Gamma Ray-Induced Small Plaque Mutants of Western Equine Encephalitis Virus

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Small plaque mutants of Western equine encephalitis virus were obtained from the surviving fractions of wild-type virus which was irradiated with gamma rays. The frequency with which small plaque mutants appeared in the surviving fraction increased with the radiation dose. These mutants were not more resistant to radiation than wild-type virus. The growth rate of a mutant, S127, was lower than that of wild-type. Clonally purified mutant virions presented two peaks in a velocity sedimentation profile; peak 1 corresponded to the peak of wild type and peak 2 moved faster than peak 1. Virions of both peaks were infectious and consistently formed small plaques in chicken embryo cells. Virions reisolated from either peak and grown in chicken embryo cells also revealed two peaks in sedimentation analysis. In the electron microscope examination peak 2 proved to consist of giant form particles, each of which contained more than one nucleoid surrounded with a common envelope. Despite this remarkable morphological difference, densities of the wild-type and S127 mutant virions were similar in cesium chloride gradients. The RNAs and proteins of mutant virions could not be distinguished from those of wild type on the basis of size or charge.

Mutations can be induced in animal viruses by treatment with chemical mutagens (7, 33). However, induction of mutants from single-stranded RNA viruses by ionizing radiation has been relatively limited thus far (33) although radio-induced mutants of Rous sarcoma virus have been reported recently by Golde (11). In the course of isolation of temperature-sensitive mutants (28) we attempted to use gamma radiation for induction of the mutants. As a result, some small plaque mutants emerged among the survivors of the radiation at relatively high rate, although temperature-sensitive mutants were not detected in the examination of a few hundred plaques. The virions of the mutants sedimented faster than those of the wild-type when analyzed in sucrose gradients, and electron microscope examination showed the morphological variations of the mutant virions to be in size and shape.

Most investigations dealing with plaque mutants in RNA viruses have been concerned primarily with their biological properties. Evidence obtained by physico-chemical and serological studies indicate that the surface structure of some of these mutants may also differ. This is reflected in differences in adsorption and

elution pattern on some chromatographic columns (9, 16), in coat proteins tested in serological studies (4, 35), and in amino acid composition of mutant proteins (22). Studies on morphological differences in mutant virions relating plaque type variations have been limited thus far.

In this investigation, we report the isolation and preliminary characterization of small plaque mutants of Western equine encephalitis (WEE) virus obtained from the surviving fractions after gamma ray irradiation. Relationship between morphological variations of the mutant virions and small plaque formation is discussed.

MATERIALS AND METHODS

Virus and cell culture. The McMillan strain of WEE virus was used. Before this experiment, it was purified by the plaque cloning technique. The procedures for the preparation of WEE virus stocks and chicken embryo (CE) monolayer cultures have been described (27, 28). Eagle minimal essential medium (MEM) supplemented with 5% calf serum was used as the cell culture medium.

Gamma ray irradiation. Virus was suspended in cell culture medium and put into glass ampoules. Those ampoules were exposed to gamma radiation from a cobalt-60 source at a dose rate of 1.4×10^4 rads

per min. They were kept cold in ice water during irradiation to minimize heat inactivation and then stored at -80°C until use.

Procedures for labeling virus. CE monolayers were infected with WEE virus at a multiplicity of 10 plaque-forming units (PFU) per cell after a 60-min incubation with MEM containing $1\text{ }\mu\text{g}$ of actinomycin D per ml. Viral RNA was labeled by incubating cells from 1 h postinoculation with fresh MEM containing ^3H -uridine ($1\text{ }\mu\text{Ci/ml}$) for 12 h (28). Viral proteins were labeled by incubating cells from 3 h postinoculation with fresh MEM containing ^3H -tyrosine ($5\text{ }\mu\text{Ci/ml}$) for 9 h. The culture fluids containing the labeled viruses were harvested and purified as described later.

Concentration and purification of viruses. Culture fluids containing viruses were clarified by centrifugation at $3,000\text{ rpm}$ for 15 min and then at $10,000 \times g$ for 15 min at 4°C . The supernatant fluid was centrifuged to pellet virus in the no. 30 rotor (Spinco, Beckman, L2-65B) at $74,000 \times g$ for 120 min at 4°C . The virus pellets were resuspended in a small volume of TNE buffer (30) (0.05 M Tris, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate, pH 7.4) and pipetted vigorously to break up aggregates. The virus suspension was then centrifuged at $10,000 \times g$ for 15 min to remove aggregates. The supernatant fluid was layered over a preformed 0 to 40% (wt/wt) linear sucrose gradient prepared in TNE buffer and centrifuged in the SW 27 rotor at $52,000 \times g$ for 2 h (rate zonal centrifugation). The visible band of virus near the middle of the tube was removed and dialyzed extensively against TNE buffer and, if necessary, rebanded in a second sucrose gradient for further purification.

Extraction of RNA. RNA was extracted from labeled virus by phenol-sodium dodecyl sulfate (SDS) at 60°C . Samples were diluted with phosphate buffered saline containing 0.15% SDS (final concentration). An equal volume of water-saturated phenol was added and the mixture was shaken for 3 min at 60°C . Two phases were separated by low-speed centrifugation; the upper aqueous phase was removed and reextracted once more with fresh phenol. RNA from the final aqueous phase was precipitated twice with 2 volumes of ice-cold ethanol. The RNA precipitate was collected by centrifugation in the cold and was resuspended in TNE buffer solution with or without 1% SDS.

Polyacrylamide gel electrophoresis. Polyacrylamide gels, 10 cm in length, were polymerized in glass tubes 0.4 cm in diameter. The gels contained 7.5% (wt/vol) acrylamide, 0.2% N,N' -bismethylene acrylamide, 0.05% ammonium persulphate, and 0.03% N,N,N',N' -tetramethylethylenediamine, in 0.1 M sodium phosphate buffer, pH 7.2, with 0.1% SDS (31). Virus samples in phosphate buffer solution were acidified with 0.1 volume of glacial acetic acid and then made 0.5 M in urea and 1% in SDS. After heating in a boiling water bath for 3 min, samples were dialyzed at room temperature overnight against 0.01 M of phosphate buffer solution with 0.5 M of urea, 0.1% of 2-mercaptoethanol and 0.1% of SDS. After dialysis $\frac{1}{10}$ volume of 60% sucrose and of bromophenol blue were added, and 200- μl samples were layered onto gels. Electrophoresis was carried out at room

temperature at 10 mA/column for 3 h. Gels were extruded from the tubes and frozen on dry ice. Frozen gels were sliced into 2-mm segments. Each slice was dissolved in 0.5 ml of 30% H_2O_2 at 80°C for 2 h and mixed with 5 ml of scintillation fluid (toluene, 1 liter; Triton X 100, 500 ml; PPO, 10 g; POPOP, 0.3 g). The radioactivity was counted in a Beckman LS-200B liquid scintillation spectrometer.

Electron microscopy. Virus samples purified by sucrose gradients were mixed with 2% solution of uranyl acetate and incubated for 1 min at room temperature. After washing once with saline, samples were stained with an aqueous 2% solution of sodium phosphotungstate at pH 7.0 for 30 s (T. Kitano, personal communication). The preparations were applied to carbon films on copper grids; they were examined in a Hitachi HU-11B electron microscope at an operating voltage of 75 kV and at a magnification of $\times 40,000$.

Hemagglutination (HA) test. The test was performed according to the procedure previously described (29) using goose erythrocytes. Hemagglutinin units (HAU) were expressed as reciprocals of the highest dilution of viruses.

Chemicals. Actinomycin D was provided by Merck, Sharp & Dohme, West Point, Pa. ^3H -uridine (20 Ci/mmol) and ^3H -tyrosine (36 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. Ribonuclease-free sucrose was purchased from Nakarai Chemicals Ltd., Japan. Acrylamide and N,N' -bis methylene acrylamide were bought from the Wako Pure Chemical Ind. Ltd., Japan.

RESULTS

Effect of gamma ray irradiation on WEE virus. The lethal and mutagenic effects of gamma rays on WEE virus were examined. When a virus suspension in cell culture medium was exposed to gamma rays, there was a linear relation between the dose of radiation and virus inactivation as seen in Table 1 (also Fig. 2). In assaying survivors, we sometimes noted small plaques among plaques of wild-type virus (Fig. 1). The frequency of small plaque formers in the surviving fraction was increased with increasing radiation dose (Table 1). Small plaques were isolated and clonally purified by three successive passages from plaque to plaque. These small plaque formers were found to be true mutants because the characters described below, especially in plaque size, were maintained

TABLE 1. Effect of gamma ray irradiation on WEE virus

Gamma ray (rad)	Survival (PFU)	Frequency of small plaques in survivors
0	$10^{7.8}$	3/2042 (0.14%)
500	$10^{6.1}$	18/1782 (1.01%)
1,000	$10^{4.4}$	24/1384 (1.73%)
1,500	$10^{2.4}$	26/ 788 (3.29%)

after several passages in CE cells and BHK-21 cells or adult mouse brains.

In order to determine whether these mutants were induced or selected by radiation, the wild type and three mutants were exposed to gamma ray irradiation and the surviving curves obtained were compared. Figure 2 indicates that the wild type and the mutants showed a similar dose-response curve, suggesting that these mutants were not obtained by selection for relative resistance to the radiation. Furthermore, the fact that frequency of mutants among survivors was increased with the radiation dose favors the hypothesis that the mutants were induced by radiation.

Biological properties of small plaque mutants. Six small-plaque mutants obtained from the irradiated virus and one spontaneous mutant were compared with wild type in regard to the biological markers which had been investigated on WEE virus in our laboratory, i.e., temperature sensitivity, heat stability, plaquing efficiency under dextran sulfate agar, and virulence for mice (26-28, 32). As summarized in Table 2, the results obtained with these mutants vary from one mutant to another but marked differences in characteristics of wild type and these mutants are not seen.

In a one-step growth experiment, growth patterns of the wild type and a small plaque mutant, S127, were compared in CE monolayer cells (Fig. 3). Mutant virus grew more slowly than wild type and the yield of the mutant was lower than that of wild type for the test period. Even at 24 h after inoculation, the yield of the mutant did not reach the level of the wild type. The total (released and cell associated) virus yield of the mutant was also lower than that of wild type. The limited growth in CE cells is a prominent characteristic of this mutant.

Sedimentation behaviors of S127 mutant. When the partially purified S127 mutant virus was examined by rate zonal centrifugation, we noted that it showed two visible bands in sucrose gradients. Therefore, mutant viruses were labeled with ^3H -uridine and analyzed by rate-zonal centrifugation to see the distribution of their radioactivity, infectivity, and HA activity. As shown in Fig. 4, two radioactive peaks of infectivity and HA activity are seen in the velocity sedimentation profile of the mutant S127. To separate these two peaks, fraction 14 of peak 1 and fractions 6 to 9 of peak 2 in Fig. 4 were collected and resedimented by rate zonal centrifugation. As seen in Fig. 5, two peaks sedimented separately corresponding to each

FIG. 1. Plaques produced with the wild type (left) and a small plaque mutant, SO92 (right) in CE cells.

FIG. 2. Surviving fractions of wild type and small plaque mutants irradiated with gamma rays. Symbols: ●, wild-type; ○, S113 mutant; ■, S118 mutant; □, S127 mutant.

TABLE 2. Biological properties of wild-type and small plaque mutants of WEE virus

Virus	EOP ^a at 42/ 37 C (%)	EOP ^b under DS-agar (%)	Heating ^c at 50 C, 30 min (%)	LD ₅₀ /PFU ^d
Wild-type	69	100	3.0	6.7/7.9 (2)
S025	29	109	3.1	6.5/7.6 (4)
S092	32	92	2.2	6.9/8.9 (3)
S113	19	120	6.8	7.1/8.0 (3)
S118	39	114	2.7	6.9/8.0 (3)
S127	66	93	0.8	6.3/7.7 (3)
S132	32	38	10.7	7.1/7.9 (2.5)
Spontaneous	90	Not done	4.4	6.8/8.6 (4)

^a Efficiency of plaquing at 37/42 C (28).

^b Efficiency of plaquing under agar containing dextran sulfate (32).

^c Surviving fraction after heating at 50 C for 30 min.

^d LD₅₀ in adult mice/PFU in CE cells. Parentheses indicate the mean survival time (days) of 10 mice inoculated with 100 LD₅₀ of each virus.

peak fraction shown in the bottom panel of Fig. 4; peak 2 moves faster, and is broader than peak 1 in both radioactivity and infectivity.

Virions in both peaks in Fig. 5 formed small plaques in CE cells and their plaques were reduced by antiserum raised in a rabbit immu-

nized with wild type virus to the same degree as those of wild type (data not shown).

In the next experiment, density of virions was examined by equilibrium centrifugation in a cesium chloride gradient. Mutant virions from fractions 6 to 14 and wild type virions from fractions 14 and 15 in Fig. 4 were collected and dialyzed to remove sucrose and then mixed in the solution of cesium chloride (starting specific gravity 1.243 g/ml). As seen in Fig. 6, densities of wild type and mutant virions were 1.234 g/cm³ and 1.226 g/cm³, respectively. In a 20 to 60% linear sucrose density gradient, both viruses showed 1.19 g/cm³ in density, although the peak of mutant was broader than that of

FIG. 3. One-step growth of the wild-type and S127 mutant of WEE virus in CE cells. CE cells were inoculated with the virus at an input multiplicity of 10 PFU per cell. After a 60-min adsorption period, the inoculated cultures were washed twice with MEM and then incubated at 37 C; duplicate plates were sampled at selected intervals. A part of culture fluid was stored after clarifying by low-speed centrifugation for released virus assay. Infected cells were collected into culture fluid with a rubber policeman and then frozen and thawed three cycles to obtain total virus. Symbols: ●, wild-type released virus; ○, wild-type total virus; ■, mutant released virus; □, mutant total virus.

FIG. 4. Sucrose gradient analysis of the wild-type and S127 mutant virions synthesized in CE cells. Twenty plates of CE cells were infected with either wild-type or S127 mutant in the presence of $1.5 \mu\text{g}$ of actinomycin D per ml and $1 \mu\text{Ci}$ of ^3H -uridine per ml. At 12 h after inoculation, the culture fluids were harvested and the labeled virions were concentrated. Concentrated virions were layered over a preformed linear 0 to 40% sucrose gradient and then centrifuged in the SW27 rotor at $52,000 \times g$ for 2 h. Fractions (1 ml) were collected and samples were assayed for acid-precipitable radioactivity (●), infectivity (Δ), and HA activity ($\square$).

wild type (data not shown). These data taken together reveal that the wild type and mutant virions are similar in density, but different in shape or size.

Electron microscope observation. In order

to confirm these observations, the virions of wild type and S127 mutant were examined in an electron microscope (Fig. 7). The wild type virions showed quite uniform enveloped spherical particles about 60 nm in diameters, as

FIG. 5. Separation of two peaks of S127 mutant in sucrose gradients. Peak 1 from fraction 14 and peak 2 from fractions 6 to 9 in Fig. 4 were collected and resedimented by rate zonal centrifugation. Fractions (1 ml) were collected and samples were assayed for acid-precipitable radioactivity (●), infectivity (Δ), and HA activity (□).

reported by Morgan et al. (21) and Grimley et al. (12), whereas the mutant virions evidently showed two size types, i.e., the regular spherical particle (peak 1) and the giant particle (peak 2), which consisted of two to four nucleoids. These giant form particles already appeared in the concentrated virus preparation before purification in sucrose (Fig. 7B). Therefore, they are not likely to be an artefact due to exposure to sucrose during purification.

Viral components of S127 mutant. The mutant virus was further examined for its RNA and protein content to compare with those of

wild type virus. Viral RNA which was extracted from the purified mutant virions was sedimented in a linear 15 to 30% sucrose gradient at the same rate as the 41S RNA extracted from wild type (Fig. 8). Viral proteins extracted from similarly purified mutant virions were analyzed by polyacrylamide gel electrophoresis. As shown in Fig. 9, viral core and envelope polypeptides migrated to positions identical with those of wild type. The molecular weight of each polypeptide was 6.0×10^4 and 3.8×10^4 , respectively, as estimated by electrophoresis with standard marker proteins. These patterns of

FIG. 6. Buoyant densities of purified WEE virions in cesium chloride density gradients. A 1.5-ml amount of ³H-uridine-labeled virions purified twice in sucrose gradients was mixed with 3.5 ml of TNE solution containing 1.6 g of cesium chloride. Centrifugation was performed in SW 50L rotor at 100,000 × g for 46 h at 4 C. Fifteen-drop fractions were collected from the bottom of the tube, and samples were assayed for acid-precipitable radioactivity (filled circles), and HA activity (open circles). Measurement of refractive index was made on all fractions, and densities at 25 C were calculated from an empirically determined relation between refractive index and density.

FIG. 7. Electron micrographs of WEE virions. $\times 100,000$. A, wild-type; B, concentrated crude S127 mutant; C, peak 1 (Fig. 5) particles of S127 mutant; D, peak 2 (Fig. 5) particles of S127 mutant.

RNA and proteins are similar to those described for group A arboviruses by Sreevalsan et al. (29) and Acheson and Tamm (2).

Genetic stability of small plaque mutants.

To determine whether these altered properties of small plaque mutants resulted from a true genotypic alteration and, if so, whether this property was stable, the following experiments were done. Viruses labeled with ^3H -uridine were sedimented in sucrose gradients under the same conditions stated above, and 30 fractions collected from each tube were assayed for radioac-

tivity and HA activity. As a result, wild type and its large plaque mutant, L49 (unpublished data), showed a single peak, whereas S118 mutant as well as S127 mutant showed 2 peaks. A spontaneous small plaque mutant derived from wild type also showed 2 peaks. However, a revertant presented a single peak corresponding to that of wild type. The revertant was obtained from a wild type sized plaque that rarely appeared after several passages of S127 mutant in CE cells.

Two clones were isolated from peak 1 (frac-

FIG. 8. Sedimentation analysis of WEE viral RNA. RNA was extracted from purified ^3H -uridine labeled virions and was sedimented in a 15 to 30% (w/w) sucrose density gradient. Centrifugation was for 12 h at 24,000 rpm in the SW 27 rotor and 1-ml fractions were collected. Arrows indicate the positions of co-sedimented 28S RNA and 18S RNA markers extracted from CE cells.

tion 12) and peak 2 (fraction 4) in Fig. 5. They were grown once in CE cells for labeling virus with ^3H -uridine, and then applied to sucrose gradients. These two clones presented two peaks again, indicating that the regular spherical particle of peak 1 is not a revertant but a mutant which is stable in the ability to reproduce particles of two types.

DISCUSSION

Since ionizing radiation causes breaks in the polynucleotide chains, arboviruses containing single-stranded RNA polynucleotides (3) are highly sensitive to radiation compared with viruses which contain double-stranded polynucleotides (10). Inactivation of the WEE virus by gamma ray followed a one-hit curve, and the 37% survival dose of this virus determined from Fig. 2 was 150 krad. This value is in agreement with those reported for single-stranded RNA viruses (10, 12).

Ionizing radiation yields highly reactive and short-lived radicals in the medium surrounding or within viruses (10, 14) and ultimately leads to alterations of viral genome through the production of reparable chain breaks or excitations in DNA or RNA molecules. Accordingly, viruses could receive a mutagenic effect other than a

killing effect of radiation which is presumably due to irreparable chain breaks. Therefore, the observed dose-dependent occurrence of small plaque mutants in the wild type WEE viruses might be explained by radiation-induced mutation. However, we should note that the wild type used here is relatively mutable in its plaque type character. After being extensively purified by plaquing, the wild type virus still contained spontaneous small plaque mutants at the rate of 0.1% (Table 1). This is not a special case in plaque-type mutations of animal viruses (7, 20, 24, 33). In view of such high mutability, it still remains a possibility that the mutants were selected by the relative resistance to radiation, although a significant difference in sensitivity to radiation between wild type and mutants was not demonstrated. A significant difference in sensitivity to gamma radiation might not be readily detectable when the majority of the infectious particles are "monoploid" in a mutant population. We would expect that preexisting mutants could be selected

FIG. 9. SDS-polyacrylamide gel electrophoresis of proteins extracted from purified WEE virions. Wild-type and S127 mutant viruses were labeled with ^3H -tyrosine. One hour before infection CE cells were incubated with 1.5 μg of actinomycin D per ml. Three hours after infection the medium was removed, and MEM with 5 μCi of ^3H -tyrosine per ml was added. At 12 h, culture fluids were harvested and virions were purified as described in the text. The virions were disrupted in 1% SDS, 0.5% urea and 0.1% 2-mercaptoethanol and electrophoresed in a 7.5% gel at 10 mA for 3 h.

by gamma rays if each genome within a "multiploid" particle is intrinsically infectious and the multiploid particles are significantly more resistant to irradiation. The fact that a spontaneous mutant closely resembled the induced mutants in biological and physical characters may also suggest this possibility.

We demonstrated two types of morphologically different particles in mutant virions by sedimentation analysis and electron micrographs. Both were infectious and formed small plaques in CE cells, and contained the complete RNAs and proteins comparable to those of wild type. Therefore, these mutants are quite different from the incomplete or defective particles recently reported for Sindbis virus (23, 25) and for other animal viruses (17). Despite this morphological difference the density of mutants was similar to that of wild type. Variant strains of some animal viruses are known to have different densities in cesium chloride from those of their parental strains (6, 19).

As shown in electron micrographs, our mutants basically consisted of a roughly spherical or somewhat polygonal nucleoid having a well-defined envelope with fine projections. However, the virions collected from peak 2 in a sucrose gradient (Fig. 5) contained more than one nucleoid surrounded by a common envelope (giant form). It is unlikely that these giant form particles are aggregates made up during the process of purification, since an attempt to break up these particles by sonication failed and, in addition, these particles were found even in the crude viral material.

Envelopment of nucleocapsids to form mature virions occurs either at the cell surface (1) or along cytoplasmic membranes (12), and accumulation of nucleocapsids around membranous vacuoles is characteristic in group A arbovirus morphogenesis (5, 8, 12, 21). Judging from our electron micrographs, we concluded that a few nucleoids were presumably enveloped by a common membrane at the final step of maturation. Similar giant forms have been noted by Klimenko et al. (18) for Venezuelan equine encephalitis virus, Higashi et al. (15) for Chikungunya virus, and Tan (34) for Semliki Forest virus in arboviruses, although these authors did not report physical properties, and plaque morphology of the viruses. The production of the two type particles of S127 mutant was constantly observed at the same rate after recloning of the mutant in CE cells or BHK cells. Furthermore, the particles which resembled those of wild type were not revertants of the mutants, because they formed small plaques in CE cells. Therefore, we concluded that the production of two particle types was a

function of the mutant genome rather than that of the host. The mechanism of the production of both types of particles is not known at the present time.

A preliminary experiment showed that infectivity of S127 mutant was about seven times lower than that of wild type when compared on the basis of RNA content. This result shows that most of the lower PFU to RNA ratio is ascribed to the multiploid nature of the particles. A particle with a few pieces of viral RNA can only make one plaque. Furthermore, these particles may have some disadvantages in the replicating process. Thus limited growth of the virus may result in developing a small plaque.

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