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Role of Protease in Mouse Hepatitis Virus-Induced Cell Fusion

Studies with a Cold-Sensitive Mutant Isolated from a Persistent Infection

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A plaque mutant was isolated from Kirsten mouse sarcoma virus-transformed BALB/c cells persistently infected with a mouse hepatitis virus strain MHV-S. While the wild type produced large plaques consisting of fused cells (fusion type) both at 39 and at 32°, the mutant produced small fusion-type plaques at 39°, and at 32°, only after longer incubation, plaques consisting of round dead cells (non-fusion type) were obtained. The mutant grew equally well at both temperatures. Thus, the mutant was cold sensitive in inducing cell fusion, but not in replication or in ultimate cell killing. The cold sensitivity was overcome by the addition of trypsin (0.04 to 0.05%) to the culture medium, but not by treating the virions with trypsin. SDS-polyacrylamide gel electrophoresis of the virion proteins failed to detect differences between the wild type and the mutant. In intracellular viral proteins immunoprecipitated with anti-MHV-S rabbit serum, a protein of 68,000 daltons (68K protein) which was present in the wild type-infected cells was absent in the mutant-infected cells, but trypsin treatment or incubation at 39° of the mutant-infected cells failed to induce the 68K protein. After six to seven undiluted passages, the mutant segregated varieties of mutants which were partially or totally reverted to the wild type in phenotype, and also those whose cell fusion induction was absent even at 39°. All these mutants failed to induce the 68K protein in the infected cells. Thus, there was no linkage between the presence of 68K protein and the fusion induction. Trypsin treatment of the infected cells enabled MHV-S to form fusion plaques on otherwise resistant cells, and MHV-2, a producer of non-fusion-type plaques, to form fusion-type plaques. Protease appears to play an important role in MHV-induced cell fusion in general.

INTRODUCTION

Mouse hepatitis virus group, a coronavirus, is one of the widespread endemic viruses in laboratory mouse colonies (Rowe *et al.*, 1963; McIntosh, 1974; Fujiwara *et al.*, 1976). Strain MHV-S isolated by Rowe *et al.* 1963) induces plaques in $SRCDF_1$ DBT cells (Hirano *et al.*, 1974) which develop by fusion of the infected cells. We previously reported that Kirsten mouse sarcoma virus-transformed BALB/c mouse cells (Ki-BALB cells) were able to be dually infected with MHV-S and mouse leukemia virus (Yoshikura and Taguchi, 1979). The Ki-BALB cells producing both viruses were maintained at 37° for nearly 2 years. Recently, we found that MHV-S released by the culture was a cold-sensitive plaque mutant. Characterization of this mutant is detailed in this report. In addition, our experiments suggested an involvement of proteolytic activities in plaque formation by MHV in general.

MATERIALS AND METHODS

Virus. MHV-S and MHV-2 were kindly provided by Dr. K. Fujiwara of this institute. Under ordinary conditions, MHV-S produced fusion-type plaques, and MHV-2 produced plaques consisting of round dead cells (non-fusion type). The viruses were serially passaged in $SRCDF_1$ DBT

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cells in the manner described by Hirano *et al.* (1974).

Cells. Ki-BALB cells (Aaronson and Weaver, 1971) were obtained from DR. Y. Ikawa of the Cancer Institute of Japan. The cells were persistently infected with MHV-S and FN-2, a helper component of Friend leukemia virus, and maintained as persistent infections for nearly 2 years. The cells were incubated at 37° and subcultured when they became confluent, about twice a week. For titrating and propagating MHV, $SRCDF_1$ DBT cells which were not producing type-C virions were used. The FV131 cell line was a ddY mouse-derived lung cell line persistently infected with Friend leukemia virus and was of untransformed morphology (Yoshikura *et al.*, 1968).

Titration. $SRCDF_1$ DBT cell cultures grown in 30-mm petri dishes were contacted with the virus in a volume of 0.1 ml for 1 hr. After the inoculum was removed, the cultures were overlaid with a medium containing 0.8% methyl cellulose, 4000 cps (Wako Pure Chemical Industries, Ltd.). Plaques were scored 24 to 40 hr after infection in formalin-fixed and crystal violet-stained cultures. The culture medium consisted of Eagle's MEM (Nissui Co.) supplemented with 7.5% calf serum heated at 56° for 30 min.

Chemicals. Trypsn (1:250) and trypsin inhibitors lyophilized from soybean were purchased from Difco laboratories and from Sigma Chemical Company, respectively. Other protease inhibitors, antipain, pepstatin, chymostatin, elastatinal, β-glucaro-δ-lactam K salt, and leupeptin, were provided by the Japanese Cancer Research Program.

Purification of virions. Culture fluid of $SRCDF_1$ cells infected with the virus for 16 hr (m.o.i., about 0.1) was clarified by centrifugation at 7000 rpm for 10 min. The virions were precipitated by ammonium sulfate (313 g/liter). The precipitate was suspended in virus standard buffer (50 m*M* Tris-HCl, pH 7.5; 100 m*M* NaCl; 1 m*M* EDTA) and sedimented to an interface of 60% sucrose (w/v) (15 ml) and 20% sucrose (w/v) (5 ml). The viral band, collected from above to avoid pellet, was diluted in the virus standard buffer, layered over a 20–60% (w/v) linear sucrose gradient, and centrifuged in a Spinco SW27 rotor for 20 hr at 24,000 rpm (4°). Gradient fractions with a buoyant density of about 1.17 g/cm^3 were collected for analysis of virion proteins.

Radioisotope labeling. Cells were labeled for 3 hr with 50 μCi/ml of [^{35}S]methionine (1140 Ci/mmol, Radiochemical Center) dissolved in methionine-free medium supplemented with 2.5% dialyzed calf serum. At the end of the labeling period, the cells were lysed, scraped into extraction buffer (1% Triton X-100; 0.5% deoxycholate; 0.1% SDS; 0.01 *M* NaH_2PO_4-Na_2HPO_4, pH 7.5; 0.1 *M* NaCl), and centrifuged at 30,000 rpm for 30 min in a Spinco SW50.1 rotor. The supernatnat was used as cell lysate.

Immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The lysate in a volume of 0.5 ml was mixed with 10 μl of normal rabbit serum and 50 μl of 10% formalin-fixed *S. aureus.* After 16 hr of incubation, the mixture was centrifuged at 2500 rpm for 2 min, and the supernatant was incubated with 5 μl of anti-MHV-S rabbit serum (gift of Dr. F. Taguchi) for 2 hr. *S. aureus* in a volume of 50 μl was added and incubated for a further 15 min. The pellets were washed three times with extraction buffer and suspended for SDS-polyacrylamide gel electrophoresis in 1% SDS, 1% 2-mercaptoethanol, 50 m*M* Tris, pH 6.8, and 10% glycerol. SDS-polyacrylamide gels were prepared and run as described by Laemmli (Laemmli, 1970).

RESULTS

Isolation of mutants. After nearly 2 years of culture at 37°, Ki-BALB cells dually infected with MHV-S and FN-2 were found to produce exclusively MHV-S, forming small plaques when titrated in $SRCDF_1$ DBT cells at 37°. No wild-type large plaques were produced. As persistent infection was frequently established by temperature-sensitive mutants (Preble and Younger, 1973; Rima and Martin, 1976; Walker, 1964), we titrated the supernatant at 32, 37, and 39°. At 32°, plaques were not produced at all, while at 39°, plaque for-

mation was as efficient as at 37°. Thus, persistently infected cultures appeared to produce an MHV-S mutant whose plaque formation was cold sensitive. Before continuing experiments, we cloned the viruses by two successive plaque purifications; Sp-11, a mutant, and 6v-1, a wild-type clone, were used in further experiments.

Plaque formations of Sp-11 and 6v-1 at varous temperatures. While the plaquing efficiency of the wild-type 6v-1 was comparable at 39 and at 32° (11×10^5 and 12×10^5 PFU/ml, respectively), Sp-11, having a titer of 1.2×10^4 PFU/ml at 39° and of 3.5×10^4 PFU/ml at 37°, formed no plaques at all at 32°. In this assay, the plaques were scored 24 hr after infection. If the Sp-11-infected cultures were kept longer at 32°, the cells remained apparently healthy for the first 4 days (viable cells of cultures infected at a m.o.i. of 0.1 and maintained in liquid medium remained constant in number in this period), but at 5 to 6 days, non-fusion-type plaques consisting of round dead cells appeared. Cell fusion was never induced by Sp-11 at 32°. Growth of Sp-11 as well as 6v-1 was comparable at 39 and 32° (Fig. 1). Thus, the cold sensitivity of the mutant affected only fusion activity and not ultimate cell killing or viral replication.

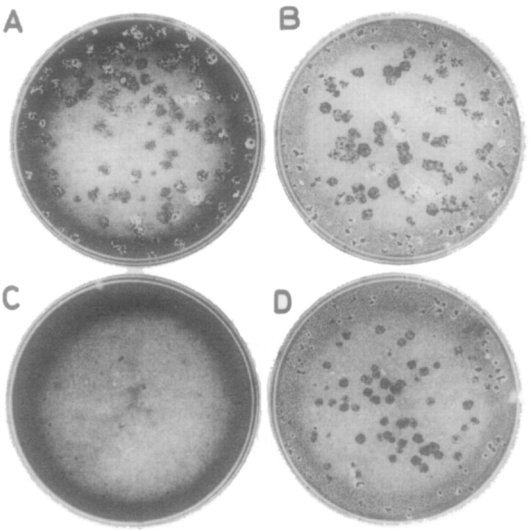

FIG. 2. Effect of trypsin on plaque size. After infection with 6v-1 or Sp-11, cultures were incubated for 48 hr at 39° in the presence or absence of trypsin (0.05%). (A) 6v-1 without trypsin. (B) 6v-1 with trypsin. (C) Sp-11 without trypsin. Minute black spots are plaques consisting of fused cells which are stained heavily with crystal violet. (D) Sp-11 with trypsin.

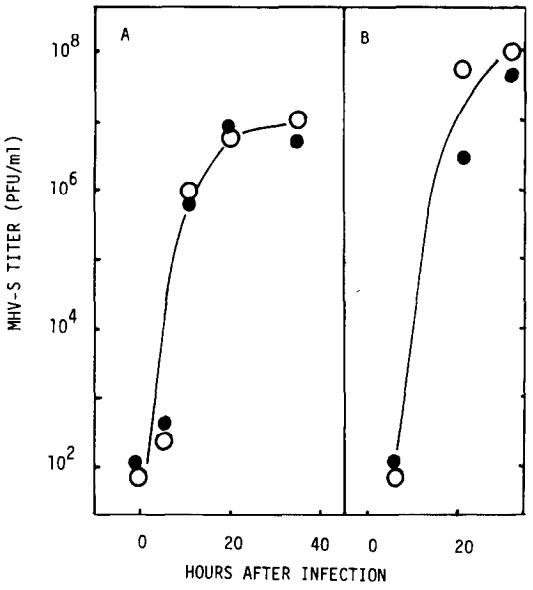

FIG. 1. Growth of Sp-11 and 6v-1 at 39 and at 32° SRCDF$_1$ DBT cells were infected with Sp-11 or 6v-1 at a m.o.i. of about 0.01 and incubated at 32 or at 39°. Medium was harvested at intervals and titrated at 37°. A shows the growth of Sp-11, and B shows that of 6v-1. Open circles indicate growth at 32°, and closed circles growth at 39°.

Effect of protease on Sp-11 plaque formation. Trypsinization of Sp-11-infected cells kept at 32° resulted in rapid cell fusion followed by cell lysis. The cold sensitivity of Sp-11-induced cell fusion appeared to be overcome by proteases. After adsorption, the cultures were overlaid with methyl cellulose medium containing trypsin (Difco, 1:250) at various concentrations. As shown in Table 1, trypsin at a concentration of 0.05% completely restored the plaquing ability of Sp-11 at 32°. At 39°, Sp-11 in the presence of trypsin gave plaques as large as 6v-1 (Fig. 2). The experiment suggested that fusion induction by Sp-11 was dependent upon proteolytic activity in the culture. For confirmation, effects of soybean trypsin inhibitor were examined. Plaque induction by trypsin (0.04%) was effectively inhibited by

TABLE 1

EFFECT OF TRYPSIN ON PLAQUE FORMATION BY Sp-11 at 32 AND AT 39°

Expt No.	Virus	Temperature[a] (°)	Concentration of trypsin (%)[b]	Obtained virus titer[c] (PFU/ml)
1	Sp-11	32	0	$<10^3$
		32	0.05	4.3×10^3
		39	0	1.4×10^3
2	Sp-11	32	0	<10
		32	0.01	0.6×10^2
		32	0.02	7.0×10^3
		32	0.04	5.1×10^5
3	Sp-11	39	0	7.6×10^4 (small plaques)[d]
		39	0.05	2.4×10^5 (large plaques)
	6v-1	39	0	2.9×10^5
		39	0.05	2.4×10^5

[a] Temperature of incubation.

[b] Trypsin concentration in methyl cellulose medium for overlay. During adsorption, trypsin was absent in all the cultures.

[c] Titers calculated from plaques counts under the respective condition.

[d] Photographic reproduction in Fig. 2.

the trypsin inhibitor. In addition, cell fusion by 6v-1 in the absence of trypsin was reduced by the agent (Table 2). Other protease inhibitors (Yuasa *et al.*, 1975), such as pepstatin, chymostatin, and leupeptin, reduced the cell fusion; antipain, elestatinal, and β-glucaro-δ-lactam were poor in the activity (data not shown).

Intracellular virus-coded proteins. The effect of trypsin was examined in an infectious center assay of Sp-11-infected cells kept at 32°. The infected cells were treated with trypsin (0.5% for 5 min), washed twice, incubated with soybean trypsin inhibitor at a concentration of 100 μg/ml for 5 min, washed once, and plated onto the untreated uninfected cell monolayer; massive cell fusion was observed even at 32°. Trypsin treatment of only uninfected cells failed to induce cell fusion. Thus, the continuous presence of trypsin was unnecessary for fusion induction, and once surface protein(s) of the Sp-11-producing cells was cleaved by the protease, the infected cells were capable of fusing with untreated surrounding uninfected cells. Therefore, we examined the effect

TABLE 2

EFFECT OF SOYBEAN TRYPSIN INHIBITOR ON FUSION INDUCTION BY 6v-1 AT 32° AND ON TRYPSIN-MEDIATED FUSION INDUCTION BY Sp-11 AT 32°

Concentration of trypsin inhibitor (μg/ml)	Experiments with	
	6v-1[a]	Sp-11[b]
0	244[c] (100)[d]	131 (100)
10	120 (49)	23 (18)
50	102 (41)	8 (6)
100	78 (32)	2 (2)
200	33 (14)	2 (2)
400	24 (10)	1 (1)

[a] After infection (m.o.i., about 0.01), cells were incubated at 32° for 24 hr in the presence of trypsin inhibitor at the concentrations indicated.

[b] After infection (m.o.i., about 0.01), cells were incubated at 32° for 48 hr in the presence of trypsin (0.05%) and trypsin inhibitor at the concentrations indicated.

[c] Fused cells in unit area (arbitrarily chosen) were counted with the microscope at a low magnification. Figures indicate the number of cells counted.

[d] Percentage control.

TABLE 3

EFFECT OF TRYPSIN ON INFECTIVITY OF 6v-1 AND Sp-11

Trypsin (%)[a]	Virus strain		
		Sp-11	
	6v-1 at 32°[b]	At 32°[b]	After shift to 39°[c]
0	1.1×10^5	$<10^2$	1.2×10^5
0.045	1.4×10^5	$<10^2$	3.7×10^4
0.225	2.2×10^4	$<10^2$	3.5×10^4

[a] Viruses harvested with serum-free medium (medium was contacted with culture for 6 hr from 16 to 24 hr after infection) were treated for 15 min with trypsin at the concentrations indicated.

[b] Titer obtained at 32° 48 hr after infection (PFU/ml).

[c] Titer obtained at 39° 24 hr after the shift from 32°, at which temperature cultures had been incubated for 48 hr.

of trypsin on MHV-coded protein in the infected cells.

The cells were infected with Sp-11 or 6v-1 at a m.o.i. of 0.1. Sp-11-infected cells were incubated for 16 or 24 hr (1) at 32° in the absence of trypsin, (2) at 32° in the presence of trypsin (0.04%), or (3) at 39° in the absence of trypsin. The 6v-1-infected cells were incubated for 16 hr at 39 or at 32° in the absence of trypsin. The cells were then labeled with [^{35}S]-methionine for 3 hr under the respective conditions. The cell lysates were immunoprecipitated with anti-MHV-S serum or with control normal rabbit serum. As shown in Fig. 3, all the preparations precipitated with the antiserum showed two major bands, A and C, which appeared to correspond to 130,000-and 60,000-dalton proteins in JHM or A59 MHV-infected cells (Bond *et al.*, 1979). Band B of 68,000 daltons (68K protein), appearing in 6v-1-infected cells, was absent in Sp-11-infected cells. However, incubation at 39° (slot 5) or treatment with trypsin (slots 2 and 4) of Sp-11-infected cells did not induce the band.

Studies with variants derived from Sp-11. After six to seven undiluted passages of Sp-11 in SRCDF$_1$ DBT cells, fusion-type plaques were produced sporadically even at 32°. Plaques were isolated for further characterization. In Table 4, the properties of some representative isolates are listed. T4a, T4c, T3e, and T3f were plaque-

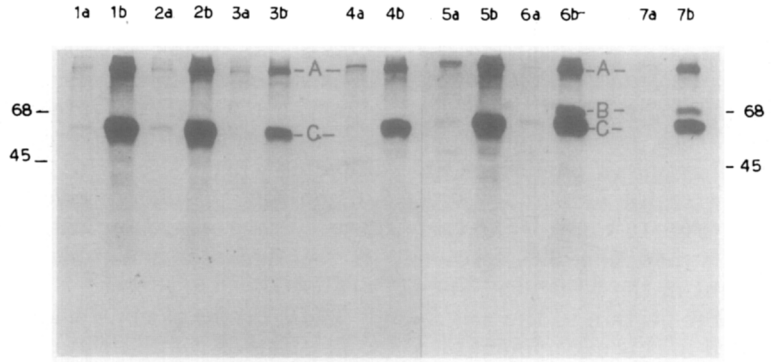

FIG. 3. Intracellular virus-specific proteins of 6v-1 and Sp-11. The infected cells were incubated as indicated below and labeled for 3 hr with 50 μCi/ml of [^{35}S]methionine under the same condition with regard to temperature and the presence of trypsin. Slots a were precipitated with normal rabbit serum, and slots b with anti-MHV-S serum. (1) Infected with Sp-11 for 24 hr at 32° without trypsin. (2) Infected with Sp-11 for 24 hr at 32° with trypsin. (3) Infected with Sp-11 for 16 hr at 32° without trypsin. (4) Infected with Sp-11 for 16 hr at 32° with trypsin (0.04%). (5) Infected with Sp-11 for 16 hr at 39°. (6) Infected with 6v-1 for 16 hr at 32°. (7) Infected with 6v-1 for 16 hr at 39°. Samples were electrophoresed on 12% polyacrylamide gels.

TABLE 4

PLAQUE PHENOTYPE OF Sp-11-DERIVED VARIANTS

Virus	Plaque phenotype[a]		Plaquing efficiency ratio[b] (at 32°/at 39°)
	At 39°	At 32°	
T4a	nf	np	<0.0002
T4c	nf	np	<0.0001
T3e	sf	np	<0.0001
T3f	sf	np	<0.0003
Lp2b	LF	sf	0.8
Lp2d	LF	sf	0.04
Lp2c	LF	LF	1.3
6v-1	LF	LF	0.6

[a] Plaque phenotype was determined 48 hr after infection. nf, non-fusion-type plaque; sf, small fusion-type plaque; LF, large fusion-type plaque; np, no plaques detected. If non-fusion-type plaques were minute, such plaques could not be detected in the assay condition.

[b] Plaque titer obtained at 32°/plaque titer obtained at 39°.

isolated at 37° in the presence of 0.05% trypsin, and L2b, L2c, and L2d at 32° in the absence of trypsin. T4a and T4c were devoid of cell fusion-inducing activity even at 39°, while T3e and T3f were close to the parental Sp-11. Lp2b and Lp2d produced small fusion plaques at 32° and relatively large plaques (diameter was about half of that of the wild-type plaques) at 39°. Though Lp2b plated equally well at both temperatures, Lp2d plated at a reduced efficiency at 32°. Lp2c was completely reverted to the wild type in phenotype. intracellular viral proteins were analyzed as described above (Fig. 4). None of the variant-infected cells had the 68K protein which was present in the wild type. Thus, the role of the 68K protein in the cell fusion was excluded in this experiment also. In this gel, band C in Fig. 3 was separated into two bands, C1 and C2. Interestingly, T3e-infected cells were free of detectable band C1.

Virion proteins. Proteins in purified virions were compared by SDS-polyacrylamide gel electrophoresis (Fig. 5). When the culture fluids were harvested, the wild type-infected cultures were full of fused cells, while the Sp-11-infected cultures were apparently healthy. The virus was purified either from culture fluids collected every 30 min with the autoharvester (Bellco Glass Inc.) for 16 hr (Fig. 5A) or from culture fluids contacted with the infected cells for 16 hr after infection (Fig. 5B). Five to six major bands, 1 (150K), 2 (100K), 3 (90K), 4 (68K), 5 (55K), and 6 (35K), were detected in both wild type and mutant. Minor bands between bands 2 and 3 were variable from one preparation to another. From molecular weight, bands 1, 3, 4, 5, and 6 may correspond to GP180, GP90, GP60, VP50, and GP38 in A59 strain (Sturman, 1977). Band 7 may correspond to GP23 (Sturman, 1977) or a 20,000-dalton protein (Bond *et al.*, 1979) in A59 strain. Trypsin digestion of purified virions resulted in reduction of bands 1, 2, and 6 to generate more intense band 3 and band D. Bands 4 and 5 were relatively unaffected by the digestion. The trypsin digestion pattern was the same in both wild type and mutant. Conversion of GP180 to GP90 by trypsin has been reported by Sturman and Holmes (1977). These studies with virion proteins failed to detect any difference between the mutant and the wild type both in polypeptide composition and in susceptibility of the polypeptides to trypsin.

Involvement of proteolysis in MHV-induced cell fusion in other cases. FV131 cells normally do not develop cytopathic changes after MHV-S infection. However, in the presence of 0.05% trypsin, the cells infected with 6v-1 or with Sp-11 developed fusion-type plaques to give fairly high titers. Viral replication was comparable in trypsin-treated and untreated cultures (Table 5).

MHV-2 induces non-fusion-type plaques consisting of round dead cells in $SRCDF_1$ DBT cells (Hirano *et al.*, 1974). The presence of 0.05% trypsin in the medium induced the rapid appearance of fusion-type plaques at 37°.

DISCUSSION

Our plaque mutant Sp-11 was cold sensitive in inducing cell fusion but not in

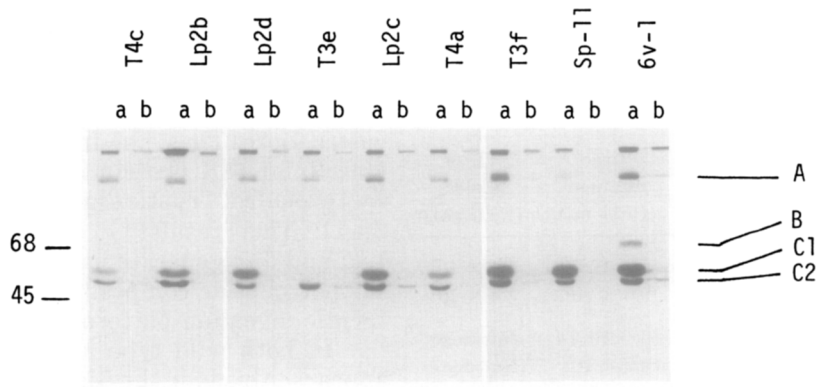

FIG. 4. Intracellular virus-specific proteins of Sp-11-derived variants. The SRCDF$_1$ DBT cells were infected with the virus for 16 hr at 37°. The cells were labeled, extracted, and immunoprecipitated as in the legend to Fig. 3. Samples were electrophoresed on 10% polyacrylamide gels. Slots a were the samples immunoprecipitated with anti-MHV-S, and slots b those immunoprecipitated with normal rabbit serum.

replication or in ultimate cell killing. The cold sensitivity was overcome by the presence of protease in the medium. We further demonstrated that protease could convert normally resistant cells sensitive to fusion plaque induction by MHV-S and

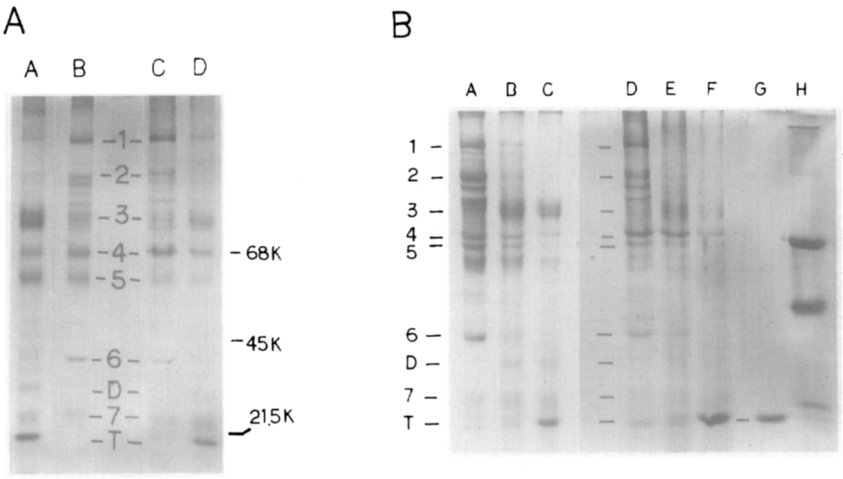

FIG. 5. Polypeptide compositions of the wild-type and Sp-11 virions. Purified virions were treated with trypsin for 5 min at 37°, mixed with an equal volume of 1% SDS, 1% 2-mercaptoethanol, 50 m*M* Tris, pH 6.8, and 20% glycerol, boiled immediately, and electrophoresed on 12% polyacrylamide gels. The gels were stained with Coomassie brilliant blue R. (A) Virions were purified from culture fluids harvested at 30-min intervals with the autoharvester from 6 to 16 hr after infection (at 32°). Slot A: Sp-11 treated with 0.01% trypsin (3x Crystallized, Sigma). Slot B: Untreated Sp-11. Slot C: Untreated 6v-1. Slot D: 6v-1 treated with 0.01% trypsin. T, trypsin, Molecular weight markers are indicated at the right (kilodaltons). (B) Virions were purified from culture fluids contacted with the infected cells for 16 hr after infection. The purified virions of the wild type harvested at 37° were kindly supplied to us by Dr. F. Taguchi. Sp-11 virions were harvested at 32°. Slot A: Untreated Sp-11. Slot B: Sp-11 treated with 0.005% trypsin. Slot C: Sp-11 treated with 0.05% trypsin. Slot D: Untreated wild type. Slot E: Wild type treated with 0.005% trypsin. Slot F: Wild type treated with 0.05% trypsin. Slot G: Trypsin. Slot G: Molecular weight markers. Bovine serum albumin (68K), chicken serum albumin (45K), and soybean trypsin inhibitor (21.5K).

TABLE 5

TRYPSIN-INDUCED PLAQUE FORMATION IN FV131 CELLS WHICH ARE OTHERWISE RESISTANT TO MHV-S PLAQUE INDUCTION

Expt	Virus[a]	Trypsin[b] (%)	Titer obtained[c] (PFU/ml)	Virus yield[d] (PFU/ml)
1	6v-1	0	<10	6.1×10^4
		0.05	9.6×10^4	3.4×10^4
2	6v-1	0	<10	Not done
		0.05	2.2×10^5	Not done
	Sp-11	0	10	Not done
		0.05	1.2×10^4	Not done

[a] Virus titers of 6v-1 and Sp-11 were about 10^6 and 10^5 PFU/dish, respectively, in $SRCDF_1$ DBT cells.

[b] Concentration of trypsin in overlaying methyl cellulose medium. During adsorption, trypsin was absent.

[c] Titers calculated from plaque counts under the respective condition.

[d] Virus titer (PFU/ml in $SRCDF_1$ DBT cells) in the supernatant of the cultures infected for 24 hr.

non-fusion-type plaques of MHV-2 to fusion type.

The role of protease in MHV plaque formation appears to be different from the similar role played by trypsin in Sendai virus infection (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; Ishida and Homma, 1978). In the later case, proteolytic cleavage of viral structural F glycoprotein caused virions infectious and capable of inducing cell fusion. In MHV case, the virions harvested from fused cell cultures and those harvested from unfused cultures, i.e., wild-type 6v-1 and mutant Sp-11 both harvested at 32°, were almost indistinguishable in SDS-polyacrylamide gels, and protease treatment of the virions failed to recover the cell fusion activity. Another important difference was that fusion induction was independent of virus growth (Fig. 1) and protease did not affect MHV infectivity (Table 3) or replication (Table 5).

Biological data indicated that proteolysis of the surface components of Sp-11-infected cells was required for cell fusion. Though the 68K protein which was present in the wild type-infected cells was absent in the mutant-infected cells, incubation at 39 or at 32° with trypsin failed to induce the 68K protein in the Sp-11-infected cells. In addition, 68K protein was absent in the cells infected with the phenotypically reverted Lp2c. Thus, the involvement of intracellular 68K protein in the cell fusion was excluded. Tryptic digestion of 6v-1 and Sp-11 virions resulted in cleavage of bands 1, 2, and 6 to generate bands 3 and D. But the polypeptide composition as well as its tryptic digestion pattern were quite similar in both wild type and mutant. This appears to exclude the possibility that proteolytic cleavage of viral polypeptides was responsible for cell fusion induction by the wild type, though it is still possible that for cell fusion to occur, only a few proteolysed MHV are enough, and in the wild type-infected cells such proteolysis could actually be brought about by a low level of cellular protease in an undetectably minor fraction of the virions on the cell surface. Another possibility is that cellular protein(s) may be interfering with MHV-mediated cell fusion, and proteolysis of such cellular materials may result in the fusion.

The effect of protease on fusion induction was not restricted to Sp-11. MHV-S was induced to form fusion-type plaques by trypsin in otherwise resistant cells, and MHV-2, a producer of non-fusion-type plaques, was induced to form fusion-type plaques by trypsin. The fact that MHV usually replicates and produces plaques better in transformed cells (Chany and Robb-Maridor, 1968; Sturman and Takemoto, 1972) may be related to this phenomenon, as transformed cells show an increased level of protease (Goldberg, 1974). The observation not only has practical value, as fusion-type plaques are more easily scored than non-fusion-type plaques, and the development of plaques was more rapid, but also is important when we consider MHV pathogenesis, i.e., local proteolytic activity may affect the pathology of the organs affected.

One of the possible mechanisms of cold sensitivity of Sp-11 may be that the protease level of $SRCDF_1$ DBT cells at 32° was much lower than at 39°, and Sp-11 was somehow more strongly dependent upon protease than the wild type. MHV-2 or the

Sp-11 variants T4a and T4c could be, in this sense, still more dependent upon protease, as it failed to induce cell fusion at 37–39° without the help of trypsin.

It was amazing that Sp-11 segregated varieties of plaque variants after repeated undiluted passages. The variants ranged from a complete revertant which produced large plaques even at 32° on one extreme to a mutant which failed to produce fusion plaques even at 39° on the other. And various intermediate ones were isolated. All these mutants lost the intracellular 68K protein which was present in the wild type, and the T3e variant lost C1 polypeptide in addition. The loss of these intracellular proteins could be due either to deletion of the coding gene or to antigenic variation which caused altered protein unreactive with the anti-MHV-S serum. From molecular weight, C1 and C2 correspond to p56 and p50 in JHM and MHV-3 (Anderson *et al.*, 1979), and it was suggested that p56 was a precursor of p50. If so, it could be argued that T3e synthesized C2 directly without synthesizing precursor C1. All these possibilities will be tested by specific antisera which are now being prepared.

ACKNOWLEDGMENTS

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