Assessment of the Efficacy of Commercially Available and Candidate Vaccines against a Pandemic H1N1 2009 Virus

Gary P. Kobinger,1,2 Isabelle Meunier,4 Ami Patel,1,2 Stéphane Pillet,4 Jason Gren,1 Shane Stebner,1 Anders Leung,1 James L. Neufeld,1 Darwyn Kobasa,1,2 and Veronika von Messling1

1Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, 2Department of Medical Microbiology, University of Manitoba, and 3National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, Manitoba, and 4Institut National de la Recherche Scientifique–Institut Armand-Frappier, University of Quebec, Laval, Quebec, Canada

Background. The emergence and global spread of the pandemic H1N1 2009 influenza virus have raised questions regarding the protective effect of available seasonal vaccines and the efficacy of a newly produced matched vaccine.

Methods. Ferrets were immunized with the 2008–2009 formulations of commercially available live attenuated (FluMist; MedImmune) or split-inactivated (Fluviral; GlaxoSmithKline) vaccines, a commercial swine vaccine (FluSure; Pfizer), or a laboratory-produced matched inactivated whole-virus vaccine (A/Mexico/InDRE4487/2009). Adaptive immune responses were monitored, and the animals were challenged with A/Mexico/InDRE4487/2009 after 5 weeks.

Results. Only animals that received the swine or matched vaccines developed detectable hemagglutination-inhibiting antibodies against the challenge virus, whereas a T cell response was exclusively detected in animals vaccinated with FluMist. After challenge, all animals had high levels of virus replication in the upper respiratory tract. However, preexisting anti–pandemic H1N1 2009 antibodies resulted in reduced clinical signs and improved survival. Surprisingly, FluMist was associated with a slight increase in mortality and greater lung damage, which correlated with early up-regulation of interleukin-10.

Conclusions. The present study demonstrates that a single dose of matched inactivated vaccine confers partial protection against a pandemic H1N1 2009 virus, and it suggests that a higher dose or prime-boost regimen may be required. The consequences of mismatched immunity to influenza merit further investigation.

Since its emergence in Mexico early in 2009, the pandemic H1N1 2009 influenza virus has resulted in >414,000 confirmed cases and ~5000 deaths worldwide, and the real numbers are likely to be considerably higher, because countries are now only required to confirm severe cases by laboratory diagnosis [1].

Even though most patients experience a disease similar to seasonal influenza, reports of severe cases are increasing [2–4]. Studies in different animal models reveal more efficient spread of the pandemic H1N1 2009 viruses to the lower respiratory tract and demonstrate increased virulence of some field isolates, suggesting that the genetic makeup of the respective strain may significantly contribute toward disease outcome [5, 6]. This observation, in combination with reports of more frequent incidents of severe disease in the Southern Hemisphere [7], also increases concerns about the fall, which is typically the period of the most severe influenza activity in the Northern Hemisphere [8, 9].

The rapid spread of the virus in countries with high seasonal influenza vaccine coverage suggests that there is little to no cross-protection conferred by these vaccines [10]. At the same time, the presence of neutralizing antibodies and the generally milder course of dis-
ease observed in individuals >60 years of age are indicative of a protective effect of prior infection with antigenetically related viruses [6].

As this pandemic unfolds, and especially in light of the emerging resistance to available antivirals [11], assessment of the safety and efficacy of available seasonal vaccines as well as matched candidate vaccines is becoming increasingly urgent. The current study evaluates in ferrets—the preferred preclinical animal model for influenza vaccine testing—3 commercially available influenza vaccines from the 2008–2009 season and 1 fully matched laboratory-produced inactivated whole pandemic H1N1 2009 virus vaccine. Immune responses were monitored, and the animals were challenged 5 weeks after vaccination with a pandemic H1N1 2009 influenza isolate that exhibits moderate to high virulence in ferrets. Viral loads, morbidity, mortality, and postchallenge immune responses were documented for 2 weeks.

MATERIALS AND METHODS

Immunization and challenge. Groups of five 16- to 20-week-old ferrets without antibodies against circulating influenza strains were immunized with one of the 2008 seasonal inactivated split vaccines (Fluviral; GlaxoSmithKline) or the cold-adapted live attenuated vaccine (FluMist; MedImmune), a swine influenza vaccine (FluSure; Pfizer), or a matched laboratory-produced inactivated vaccine (pH1N1Iinact). The latter vaccine consisted of a Madin-Darby canine kidney (MDCK) cell–produced whole-virus preparation of A/Mexico/ InDRE4487/2009 (MX10; H1N1) that was isolated during the ongoing H1N1 influenza outbreak, purified by ultracentrifugation, subsequently inactivated by addition of formalin to a final concentration of 0.1%, and incubated for 3 days at 4°C (Fisher Scientific). The animals received the recommended dose of the respective commercial vaccines or a dose containing 15 μg of hemagglutinin (HA) of the experimental vaccine. With the exception of FluMist, which was inoculated intranasally, all vaccines were injected in the gluteal muscle at the recommended dose for humans or pigs, respectively. Five weeks later, the animals were challenged intranasally with 10³ 50% tissue culture infectious doses (TCID₅₀) of MX10. Clinical signs, body temperature, and weight were assessed daily, and animals were euthanized based on clinical evaluation or at the end of the study on day 16.

Virus quantification and pathology. Nasal washes were collected on days 1, 3, 6, 9, and 16 after challenge, and virus titers were quantified by limiting dilution. In brief, 10-fold serial dilutions were incubated on MDCK cells with 6 replicates per dilution. At 72–96 h after infection, the plates were scored for cytopathic effect, and the TCID₅₀ virus titers were calculated using the method of Reed and Muench [12]. RNA was isolated, and viral copy numbers were quantified using real-time reverse-transcription polymerase chain reaction (RT-PCR). Tissues preserved in RNalater were homogenized using a bead mill homogenizer for extraction of total RNA. RNA was isolated from nasal washes and swabs, by use of the QIAamp Viral RNA Mini Kit (Qiagen), and from tissues, by use of the RNeasy Mini Kit (Qiagen). The H1N1 virus was detected by quantitative real-time RT-PCR performed using the LightCycler 480 RNA Master Hybridization Probes (Roche) assay targeting the HA gene (nucleotide position 714–815; GenBank accession number GQ160606). Reaction conditions were as follows: at 63°C for 3 min; at 95°C for 30 s; and 45 cycles at 95°C for 15 s and at 60°C for 30 s with the use of a Lightcycler 480 (Roche). The low detection limit for this H1N1 assay is 0.1 pfu/mL. The primer sequences are as follows: HAF, GGATCAAAGGGAATGAATATT; HAR, AATGCAAAAGGATCAAGAAGAAGGAATGAACTATT; and HAP, CGGGAGACAAAATACCCAGCAGCAAC.

After euthanasia, necropsy was performed for all animals, and photographs of their lungs were taken before the lungs were harvested for histopathologic analysis. Lungs were inflated by slow injection of ~5 mL of phosphate-buffered saline (PBS; Invitrogen) in the trachea, and formalin-fixed and paraffin-embedded tissue sections were stained with hematoxylin-eosin.

Immune response assessment. Serum samples were collected on days 3, 7, 10, 14, and 21 after vaccination and were analyzed for the presence of hemagglutination-inhibiting (HAI) antibodies against MX10 and the seasonal H1N1 strain A/Brisbane/59/2007. HAI antibody titers are expressed as the reciprocal of the highest serum dilution that inhibits hemagglutination of turkey red blood cells. On day 10 after vaccination, heparinized blood was collected for proliferation assays. In brief, peripheral blood mononuclear cells were isolated by ficoll-hypaque (GE Healthcare) gradient purification and cultivated in the presence of overlapping peptide pools covering the nucleocapsid (NP), neuraminidase (NA), and HA proteins of the related H1N1 strain A/Brevig Mission/1918. The proliferation response was measured by adding 5-bromo-2-deoxyuridine (BrdU) to the peptide-exposed peripheral blood mononuclear cells after 72 h. The next day, cells were fixed, and BrdU incorporation was quantified by immunostaining performed using a chemoluminescent substrate (Roche). The proliferation index is expressed as the ratio of BrdU incorporation measured for the respective influenza peptide pool and for an Ebola virus peptide as negative control. Messenger RNA profiles of cytokines, including interferon (IFN)–α, IFN-γ, interleukin (IL)–6, and IL-10, were generated from nasal wash RNAs isolated on days 1, 3, 6, and 9 after infection and from RNA isolated from the right and left lung, respectively, of animals euthanized on day 9. The assays were performed using the primers and method outlined elsewhere [13].
RESULTS

Failure of seasonal vaccines to elicit HAI antibodies recognizing the pandemic H1N1 2009 virus. HAI antibody titer kinetics were monitored for 21 days after immunization, because titers of >40 reciprocal dilutions remain the reference standard that is predictive of protective immunity elicited by influenza vaccine candidates [14]. HAI antibody titers against the pandemic H1N1 2009 MX10 isolate were detected at day 7 in ferrets receiving the laboratory-produced matched vaccine pH1N1inact or the swine influenza vaccine FluSure, reaching titers >40 on day 7 or 10, respectively. FluSure or pH1N1inact did not generate detectable HAI antibody titers against the seasonal H1N1 strain A/Brisbane/59/2007 included in conventional seasonal influenza vaccines, such as Fluviral or FluMist. In contrast, between day 7 and day 10, both seasonal vaccines elicited HAI antibody titers against A/Brisbane/59/2007 that were >40, whereas no cross-reactive response to MX10 was detected before challenge (Figure 1B). None of the vaccines elicited an IFN-γ enzyme-linked immunospot response upon stimulation with overlapping peptides covering the NP, NA, and HA proteins of H1N1 strain A/Brevig Mission/1918. These proteins share 94%, 87%, and 86% amino acid identity with the respective MX10 proteins, which may have contributed to the weak response observed. However, increased proliferation activity in response to NP peptide pools was detected in animals immunized with FluMist, indicating a cross-reactive T cell response (Figure 1C).

Correlation of presence of HAI antibodies with milder disease and improved survival. Upon intranasal challenge with MX10, all vaccinated animals displayed a 1-day delay in the onset of fever, and they then followed a course comparable to that of nonvaccinated controls (Figure 2A). Clinically, animals immunized with the swine vaccine FluSure demonstrated more complete protection with mild and transient signs of disease, less weight loss in the first week than in the other groups, and 100% survival. The matched pH1N1inact vaccine also resulted in reduced weight loss, clinical signs of disease, and improvement of the survival rate from 50% to 80%, compared with observations in naïve controls (Figure 2B–D). In contrast, there were no statistically significant differences in recorded clinical signs of disease, weight loss, or survival rate between the animals given Fluviral and the control groups, over the course of the experiment after challenge (P > .05). The group of ferrets vaccinated with FluMist showed a slight improvement in average body weight at days 5 and 6 after challenge. However, weight loss increased from day 6 to day 9, at which point clinical signs of disease, including nasal seromucous exudates, shallow and labored breathing, and reduced activity forced euthanasia for 4 of the 5 animals given FluMist, resulting in a small increase in the mortality rate, compared with that noted for the un-
vaccinated control group. This increase in the mortality rate associated with mismatched FluMist immunization was also observed in a second experiment with 4 animals, although the increase was not statistically significant (data not shown).

At the respective times of euthanasia, gross pathological evaluation of lungs demonstrated minimal lesions in all 5 animals vaccinated with FluSure, including 1 animal euthanized on day 9, without reaching experimental end points to obtain, on a timely basis, tissue samples matched to those obtained from the other groups (Figure 3). Animals vaccinated with pH1N1inact showed a slight improvement, with smaller lesions noted in 3 of the 5 ferrets. Three of 4 control animals had severe lesions with hepatization, hemorrhages, and widespread alveolitis and bronchiolitis, which were comparable to lesions observed in 4/5 or 5/5 ferrets vaccinated with Fluviral or FluMist, respectively.

**Association between protection and lower infectious viral loads early after infection.** All groups reached nasal infectious titers of $\sim10^4$ TCID$_{50}$ at day 1 after challenge, with the exception of the animals immunized with the swine vaccine FluSure, which had a 10-fold lower titer (Figure 4A). The group vaccinated with FluMist maintained relatively high levels of virus replication through day 3, whereas the other groups experienced titer decreases of $\geq 20$-fold. With the exception of one animal in the control group, no infectious viral particles were detectable by titration in the nasal washes of animals on day 6 or later, although viral RNA could be detected by real-time RT-PCR until the end of the experiment (Figure 4B).

**Correlation between moderate levels of IL-6 in nasal wash cells at day 9 after infection and partial protection.** Animals immunized with pH1N1inact or FluSure experienced the lowest IFN-α levels and up-regulation of IFN-γ during the early stage of the infection. Moderately elevated levels of IL-6 were detected later in the course of disease—at day 9 exclusively in these 2 groups, which showed evidence of protection (Figure 5). In contrast, the group vaccinated with FluMist exhibited the highest average of mRNA transcripts for IFN-α, up to day 6, and for IFN-γ, at day 3, possibly reflecting a better cellular response. Of interest, at day 3, levels of IL-10 transcripts were significantly higher in the upper airway of animals vaccinated with FluMist, and at day 9 after infection, they were slightly increased in the lower airway, compared with observations in control animals and animals vaccinated with pH1N1inact or FluSure (Figure 5). No differences in tumor necrosis factor-α and IL-8 levels were observed between the groups (data not shown).
DISCUSSION

The availability of an efficient vaccine is essential to alleviate the effect of the ongoing influenza pandemic. A possible intervention strategy to mitigate the 2009 fall influenza season in the Northern Hemisphere was to initially perform mass immunization with the seasonal vaccine, followed by mass immunization with the fully matched pandemic H1N1 2009 vaccine as soon as would become available. To direct a concerted public health response to control the spread of the virus, the efficacy of a newly produced matched inactivated vaccine, as well as that of already available inactivated and live attenuated vaccines, has to be assessed. Toward this end, we compared the antibody and cellular responses elicited by 2 seasonal vaccines (Fluviral and FluMist), the commercial swine vaccine FluSure, and a laboratory-produced matched inactivated whole-virus preparation. We found that only the swine and matched vaccines resulted in production of HAI antibodies against the pandemic H1N1 2009 virus, whereas only FluMist triggered a cross-reactive cellular response. Intranasal challenge with the virulent Mexican isolate MX10, similar to MX/4482, which also leads to a 50% mortality rate among naive animals [6], revealed that none of the vaccines was able to confer complete protection after only one immunization. However, FluSure was associated with the best reduction in morbidity and complete protection from mortality, whereas the matched inactivated vaccine resulted in moderate clinical improvement and reduced mortality. As was expected from undetectable HAI antibody titers, animals vaccinated with Fluviral or FluMist did not experience a beneficial effect, compared with unvaccinated control animals.

The partial protection observed in animals vaccinated with one dose of the matched inactivated vaccine, despite the detection of an HAI antibody response within the protective range, indicates that protection from aggressive isolates may require more than a single immunization, which would put an additional strain on vaccine availability. The use of a more virulent challenge strain enables assessment of vaccine efficacy in a worst-case scenario. However, the disease severity associated with currently circulating pandemic H1N1 2009 strains in most patients is more similar to that associated with seasonal influenza [3]. It is thus possible that a single 15-µg dose of a matched inactivated vaccine will be sufficient to confer protection against most pandemic H1N1 2009 strains, especially in individuals with some levels of cross-protection due to previous influenza infection. The efficiency of a commercially available swine vaccine indicates that this product would be adequate to protect animals, including pig herds, which could minimize interspecies transmission and maybe limit evolution of the virus. The FluSure vaccine consists of an inactivated H1N1 and H3N2 type A field isolate formulated with Amphigen (Pfizer) as an adjuvant [15], indicating that the use of this and other adjuvants merits a more in-depth evaluation in the context of the development of improved human influenza vaccines.

A curious observation is the more severe cases of disease and the higher mortality rate noted for animals vaccinated with FluMist, which correlated with slightly more infectious virus in the nasal washes of this group at day 3. This correlation...
between the infectious viral load in the upper respiratory tract and the clinical outcome was in fact observed for all groups in the present study, confirming previous reports that the infectious viral load in the airway is predictive of levels of protection in vaccinated ferrets after challenge with respiratory viruses, including severe acute respiratory syndrome-associated coronavirus and influenza [16, 17]. On the other hand, data from this study also indicate that weight loss was, on average, more severe for the unvaccinated control animals than for any other vaccinated group of animals. The present study was designed to evaluate protective efficacy after vaccination and not the possible subtle negative effects caused by immunization with mismatched influenza antigens. Larger study group sizes will be necessary to conclusively address this question with an appropriate degree of statistical confidence. Antibody-mediated enhancement of influenza infection, including subtype–cross-reactive, nonneutralizing antibodies, has been previously described in cultured cells [18–20]. This mechanism has never been directly associated with a worsened clinical condition in animal models of influenza infection, although a recent study reported that maternally derived antibodies possibly enhanced swine influenza virus–induced pneumonia in pigs [21]. These observations further support the need for a more detailed evaluation of the efficacy of influenza vaccine in controlled experimental conditions where various levels of preexisting immunity to mismatched influenza antigens could be studied.

All animals demonstrated a strong induction of the proinflammatory cytokine IL-6 at day 6; this level remained elevated at day 9 only in the 2 groups of ferrets showing noticeable protection. Animals vaccinated with FluMist, the only group that mounted a strong cross-reactive cellular response, had the highest IFN-γ response. However, this response was not sufficient to control the disease. In fact, the strong expression of IL-10, an anti-inflammatory cytokine, detected in that group on day 3 may have suppressed an appropriate inflammatory response, including IL-6 expression, and temporarily favor virus replication, as previously demonstrated in pigs infected with foot-and-mouth disease [22]. There are reports showing the negative effect of IL-10 on influenza virus–infected mice and pigs [23, 24], and increased IL-10 production correlated with a low antibody response in elderly individuals after influenza vaccination [25]. Evaluating the response of cytokines, including IL-6 and IL-10, at early time points in patients may help predict unfavorable outcome and allow for better allocation of resources to individuals requiring more intensive clinical intervention.

The present study reports the immune responses and protective efficacy of commercially available vaccines and one lab-
oratory-produced matched vaccine with regard to prevention of pandemic H1N1 2009 infection in ferrets. The findings of this study may help to guide ongoing preparations for the influenza season in the Northern Hemisphere.

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References

1. World Health Organization (WHO). Pandemic (H1N1) 2009—update 60. Geneva, Switzerland: WHO, 31 July 2009.
2. Perez-Padilla R, de la Rosa-Zamboni D, Ponce de Leon S, et al. Pneumonia and respiratory failure from swine-origin influenza A (H1N1) in Mexico. N Engl J Med 2009;361:680–9.
3. Galwankar S, Clem A. Swine influenza A (H1N1)1 strikes a potential for global disaster. J Emerg Trauma Shock 2009;2:99–105.
4. Chowell G, Bertozzi SM, Colchero MA, et al. Severe respiratory disease concurrent with the circulation of H1N1 influenza. N Engl J Med 2009;361:674–9.
5. Neumann G, Noda T, Kawaoka Y. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature 2009;459:931–9.
6. Maines TR, Jayaraman A, Belser JA, et al. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science 2009;325:484–7.
7. Depoortere E, Mantero J, Lenglet A, Kreidl P, Coulombier D. Influenza A(H1N1)v in the Southern Hemisphere—lessons to learn from Europe? Euro Surveill 2009;14:19,246.
8. Paget J, Marquet R, Meijer A, van der Velden K. Influenza activity in Europe during eight seasons (1999–2007): an evaluation of the indicators used to measure activity and an assessment of the timing, length and course of peak activity (spread) across Europe. BMC Infect Dis 2007;7:141.
9. Brammer TL, Murray EL, Fukuda K, Hall HE, Klimov A, Cox NJ. Surveillance for influenza—United States, 1997–98, 1998–99, and 1999–00 seasons. MMWR Surveill Summ 2002;51:1–10.
10. Dawood FS, Jain S, Finelli L, et al. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009;360:2605–15.
11. Centers for Disease Control and Prevention. Oseltamivir-resistant novel influenza A (H1N1) virus Infection in two immunosuppressed patients—Seattle, Washington, 2009. MMWR Morb Mortal Wkly Rep 2009;58:893–6.
12. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg 1938;27:493–7.
13. Svitek N, Rudd PA, Obojes K, Pillet S, von Messling V. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. Virology 2008;376:53–9.
14. Ross TM, Mahmood K, Crevar CJ, Schneider-Ohrum K, Heaton PM, Bright RA. A trivalent virus-like particle vaccine elicits protective immune responses against seasonal influenza strains in mice and ferrets. PLoS One 2009;4:e6032.
15. Flusure XP. http://www.flusurexp.com. Accessed 1 February 2010.
16. Kobinger GP, Figueredo JM, Rowe T, et al. Adenovirus-based vaccine prevents pneumonia in ferrets challenged with the SARS coronavirus and stimulates robust immune responses in macaques. Vaccine 2007;25:5220–31.
17. Song MS, Oh TK, Pascua PN, et al. Investigation of the biological indicator for vaccine efficacy against highly pathogenic avian influenza (H5N1) virus in mice and ferrets. Vaccine 2009;27:3145–52.
18. Ochiai H, Kurokawa M, Hayashi K, Niwayama S. Antibody-mediated growth of influenza A NWS virus in macrophagelike cell line P388D1. J Virol 1988;62:20–6.
19. Ochiai H, Kurokawa M, Matsui S, et al. Infection enhancement of influenza A NWS virus in primary murine macrophages by anti-hemagglutinin monoclonal antibody. J Med Virol 1992;36:217–21.
20. Tamura M, Webster RG, Ennis FA. Subtype cross-reactive, infection-enhancing antibody responses to influenza A viruses. J Virol 1994;68:3499–504.
21. Kitikoon P, Nilubol D, Erickson BJ, et al. The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol 2006;112:117–28.
22. Diaz-San Segundo F, Rodriguez-Calvo T, de Avila A, Sevilla N. Immunosuppression during acute infection with foot-and-mouth disease virus in swine is mediated by IL-10. PLoS One 2009;4:e5659.
23. Kim HM, Lee YW, Lee KJ, et al. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. J Virol 2008;82:4265–74.
24. McKinstry KK, Strutt TM, Buck A, et al. IL-10 deficiency unleashes an immune response to influenza infection in two immunosuppressed patients—Seattle, Washington, 2009. MMWR Morb Mortal Wkly Rep 2009;58:893–6.
25. Kobinger GP, Figueredo JM, Rowe T, et al. Adenovirus-based vaccine prevents pneumonia in ferrets challenged with the SARS coronavirus and stimulates robust immune responses in macaques. Vaccine 2007;25:5220–31.
13. Svitek N, Rudd PA, Obojes K, Pillet S, von Messling V. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. Virology 2008;376:53–9.
14. Ross TM, Mahmood K, Crevar CJ, Schneider-Ohrum K, Heaton PM, Bright RA. A trivalent virus-like particle vaccine elicits protective immune responses against seasonal influenza strains in mice and ferrets. PLoS One 2009;4:e6032.
15. Flusure XP. http://www.flusurexp.com. Accessed 1 February 2010.
16. Kobinger GP, Figueredo JM, Rowe T, et al. Adenovirus-based vaccine prevents pneumonia in ferrets challenged with the SARS coronavirus and stimulates robust immune responses in macaques. Vaccine 2007;25:5220–31.