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A Protease Activation Mutant, MVCES1, as a Safe and Potent Live Vaccine Derived from Currently Prevailing Sendai Virus

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Sendai virus fresh isolates were shown to be antigenically different from the prototype Fushimi strain that had long been passaged in embryonated chicken eggs. Phylogenetic analysis of the hemagglutinin-neuraminidase genes also revealed the difference between these two virus groups. Both trypsin-resistant and elastase-sensitive mutations were additionally introduced to an LLC-MK_2-cell-adapted and attenuated mutant derived from one of the fresh isolates. This protease activation mutant (MVCES1) showed the same antigenicity as the fresh isolates, and as a result of a single cycle of growth in lungs, it could confer better protection on mice against challenge infection with the currently prevailing Sendai virus than TR-5, which is a trypsin-resistant mutant derived from the Fushimi strain. The eligibility of MVCES1 as an attenuated live vaccine of Sendai virus is discussed.

Sendai virus, a member of the genus *Paramyxovirus*, has two glycoproteins, the hemagglutinin-neuraminidase (HANA) and fusion (F) proteins (12, 27). HANA protein is involved in cell-binding and neuraminidase activities, while F protein is involved in penetration of the virus by envelope fusion (10, 34, 44). F protein is synthesized as an inactive precursor and subsequently activated by proteolytic cleavage into F_1 and F_2 subunits by trypsin-like proteases present in host cells (8–11, 28, 35). The activating proteases cleave F protein at residue 116 and expose a hydrophobic amino acid sequence necessary to the envelope fusion at the N-terminal end of the F_1 subunit (4, 13, 16). Accordingly, the activation of F protein is indispensable for Sendai virus to express its infectivity, and the presence of the activating proteases in host cells or organs is a prime factor that permits multicycle replication of the virus (10, 28, 29, 34, 36, 37, 39, 40). Kido et al. (20, 32) isolated a serine protease from bronchiolar epithelial Clara cells in rat lungs which was shown to be an activating enzyme of Sendai virus. On the other hand, we have demonstrated previously that the sensitivity of F protein to such proteases is another factor that determines Sendai virus replication in mouse lungs and hence the virulence for mice: a trypsin-resistant (TR) mutant that had a single amino acid substitution at the cleavage site was resistant to activation by the activating protease in mouse lungs and, therefore, was avirulent because of the lack of ability to replicate in mouse lungs in a multistep manner (15, 28, 41).

Sendai virus is a common causative agent of respiratory tract infection in rodents such as mice and rats (2, 5, 14, 24, 31). Epidemic as well as endemic infection with the virus in animal laboratories often results in inestimable losses and causes unexpected deviations in the results of experiments using those animals. Therefore, control of Sendai virus infection is an urgent matter. In previous reports, we showed that TR mutants TR-2 and TR-5 conferred protection on mice against challenge infection with wild-type Sendai virus (41, 42), and we proposed a possible use of a TR mutant as an attenuated live vaccine (25). Recently, we have isolated several strains of Sendai virus from enzootic infections of mice in different animal laboratories. Those isolates appear to be antigenically different from the prototype Sendai virus strains that have long been passaged in eggs. Although it is possible that cross-reactive cytotoxic T cells contribute to some extent to protection against Sendai virus infection, it would be better if the antigenicity of the HANA protein of the vaccine were closer to those of the currently prevailing virus strains. The purpose of this study was to establish a more potent and safer vaccine against the currently prevailing Sendai virus than vaccines with TR mutants. Although the fresh isolates of Sendai virus were highly virulent, their mouse pathogenicity became decreased after the isolates were passaged several times in LLC-MK_2 cells. We found that one of the plaque-purified mutants among such isolates was highly attenuated and never killed mice. Although the mechanism of attenuation of the mutant had not yet been studied thoroughly, our preliminary studies suggested that a mutation(s) in a gene(s) other than the F gene was responsible for the attenuation. We then introduced an additional mutation into the F gene of the attenuated mutant described above so that F protein could not be activated by the activating protease in mouse lungs. The possibility that such a double mutant reverts to generation of virulent progeny would be very low compared with that for a single mutant such as TR-5. In this paper, we describe the eligibility of such a double mutant for a safe and potent live vaccine against the currently prevailing Sendai virus.

The Ohita-M strain (Oh-M) of Sendai virus was isolated from mice during an epidemic in an animal laboratory by inoculating the lung homogenate of an infected mouse intranasally into 3-week-old male BALB/c mice (Clea Japan Inc.). The Tokyo-M and Kyoto-M strains were also isolated from different areas of Japan. Oh-M1 is one of the plaque-purified clones of Oh-M grown in LLC-MK_2 cells in the presence of 1 μg of trypsin per ml. After several passages of Oh-M1 in LLC-MK_2 cells, mutants that form clearer plaques began to appear and increased in number as the passages went on. One such mutant was cloned and designated Oh-MVC11 and was stably maintained in LLC-MK_2 cell cultures. One 50% lethal dose (LD_{50}) of Oh-M1 corresponded to 4.0×10^1 cell-infecting units (CIU) per mouse, while that of Oh-MVC11 corresponded to over 8.0×10^5 CIU per mouse. Although Oh-MVC11 did not show lethality to mice even at the highest

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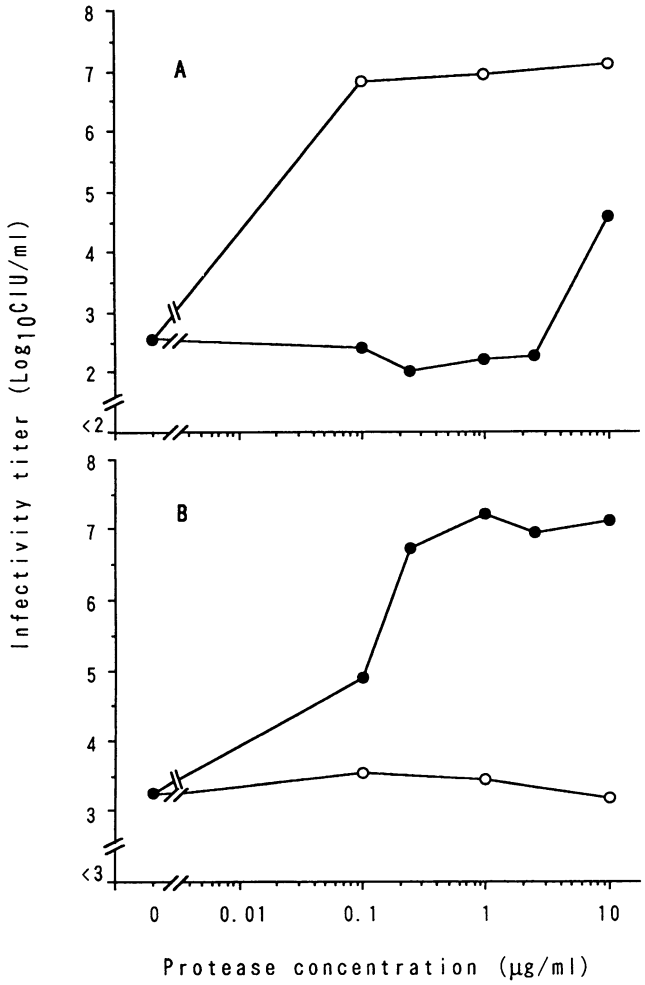

FIG. 1. In vitro protease sensitivities of Oh-MVC11 (A) and MVCES1 (B). Inactive Oh-MVC11 and MVCES1 grown in LLC-MK_2 cells were incubated with various concentrations of trypsin (○) or elastase (●) at 37°C for 10 min. The infectivity was determined by the immunofluorescent-cell counting method (20).

titer tested, a slight decrease in the body weights of the test mice was observed (data not shown). A protease activation mutation was then introduced into Oh-MVC11 by the following method. A 0.5-ml sample of Oh-MVC11 in a plastic dish (15 mm in diameter) was UV irradiated at a distance of 60 cm (10 ergs/mm^2/s) for 25 s; irradiation was followed by inoculation into LLC-MK_2 cells and five successive passages in the presence of 0.1 μg of elastase per ml. The elastase-sensitive mutants thus obtained were plaque purified, and one of the clones, MVCES1, was used for further study.

The protease sensitivity of the virus was assayed by the method described previously (41), with a slight modification. Inactive virus grown in LLC-MK_2 cells in the absence of any protease was treated with various concentrations of trypsin or elastase at 37°C for 10 min in a phosphate-buffered saline. The activated virus was assayed for infectivity by the immunofluorescent-cell counting method (19). Figure 1 shows the sensitivities of Oh-MVC11 and MVCES1 to elastase and trypsin. Oh-MVC11 was activated by trypsin to the maximum level at a concentration of 0.1 μg/ml, whereas it was less efficiently activated by elastase even at a concentration of 10 μg/ml. In contrast, MVCES1 was activated by elastase, with maximum recovery at a concentration of 0.2 μg/ml, but was not activated at all by trypsin. The Western blot (immunoblot) analysis revealed cleavage of the F protein of MVCES1 by elastase (data not shown).

To provide genetic bases for the TR of MVCES1, the F genes of both Oh-MVC11 and MVCES1 were sequenced. The nucleotide sequence of Oh-MVC11 was somewhat different from those of the laboratory-adapted, prototype strains of Sendai virus, such as the Fushimi, Z, and Harris strains: the homology of the nucleotide sequences between Oh-MVC11 and the prototype strains was about 86%, and a considerable number of possible amino acid substitutions were identified (Table 1). When the sequences of Oh-MVC11 and MVCES1 were compared, two nucleotide changes, G→T and T→C, were observed at positions 400 and 403 of the F gene, respectively, resulting in the amino acid substitutions of Ile for Arg at residue 116 and Ser for Phe at residue 117. The amino acid substitution of Ile for Arg at residue 116, the cleavage site of the F protein, was also demonstrated with the TR mutants derived from the Fushimi strain (17).

Our preliminary results obtained with an enzyme-linked immunosorbent assay revealed that the antigenicity of the HANA protein of the egg-passaged prototype Fushimi strain of Sendai virus was different from that of fresh isolates such as Oh-M, Tokyo-M, and Kyoto-M. Since HANA protein is a principal target of the humoral immune response and cytotoxic T lymphocytes (1, 6, 7, 22, 23, 41), it is reasonable to assume that a vaccine strain would exert excellent protective effects against the currently prevailing Sendai virus if its HANA protein were antigenically closer to that of the fresh isolates. Therefore, we performed antigenic analysis by hemagglutination inhibition (HI) and neutralization (NT) assays using a mouse antiserum prepared against Oh-M1. HI antibody was measured by the standard microtitration method using chicken erythrocytes. For NT, serial twofold dilutions of the serum were incubated with an equal volume of Sendai virus for 1 h at 37°C, and the remaining infectivity was assayed by the immunofluorescent-cell counting method. The NT activity was expressed by the reciprocals of the serum dilutions that caused 50% reduction of the infectivity. As shown in Table 2, HI and NT titers of the antiserum were almost the same against Oh-M1, Oh-MVC11, and MVCES1 as well as against the Tokyo-M and Kyoto-M strains, showing that MVCES1 still possessed the original antigenicity of the Sendai virus fresh isolates. On the contrary, HI and NT titers against the prototype Fushimi strain and its derivative, TR-5, were lower than those against Oh-M1 and its derivatives. The nucleotide sequences of the HANA genes of the fresh isolates were determined as described previously (33), and phylogenetic analysis was performed by using the unweighed pairwise grouping method (21) to compare the sequences of the fresh isolates and the laboratory-adapted, prototype strains, such as the Fushimi, Harris, and Z strains (3, 26, 30). All of the fresh isolates obtained from mice in different areas of Japan were nearly identical with each other but clearly different from the laboratory-adapted strains (Fig. 2). On the basis of the predicted amino acid sequences of HANA protein, phylogenetic analysis supported the observation stated above. The observed differences between the fresh isolates and the laboratory-adapted strains may be due to genetic alterations that have accumulated during repeated passages in chicken eggs, as demonstrated previously (18). The actual sequences responsible for the antigenic difference described above have yet to be determined.

To see the vaccine effect of MVCES1 in comparison with

TABLE 1. Amino acid differences between the F proteins of the laboratory strains and the fresh isolates of Sendai virus

Virus	Amino acid residue of F protein[a]																				
	2	3	8	13	14	16	64	97	107	108	109	111	112	116	117	165	215	216	334	336	378
Fushimi[b]	T	A	S	T	S	L	V	E	T	Q	N	G	V	R	F	I	S	G	D	T	S
Z[c]	—	—	—	—	—	—	—	—	—	—	—	—	A	—	—	—	—	—	—	—	—
Harris[d]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	V	F	—	—	—	—
Oh-MVC11[e]	A	T	V	A	L	S	I	R	M	T	G	D	—	—	—	—	—	E	N	A	N
MVCES1[f]	A	T	V	A	L	S	I	R	M	T	G	D	—	I	S	—	—	E	N	A	N
Hamamatsu[g]	A	T	V	A	L	S	—	—	M	T	S	D	—	—	—	—	—	E	N	A	N

Virus	Amino acid residue of F protein[a]																					
	379	394	397	398	432	439	440	461	469	472	478	498	499	501	509	511	526	532	535	536	539	562
Fushimi[b]	L	C	S	T	V	R	G	I	D	N	K	R	E	V	V	I	K	G	D	D	P	A
Z[c]	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Harris[d]	—	R	—	—	—	—	—	V	—	—	—	—	—	—	—	—	—	—	—	E	—	—
Oh-MVC11[e]	I	—	—	—	I	E	R	V	A	D	R	G	A	L	I	V	R	S	A	G	S	T
MVCES1[f]	I	—	—	—	I	E	R	V	A	D	R	G	A	L	I	V	R	S	A	G	S	T
Hamamatsu[g]	—	—	W	R	I	K	—	V	A	D	R	G	A	L	I	V	R	S	A	G	S	T

[a] —, residue identical to that of the Fushimi strain. For the Fushimi, Z, Harris, and Hamamatsu strains, the trypsin cleavage site in vitro was between residues 116 and 117.
[b] Data from reference 17.
[c] Data from reference 43.
[d] Data from reference 4.
[e] The trypsin cleavage site was not determined.
[f] The elastase cleavage site was not determined.
[g] Data from reference 45.

TABLE 2. Antigenic analysis of Sendai virus

Virus	Titer in anti-Oh-M mouse serum[a]	
	HIU/ml	NT 50/ml[b]
Oh-M1	5,120	1,100
Oh-MVC11	5,120	900
MVCES1	5,120	1,200
Tokyo-M	2,560	980
Kyoto-M	5,120	1,000
Fushimi	640	260
TR-5	640	360

[a] Hyperimmune mouse serum prepared against Oh-M1 strain was used.
[b] Serial twofold dilutions of the serum were incubated with an equal volume of each strain of Sendai virus at 5.4×10^4 CIU/ml, and the remaining infectivity was assayed.

that of TR-5, specific-pathogen-free, 3-week-old male ICR mice weighing 8 to 11 g were inoculated intranasally with serial 10-fold dilutions of elastase-activated MVCES1 or chymotrypsin-activated TR-5. Mice inoculated with MVCES1 even at the highest titer exhibited neither the clinical symptoms nor a loss of body weight, while mice inoculated with Oh-MVC11 showed a slight body weight loss (data not shown), suggesting that more attenuation had been achieved with MVCES1 by the introduction of a protease activation mutation into Oh-MVC11. Three weeks after vaccination, the mice were challenged with 50 LD_{50}s of Oh-M1, Tokyo-M, and Kyoto-M; weighed daily; and observed for clinical symptoms. As shown in Table 3, all of the mice inoculated with 2.5×10^5 CIU of MVCES1 survived challenge infection not only with Oh-M but also with Tokyo-M and Kyoto-M, while two to three of six mice inoculated with the same dose of TR-5 died of pneumonia. Similarly, three to five of five mice inoculated with 2.5×10^4 CIU of MVCES1 survived challenge infection, while almost all

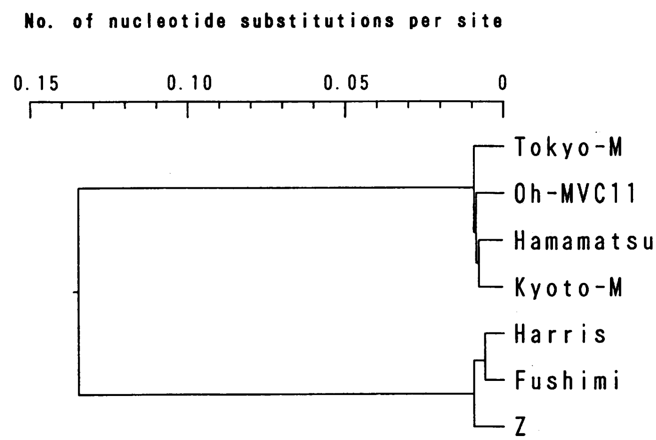

FIG. 2. Phylogenetic analysis of the HANA genes of the fresh isolates (Tokyo-M, Oh-MVC11, Hamamatsu, and Kyoto-M) and the laboratory-adapted strains (Harris, Fushimi, and Z) of Sendai virus. On the basis of sequence alignment, the number of nucleotide substitutions per site was estimated and a phylogenetic tree for various Sendai virus strains was constructed by using the unweighed pairwise grouping method (21). The nucleotide sequences of the Oh-MVC11, Tokyo-M, and Kyoto-M strains have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession numbers D26475 to D26479. The nucleotide sequences of the Fushimi (31), Z (26), Harris (3), and Hamamatsu (45) strains were obtained elsewhere.

TABLE 3. Protection of mice vaccinated with MVCES1 or TR-5 from challenge infection of the fresh isolates of Sendai virus

Inoculum dose of vaccine strain (CIU/mouse)	No. of mice surviving/no. of mice challenged with:					
	Oh-M		Tokyo-M		Kyoto-M	
	MVCES1	TR-5	MVCES1	TR-5	MVCES1	TR-5
2.5×10^5	6/6	4/6	6/6	3/6	6/6	4/6
2.5×10^4	3/5	0/5	4/6	0/6	5/6	1/6
2.5×10^3	1/5	0/5	2/6	0/6	2/6	0/6
2.5×10^2	1/5	0/5	1/6	0/6	1/6	0/6
2.5×10^1	0/5	0/5	0/6	0/6	0/6	0/6

of the mice inoculated with the same dose of TR-5 died of pneumonia. These results demonstrate that MVCES1 is superior to TR-5 in terms of the ability to elicit protective immunity against the currently prevailing Sendai virus.

In previous studies, we have demonstrated that the TR mutants, TR-2 and TR-5, derived from the prototype Fushimi strain conferred protection on mice against challenge infection with the virulent Sendai virus (41, 42), and we have proposed the applicability of the protease activation mutant as an attenuated vaccine for various strains of mice (25). Although the TR mutants elicited better protective immunity than a conventional ether-inactivated vaccine (25), this study has clearly demonstrated the antigenic differences between the Fushimi strain as well as other laboratory-adapted strains of Sendai virus and the currently prevailing Sendai viruses. From a practical point of view, the antigenicity of a vaccine strain should match that of the currently prevailing virus strains in the field. MVCES1 protected mice from challenge infection with the currently prevailing Sendai virus strains, such as Oh-M1, Tokyo-M, and Kyoto-M, more effectively than TR-5, which had been derived from the prototype Fushimi strain (Table 3). We observed that MVCES1 elicited higher titers of NT antibodies against the fresh isolates than did TR-5 (data not shown). It is likely that the observed better protection by MVCES1 against challenge infection is attributable partly, if not solely, to a higher neutralizing activity in the serum. The role of cell-mediated immunity induced by MVCES1, however, remains to be investigated.

In our previous study, the TR mutants TR-2 and TR-5 were obtained by passaging the Fushimi strain in the presence of chymotrypsin, and the mechanism for the selection of the mutants was already elucidated: a single point mutation at residue 116 from Arg to Ile was responsible for both TR and increased sensitivity to chymotrypsin (16). In this study, elastase was used instead of chymotrypsin to obtain a TR mutant derived from Oh-MVC11, since chymotrypsin was not effective for that purpose probably because of the difference in the overall three-dimensional structure of the F proteins of Oh-MVC11 and the Fushimi strain (Table 1). The elastase sensitivity of all of the elastase-sensitive mutants reported so far has been explained by the substitution of Ile for Arg at the cleavage site of the F protein. This substitution simultaneously endows the mutants with TR (13, 17, 43). The same mutation was shown to have occurred in the F protein of MVCES1, and the substituted Ile at residue 116 was supposed to be the target amino acid for cleavage by elastase. The significance of the additional mutation (from Phe to Ser) at residue 117 next to the cleavage site remains to be clarified.

A possible disadvantage for TR mutants as a live vaccine is that they are single-point mutants and the emergence of a pathogenic revertant(s) could occur, although such a revertant has never been recovered from the mice vaccinated with the TR mutants. On the other hand, Oh-MVC11, the parental virus of MVCES1, is already highly attenuated because of a mutation(s) in a gene(s) other than the F gene. Introduction of an additional mutation into the cleavage site of the F protein of Oh-MVC11 resulted in the generation of the more attenuated virus MVCES1, whose replication in mouse lungs was limited to a single cycle, as was the case with the TR mutants. Such multiple mutations provide MVCES1 with a warrant of safety in terms of possible emergence of a virulent revertant(s). Thus, MVCES1 is a good candidate for a Sendai virus vaccine on the basis of its antigenic similarity to the currently prevailing virus and its inability to generate a virulent revertant(s).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper will appear in DDBJ, EMBL, and GenBank nucleotide sequence data bases with the following accession numbers: D17334, D17335, and D26475 to D26479.

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