Microbiol. Immunol.
Vol. 27 (5), 445–454, 1983

Characterization of Small Plaque Mutants of Mouse Hepatitis Virus, JHM Strain

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(Accepted for publication, March 30, 1983)

Abstract Two small plaque mutants designated as 1a and 2c were isolated from DBT cells persistently infected with the JHM strain of mouse hepatitis virus. Unlike the wild type JHM, these two mutant viruses grew more slowly with no prominent cell fusion. The buoyant densities of the mutants were slightly lower and 2c was revealed to have fewer peplomers than JHM by electron microscopy. The purified JHM contained five polypeptides with molecular weights (M.W.) of 260,000, 105,000 (GP105), 65,000, 60,000 (P60), and 23,000 (GP23). In addition to two polypeptides, P60 and GP23, which were common to JHM and the mutants, 1a was found to contain three other specific polypeptides with M.W. of 180,000 (GP180), 110,000, and 95,000 (GP95), while 2c had GP180, GP105, GP95, and one with a M.W. of 175,000. All of these polypeptides were shown to be glycosylated except for P60. After bromelain treatment, all these viruses lost the peplomers and contained P60 and another new 18,000 dalton polypeptide.

Mouse hepatitis virus (MHV) is a member of the coronavirus group, and a variety of virulences and organ tropism are known among MHV strains (10, 19, 26). An MHV strain, JHM, causes acute or chronic encephalitis and demyelination in mice and rats (7, 16, 29), and it may provide an experimental model for virus-induced disorders of the central nervous system. In this context, chronic JHM infection in the central nervous system seems to be of interest. As an approach to understanding the viral persistency *in vivo*, we previously established and studied an *in vitro* persistent infection with JHM in a SR-CDFl-DBT (DBT) cell line derived from a mouse brain tumor (8, 9, 12, 15). From this persistently infected cell culture, two small plaque viral mutants were isolated.

In this report, we attempted to characterize the structural proteins and biological activities of these mutants in comparison with the original JHM and found that these mutants possessed different peplomer-forming glycoproteins with respect to molecular weights (M.W.) and amounts and that they showed reduced cell fusion capacities.

MATERIALS AND METHODS

Virus and cell cultures. Plaque-purified JHM was propagated on DBT cells in the manner described previously (8). DBT cells were grown in Eagle's minimal essential medium (MEM, Nissui, Tokyo) containing 10% calf serum and 10% tryptose phosphate broth (Difco, Detroit, Michigan). In the maintenance medium (MM) for virus harvesting, serum content was reduced to 5%.

Several small plaque mutants were cloned by three successive plaque purifications from DBT cells persistently infected with JHM strain of MHV after nearly 1 year of culturing (9). Among them, two virus clones, la and 2c, propagated on DBT cells were subjected to virological characterization.

Virus assay. DBT cell cultures grown in 60 mm Petri dishes were inoculated with 0.2 ml of virus samples. After adsorption at 37 C for 60 min, the cultures were overlaid with 5 ml of MM contaning 0.8% agar (Agar Noble, Difco). After 48 to 72 hr of incubation at 37 C, the cultures were overlaid with 5 ml of MM containing 0.8% agar and neutral red (1: 10,000). Plaques were counted 6 to 8 hr later.

Radiolabeling of the virus. DBT cells in 60 or 35 mm plastic dishes were inoculated with JHM at a multiplicity of infection (m.o.i.) of 1.0. After adsorption for 60 min at 37 C the inoculum was replaced with a labeling medium, *i.e.*, MEM containing 10% dialyzed calf serum, and 100 μCi/ml of [5, 6-^{3}H]uridine (42 Ci/mmol, Amersham) or D-[1,6-^{3}H(N)]glucosamine hydrochloride (20.2 Ci/mmol, New England Nuclear). In case of mutants, the medium was replaced by labeling medium including radioisotopes after 12 hr of infection. L-[4,5-^{3}H]leucine (65 Ci/mmol, Amersham) labeling was achieved in a similar manner, but leucine-free MEM was used in place of MEM. The culture media were harvested 12 hr after addition of radioisotopes.

Virus purification. The culture supernatants from infected cells were clarified by centrifugation. The supernatants were then placed on discontinuous sucrose gradients consisting of 60, 30, and 20% sucerose (3: 2: 2 by volume, respectively) in NTE buffer consisting of 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.2, and 0.001 M EDTA. After centrifugation at 27,000 rpm for 3 hr at 4 C in a Beckman SW 28.1 rotor, a virus band between 60 and 30% sucrose was taken and diluted three-fold with NTE buffer and the dilution was then subjected to 20 to 60% continuous sucrose gradients. After centrifugation at 27,000 rpm for 18 hr at 4 C, each fraction was collected from the bottom and examined for radioactivity and virus infectivity. Fractions showing high radioactivity were pooled, diluted with NTE buffer, and pelleted by centrifugation at 45,000 rpm for 60 min at 4 C in a SW 50.1 rotor.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). The discontinuous SDS-polyacrylamide system of Laemmli (13) was used. The separating gel was a slab of $15 \times 14 \times 0.1$ cm with a linear gradient gel of 7.5 to 15% polyacrylamide, pH 8.8, and stacking gel of 5% polyacrylamide, pH 6.8. Samples were solubilized in a sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 5% 2-merca-

ptoethanol, and 0.1% bromophenol blue) and heated at 100 C for 2 min. After electrophoresis the gels were fixed with 10% acetic acid in 40% methanol, stained with Coomassie brilliant blue G-250 (Nakarai Chemical, Tokyo) and then processed by the fluorographic method of Bonner and Laskey (2). The molecular weight was determined by comparison with marker polypeptides, *i.e.* lysozyme (M.W.: 14,300), trypsinogen (24,000), pepsin (34,700), ovalbumin (45,000), bovine serum albumin (66,000), and crosslinked polymers of hemocyanin (70,000, 140,000, 21,000, and 28,000) (Sigma, Saint Louis).

Bromelain treatment. After discontinuous sucrose gradient centrifugation partially purified virus was incubated at 37 C for 2 hr with a final concentration of 1.3 mg/ml of bromelain (Wako Pure Chemical, Tokyo) in 0.1 M Tris-HCl, pH 7.2, 0.001 M EDTA, 0.01 M NaCl, and 0.005 M dithiothreitol. The treated virus suspension was centrifuged at 2,500 rpm for 10 min. Then the supernatant was loaded immediately on a continuous sucrose gradient of 20 to 60% and centrifuged to equilibrium as described above. The peak fractions were pelleted and analyzed by SDS-PAGE.

Electron microscopy. Samples were plated on carbon-coated grids, negatively stained with 2% phosphotungstic acid at pH 7.0 and observed by a JEM 100B transmission electron microscope.

RESULTS

Growth and CPE on DBT Cells

Plaques of JHM, 1a and 2c on DBT cells 72 hr postinfection are shown in Fig. 1 JHM produced plaques of 3.97 ± 0.63 mm in diameter, while 1a and 2c produced plaques of 0.61 ± 0.29 mm and 1.36 ± 0.40 mm in diameter, respectively.

The growth kinetics of JHM, 1a and 2c in DBT cells are shown in Fig. 2. The progeny virus of JHM appeared 4 to 5 hr postinoculation and increased in titer exponentially from 6 to 10 hr, reaching 10^7 PFU/0.2 ml after 8 to 12 hr.

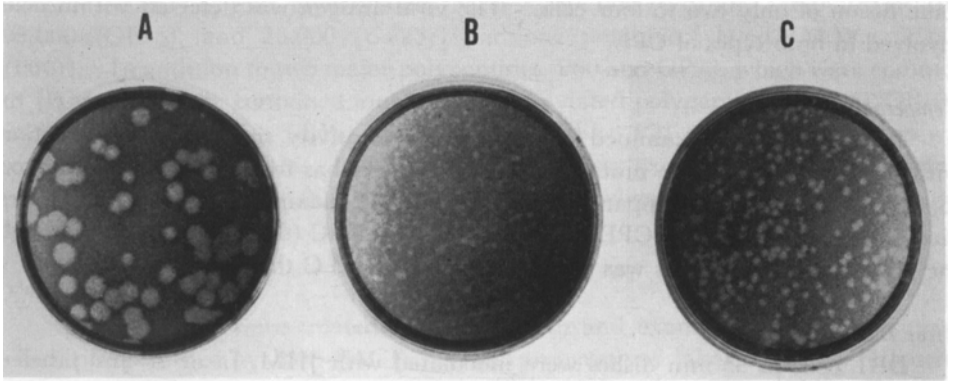

Fig. 1. Difference in plaque size on DBT cells. After inoculation with JHM(A) 1a (B), and 2c (C), cultures were incubated for 72 hr and stained with neutral red.

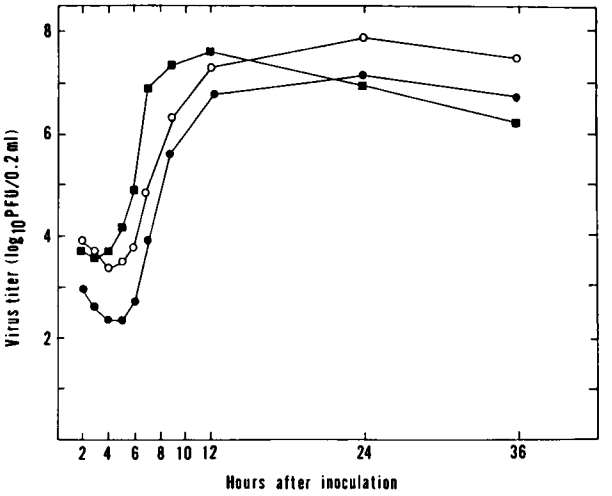

Fig. 2. Growth of JHM (■), 1a (○), and 2c (●) after inoculation of DBT cells at an m.o.i. of about 10 and incubation at 37 C for 36 hr.

With 1a and 2c, the exponential increase of virus titer occurred 6 hr postinoculation reaching a plateau after 12 to 24 hr. In general, the maximum titer of 1a was about 5 to 10 fold higher than that of JHM or 2c.

Syncytium formation was first noticed 4 to 5 hr after JHM infection and became more extensive thereafter. Almost all the cells fused 9 to 12 hr postinoculation and the syncytia then became detached. After inoculation of 1a the syncytia were detectable about 9 to 10 hr postinoculation. However, 30 to 50% of the cells still remained unfused after 36 hr, although the remaining poulation also finally exhibited a rounding type of CPE and became detached. Viral antigens were markedly demonstrated in both fused and rounded cells by immunofluorescence. After inoculation with 2c mainly rounding type CPE occurred, although there was some fusion of only two to four cells. The viral antigen was detected within cells involved in both types of CPE.

Temperature Sensitivity

The mutants were examined for temperature sensitivity, since persistent infection with temperature sensitive mutants in tissue cultures has frequently been observed (5, 18). There was no apparent difference in the maximum yields of the three viruses or in the resulting CPE at 33 C, 37 C, and 39 C (data not shown) although the growth of these viruses was somewhat slower at 33 C than at 37 C and 39 C.

Virus Buoyant Densities

DBT cells in 35 mm dishes were inoculated with JHM, 1a or 2c and labeled with [^{3}H]uridine 100 μCi/ml for 12 hr, and the viruses were purified. As illustrated in Fig. 3, the three viruses exhibited a single radioactive peak which cor-

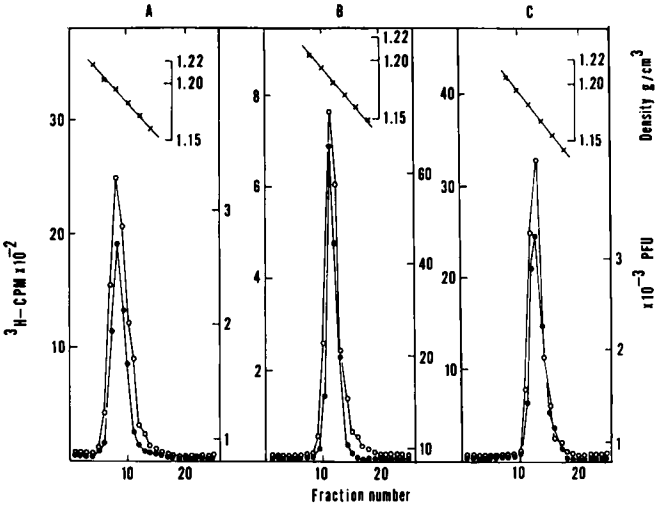

Fig. 3. Buoyant densities of JHM (A), 1a (B), and 2c (C). Virus infectivity (●) and [^{3}H]-uridine incorporation (○) of fractions after continuous sucrose gradient centrifugation for 18 hr.

responded to the peak of infectivity, although the buoyant densities of JHM, 1a and 2c were different, *i.e.* 1.196 g/cm^3, 1.185 g/cm^3, and 1.175 g/cm^3, respectively.

Viral Proteins

The purified [^{3}H]leucine and [^{3}H]glucosamine labeled viruses were compared by SDS-PAGE. Polypeptide patterns are shown in Fig. 4. In JHM, five major polypeptides were detected, four glycosylated [M.W.: 260,000 (GP260), 105,000 (GP105), 65,000 (GP65), and 23,000 (GP23)] and one non-glycosylated [M.W.: 60,000 (P60)]. In some cases, non-glycosylated 21,000 dalton polypeptide (P21) and two separated forms of GP105 were detected. In 1a five major polypeptides were detected, *i.e.*, four glycosylated [M.W.: 180, 000 (GP180), 110,000 (GP110), 95,000 (GP95), and 23,000 (GP23)] and one non-glycosylated [M.W.: 60,000 (P60)]. In addition to two major polypeptides, P60 and GP23, which were common to JHM and 1a, 2c contained four minor glycosylated polypeptides, GP180, GP105, GP95, and one with a M.W. of 175,000 (GP175). P21 of 2c seen in Fig. 4 was sometimes not detectable. Among the three viruses examined there were diffuse patterns of 40,000 to 45,000 dalton polypeptides when samples were tested after long storage at −20 C.

Effect of Bromelain

Virus samples were treated with bromelain and examined for localization of virus polypeptides by SDS-PAGE. Virus preparations labeled with [^{3}H]leucine were repurified after bromelain treatment by centrifugation to equiliblium. There was a slight shift towards low densities from 1.196 to 1.181 g/cm^3 and from 1.185

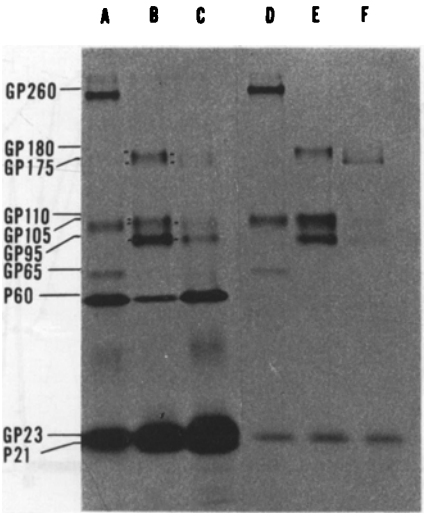

Fig. 4. Polypeptide composition of JHM (A, D), 1a (B, E), and 2c (C, F). Viruses were labeled with [^{3}H]leucine (A, B, and C) and [^{3}H]glucosamine (D, E, and F). Purified viruses were treated with sample buffer and boiled for 2 min. Electrophoresis was performed on 7.5 to 15% polyacrylamide gels.

to 1.182 g/cm^3 in JHM and in 1a, respectively. In 2c, however, a slight shift towards high densities from 1.175 to 1.180 g/cm^3 was observed. After bromelain treatment, the polypeptides of these three viruses showed almost identical patterns, with disappearance of high M.W. glycosylated polypeptides and appearence of a single new polypeptide with a M.W. of 18,000 (P18) (Fig. 5). P60 was unaffected by bromelain treatment.

Electron Microscopy

As shown in Fig. 6, JHM and 1a were found to have a number of club-shaped surface peplomers which were much less in numbers in 2c. After treatment with bromelain, the surface peplomers were completely removed from virions of all three viruses.

DISCUSSION

The virus released from DBT cells persistently infected with JHM formed small plaques on DBT cells. From the small plaque formations, two types of mutants, 1a and 2c, were cloned and their structural proteins and biological activities were studied.

Many viruses isolated from persistently infected cell cultures were shown to possess properties different from those of the parent virus. Stohlman et al (22, 23) reported that cold-sensitive mutants were rescued by fusing with permissive cells from virus-nonproducing clones of neuroblastoma cells latently infected with

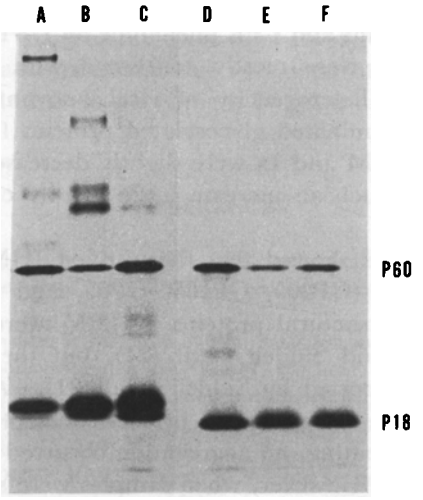

Fig. 5. Polypeptide analysis of nontreated JHM (A), 1a (B), and 2c (C) and bromelain treated JHM (D), 1a (E), and 2c (F). Partially purified viruses were treated with bromelain at a final concentration of 1.3 mg/ml at 37 C for 2 hr, repurified by continuous sucrose gradient and subjected to SDS-PAGE.

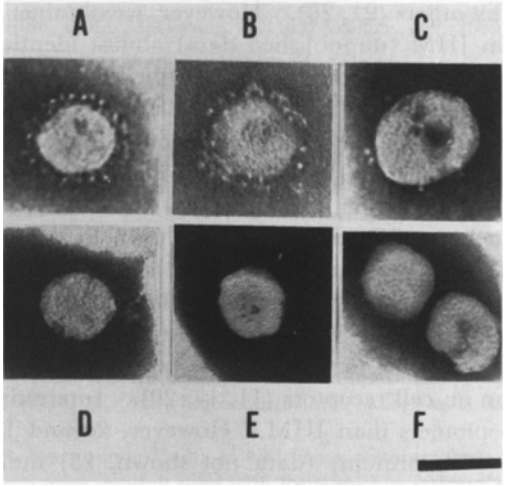

Fig. 6. Electron microscopy of negatively stained untreated JHM (A), 1a (B), and 2c (C) and bromelain treated JHM (D), 1a (E), and 2c (F). The bar represents 100 nm.

JHM. Yoshikura and Tejima (30) obtained a cold-sensitive mutant with CPE activity from Kirsten sarcoma virus-transformed BALB/C cells which were persistently infected with MHV-S. Mutants 1a and 2c, however, grew equally well at 33 C and 39 C producing CPE with equal efficiency.

The prominent differences between parental JHM and its mutant 1a or 2c were in the structural proteins and CPE phenotype on DBT cells. The differences in virus structural proteins were mostly concerned with the envelope proteins, presumably reflecting the heterogeneity of viral buoyant densities. After bromelain treatment which eliminated glycosylated proteins from the virion surface, the buoyant densities of JHM and 1a were slightly decreased, while the 2c density increased unexpectedly. Such an increase in 2c density can not be explained at present.

Analysis by SDS-PAGE showed that the purified JHM contained five major proteins, four glycosylated (GP260, GP105, GP65, and GP23) and one non-glycosylated (P60). The structural proteins of JHM were reported by Bond et al (1), Wege et al (28), and Siddell et al (21), but the results described here mostly agree with those reported by Siddell et al (21). Although some workers (21, 28) noted the presence of aggregated proteins on top of the gels and the loss of some virus proteins by heating, no aggregation occurred on top of the gels after heating in our experiments. However, when samples were subjected to electrophoresis without boiling, GP260 was converted to 220,000 dalton glycoprotein (GP220), whereas there was no change in the other four proteins (data not shown). This heating effect on M.W. of GP260 was similar to that observed with GP170 of JHM by Siddell et al (21). We could not fully explain the difference in M.W. of peplomer forming glycoproteins, especially between our GP220 or GP260 and GP170 as described by others (21, 28). However, we obtained a T_1 fingerprinting pattern of RNA from JHM (unpublished data) almost identical to that described by Lai and Stohlman (14) but not to that described by Wege et al (27). Genetic mutation and/or the selection of virus sources might have occurred during the passage of each virus, affecting the M.W. of peplomer forming glycoproteins.

It is interesting that glycosylated proteins of the mutant viruses were so different from those of the wild type JHM in terms of M.W. Except for GP23 all of them are assumed to be surface peplomers as shown in the bromelain teratment. GP23 of 1a, 2c and JHM corresponds to GP23 of MHV-A59 (24), and the P18 produced after bromelain treatment in the present study may be an undigested portion of GP23 as shown in MHV-A59 by Sturman (24).

High M.W. glycoproteins forming MHV peplomers are thought to be important for adsorption on cell receptors (11, 17, 20). Interestingly, 2c had a much smaller number of peplomers than JHM. However, 2c and JHM showed similar specific infectivities (PFU/protein) (data not shown, 25) indicating that a small number of peplomers is sufficient for virus infectivity. Peplomer-forming proteins were also thought to be crucial in the induction of cell fusion (3, 11, 20). The reduced fusion activities of 1a and 2c in DBT cells as compared with JHM may result not only from the change of peplomer-constituting proteins but also from the quantities of these proteins produced in infected cells. The mutant 2c, which is less active in fusion, was found to contain a much smaller amount of peplomer-constituting proteins in infected cells (data not shown).

Recently, progress has been made in understanding the induction of demyeli-

nation in mice using the ts mutant of JHM (4, 6). After intracerebral inoculation 1a and 2c showed lower virulence for mice than JHM, causing progressive demyelination without remarkable neuronal degeneration (unpublished data). For this reason, infection of 1a and 2c in mice might be good models for the study of virus-induced demyelination.

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(Received for publication, January 28, 1983)