Attenuating Mutations of the Matrix Gene of Influenza A/WSN/33 Virus

Teresa Liu and Zhiping Ye*

*Laboratory of Pediatric and Respiratory Viral Diseases, Division of Viral Products,
Office of Vaccines Research and Review, Center for Biologics Evaluation and
Research, Food and Drug Administration, Bethesda, Maryland*

Received 2 August 2004/Accepted 7 September 2004

The matrix protein (M1) of influenza virus plays an essential role in viral replication. Our previous studies have shown that basic amino acids 101RKLKR105 of M1 are involved in RNP binding and nuclear localization. For the present work, the functions of 101RKLKR105 were studied by introducing mutations into the M gene of influenza virus A/WSN/33 by reverse genetic methods. Individual substitution, R101S or R105S, had a minimal effect on viral replication. In contrast, the double mutation R101S-R105S was synergistic and resulted in temperature sensitivity reflected by reduced viral replication at a restrictive temperature. To investigate the in vivo effect on infection, BALB/c mice were infected with either A/WSN/33 wild-type (Wt) or mutant viruses and assessed for signs of illness, viral replication in the lungs, and survival rates. The results from mouse studies indicated that the R101S-R105S double mutant virus was strongly attenuated, while single mutant viruses R101S and R105S were minimally attenuated compared to A/WSN33 Wt under the same conditions. In challenge studies, mice immunized by infection with R101S-R105S were fully protected from lethal challenge with A/WSN/33. The replication and attenuating properties of R101S-R105S suggest its potential in development of live influenza virus vaccines.

The genome of influenza A virus consists of eight distinct segments of negative-sense RNA coding for at least 10 viral proteins. The matrix protein (M1), one of the products of the seventh gene segment, is a major structural component of the virion and has multiple functions in viral replication. The dissociation of M1 from ribonucleoprotein (RNP) is an early step preceding entry of viral RNPs into the cytoplasm of the host cell (3, 9, 22). Dissociation of M1 from RNP is triggered by transport of H⁺ ions across the viral membrane by M2, a protein translated as a second, spliced mRNA derived from the seventh gene segment (9, 17, 31). During early viral replication, newly synthesized M1 is transported from its cytoplasmic translation site into the nucleus (24) via a nuclear localization signal in M1 at amino acids 101RKLKR105 (37, 40). Later in the replication cycle, accumulation of M1 occurs in the cytoplasm coincident with the export of RNPs from the nucleus (3, 4, 11, 15, 22, 27). The binding of RNA, nucleoprotein (NP), and M1 is required for the maturation and transport of M1/RNP complexes from the nucleus to the cytoplasm (12, 22), and M1 prevents RNP from reentering the nucleus (22). M1 also binds to NS2 (34, 36) to facilitate nuclear export of the viral RNP (23). In the cytoplasm, M1 interacts with HA, NA, M2, and lipid membranes during budding of new virions from the cell surface (2, 7, 8, 16, 18, 26, 39).

The domain of the nuclear localization signal of M1, containing the basic amino acids 101RKLKR105, has been shown to bind viral RNA (6, 32, 37, 39), NS2 protein (1), and NP during maturation and preparation of RNPs for export to the cytoplasmic compartment (12). In the absence of M1, viral

RNP can spontaneously assemble into circular structures but does not assemble into the fully mature helical form (12). M1s vary in RNP-binding affinity, which affects viral growth potential (21). Studies indicate that replacement of the R's of 101RKLKR105 with S's interferes with viral growth and introduces temperature sensitivity (19).

The M gene of influenza A virus is reported to be responsible for mouse lung virulence and growth (28, 33). In order to further describe the effects of mutations in 101RKLKR105, mutant A/WSN/33 viruses generated by reverse genetic methods were tested in a mouse model to assess replication and possible attenuation compared to the original fully virulent A/WSN/33. We also explored additional M1 and M2 mutations including those identified during passage of influenza A viruses in the laboratory such as the temperature-sensitive strain A/WSN/33 *ts51* or cold-adapted variant of strain A/Ann Arbor/6/60 (10).

Generation of mutant A/WSN/33 viruses. In previous studies we demonstrated that replacement in M1 of single amino acids of the zinc finger motif (C148S) or 101RKLKR105 (R101S or R105S) had no major effect on viral replication (19). However, the double mutant R101S-R105S resulted in introduction of the *ts* phenotype in A/WSN/33. 101RKLKR105, which serves as the nuclear localization signal for M1, binds both RNA and RNP. The zinc finger motif, located between amino acids 148 and 162 of M1, binds RNA. A/WSN/33 *ts51* is a previously described temperature-sensitive variant of A/WSN/33 containing a mutation of M1 characterized by the amino acid substitution F79S (35). The M gene of the cold-adapted variant of A/Ann Arbor/6/60 has been identified as a contributor to the attenuation phenotype of the strain but not to the *ts* or cold-adapted phenotypes of the strain (5, 29). Compared to the original A/Ann Arbor/6/60 influenza virus, the M gene of the

* Corresponding author. Mailing address: Bldg. 29A, Rm. 2B17, 8800 Rockville Pike, Bethesda, MD 20982. Phone: (301) 435-5197. Fax: (301) 480-3157. E-mail: yez@cber.fda.gov.

cold-adapted variant of A/Ann Arbor/6/60 contains an amino acid substitution (A86S) of the M2 protein (5, 6, 10). To assess the effects on viral replication and attenuation of amino acid substitutions including those in 101RKLKR105 and the F79S and V86S (M2) mutations in the M gene of A/WSN/33, nucleotide sequences were altered by site-directed mutagenesis. Recombinant influenza viruses carrying M gene mutations were generated by reverse genetic techniques described previously (19). The viruses generated by reverse genetics were first plaque purified in MDCK cells and subsequently amplified in the allantoic cavities of 9-day-old embryonated eggs.

Characterization of mutant A/WSN/33 viruses. The replication potential of the recombinant viruses was assessed initially by monitoring the hemagglutination (HAU) titers of the output virus in culture fluids of the transfected cells. Posttransfection titers measured by HAU are shown in Fig. 1A. The deletion mutant Δ IRKLKR had no detectable HAU titer and was apparently a lethal mutation (19), but several of the single amino acid mutants [R101S, R105S, V86S(M2) and C148S] and the double amino acid mutants [C148S-V86S(M2) and R101S-V86S(M2)] gave results similar to those for A/WSN/33 wild type (Wt). The double mutant R105S-V86S had a relatively reduced HAU titer compared with that of A/WSN/33 Wt. The double mutant virus R101S-R105S had the lowest titer of HAU, which was undetectable until 144 h posttransfection. The single amino acid mutant F79S, which mimics the A/WSN/33 *ts51* virus, gave HAU titers intermediate between those of A/WSN/33 Wt and the R101S-R105S double mutant. The double mutant virus F79S-V86S(M2) resulted in titers of HAU similar to those of F79S.

The mutant viruses in the culture fluids of the transfected cells were purified by plaque plating on MDCK cells and amplified in the allantoic cavities. Cell monolayers were infected with purified M gene mutant viruses at a multiplicity of infection of approximately 0.001. Viral titers in the supernatant of infected cells were measured by plaque assay. Figure 1B shows the growth curve of multiple cycles of mutant A/WSN/33 viruses in MDCK cells. The single amino acid mutant V86S(M2) gave results similar to those of A/WSN/33 Wt. The double mutant R105S-V86S(M2) had a reduced virus titer compared with A/WSN/33 Wt. R101S-R105S had the lowest titer among the mutant viruses. F79S-V86S(M2) gave virus titers intermediate between those of V86S(M2) and the R101S-R105S double mutant. These results indicate that the delayed detection of hemagglutinin (HA) and lower HA titer during virus rescue (Fig. 1A) are an indication of a growth disadvantage compared with the multiple cycles of the viruses in MDCK cells (Fig. 1B).

Figure 2 shows the results of plaque assays of the recombinant viruses compared to A/WSN/33 Wt at 33, 37, and 39.5°C. All of the M mutant viruses yielded virus titers of 1×10^6 to 7.2×10^6 /ml at 33°C (data not shown). At 39.5°C (Fig. 2A), which is the restrictive temperature for A/WSN/33 *ts51* (35), virus titers were reduced by over 100-fold for the single amino acid mutant F79S and for the double amino acid mutants R101S-R105S and F79S-V86S(M2). Virus titers at 39.5°C were reduced by less than 10-fold for single amino acid mutants C148S, R101S, R105S, and V86S and double amino acid mutants R101S-C148S, C148S-V86S(M2), and R101S-V86S(M2), while double mutant R105S-V86S(M2) had more than a 10-fold reduction in virus titer. At 37°C (Fig. 2B), virus titers were

FIG. 1. (A) HA titers of the output mutant A/WSN/33 viruses in the initial culture fluids of the transfected cells. 293T/MDCK cells were transfected with a modified reverse genetic system to rescue M gene mutant viruses. The titers of viruses in supernatant of the transfected cells were measured by HAU, in the course of 48 to 144 h posttransfection. (B) Growth curves of purified mutant A/WSN/33 viruses in the culture fluids of virus-infected MDCK cells. The mutant viruses in panel A were purified by plaque plating on MDCK cells and amplified in the allantoic cavities. Confluent cell monolayers in 12-well plates were infected with A/WSN/33 Wt or with the mutant A/WSN/33 viruses at a multiplicity of infection of 0.001 at 37°C. At the indicated time points, infectious particles present in the culture fluids were titrated by plaque assay in MDCK cells.

reduced by approximately 10-fold for the double amino acid mutants R101S-R105S, F79S-V86S(M2), and R105S-V86S(M2). Mutant F79S formed small plaques at 39.5°C and turbid plaques at 37°C. Mutant R101S-R105S formed no plaques (even at the lowest input dilution for plaquing, 1:10) at 39.5°C, small plaques at 37°C, and turbid plaques at 33°C (data not shown). The virus titers and plaque morphologies indicated that M gene mutations have additive effects in relation to *ts* phenotype. However, introduction of R101S-R105S had more profound effects on replication than did other single or double amino acid mutations including the F79S mutation previously described for A/WSN/33 *ts51*. It is also confirmed that substitution of a Ser for a Val at amino acid position 86 had a minimum effect on *ts* phenotype (14).

FIG. 2. Temperature sensitivity of M gene mutant viruses. The temperature-sensitive phenotype (*ts*) of the recombinant viruses was determined by plaque assay on MDCK cells at 33, 37, and 39.5°C. MDCK cells in 12-well plates were infected with 200 μ l of 10-fold serially diluted virus and adsorbed at 37°C for 60 min. The inocula were removed and replaced with 1.5 ml of Eagle's minimal essential medium containing 0.7% agarose and 1 μ g of TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-trypsin/ml. The infected cells were incubated at 33, 37, or 39.5°C. After 3 days' incubation, the cells were fixed with 100% methanol and stained with 0.1% crystal violet. The titer reduction at 39 to 33°C (A) or 37.5 to 33°C (B) was compared to that of Wt.

Replication of M1 mutants in mouse lung. The replication of A/WSN/33 M1 mutants in mouse lung was studied by inoculating groups of 4-week-old mice intranasally with 5×10^4 PFU of one of the mutants or A/WSN/33 Wt. Four days postinfection, lung tissue was collected for measurement of virus titer by plaque assay in MDCK cells. As shown in Table 1, A/WSN/33 Wt replicated in mouse lung and resulted in approximately $6.8 \log_{10}$ PFU/g of lung tissue. Single amino acid alterations in 101RKLKR105 at either position 101 or 105 had no effect on the virus titer in lung (approximately 7.2 and 6.5 $\log_{10}$ PFU/g, respectively). Single amino acid mutant F79S or V86S(M2) also showed no reduction of virus titer in mouse lung (approximately 7.0 and 6.8 $\log_{10}$ PFU/g, respectively). Similarly, double amino acid mutants R101S-C148S and C148S-V86S(M2) did not reduce viral growth (approximately 7.2 and 6.3 $\log_{10}$ PFU/g, respectively). Although the double mutants R105S-V86S(M2) and F79-V86S(M2) reduced virus titers slightly (approximately 5.3 and 5.9 $\log_{10}$ PFU/g, respec-

TABLE 1. Virus titers in lung tissues of mice infected with recombinant influenza viruses^a

M mutant	No. of animals	Virus titer in lung ($\log_{10}$ PFU/g $\pm$ SE)
Wt	12	6.8 ± 1.02
R101S	12	7.2 ± 0.40
R105S	11	6.5 ± 0.34
F79S	12	7.0 ± 0.29
R101S-R105S	12	3.6 ± 0.78
R101S-C148S	11	7.2 ± 0.53
M2-V86S	12	6.8 ± 1.49
C148S-V86S(M2)	11	6.3 ± 1.60
R105S-V86S(M2)	10	5.3 ± 0.62^b
F79S-V86S(M2)	11	5.9 ± 1.43^b

^a Analysis of attenuation of the viruses was carried out as described previously (30) with minor modification. Briefly, following isoflurane inhalation, 4-week-old female BALB/c mice were inoculated intranasally with 50 μ l of 5×10^4 PFU of reverse genetics-generated recombinant viruses. Four days after virus inoculation, mice were euthanized, and the lungs and attached large airways were collected, weighed, and frozen at -70°C until used for further study. For virus titration, a volume of $2 \times$ minimal essential medium equivalent to the total lung tissue mass was added to lung tissue for homogenization. The quantity of virus in individual lung homogenates was determined using monolayer cultures of MDCK cells in 12-well plates, and the titers, were expressed as $\log_{10}$ PFU per milliliter. Student's *t* test was used to compare lung titers of the double mutant viruses with that of Wt.

^b $P < 0.05$.

tively), replication of double mutant R101S-R105S showed the largest reduction in virus titer (3.6 $\log_{10}$ PFU/g of lung tissue), approximately 2 to 3 logs lower than A/WSN/33 Wt. These results indicated that the double amino acid mutant R101S-R105S has the *ts* phenotype not only in vitro but also in vivo and suggested that R101S-R105S might also have an attenuation phenotype.

Morbidity and mortality associated with M1 mutant virus infection in mice. To further examine the potential attenuating effects of M mutations, groups of BALB/c mice were infected with 5×10^4 PFU of individual M1 mutant viruses. Animals receiving A/WSN/33 Wt or phosphate-buffered saline (PBS) served as positive or negative controls, respectively, for comparison. Mice were observed daily for 14 days for changes in body weight. As shown in Fig. 3, mice became ill and weight loss was evident starting on day 3 after virus infection except for mice receiving PBS (data not shown) and mice receiving the double mutant R101S-R105S. The body weight of mice receiving other mutants declined in parallel with A/WSN/33 Wt, and mice began to die as early as day 5 postinfection. The survival curves for infected mice are shown in Fig. 4. The survival curves fell into three general groups. The group of viruses associated with rapid death, which started on days 5 to 7, included A/WSN/33 Wt and mutants V86S, R101S, R101S-C148S, and F79S. By day 11, all of the mice in these virus groups were dead. The second group of viruses associated with some survival and less rapid death included mutants R105S, R105S-V86S(M2), and F79S-V86S(M2). The third group, associated with no death, included only the mutant R101S-R105S. These results indicated that the double amino acid mutant R101S-R105S is fully attenuated in mice. These results also suggested that viruses with the R105S mutation alone or in combination with V86S(M2) are partially attenuated in mice.

Protective effect of R101S-R105S mutant virus in mice. To investigate whether infection with the double amino acid mu-

FIG. 3. Percent loss of body weight of mice infected with M mutant viruses. Four-week-old BALB/c mice (six mice/group) were infected under isoflurane anesthesia with 5×10^4 PFU of Wt or M gene mutant viruses/50 μ l. Mice and body weight were observed and measured for 14 days. The body weights were compared with the body weights on day 0 after infection with viruses.

tant R101S-R105S could protect against a lethal challenge of A/WSN/33 Wt virus, mice (six per group) were infected by intranasal administration under isoflurane anesthesia of R101S-R105S at doses ranging from 5×10^2 to 5×10^5 PFU per animal. Animals receiving 5×10^4 PFU of A/WSN/33 Wt or PBS served as positive or negative controls, respectively. The mice were monitored daily for changes in body weight and survival. As expected, all of the mice infected with A/WSN/33 Wt died by day 9. No body weight loss or death was observed for the mice immunized with any dose of the R101S-R105S mutant virus (data not shown). Fourteen days after the initial infection, surviving mice were challenged with 5×10^5 PFU of A/WSN/33 Wt (10 times the dose given in the preliminary round of infection) and were monitored for signs of illness and death for an additional 14 days. As shown in Fig. 5, all six

FIG. 4. Survival rate of mice infected with M mutant viruses. Four-week-old BALB/c mice (six mice/group) were infected under isoflurane anesthesia with 5×10^4 PFU of Wt or M gene mutant viruses/50 μ l. Mice were monitored for 14 days.

FIG. 5. Protection of mice infected with mutant influenza viruses and challenged with a lethal dose of Wt virus. Mice (six/group) were immunized intranasally with 5×10^5 , 5×10^4 , 5×10^3 , or 5×10^2 (5.7, 4.7, 3.7, and 2.7 $\log_{10}$, respectively) PFU of R101S-R105S virus/50 μ l. Animals receiving PBS served as a positive control. Two weeks after immunization, mice were challenged under isoflurane anesthesia with 5×10^5 PFU of Wt virus/50 μ l. The virus-challenged mice were monitored for body weight.

animals from the control group (mice receiving PBS in the preliminary round of infection) infected with A/WSN/33 Wt were dead by day 9 postinfection. In contrast, 100% of mice infected with any dose (even the lowest dose) of R101S-R105S survived the lethal challenge with A/WSN/33 Wt. Although mice infected with the lowest dose of R101S-R105S became ill and lost approximately 10 to 15% of body weight, all of the mice regained the body weight by day 12. Mice in other groups receiving R101S-R105S also experienced illness demonstrated by transient reduction in body weight, but all regained weight by the end of the experiment and all survived. These results indicated a high level of protective efficacy of R101S-R105S against lethal influenza virus challenge.

In previous studies of influenza A viruses, we examined the role of 101RKLKR105 and the zinc finger motif in viral assembly and replication. Of multiple mutant A/WSN/33 viruses engineered and evaluated, the double mutant virus R101S-R105S has been found to be one of the most informative in relation to understanding the functions of M1. We have shown that R101S-R105S exhibits weaker binding of M1 to RNPs and leads to reduced amounts of M1 relative to NP in viral particles (20), which suggests that mutations introduced in 101RKLKR105 might be used to control viral replication and alter viral virulence. To further our understanding, we have analyzed viral replication, illness, and mortality after virus infection in an animal model and have now demonstrated that the double mutant R101S-R105S in the otherwise complete background of virulent A/WSN/33 has not only the *ts* phenotype in vitro but also the attenuation phenotype in vivo. One of the M gene mutations can confer the *ts* phenotype (e.g., F79S of A/WSN/33 *ts51*), and the other mutation gives no attenuation (e.g., V86S of cold-adapted A/Ann Arbor/6/60). However when two mutations were brought together in one virus [F79S-V86S(M2)], both *ts* and attenuation phenotypes were observed in vivo. The combination of F79S and V86S or R105S and V86S also confers a degree of *ts* and attenuation but not to the same extent as R101S-R105S. However, none but the double

mutation R101S-R105S appears to be truly attenuating. The *ts* phenotype and attenuation phenotype of R101S-R105S appear to be due to a large contribution from R105S, since R105S alone demonstrates a slight reduction in replication at higher temperatures and is partially attenuating in mice. Data from Hui et al. (13) also indicate that the amino acid at position 105 is critical in viral replication. It is of interest that it was not possible to produce a double mutant including F79S with either R101S or R105S (data not shown). Incompatibilities of F79S with either R101S or R105S may relate to the fact that R101S and R105S disrupt one RNP-binding domain of M1 (i.e., 101RKLKR105) and that the F79S mutation is near the amino-terminal 76 amino acids of M1, which we have documented as being capable of binding RNP in the absence of 101RKLKR105 (38).

Our studies highlight the fact that the *ts* phenotype and attenuation phenotype are not necessarily concordant results of a given mutation. Although infection of animals with viruses that bear the *ts* phenotype may result in illness that is milder (25) than illness due to infection with Wt virus, F79S, a surrogate of a naturally occurring chance discovery M mutant (A/WSN/33 *ts51*), did not demonstrate attenuation in mice. The lack of attenuation in mice may be due to the fact that mouse body temperature (37°C) did not inhibit replication of F79S, which is reduced in replication by 100-fold only at 39.5°C. In contrast, R105S, which was partially attenuated in mice compared with F79S, would not generally be considered *ts*, even though it demonstrates some reduction in virus titer at higher temperatures. (Fig. 2 and 4).

M1 protein plays an important role in particle assembly and viral replication. Both the dissociation of M1 from RNP in the early phase of the infection and the association of M1 and RNP in the late phase of the infection are required for sufficient viral replication. It is believed that the association of M1 with RNP leads to translocation of RNP from nucleus to cytoplasm. The basic amino acid sequence (RKLKR) of M1 plays an important role for M1 protein. It has a function in viral replication by translocation of M1 from the cytoplasm into the nucleus, and it interacts with RNP. In addition to acting as an essential component to promote the transport of RNP out of the nucleus, the RNP binding of M1 has a structural role in the final viral assembly. Our previous studies (12) demonstrated that vRNA and M1 together promote the self-assembly of influenza virus NP into the quaternary helical structure similar to the typical viral RNP, that the RNP-binding activity of the M1 protein affects the replication efficiency of the viruses, and that M1 that binds more strongly to RNP is found in viruses that replicate more efficiently (19). Our recent studies indicate that weakened RNP-binding activity of the M1 protein may partially contribute to the *ts* phenotype of the viruses. Current studies of the M1 mutant viruses in animal models indicate that attenuated influenza virus can be gained by replacing both Args with Ser at positions 101 and 105 of M1 protein and that a single mutation from *ts51* and A/Ann Arbor/6/60 may be enhanced by combination of the two mutations. The advantage of the attenuated mutant viruses generated by introducing the mutation into the functional domains such as the RNP-binding domain and the nuclear localization signal of M1 is that the lesion is located in a single gene of the resulting virus. Since the "lesion" is related to the defect of the func-

tional domain of the virus, such a lesion can be used as a "signature" to evaluate an attenuated virus during preparation of a vaccine.

In summary, attenuated influenza viruses were generated using a reverse genetics method by replacing the matrix gene with a mutated M gene in the virulent influenza virus strain. Replication and attenuation of resulting viruses were evaluated in mice. Viruses containing the mutated M gene were attenuated in vivo and have the potential to be candidates for live attenuated influenza virus vaccines. Alternatively, this approach may be used to further attenuate present live influenza virus vaccine strains for use in naïve or immunocompromised human populations.

We thank Roland Levandowski for critically reading and improving the manuscript and Michael Klutch at CBER, FDA, for DNA sequencing.

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