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Influenza A Virus with Defective M2 Ion Channel Activity as a Live Vaccine

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We propose a rational approach to the design of live virus vaccines against influenza infection by alteration of the influenza A virus M2 protein, which is responsible for ion channel activity. Previously we demonstrated that a mutant A/WSN/33 (H1N1) influenza virus with defective M2 ion channel activity did not show appreciable growth defects in cell culture, although its growth was attenuated in mice (T. Watanabe, S. Watanabe, H. Ito, H. Kida, and Y. Kawaoka, 2001, *J. Virol.* 75, 5656–5662). Here, we show that this M2 ion channel defective mutant virus, the M2del29-31, protected mice against challenge with lethal doses of influenza virus, indicating the potential of incorporating this M2 alteration in a live influenza vaccine as one of the attenuating mutations. © 2002 Elsevier Science (USA)

Keywords: influenza A virus; M2 ion channel; live-attenuated vaccine.

INTRODUCTION

Influenza A virus is a highly infectious respiratory pathogen of birds and mammals, including humans (Wright and Webster, 2001). It often produces significant morbidity and mortality in humans and domestic animals, resulting in global economic burden. Current methods of immunization against influenza A virus include parenteral administration of inactivated influenza virus vaccines, which generally show 70–90% efficacy in reducing the incidence of clinical illness in healthy subjects, if the antigenicities of the circulating strains of virus match that of the vaccine strain (Cox and Subbarao, 1999). In some instances, however, immunization with inactivated vaccines is associated with very low protection rates or short protection periods, because mucosal immunity and cytotoxic T cell response are limited. It may also be associated with adverse reactions such as pain, tenderness, myalgia, and, rarely, anaphylactic reaction to chicken egg proteins introduced during vaccine production in embryonated eggs.

Another promising approach to vaccination is the use of cold-adapted live-attenuated influenza viruses (Maassab and Bryant, 1999). These vaccines have shown considerable promise in ongoing clinical trials, especially in young children, who are poor responders to inactivated vaccines due to the lack of immune memory of influenza virus (Belshe *et al.,* 1998). However, preliminary results indicate that live virus vaccines may not be significantly more effective than the best inactivated vaccine, especially in adults (Edwards *et al.,* 1994), leaving room for further improvement.

We propose an alternative approach to the design of live virus vaccines, one that relies on alteration of the influenza A virus M2 protein, whose transmembrane (TM) domain is responsible for the protein's ion channel activity (Duff and Ashley, 1992; Holsinger *et al.,* 1994; Pinto *et al.,* 1992; Sugrue *et al.,* 1990). M2 ion channel activity functions at an early stage of the viral life cycle between the steps of virus penetration and uncoating (Helenius, 1992). Specific changes in the M2 protein TM domain alter the kinetics and ion selectivity of the channel. An M2 mutant protein with deletions of residues from positions 29 to 31 in the TM domain ameliorated functional ion channel activity, as assayed by a two-electrode voltage-clamp procedure (Holsinger *et al.,* 1994). We previously showed that the M2del29-31 virus, which contains the A/Udorn/72 (H3N2) M gene carrying this TM mutation and the other genes from A/WSN/33 virus, did not show appreciable growth defects in cell culture, although its growth was attenuated in mice (Watanabe *et al.,* 2001). In mice infected with wild-type virus, nearly 10^4 plaque-forming units (PFU) of virus were present in the nasal turbinates and nearly 10^7 PFU of virus were in the lungs, while no virus was recovered from the nasal turbinates of mice infected with this M2 mutant and 1 log less virus was found in the lungs. Here, we describe experiments in which this M2 ion channel-defective mu-

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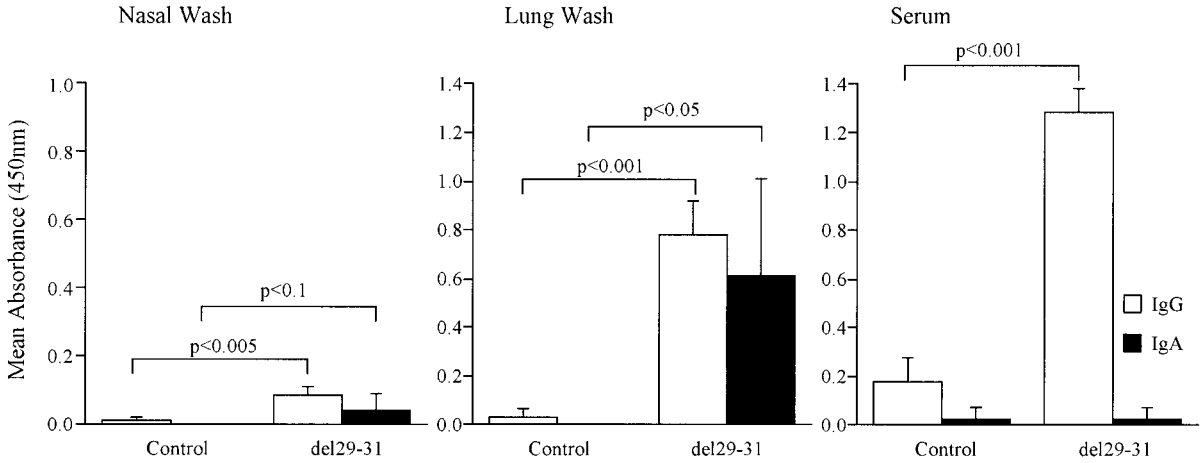

FIG. 1. Virus-specific antibodies in samples from vaccinated mice. Sera, trachea-lung washes, and nasal washes from four mice from each group were obtained before virus challenge. IgG and IgA in the samples of individual mice were detected by ELISA, as described under Materials and Methods. Results are expressed as the mean absorbances (±SD) of undiluted samples (trachea-lung and nasal washes) or 1:100 diluted samples (sera). Differences between responses to control and the M2del29-31 virus were tested for statistical significance by Student's *t* test.

tant protected mice against challenge with lethal doses of influenza virus.

RESULTS

Attenuation of M2del29-31 virus in mice

Previously, we demonstrated that replication of M2del29-31 virus in the lungs was more than 10-fold lower than that of wild-type WSN-UdM virus and the former was not recovered in nasal turbinates from any of the infected mice with 2×10^3 PFU of WSN virus intranasally (Watanabe *et al.,* 2001). To further characterize the attenuated phenotype of the M2del29-31 virus in mice, we compared LD_{50} values of the M2del29-31 and its parent WSN-UdM viruses and found that the LD_{50} of the former was 6.3 log PFU, while that of the latter was 5.8.

Antibody responses of mice immunized with the M2del29-31 virus

To test the efficacy of M2del29-31 virus as a vaccine, we intranasally inoculated BALB/c mice with 2×10^3 PFU of the virus. Body weight was monitored daily for 2 weeks, and mice infected with this dose of M2del29-31 virus did not lose weight (data not shown). Two weeks after infection, four mice were sacrificed to obtain sera, trachea-lung washes, and nasal washes. Immunoglobulin G (IgG) and IgA antibodies were measured in these samples with an enzyme-linked immunosorbent assay (ELISA) (Fig. 1). Both IgG and IgA values in nasal and trachea-lung washes from mice immunized with the M2del29-31 virus were higher than in control mice. The IgA response was negligible in serum, but IgG production was clearly higher in mice inoculated with M2del29-31. Neither Ig response was appreciable in control mice. Thus, the M2del29-31 virus lacking M2 ion channel activity efficiently induced antibody responses in a murine model.

Protective efficacy of the M2del29-31 virus

The efficacy of vaccination with the M2del29-31 virus was assessed at 2 weeks, 1 month, or 3 months postimmunization by intranasal challenge under anesthesia, with 100 LD_{50} doses of wild-type WSN virus. Virus titers were determined by titration on Madin–Darby canine kidney (MDCK) cells, using lung samples collected from four mice on Day 3 postchallenge. The remaining animals were observed for clinical signs and symptoms of disease for 14 days after challenge. In contrast to the fate of control mice, those immunized with the M2del29-31 virus were protected against lethal challenge with WSN virus (Table 1). All mice in the M2del29-31 virus group survived, even when challenged 3 months after vaccination. Moreover, their body weights were not appreciably affected by virus challenge, in contrast to control groups, whose weights decreased rapidly postchallenge (Fig. 2). The lung titers from immunized mice after challenge with a 100 LD_{50} dose of wild-type WSN virus were below the limit of detection by plaque assay (less than 10^2 PFU/g), in contrast to control mice, which had over 10^7 PFU in lung tissue after the same WSN challenge (Table 1). Thus, the M2del29-31 virus effectively protected mice against lethal influenza virus infection.

DISCUSSION

Promising live virus vaccine candidates must satisfy the following criteria: growth to high titers in a suitable

TABLE 1

Protection against Virus Challenge in Mice Immunized with M2del29-31 Virus[a]

Testing interval postimmunization	No. of survivors/ No. tested[b]	Virus titer in lungs [$\log_{10}$(PFU/g)]
2 Weeks		
Control[c]	0/4	7.5 ± 0.1
M2del29-31	4/4	<2[d]
1 Month		
Control	0/4	7.4 ± 0.1
M2del29-31	4/4	<2[d]
3 Months		
Control	0/4	7.2 ± 0.1
M2del29-31	4/4	<2[d]

[a] Mice were challenged intranasally, under anesthesia, with 100 LD_{50} doses of wild-type WSN virus at 2 weeks, 1 month, or 3 months postvaccination. Virus titers were determined in lung tissue at 3 days postchallenge.

[b] Mice were monitored for 14 days after challenge.

[c] Control mice were mock-vaccinated with phosphate-buffered saline.

[d] No virus was detected in the lungs from any of the infected mice (less than 10^2 PFU/g). Significantly different from the control ($P < 0.001$) by Student's *t* test.

preparative medium, attenuation in the host, genetic stability, and consistent immunogenicity. The M2del29-31 virus, despite having an apparent defective M2 ion channel activity, replicates efficiently in cell culture. It also shows genetic stability and attenuation in mice (Watanabe *et al.,* 2001) and was clearly immunogenic in our animal model. Unlike inactivated virus, which did not induce protective immunity in mice when intranasally inoculated three times with 16 hemagglutinating units of virus (equivalent to 10^8 particles; Desselberger, 1975) (Watanabe *et al.,* 2002), the M2del29-31 virus provided complete protection to animals (Table 1). A similar level of protection has also been shown with virus containing alterations in NS1 protein (Talon *et al.,* 2000). Thus, the M2del29-31 virus has considerable potential for use in a live vaccine preparation.

We previously demonstrated that the chimeric mutant M2HATM virus, in which the M2 TM domain was replaced with that from the HA of WSN virus, was highly attenuated in mice (Watanabe *et al.,* 2001), although it replicated reasonably well in cell culture. Use of this chimeric virus as a live vaccine would greatly reduce the likelihood of the emergence of revertant viruses. However, when tested as a vaccine in mice, the M2HATM virus failed to protect any animals from lethal challenge with WSN virus and failed to induce an immune response in immunized mice (data not shown). These results indicate that the M2HATM virus cannot replicate in mice, and hence, that the M2 TM domain is a vital component of the influenza A virus life cycle *in vivo*. The lack of replication, and thus protection, in mice of the M2HATM virus underscores the necessity of introducing attenuating, not abrogating, mutations in the vaccine strain. Indeed, for attenuated live vaccines to be effective, they must replicate to a sufficient level to induce an effective immune response, without causing severe disease symptoms. The LD_{50} of the M2del29-31 virus was less than 1 log greater than that of the wild-type strain, permitting sufficient replication for induction of protective immunity, without causing significant disease symptoms, e.g., loss of body weight.

Recently, we (Neumann *et al.,* 1999) and others (Fodor *et al.,* 1999) established a reverse genetics system for generating infectious influenza virus entirely from cDNAs. The ability to manipulate the viral genome without technical limitations has profound implications for the study of viral life cycles and their regulation, the function of viral proteins, and the molecular mechanisms of viral

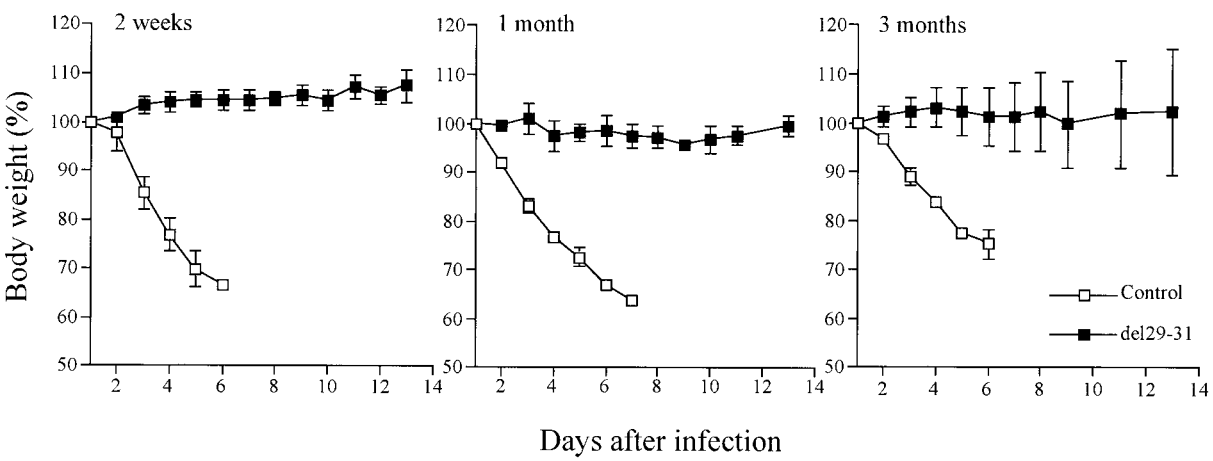

FIG. 2. Body weights of immunized mice after challenge with wild-type virus. Control mice and mice immunized with the M2del29-31 virus lacking M2 ion channel activity were challenged with 100 LD_{50} doses at 2 weeks, 1 month, or 3 months postvaccination. Values are mean weights (±SD).

pathogenicity—all of which are significant factors in the control of influenza infection. Reverse genetics enables one to modify cold-adapted vaccines or to produce different "master" influenza vaccine strains. Although they have proven to be extremely stable in clinical trials (Cha *et al.,* 2000), cold-adapted vaccine strains possess a limited number of amino acid changes, raising concern over the emergence of virulent revertants (Herlocher *et al.,* 1993). Ideally, live-attenuated virus vaccines would have multiple attenuating mutations in the genes that encode internal proteins. NS1 mutant viruses are highly attenuated in mice because they lack interferon antagonist activity while retaining the ability to induce protective immunity against influenza virus challenge (Talon *et al.,* 2000). Hence, by combining attenuating mutations such as those in viruses reported by Talon *et al.* (2000) and ours, one could produce an improved master influenza virus as a first step in the production of safe live influenza vaccines. Continued progress in understanding the functions of viral proteins should allow the introduction of multiple mutations in live vaccine strains, in addition to those in the NS and M genes, thereby reducing the likelihood of revertant virus generation.

MATERIALS AND METHODS

Cells and viruses

MDCK cells were maintained in minimal essential medium (MEM) containing 5% newborn calf serum at 37°C in 5% CO_2. WSN-UdM and M2del29-31 viruses, which contain the M gene from the A/Udorn/72 (H3N2) virus and the other genes from A/WSN/33 virus, were propagated in MDCK cells (Watanabe *et al.,* 2001).

Experimental infection

Four-week-old female BALB/c mice, anesthetized with sevoflurane, were infected with approximately 2×10^6 PFU (in 50 μl) of the WSN-UdM (wild-type) and M2del29–31 viruses. Body weights of mice infected with these viruses were monitored for 14 days after challenge.

Immunization and protection tests

BALB/c mice (4-week-old female) were intranasally immunized with 2×10^3 PFU (in 50 μl) of the M2del29-31 virus. On the second week, four mice were sacrificed to obtain sera, trachea-lung washes, and nasal washes. Two weeks, one month, or three months after the last vaccination, immunized mice were challenged intranasally, under anesthesia, with 100 LD_{50} doses of the wild-type WSN virus. For determination of virus titers in lung, tissues were harvested at Day 3 and were homogenized and titrated on MDCK cells. The remaining animals were observed for clinical signs and symptoms of infection for 14 days after challenge.

Detection of virus-specific antibody

Serum samples were examined for antibody by an ELISA (Kida *et al.,* 1982). In this assay, the wells were coated with purified WSN virus after treatment with 0.05 M Tris–HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6 M KCl at room temperature and diluted in PBS. After incubation of virus-coated plates with test serum samples, bound antibody was detected with rabbit anti-mouse IgA (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) and goat anti-mouse IgG (Boehringer Mannheim, Germany) conjugated to horseradish peroxidase.

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REFERENCES

Belshe, R. B., Mendelman, P. M., Treanor, J., King, J., Gruber, W. C., Piedra, P., Bernstein, D. I., Hayden, F. G., Kotloff, K., Zangwill, K., Iacuzio, D., and Wolff, M. (1998). The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. *N. Engl. J. Med.* **338,** 1405–1412.

Cha, T. A., Kao, K., Zhao, J., Fast, P. E., Mendelman, P. M., and Arvin, A. (2000). Genotypic stability of cold-adapted influenza virus vaccine in an efficacy clinical trial. *J. Clin. Microbiol.* **38,** 839–845.

Cox, N. J., and Subbarao, K. (1999). Influenza. *Lancet* **354,** 1277–1282.

Desselberger, U. (1975). Relation of virus particles counts to the hemagglutininating activity of influenza virus suspensions measured by the HA pattern test and by use of the photometric HCU method. *Arch. Virol.* **49**, 365–372.

Duff, K. C., and Ashley, R. H. (1992). The transmembrane domain of influenza A M2 protein forms amantadine-sensitive proton channels in planar lipid bilayers. *Virology* **190,** 485–489.

Edwards, K. M., Dupont, W. D., Westrich, M. K., Plummer, W. D., Jr., Palmer, P. S., and Wright, P. F. (1994). A randomized controlled trial of cold-adapted and inactivated vaccines for the prevention of influenza A disease. *J. Infect. Dis.* **169,** 68–76.

Fodor, E., Devenish, L., Engelhardt, O. G., Palese, P., Brownlee, G. G., and Garcia-Sastre, A. (1999). Rescue of influenza A virus from recombinant DNA. *J. Virol.* **73,** 704–712.

Helenius, A. (1992). Unpacking the incoming influenza virus. *Cell* **69,** 577–578.

Herlocher, M. L., Maassab, H. F., and Webster, R. G. (1993). Molecular and biological changes in the cold-adapted "master strain" A/AA/6/60 (H2N2) influenza virus. *Proc. Natl. Acad. Sci. USA* **90,** 6032–6036.

Holsinger, L. J., Nichani, D., Pinto, L. H., and Lamb, R. A. (1994). Influenza A virus M2 ion channel protein: A structure-function analysis. *J. Virol.* **68,** 1551–1563.

Kida, H., Brown, L. E., and Webster, R. G. (1982). Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. *Virology* **122,** 38–47.

Maassab, H. F., and Bryant, M. L. (1999). The development of live attenuated cold-adapted influenza virus vaccine for humans. *Rev. Med. Virol.* **9,** 237–244.

Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D. R., Donis, R., Hoffmann, E., Hobom, G., and Kawaoka, Y. (1999). Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* **96,** 9345–9350.

Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992). Influenza A virus M2 protein has ion channel activity. *Cell* **69,** 517–528.

Sugrue, R. J., Bahadur, G., Zambom, M. C., Hall-Smith, M., Douglas, A. R., and Hay, A. J. (1990). Specific structure alteration of the influenza haemagglutinin by amantadine. *EMBO J.* **9,** 3469–3476.

Talon, J., Salvatore, M., O'Neill, R. E., Nakaya, Y., Zheng, H., Muster, T., Garcia-Sastre, A., and Palese, P. (2000). Influenza A and B viruses expressing altered NS1 proteins: A vaccine approach. *Proc. Natl. Acad. Sci. USA* **97,** 4309–4314.

Watanabe, T., Watanabe, S., Ito, H., Kida, H., and Kawaoka, Y. (2001). Influenza A virus can undergo multiple cycles of replication without M2 ion channel activity. *J. Virol.* **75,** 5656–5662.

Watanabe, T., Watanabe, S., Gabriele, N., Kida, H., and Kawaoka, Y. (2002). Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. *J. Virol.* **76,** 767–773.

Wright, P. W., and Webster, R. G. (2001). Orthomyxoviruses. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Lippincott-Raven Publishers, Philadelphia.