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An M2 Cytoplasmic Tail Mutant as a Live Attenuated Influenza Vaccine against Pandemic (H1N1) 2009 Influenza Virus

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Abstract

The 2009 influenza pandemic brought home the importance of vaccines in infection control. Previously, we demonstrated an M2 cytoplasmic tail mutant H5N1 influenza virus could serve as a live-attenuated vaccine. Here, we adapted that strategy, generating a mutant pandemic (H1N1) 2009 virus that grew well in cell culture, but replicated less well in mice than did wild-type virus. The mutant virus elicited sterile immunity in mice, completely protecting them from challenge with a pandemic (H1N1) 2009 virus. Our results indicate that M2 cytoplasmic tail mutants are suitable for live-attenuated vaccines against pandemic viruses.

1. Introduction

In the Spring of 2009, a new pandemic influenza virus (pandemic (H1N1) 2009) spread around the world in a matter of months. Although this virus is not as virulent as highly pathogenic H5N1 avian influenza viruses, it transmits readily among people, resulting in widespread hospitalizations due to infection and more than 18,000 deaths worldwide.

To combat pandemic influenza, there are three basic strategies: 1) containment, 2) antiviral medications, and 3) vaccines. When the 2009 influenza pandemic began, some countries tried to isolate infected individuals to minimize exposure. However, this policy failed, as the number of infected individuals grew, and epidemiological tracking quickly became impossible. The second strategy, anti-viral medications, is currently the most powerful. Two types of anti-influenza virus medications are available: M2 ion channel inhibitors and neuraminidase inhibitors. Because pandemic (H1N1) 2009 influenza viruses are already resistant to the M2 ion channel inhibitors amantadine and rimantadine [1], health care

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providers have relied on neuraminidase inhibitors. In October 2009, the U.S. Food and Drug Administration (FDA) issued an emergency use authorization (EUA) for the investigational antiviral drug peramivir - a decision that reflects the limited therapeutic options currently available to combat these pandemic influenza viruses.

Immediately after the pandemic (H1N1) 2009 virus emerged, governments, researchers, and the pharmaceutical industry initiated the development of pandemic (H1N1) 2009 vaccines. However, there were difficulties making seed vaccine viruses for the manufacture of inactivated vaccines because the viruses did not grow well in eggs, resulting in low virus yields. Live-attenuated pandemic (H1N1) 2009 vaccine relies on a production procedure that differs from that of inactivated vaccines, such that these vaccines can be produced more quickly and in greater abundance [2;3]. However, only one live-attenuated influenza vaccine is approved in the United States. This has prompted researchers and industry to develop other promising live-attenuated influenza vaccines based on other mechanisms of attenuation.

The influenza A virus M2 protein is multifunctional. Its best-characterized function is its ion channel activity, which acts at an early stage of the virus life cycle between virus penetration and uncoating [4;5]. The M2 cytoplasmic tail domain also has an important role in viral assembly and morphogenesis [6–8]. Deletion of this portion of H5N1 highly pathogenic influenza virus causes attenuation in mice, demonstrating the potential of M2 tail mutants as live-attenuated vaccines against H5N1 influenza virus infections [9].

Here, we made use of this M2 tail mutations for the development of a live-attenuated pandemic (H1N1) 2009 influenza vaccine and assessed its attenuation and protective efficacy in mice.

2. Materials and Methods

2.1. Cells

293T human embryonic kidney cells, Madin-Darby canine kidney (MDCK), and M2-expressing MDCK (M2CK) [9;10] cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, in minimal essential medium containing 5% newborn calf serum, and in minimal essential medium containing 10% fetal calf serum, respectively. All cell types were maintained at 37°C in 5% CO₂.

2.2. Plasmid construction

The cDNA of influenza A/California/04/09 (H1N1; CA04) was synthesized by reverse transcription of viral RNA and amplified by PCR with oligonucleotides, as described by Hoffmann et al. [11] and then cloned between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, as previously described [12;13]. A plasmid that expresses the CA04 M segment coding the M2 protein lacking 11 amino acids was constructed by insertion of two stop codons, as previously described by Watanabe et al. [9].

2.3. Viruses

Influenza A/Wisconsin/WSLH34939/09 (H1N1; WSLH34939), a clinical isolate, was passaged twice in MDCK cells. CA04 virus and a recombinant CA04 virus that possesses the M2 protein lacking 11 amino acids of its cytoplasmic tail (CA04M2del11) were generated by transfection of plasmids into 293T cells and amplified in MDCK cells. All viruses were titrated in MDCK cells and kept at –80°C until use.

2.4. Immunization and virus challenge

Six-week-old female BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine, USA), anesthetized with isoflurane, were infected intranasally with 10^4 or 10^5 plaque-forming units (PFU) of wild-type CA04 or CA04M2del11 virus in 50 μ l PBS. Mock-infected control mice received 50 μ l PBS only. Animal body weight was monitored for 14 days after infection. On days 3 and 6 post-infection, 3 mice per group, with the exception of the PBS control group, were euthanized and their lungs and nasal turbinates were collected for virus titer determinations. On day 27 post-infection, 3 mice per group were euthanized and their trachea-lung washes, nasal washes, and sera were collected for antibody titer determinations. On day 28 post-infection, mice were intranasally challenged with 10^6 PFU of WSLH34939 and their body weights observed for the following 14 days. On days 3 and 6 post-challenge, 3 mice per group were euthanized and their lungs and nasal turbinates were collected for virus titer determinations.

2.5. Virus-specific antibody detection

Immunoglobulin G (IgG) and IgA antibody titers were measured in sera, trachea-lung washes, and nasal washes of immunized mice by use of an enzyme-linked immunosorbent assay (ELISA). In this assay, the wells were coated with purified recombinant HA protein from CA04 (Protein Sciences, Meriden, Connecticut, USA). After the HA-coated plates were incubated with test samples, bound antibody was detected with a goat anti-mouse IgA and a goat anti-mouse IgG (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Maryland, USA) conjugated to horseradish peroxidase. Neutralizing antibody titers in serum samples from immunized mice were also evaluated. The sera were treated with receptor-destroying enzyme (Denka-Seiken, Tokyo, Japan) to destroy non-specific inhibitors of influenza virus binding. After inactivation of the receptor-destroying enzyme by incubation at 56°C for 30 min, a hemagglutination inhibition assay was performed with CA04 and WSLH34939 and turkey red blood cells (Rockland Immunochemicals Inc., Gilbertsville, Pennsylvania, USA).

3. Results

3.1. Generation of the recombinant CA04M2del11 virus and its growth in cell culture

We successfully generated a recombinant CA04 virus (CA04M2del11) that possesses an 11 amino acid deletion of its M2 cytoplasmic tail from plasmids and amplified it in MDCK cells twice. The stock CA04M2del11 virus titer was 7.4×10^6 PFU/ml and the M segment sequence of the virus RNA was confirmed to possess the two stop codons that were introduced to delete the terminal 11 amino acids of the M2 cytoplasmic tail.

To assess the growth properties of the recombinant virus, CA04M2del11 and wild-type CA04 viruses were inoculated into MDCK cells and M2CK cells, and virus titers were measured every day for 4 days. Although the titers of CA04M2del11 in both MDCK and M2CK cells were slightly lower than those of CA04 at 24 hours post-infection, CA04M2del11 replicate well in both cell types and reached peak titers slightly higher than those of CA04 at 48 hours post-infection in MDCK cells and at 72 hours post-infection in M2CK cells (Fig. 1.). The recombinant virus produced comparable peak titers in MDCK cells ($10^{7.9}$ PFU/ml) and in M2CK cells ($10^{7.8}$ PFU/ml). These results demonstrate that the production of a large quantity of CA04M2del11 virus in cell culture for vaccine development would not be an issue.

3.2. Attenuation of the recombinant CA04M2del11 virus in mice

To determine the virulence of the CA04M2del11 virus, 6-week-old female BALB/c mice were inoculated with 10^4 or 10^5 PFU of the recombinant virus, wild-type CA04 virus, or PBS as a negative control, and their body weights and survival were monitored for 14 days

post-infection. As shown in Fig. 2., mice inoculated with the CA04M2del11 virus lost less body weight than those inoculated with wild-type CA04. Eight of the 15 mice infected with 10^5 PFU of the wild-type virus were euthanized on days 5 and 6 because of the severity of their symptoms (lost more than 25% of their body weight), whereas the survival rates for the other four groups were 100%. On days 3 and 6 post-infection, lung and nasal turbinate samples were assessed for virus. As shown in Table 1, CA04M2del11 virus grew less well than wild-type CA04 in both lungs and nasal turbinates regardless of dose or time-point. These data indicate that the recombinant CA04M2del11 virus is attenuated in mice.

3.3. Antibody responses of mice immunized with the CA04M2del11 virus

To test the efficacy of the CA04M2del11 virus as a vaccine, we intranasally immunized mice with 10^4 or 10^5 PFU of the recombinant virus, wild-type CA04 virus, or PBS as a negative control. Twenty-seven days later, IgG and IgA levels in sera, trachea-lung washes, and nasal washes were measured by ELISA (Fig. 3. A–C). IgA levels in both the trachea-lung and nasal washes were appreciably higher in mice immunized with the CA04M2del11 and wild-type CA04 viruses than in those treated with the PBS control. IgG levels in the trachea-lung washes were also higher in the immunized mice compared to the control mice. The IgA response was negligible in serum, regardless of the dose or strain of virus.

Next, to examine whether the antibodies detected by ELISA affected viral replication of the pandemic (H1N1) 2009 influenza virus, we tested the hemagglutination inhibition (HI) activity of the serum samples against wild-type CA04 (homologous virus) and WSLH34939 (a pandemic (H1N1) 2009 influenza virus that is more virulent than CA04 [14], which we used for our mouse challenge study [see below]) (Fig. 3. D and E). All serum samples from mice immunized with the viruses showed appreciable HI activity against both virus strains. These data suggest that the CA04M2del11 virus elicits appreciable antibody responses in immunized mice.

3.4. Protective efficacy of the CA04M2del11 virus in mice

Virus-immunized mice were intranasally challenged 28 days after vaccination with 10^6 PFU of WSLH34939. We used this strain for our challenge study because it was the most virulent pandemic (H1N1) 2009 strain available to us. Unlike mock-immunized control mice, all CA04M2del11- and wild-type CA04-immunized mice showed no weight loss (Fig. 4.) or clinical symptoms after the challenge (data not shown). By contrast, all of the control naïve mice lost a substantial amount of body weight and showed clinical symptoms, including rough fur, quietness, and loss of appetite. In fact, 3 of 8 mice had to be euthanized on days 5 and 6 post-challenge due to the severity of their symptoms (loss of >25% of their body weight). We also determined the virus titers in the lungs and nasal turbinates of the mice challenged with WSLH34939 on days 3 and 5 post-challenge (Table 2). High virus titers were recovered from all samples from the mock-immunized control group on both days. By contrast, no virus was detected in any organs from the CA04M2del11- and wild-type CA04-vaccinated groups (Table 2). Taken together, our results show that the CA04M2del11 virus can confer protective immunity to mice against pandemic (H1N1) 2009 influenza virus.

4. Discussion

Influenza pandemics are both unpredictable and unpreventable. When one occurs, it influences worldwide public health, as well as economic and social activities. Pandemic planning and mitigation strategies show that vaccination can reduce the impact of a pandemic virus and that delays in the implementation of such strategies can significantly erode their effectiveness [15]. To protect populations from pandemics, mass vaccination is one of the best approaches. Currently, most pandemic vaccination relies on the use of

inactivated vaccines. The 2009 influenza pandemic underscored the limitations of the current vaccine production process, where only 45 million doses of novel H1N1 vaccine were available by mid-October, instead of the projected 120 million in the United States. Alternative vaccines and vaccine production systems are, therefore, needed.

In this study, we successfully showed that CA04M2del11 live-attenuated vaccine virus provides protective efficacy to pandemic (H1N1) 2009 influenza virus in mice. This M2 cytoplasmic tail deletion approach to viral attenuation and immunization has already proven effective for H5N1 virus infections [9]; our study thus demonstrates the universal power of this approach for pandemic influenza vaccine production. The potential advantages of using live-attenuated vaccines against pandemic influenza include rapid induction of mucosal and systemic humoral and cell-mediated immune responses and broad cross-protection against antigenically distinct viruses.

Live-attenuated influenza vaccines are powerful tools for pandemic influenza prevention in humans since they stimulate an immune response in naïve individuals after only a single dose of vaccine. Inactivated vaccines, which are designed to boost pre-existing immunity, may not be as effective when a population is serologically negative for the pandemic strain. Live-attenuated vaccines may give better immunity to naïve populations because they replicate *in vivo* like wild-type virus, targeting similar organs and cells as the wild-type, and triggering local immune responses. However, the sole, currently approved cold-adapted, live-attenuated influenza vaccine seems not to work as well as inactivated vaccines in adults [16], possibly because it is too attenuated. Indeed, mice had to be immunized at least twice with cold-adapted virus to induce protective immunity [17;18]. By contrast, our M2del11 live-attenuated influenza vaccine candidate generated protective immunity comparable to that of the wild-type virus with only a single dose.

Reversion to its pathogenic form is always a concern for live attenuated vaccines. To reduce the possibility that the virus regains its wild-type pathogenicity, it would be ideal for live vaccine strains to have multiple attenuating mutations. However, each attenuating mutation should not attenuate the virus extensively, so that it retains immunogenicity. Since the M2 tail deletion attenuates the pathogenicity of its parent virus but still allows sufficient replication *in vitro* and *in vivo*, it is an excellent candidate to be introduced into live vaccine strains.

Because live-attenuated vaccine strains replicate less efficiently than wild-type viruses in recipients, large amounts of virus inoculum are required to stimulate immune responses. For example, the FDA-approved live-attenuated vaccine FluMist® contains $10^{6.5-7.5}$ fluorescent focus units (FFU) of live-attenuated virus in a single 0.2 ml dose. Another live-attenuated influenza vaccine candidate, an influenza virus that lacks the NS1 gene, needs a median tissue culture infective dose (TCID₅₀) of more than 10^7 to induce virus-specific antibody responses in humans [19]. Therefore, live-attenuated vaccine strains must replicate well in *in vitro* systems for adequate vaccine production. Our M2del11 live-attenuated influenza vaccine candidate grows as well in mammalian cells as the wild-type parent virus. This characteristic makes it suitable for cell-based manufacturing; especially during an impending pandemic where rapid large-scale vaccine production is needed. Moreover, we have established M2 protein-expressing cells that support the growth of M2-deficient influenza viruses [9;10] to high titers. In these cells, an influenza A virus lacking the M2 protein [10] was stably passaged more than 10 times. After 10 passages, the virus still contained the 2 stop codons that were inserted to prevent the expression of a major part of the M2 protein (Dr. Shinji Watanabe, personal communication, data not shown). These data suggest that the M2del11 live-attenuated virus could be prepared efficiently and safely in these M2-expressing cells.

In conclusion, although influenza pandemics can be devastating, seasonal influenza should not be overlooked as it continues to cause a sizeable death toll each year. The M2del11 live-attenuated influenza vaccine candidate exhibited its efficacy in two subtypes of virus, a highly pathogenic H5N1 virus [9] and a pandemic (H1N1) 2009 virus (this study). Therefore, it is reasonable to believe that M2del11 live-attenuated influenza vaccines will also be efficacious against seasonal influenza. Further studies, including the use of a ferret model and comparisons with currently approved live vaccines, are warranted to extend the current studies regarding the immunogenicity, attenuation, and lack of transmissibility of the M2del11 candidate.

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Fig. 1. Growth properties of CA04 and CA04M2del11 viruses in M2-expressing MDCK (M2CK) and MDCK cells. M2CK and MDCK cells were inoculated with the viruses at a multiplicity of infection of 0.001. Virus titers in the supernatant of M2CK (left) and MDCK (right) cells at various time points post-infection were determined by using MDCK cells. Data are reported as the average titer $\pm$ standard deviations ($n = 3$).

Fig. 2.

Attenuation of CA04M2del11 virus infection in mice. Mice were infected with 10^5 or 10^4 PFU of recombinant CA04M2del11 or wild-type CA04 virus, and their body weights monitored for 14 days. Data are reported as the mean change in body weight $\pm$ standard deviations ($n = 11$). Four mice infected with 10^5 PFU of CA04 virus were euthanized on days 5 and 6 post-infection (i.e., a total of 8 of the 15 infected mice) because of the severity of their symptoms (loss of $> 25\%$ of their body weight).

Fig. 3.

Virus-specific serum and mucosal antibody responses in mice immunized with the CA04M2del11 virus. Samples from each group were obtained 27 days post-immunization. IgG and IgA levels in sera (A), trachea-lung washes (B), and nasal washes (C) from pooled samples ($n = 3$) were detected by ELISA. Values are expressed as absorbance of samples diluted 1:1000 (sera), 1:100 (trachea-lung washes), and 1:10 (nasal washes). Serum samples were treated with receptor-destroying enzyme and hemagglutination inhibition activity against CA04 and WSLH34939 was determined (D and E). Dots represent individual values.

Fig. 4. Protection of mice immunized with the CA04M2del11 virus from WSLH34939 challenge. Mice were immunized with 10⁵ or 10⁴ PFU of recombinant CA04M2del11 or wild-type CA04 virus, or PBS as a negative control. They were then challenged with 10⁶ PFU of WSLH34939 on day 28 post-immunization and their body weights monitored for 14 days. Data are reported as the mean change in body weight $\pm$ standard deviations (n = 8).

TABLE 1

Replication of CA04M2del11 in mouse respiratory organs.

Virus	Infection dose (PFU/mouse)	Virus titer (mean log ₁₀ PFU/g ± SD) ^a				Survival (survived/total)
		Day 3 post-inoculation		Day 6 post-inoculation		
		Lungs	Nasal turbinates	Lungs	Nasal turbinates	
CA04M2del11	10 ⁵	6.8 ± 0.2	5.6 ± 0.2 ^b	5.5 ± 0.3	6.9 ± 0.2 ^c	15/15
	10 ⁴	6.6 ± 0.1 ^d	4.6 ± 1.1 ^e	5.7 ± 1.3	6.9 ± 0.5	15/15
CA04	10 ⁵	7.1 ± 0.8	6.7 ± 0.3 ^b	6.1 ± 0.1	7.8 ± 0.2 ^c	7/15
	10 ⁴	7.3 ± 0.1 ^d	6.7 ± 0.2 ^e	6.5 ± 0.2	7.5 ± 0.2	15/15

^a Mice were infected with 100,000 or 10,000 PFU of CA04M2del11 or wild-type CA04 virus. Organ samples were taken from three mice per group at the indicated days post-inoculation. Virus titers were determined in MDCK cells.

^{b,c,d,e} Virus titer differences between two groups were tested for statistical significance by use of the Student's *t* test and resulted in *p* < 0.02.

TABLE 2
Replication of WSLH34939 in immunized mouse respiratory organs after challenge.

Virus	Infection dose (PFU/mouse)	Virus titer (mean log ₁₀ PFU/g ± SD) ^a				Survival (survived/total)
		Day 3 post-challenge		Day 6 post-challenge		
		Lungs	Nasal turbinates	Lungs	Nasal turbinates	
CA04M2del11	10 ⁵	ND ^b	ND	ND	ND	8/8
	10 ⁴	ND	ND	ND	ND	8/8
CA04	10 ⁵	ND	ND	ND	ND	8/8
	10 ⁴	ND	ND	ND	ND	8/8
PBS		7.3 ± 0.3	5.9 ± 0.3	7.8 ± 0.3	7.3 ± 0.2	5/8

^aThree mice from each group were euthanized on days 3 and 6 post-challenge for virus titration.

^bND, not detected in all three mice.