizes the data obtained in this study. Details of the results are presented below. Several cultural properties of the three strains of measles virus studied were markedly different. The Halle virus proved to be the most cytolytic of the three strains, causing a pronounced CPE as early as 24 h after infection. This CPE was characterized by an extensive formation of syncytia which fused virtually all cells of the monolayers within 3 days. In general, the huge syncytia produced by the Halle strain detached from the monolayers on day 5

TABLE 1. *Summary of some of the biological characteristics of Halle, Dean, and Edmonston strains of measles virus*

Virus	CPE	Infectivity	HA	FA	Antigenicity for rats	EM
Hallé (SSPE)	Rapid, syncytia	Rapid, high	Rapid, high	Rapid, cytoplasmic	HI and CF antibodies	Classical morphogenesis
Dean (SSPE)	Slow, pyknosis	Slow, low	Slow, low	Slow, nuclear	None	Classical morphogenesis
Edmonston (wild)	Slow, syncytia	Slow, low	None	Rapid, cytoplasmic	HI antibodies	Classical morphogenesis

postinfection, leaving only a few cells scattered on the petri dish surfaces.

The Dean strain showed a latent period lasting about 6 days, followed by 5 days of slow development of CPE. One striking feature of the CPE produced was that it totally differed from the classical giant cell formation or spindle-like cells caused by regular measles virus (10). The cells became pyknotic and then rounded in a manner similar to the CPE characteristic of most picornaviruses (11).

Monolayers infected with Edmonston virus started to show polykaryocyte formation after 6 days of incubation. These evolved into typical giant cells containing 10 to 30 nuclei by the 8th day postinoculation. The giant cells rounded up and sloughed from the culture surface, leaving islands of intact cells connected by a few cytoplasmic bridge processes. Figures 1 through 4 illustrate these observations.

Virus infectivity. Free infectious virus as estimated by TCID₅₀ was detected as early as 24 h in the fluids of Vero cultures infected with the Halle virus. Maximum titer of $10^{6.7}$ /ml was achieved at 3rd day postinfection; after the 5th day, when the majority of the cells had been destroyed by the virus action, infectivity de-

creased progressively, possibly due to thermal inactivation of the infectious particles.

Detectable virus in fluids of cultures infected with the Dean strain was found only 6 days after inoculation. Between day 8 and day 11 the titer remained stable between a value of $10^{5.0}$ and $10^{5.2}$.

The growth curve of the Edmonston strain paralleled that of the Dean virus with slightly lower titers.

Figure 5 gives TCID₅₀ values obtained at 24-h intervals with each of the three strains included in this study.

Virus hemagglutinin. As shown in Fig. 6 only the Halle strain produced high HA titers with monkey erythrocytes. The Dean virus proved to be a poor hemagglutinating antigen producer and the Edmonston strain showed no HA activity.

Immunofluorescence assay. With the Halle virus, fluorescence could be readily discerned 10 h after infection. Beyond 24 h postinfection the number of fluorescing cells increased as foci of fusion became more numerous. The fluorescence was homogeneous and more intense in the perinuclear area of the cytoplasm although some nuclear staining was observed. At 48 h of

FIG. 1. Phase microscopy of normal Vero cells after 12 days in culture; $\times 400$.

FIG. 2. Phase microscopy showing extensive syncytial formation in Vero cell monolayer 2 days after infection with Halle virus; $\times 400$.

FIG. 3. Phase-contrast microscope reveals giant cell produced in Vero monolayer by the Edmonston virus after 8 days of incubation; $\times 400$.

FIG. 4. Phase-contrast micrograph of the peculiar CPE produced by the Dean virus in Vero cell culture after 8 days of incubation; $\times 400$.

FIG. 5. Growth curves of two SSPE isolates and the Edmonston strain of measles virus in Vero cells.

incubation the specific immunofluorescence was evenly distributed in the cytoplasm of the syncytia (see Fig. 7). Most fused areas sloughed from the culture surface but viral antigen could still be detected in the remaining cells.

In contrast, the immunofluorescence observed in monolayers infected with the Dean virus was somewhat different from that produced by the Halle strain. The FA became discernible only by the 4th day postinfection and was found to be predominantly intranuclear and confined to small irregular packets. This nuclear staining persisted throughout the subsequent observation period (see Fig. 8).

FIG. 6. Hemagglutinin production by two SSPE isolates and the Edmonston strain of measles virus in Vero cells.

The Edmonston virus produced foci of fluorescence which could be detected 1-day postinoculation. The antigen appeared only in small areas and was found predominantly in the cytoplasm near the nuclei. By day 9 after infection, when giant cell formation was evident, the specific staining was more intense and diffuse in both mononuclear and polynuclear cells. Some intranuclear fluorescence was noted. Figure 9 shows the Edmonston strain-specific immunofluorescence at day 10 postinfection.

A quantitative evaluation by the immunofluorescent-cell counting procedure (6) revealed that at peak of infection the Halle virus in-

FIG. 7. Extensive cytoplasmic fluorescence in polykaryocyte produced in Vero cells by the Halle virus 2 days postinoculation. $\times 640$.

FIG. 8. Vero cells inoculated with the Dean virus show rounding and specific intranuclear immunofluorescence 10 days after infection. $\times 640$.

FIG. 9. Fluorescent staining observed in Vero cells 10 days postinoculation with the Edmonston strain of measles virus. $\times 640$.

FIG. 10. Immunofluorescent-cell counting assay of two SSPE isolates and the Edmonston strain of measles virus in Vero cells.

involved 90% of the cells in the monolayer while the Dean virus was detected in 70% of the cells, and the Edmonston strain involved approximately 80% of the cell sheet. This finding and the percentage of cells with virus fluorescence at different times of the observation period are shown in Fig. 10.

Antigenicity for rats. A single injection with the Halle virus induced high levels of CF and HI antibodies after 3 weeks. In contrast, an equiva-

FIG. 11. Antibody response elicited in rats by two SSPE isolates and the Edmonston strain of measles virus.

lent dose of the Dean virus failed to produce an antibody response, whereas an equal dose of the Edmonston virus elicited a low level of HI antibodies but no CF antibodies. Since all antigen preparations used were UV-killed virus combined with complete Freund adjuvant, neither infection nor virus multiplication occurred in the rats, and thus the resulting antibody production represented a true primary response to biologically inert measles virions. Figure 11 illustrates the different antigenic strength observed for each virus under study.

Electron microscopy. The morphogenesis of

FIG. 12. *Electron microscopy aspect of Vero cells 43 h after infection with the Halle virus. Numerous complete virus particles are seen between two cells. The two nuclei on bottom contain nuclear bodies with smooth tubules. $\times 15,000$.*

the three strains was essentially identical and similar to what has been described previously with the Edmonston strain in other cell lines and in nervous tissue cultures (12-14, 19). However, the signs of viral propagation were more pronounced with the SSPE Halle virus than with the Edmonston virus. Intracytoplasmic complexes of "fuzzy" nucleocapsids were seen first either gathered in a juxtanuclear inclusion or dispersed in the cytoplasm. Simultaneously with the appearance of cytoplasmic microtubules, a dense inner lining of the cell membranes was observed, followed by membrane fusion and giant cell formation. In this inner lining, rows of evenly spaced filaments were observed; the modified membrane was subsequently seen budding to form the complete particles containing the nucleocapsids (Fig. 12 and 13). Smooth nucleocapsids similar to those observed in SSPE brain (17) were seen dispersed in the nucleus or forming small aggregates in a nuclear body (Fig. 12). With the Dean strain, the nucleocapsids inclusions were rather frequent in the nuclei of giant cells, whereas nuclear bodies were seen in mononuclear infected cells. At later stages crystalline aggregates of intranuclear tubules were seen (Fig. 14); these would eventually erupt into the cytoplasm with the disruption of the nuclear membrane (Fig. 15).

DISCUSSION

Some biological characteristics of two SSPE measles isolates and the Edmonston strain were considerably different. The two SSPE viruses showed divergent cultural properties. The principal interpretation that emerges from this finding is that SSPE isolates are not necessarily genetically homogeneous variants of measles and that none of the markers examined distinguish these strains from classical laboratory-adapted strains of measles such as the Edmonston virus. A similar observation was reported by Schumacher et al. (23), who suggested that the occurrence of strain differences is probably dependent upon the type of host cell utilized, temperature of cultivation, and the passage history.

Our observations support previous reports on the great variability of measles virus properties (10, 23). It appears that the characteristics displayed by a given strain of the virus can be converted into those of a different strain by changes on the passage conditions. This diversity of behavior is probably shared by SSPE measles virus once rescued from its latent state and propagated in vitro.

FIG. 13. *Electron microscopy aspect of Vero cell 12 days postinoculation with the Edmonston strain. A typical complete particle. $\times 84,000$.*

The Dean virus displayed a slow rate of replication, relatively low yield of infectious particles, undetectable hemagglutinating activity, a very singular CPE associated with intranuclear fluorescent inclusions, and extremely poor antigenic properties. In this connection it resembled the UP measles strain described by Norrby and co-authors (13).

The Halle virus proved to be extremely cytolytic, producing extensive syncytia with predominantly intracytoplasmic fluorescence and unusually high yield of infectious virus carrying hemagglutinin. This SSPE isolate was found to have excellent antigenic activity.

The Edmonston strain had some properties which were shared by the Dean virus and some other characteristics which were common to the Halle virus. For example, the long latent period, the slow rate of replication, the comparatively low infectious titer, and the lack of hemagglutinin were features observed both with Dean and Edmonston viruses. On the other hand the polykaryocyte formation, the type of immunofluorescence noted, and the ability to elicit antibody in rats were properties common to the Halle and Edmonston strains only.

The fine structure of products of viral synthesis was rather similar among the different strains studied. In contrast to observations of others (15, 18), the presence of incomplete

FIG. 14. *Electron microscopy aspect of Vero cell 90 h after infection with Halle virus. Intranuclear tubules and crystalline aggregates. $\times 75,000$.*

FIG. 15. *Electron microscopy aspect of Vero cells 9 days after inoculation with the Edmonston virus. Intracytoplasmic complexes of striated nucleocapsids in a degenerating cell. $\times 75,000$.*

particles did not appear as a characteristic of the SSPE strains in our study, and the late nuclear tubular inclusions were no larger with SSPE strains than with the Edmonston strain.

The data presented here indicate that, whatever peculiar circumstances are responsible for the unique defective state of the virus encountered in SSPE tissue, such circumstances are no

longer present once the agent is rescued by cocultivation techniques; the recovered virus returns to its ordinary condition expressing itself as complete conventional measles virus, displaying the strain differences which are common to this virus.

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