Preclinical efficacy of a prototype DNA vaccine: Enhanced protection against antigenic drift in influenza virus

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Vaccination with plasmid DNA expression vectors encoding foreign proteins elicits antibodies and cell-mediated immunity and protects against disease in animal models. We report a comparison of DNA vaccines, using contemporary human strains of virus, and clinically licensed (inactivated virus or subvirion) vaccines in preclinical animal models, to better predict their efficacy in humans. Influenza DNA vaccines elicited antibodies in both non-human primates and ferrets and protected ferrets against challenge with an antigenically distinct epidemic human influenza virus more effectively than the contemporary clinically licensed vaccine. These studies demonstrate that DNA vaccines may be more effective, particularly against different strains of virus, than inactivated virus or subvirion vaccines.

The ability of directly injected DNA expression vectors coding for viral proteins to elicit protective immune responses in laboratory models has suggested that such an approach might be clinically useful for the vaccination of humans[1–5]. Both antibody and cell-mediated immune responses have been demonstrated, indicating that DNA vaccination may induce an integrated humoral and cellular response similar to that induced by live viruses; thus DNA vaccines may serve as potentially safer alternatives to immunization with certain live attenuated viruses (namely, human immunodeficiency virus (HIV)) and may provide an increased breadth of protection relative to inactivated virus vaccines[1–5]. To assess the potential human applications of DNA vaccines, we used current circulating human strains of influenza virus in preclinical animal models and compared the efficacy of DNA vaccination with that of conventional widely used clinical vaccines. Our results indicate that DNA vaccines appear promising, as a means of providing a greater breadth of protection of humans against influenza in comparison with whole inactivated virus vaccine, and for vaccination against other diseases.

One means by which viruses escape host antibody responses and maintain epidemic status in a host population is through alterations of their surface antigenicity. Influenza virus provides a useful model for studying methods of vaccination against viruses with antigenically variable surface proteins, because frequent point mutations (antigenic drift) in antigenically important regions of the surface glycoprotein haemagglutinin (HA) result in substantial antigenic variation. This variation reduces the susceptibility of the virus to neutralization by antibodies induced by previous influenza virus infections or immunizations and limits the efficacy of conventional inactivated-virus influenza vaccines[6,7]. In contrast, the major internal proteins of influenza have remained relatively constant over the last 60 years[8,9]. Therefore, a vaccine that induced cell-mediated immune (CMI) responses against conserved internal proteins, although operating through a different immunological mechanism, may provide broader protection (protection against illness caused by a relatively greater range of antigenic variants) than would vaccines that elicit only type-specific neutralizing antibodies. For example, when antigenic drift decreases the efficacy of neutralizing antibodies, CMI responses could restrict viral replication and accelerate viral clearance and recovery of the host. An ideal vaccine thus might provide type-specific antibodies to protect against homologous virus and CMI responses to provide a defence against antigenic variants.

From 1990 to 1993, commercially available inactivated-virus vaccines contained as their H3N2 component the epidemic strain A/Beijing/353/89 (Table 1). This strain and closely related H3N2 strains, such as A/Hawaii/01/91, continued to circulate in human populations until late in the 1992–93 flu season. At that time a significant antigenic drift event led to the emergence of a new complex of epidemic strains (for example, A/Georgia/03/93 and A/Beijing-/32/92)[7,11]. In highly vaccinated human populations, the inactivated-virus A/Beijing/353/89 vaccine provided significantly lower cross-reactive antibody titres, and less protection from influenza disease, against antigenic drift variants such as A/Georgia/03/93 (refs 7, 11). Hence, these influenza virus strains formed the basis of our preclinical studies to determine whether a DNA vaccine that induced both antibody against the surface protein HA and CMI responses against the internal proteins nucleoprotein and matrix protein (M1), offers an improved breadth of protection compared with the contemporary inactivated virus vaccine.

Table 1 Influenza H3N2 strains in human populations, 1990–94

Years	Vaccine H3N2 Strain (USA)	Epidemic H3N2 Strains (Examples)
1990–92	A/Beijing/353/89	A/Beijing/353/89 A/Hawaii/01/91
1992–93	A/Beijing/353/89	A/Beijing/32/92 A/Georgia/03/93
1993–94	A/Beijing/32/92	A/Beijing/32/92 A/Georgia/03/93

References 7, 11.

DNA vaccine vs. inactivated virus in non-human primates

Because one requirement for the DNA vaccine was that it induce antibodies against HA, we tested this activity in non-human primates in comparison with the currently licensed vaccine. Anti-A/Beijing/353/89 haemagglutination-inhibiting (HI) antibodies were induced in African Green Monkeys injected with commercially obtained inactivated virus vaccines (1992–93 formulation), or with DNA that encoded the corresponding HA, nucleoprotein and M1 genes (Fig. 1). The HI antibody titres induced by DNA vaccines at doses as low as 10 μg of each construct (the lowest dose tested) were similar to, or substantially better than, those induced by injection of the full human dose of whole inactivated virus vaccine, or split virus vaccine, respectively.

Ferret influenza challenge model

Non-human primates are limited in their ability to be productively infected with human influenza strains[12–15]. In contrast, ferrets can produce sustained high levels of viral shedding in nasal washes following infection with a wide variety of human clinical isolates of influenza viruses[16,17]. Ferrets are considered to be the preclinical model of choice for studies of resistance to infection by human strains of influenza virus and are widely employed in evaluations of the effects of antigenic drift on the efficacy of vaccines[16,17]. The antibody responses of ferrets to influenza infection parallel those of humans in their ability to distinguish between antigenic variants[6,7]. In challenge studies in humans, the duration of virus shedding and peak level of virus shed are thought to correlate with vaccine efficacy[18–20]. Therefore, we used influenza virus infection in the ferret, as a preclinical model, to test the efficacy of a DNA vaccine against challenge with recent clinically significant antigenic drift variants within the H3 subtype.

Homologous protection in ferrets by a DNA vaccine

Protection against homologous challenge was investigated initially in ferrets immunized with DNA encoding HA from A/PR/8/34. Following homologous challenge, viral shedding was significantly reduced in DNA-vaccinated ferrets ($P < 0.0001$) (at challenge, geometric mean HI antibody titre [GMT] = 119); three of seven ferrets given HA DNA shed no detectable virus after challenge (Fig. 2). We next investigated the effect of immunization with HA DNA from A/Georgia/03/93, a recent clinical isolate, on viral shedding following homologous challenge with A/Georgia/03/93. Viral shedding was not detected after day 3, and overall shedding was very significantly reduced in comparison with non-immune ferrets ($P < 0.0001$) (Fig. 3). Immunization with HA DNA from A/Georgia/03/93 induced HI antibodies (GMT = 127 at challenge), and a significant reduction in the duration of viral shedding (Fig. 3), although the peak level of viral shedding on day 2 was not substantially decreased in contrast to the previous finding with A/PR/8/34. Therefore, the ability of serum HI antibody alone to prevent completely virus shedding in ferrets, as in humans, may vary for different influenza strains. Not surprisingly, ferrets immunized with HA DNA from A/Beijing/353/89 or from the Beijing 89-like strain A/Hawaii/01/91 had less extensive reductions in viral shedding, with viral shedding detectable until day 5, when they were challenged with the antigenic drift variant A/Georgia/03/93 (Fig. 3). Thus, as expected, immunization with HA DNA alone was most effective when the immunogen exactly matched the challenge strain.

Cross-strain protection in ferrets by a DNA vaccine

As the most critical aspect of these studies, we compared the breadth of protection provided by combined immunization with HA and internal protein DNAs with that of the current killed virus vaccine. We immunized ferrets with HA DNA from the Beijing 89-like strain A/Hawaii/01/91, combined with nucleoprotein and M1 DNAs from A/Beijing/353/89, and measured viral shedding following challenge with the antigenic drift variant A/Georgia/03/93. Combined DNA immunization reduced viral shedding significantly ($P = 0.0008$ compared with that observed following immunization with the 1992–1993 licensed inactivated influenza virus vaccine containing A/Beijing/353/89; Fig. 4), to levels that were similar ($P = 0.24$) to those seen in ferrets immunized with the homol-

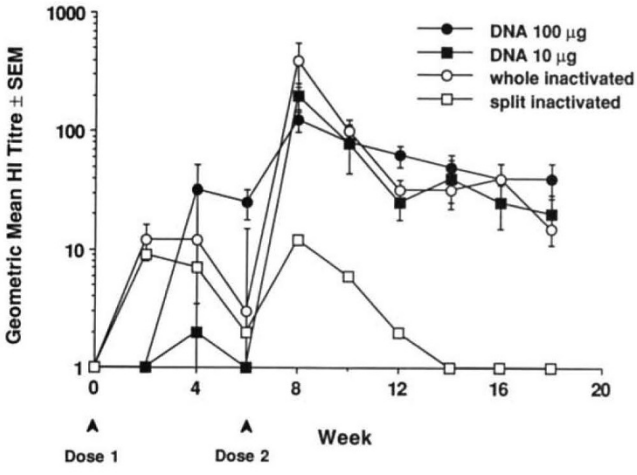

Fig. 1 Comparison of HI antibody responses of African Green Monkeys immunized with whole inactivated virus or DNA. Animals were injected twice with a mixture of five DNAs (HA from A/Beijing/353/89, A/Texas/36/91, and B/Panama/45/90 and nucleoprotein and M1 from A/PR/8/34) at a dose of 100 μg (filled circles) or 10 μg (filled squares) μg/construct, or twice with a full human dose (15 μg per strain) of commercially obtained (1992–93 formulation) whole inactivated (open circles) or split inactivated (open squares) vaccine containing the HA from the same three strains expressed as high-yielding reassortants with A/PR/8/34. The commercial whole inactivated vaccine is a reassortant virus that contains the internal proteins of A/PR/8/34, and therefore the nucleoprotein and M1 from A/PR/8/34 were also included in the DNA vaccine. HI antibody titres against A/Beijing/353/89 are shown.

Fig. 2 Reduction in viral shedding after challenge in ferrets immunized with DNA coding for the HA of A/PR/8/34. Ferrets were immunized intramuscularly with control DNA (open circles), or DNA encoding influenza A/PR/8/34 HA (filled circles) on days 0, 21, and 121, and were challenged intranasally with 10 times the 75% infectious dose for ferrets of A/PR/8/34 on day 148.

ogous HA DNA from A/Georgia/03/93 (Fig. 3). Combined DNA immunization also was more effective than immunization with HA (Fig. 3) or internal protein (not shown) DNAs alone ($P = 0.0027$, grouped comparison), although each of these separately did mediate a limited decrease in viral shedding. Likewise, when DNA encoding the HA from A/Beijing/353/89 was given in combination with nucleoprotein and M1 DNAs, the combined DNA vaccine allowed significantly less ($P = 0.02$) virus shedding than the inactivated virus vaccine (not shown). The inactivated virus vaccine also contains HA, nucleoprotein and M1, but as exogenous proteins, rather than as endogenously expressed antigens. Thus endogenous expression of the antigens may play an important role in the relatively greater efficacy of the DNA vaccine.

Discussion

Vaccination with DNA offers a number of potential advantages over immunization with whole inactivated virus, subunit, and recombinant vaccines. First, cellular immune responses induced by DNA vaccines against conserved internal proteins of a virus together with antibodies to viral surface proteins have the potential to provide protection against antigenic variants that differ in their surface proteins. As demonstrated for influenza, the protection seen against a recent clinical strain was better than that provided by the currently licensed inactivated virus vaccine, which loses protective efficacy as newly arising virus strains diverge from the original vaccine strain. Second, DNA immunization allows the use of sequences derived directly from human clinical specimens (or, in the case of influenza, after only limited passage in mammalian cell culture). This would avoid the selection of mutants divergent from the clinical strains. In some influenza strains, for example, mutants selected experimentally by growth in eggs differ in antigenicity from field strains[21,22]. As another example, antibodies raised against laboratory strains of HIV-1 may not neutralize recent clinical isolates[23]. Although it is not known whether the selection of mutant strains contributes to the limited efficacy of the current influenza vaccines, these observations underscore the point that DNA vaccines can generate the protein encoded by the exact sequence of the clinical viral isolate. In our studies, when the HA from the egg-passaged strain A/Beijing/353/89 was used, the level of protection was slightly, although not statistically significantly, lower than that obtained with HA DNA from the mammalian cell-passaged strain A/Hawaii/01/91. The sequences of the HAs of the two strains are known; they differ by only two point mutations. Both of the point mutations are thought to have been selected by growth of the A/Beijing/353/89 strain in eggs. This suggests that in some instances it might be possible to increase the efficacy of influenza vaccines by using HA sequences obtained from mammalian cell-passaged virus, thereby avoiding the selection of antigenic variants by growth of the virus in eggs. The third advantage of vaccination with DNA is that viral surface proteins produced by somatic cells of the host would be subjected to folding, assembly and post-translational modifications that are more similar to those found in native virus, resulting in the presentation of epitopes that also may be more similar to those found in native virus, compared with viral proteins produced in *Escherichia coli*, yeast, or insect cells. Antigens made by recombinant means may require artificial refolding in some instances to provide proper antigenicity. Endogenous production of protein immunogens by the host also avoids the need for formalin inactivation of whole-virus vaccines, which may modify amino acid side chains in key epitopes.

Both DNA vaccines and live attenuated virus vaccines can elicit CMI responses and may present viral surface proteins in a more native form than would inactivated or subunit vaccines. The safety and efficacy of DNA vaccines in humans are as yet unproven. Aspects of this technology, such as concerns over the potential for the induction of tolerance, clinically significant immune responses against the injected DNA, or integration of the injected DNA, require careful study. Thus far, DNA vaccines have induced robust primary and secondary immune responses in a variety of animal systems[1-5]. The experience with live DNA virus vaccines (for example, for smallpox[24], adenovirus[25], and most recently varicella[26]) points toward the anticipated safety of DNA vaccines.

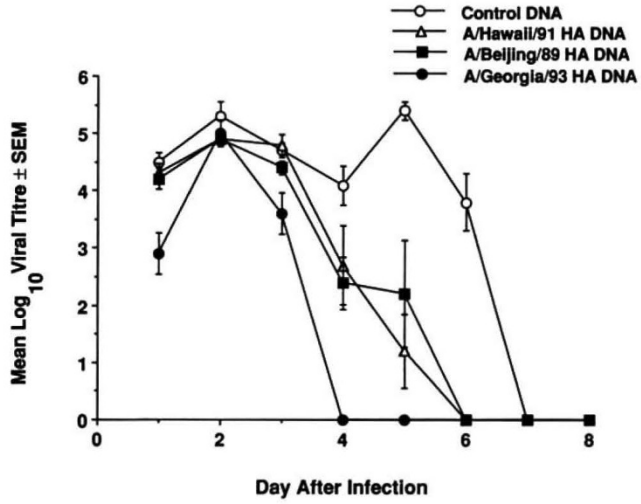

Fig. 3 Effect of immunization with HA DNA on viral shedding after challenge with an antigenic drift variant. Ferrets were immunized twice with control DNA (open circles), or with DNA coding for HA of A/Beijing/353/89 (filled squares), A/Hawaii/01/91 (open triangles), or A/Georgia/03/93 (filled circles) and challenged with A/Georgia/03/93 two weeks after the second immunization.

Moreover, DNA vaccines have several potential advantages over live attenuated virus vaccines. First, plasmid DNA is not subjected to the same selective pressures to optimize infectivity for a particular host cell or culture system, which may occur when a virus is propagated *in vitro*, and which may result in changes in antigenicity, as described above for influenza. Thus a DNA vaccine could be expected to yield a highly reproducible immunogen over time. Second, intramuscular injection of DNA may provide a sustained production of protein[27,28] that may result in increased duration of protective immunity as well as a greater opportunity to induce and expand cell-mediated immune responses. In contrast, virus replication after infection with attenuated influenza virus vaccines may last for only one or two days[20,29]. In our laboratory studies, HI antibodies induced by DNA immunization persisted for one year (to date) in mice and were at that time able to fully protect the mice from a lethal challenge with homologous influenza virus (J.B.U., J.J.D., M.A.L. *et al.*, unpublished observations). Finally, direct injection of DNA has not to date yielded immune responses above those of controls against the injected DNA expression vector itself (J.B.U. and C. DeWitt, unpublished observations), whereas immune responses against attenuated viral vectors or their wild-type parents are known to limit the utility of these vectors for repeated immunizations[30–32]. Additionally, certain live attenuated vaccines (such as polio, vaccinia, or HIV) may be pathogenic, especially in immunocompromised subjects[33,34], whereas others such as adenovirus, vaccinia, and human papillomavirus may downregulate immune responses by interfering with the expression of MHC class I molecules on infected cells during the virus life cycle.

DNA vaccines also have been shown to induce cytotoxic T cell (CTL) responses efficiently in mice, and mice immunized with nucleoprotein DNA from an H1 influenza strain were protected against disease produced by a strain of the H3 subtype (cross-strain protection)[1]. This is in contrast to inactivated virus and split virus vaccines which generally induce weak cross-strain protection (Fig. 4 and refs 7, 11). An alternative approach to the induction of cross-strain protection, using a fusion protein including the HA2 and non-structural protein-1 of influenza virus, was able to induce CTLs in mice against a peptide epitope of HA2 (ref. 35). However, the CTL epitope of the HA2 polypeptide was not sufficiently well conserved to provide cross-strain protection from the H1 to H3 subtype in mice, although cross-strain protection from H1 to H2, two more closely related viral subtypes, was shown[36]. Administration of this fusion protein to humans did not appear to induce $CD8^+$ MHC class I-restricted CTLs, but did provide limited protection against challenge with a second strain from within the H1 subtype, possibly as a result of $CD4^+$ MHC class II-restricted T cells[37]. DNA vaccines, therefore, would be expected to offer distinct advantages over whole inactivated virus, recombinant, subunit, and live attenuated vaccines.

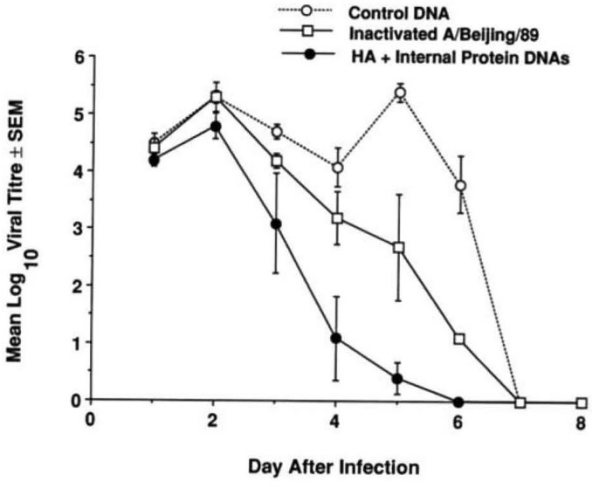

Fig. 4 Comparison of protection against an antigenic drift variant by immunization with whole inactivated virus vaccine or a combination of HA, nucleoprotein and M1 DNA. Ferrets were immunized twice with control DNA (open circles, representing the same control group as in Fig. 3), with a mixture of 3 DNAs coding for the HA from A/Hawaii/01/91 and the nucleoprotein and M1 of A/Beijing/353/89 (filled circles), or with commercially obtained whole inactivated influenza virus vaccine (1992–93 formulation) (open squares). The ferrets were challenged with A/Georgia/03/93 two weeks after the second injection.

Previous demonstrations of the efficacy of DNA vaccines have been in laboratory animal models, using adapted strains of virus. In this study, we have shown that DNA vaccination is effective against human isolates of virus in preclinical models, even where clinically significant antigenic variation has substantially reduced the effectiveness of a conventional inactivated vaccine.

Methods

Influenza genes. Haemagglutinin and nucleoprotein from influenza A/PR/8/34 were cloned into the V1J vector as previously described[2]. Viral stocks of the A/Hawaii/01/91, A/Beijing/353/89, and A/Georgia/03/93 strains were provided by Nancy Cox, Influenza Branch, Centers for Disease Control and Prevention. The nucleoprotein gene from A/Beijing/353/89 was cloned from cDNA prepared from viral RNA. The M1 and HA genes (other than A/PR/8/34) were cloned from viral RNA by RT–PCR. Plasmid DNA was prepared as previously described[2].

Immunizations. Young adult (2.2–7 kg body mass) seronegative male African Green Monkeys (three per group) were injected intramuscularly in one deltoid and one quadriceps with 0.5 ml of inoculum per site containing the indicated dosages of DNA. Control monkeys received a full human dose (15 μg of each HA antigen) of licensed influenza vaccine (Fluzone Whole Virion or Subvirion, Connaught Laboratories Inc., Swiftwater, Pennsylvania; trivalent, 1992–93 formulation). Male ferrets aged 12–14 weeks were obtained from Marshall Farms (North Rose, New York), and were screened for antibodies to influenza A nucleoprotein and HA. Only animals that were seronegative for influenza A virus were used in experiments. For studies on A/PR/8/34, ferrets were immunized intramuscularly with control DNA, or DNA encoding influenza A/PR/8/34 HA on days 0, 21 and 121 and were challenged intranasally with 10 times the 75% infectious dose for ferrets of A/PR/8/34 on day 148. Immunized animals received 1 mg of HA DNA; controls received 1 mg of control DNA (vector without an inserted coding region). For studies using current H3N2 influenza virus strains (A/Georgia/03/93, A/Hawaii/01/91, and A/Beijing/353/89) ferrets were injected on days 0 and 42 and then challenged on day 56. Animals were given 1 mg of individual DNAs or 2 mg of combined HA, nucleoprotein and M1 DNAs (660 μg of each construct) intramuscularly at each injection. Controls received 1 mg of control DNA (vector without a coding region). The dosage levels of DNA were selected arbitrarily. Ferrets immunized with licensed whole inactivated influenza vaccine (Fluzone Whole Virion, Connaught Laboratories Inc.; trivalent, 1992–93 formulation) received a full human dose (15 μg HA equivalent for each strain) intramuscularly on days 0 and 42. Two immunizations were

given as that is the recommended clinical protocol for immunization of humans without previous exposure to influenza virus.

Ferret challenge model. Immunized ferrets were challenged with 10 times the 75% infectious dose for ferrets of A/PR/8/34 (maintained in chicken eggs) or A/Georgia/03/93 (maintained in MDCK cells). To determine the amount of virus shed during the infections, nasal wash fluid was collected daily by instillation of 3 ml of PBS and collection of the expressed fluid. Viral titres were determined on MDCK cell monolayers in 96-well tissue culture plates, as previously described[1]. Chicken red blood cells (RBC; Hazleton Research Products, Hazleton, Pennsylvania) were used for haemagglutination reactions with culture supernatants from the assay plates. Fifty per cent infectivity ($TCID_{50}$) end points were interpolated from triplicate titrations. Data is represented as mean log_{10} virus titre versus days after infection. Viral titres were compared for immunized and control ferrets for individual days by the *t*-test for two means. Viral titres were compared for entire data sets, consisting of nasal wash viral titres from days 1–6, by repeated measures analysis of variance. Differences between groups were analyzed by pairwise comparisons unless otherwise indicated. Statistical analyses were performed using StatisticaMAC software (StatSoft, Inc., Tulsa, OK).

Haemagglutination inhibition test. Haemagglutination inhibiting antibodies were determined using four HA units of selected virus strain and chicken RBCs. For A/PR/8/34, sera were tested directly; for other strains, sera were incubated overnight with receptor-destroying enzyme (RDE, Sigma) and heated for 30 min at 56 °C. HI antibody responses induced in ferrets by A/Beijing/353/89 HA DNA and by licensed vaccine containing whole inactivated A/Beijing/353/89, respectively, were GMT of 138 for HA DNA and 45 for inactivated virus against the homologous strain, and GMT of 40 for animals given Beijing/353/89 HA DNA and 20 for animals given inactivated virus against A/Hawaii/01/91. Ferrets immunized with A/Hawaii/01/91 HA DNA had a GMT of 71 against the homologous strain and 40 against the related strain A/Beijing/353/89. Although antibody against the antigenic drift variant A/Georgia/03/93 was detected in these sera by ELISA, none of the antisera reacted well in HI assays against A/Georgia/03/93. Sera from ferrets immunized with A/Georgia/03/93 HA DNA had a GMT of 127 against Georgia but minimal HI activity against the two older strains.

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1. Ulmer, J.B. *et al.* Heterologous protective immunity to influenza A by intramuscular injection of DNA encoding a conserved viral protein. *Science* **259**, 1745–1749 (1993).
2. Montgomery, D.L. *et al.* Heterologous and homologous protection against influenza A by DNA vaccination: Optimization of DNA vectors. *DNA Cell Biol.* **12**, 777–784 (1993).
3. Wang, B. *et al.* Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4156–4160 (1993).
4. Cox, G.J.M., Zamb, T.J. & Babiuk, L.A. Bovine Herpesvirus 1: Immune responses in mice and cattle injected with plasmid DNA. *J. Virol.* **67**, 5664–5667 (1993).
5. Xiang, Z.Q. *et al.* Vaccination with a plasmid carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* **199**, 132–140 (1994).
6. Both, G.W., Sleigh, M.J., Cox, N.J. & Kendal, A.P. Antigenic drift in influenza virus H3 haemagglutinin from 1968 to 1980: Multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. *J. Virol.* **48**, 52–60 (1983).
7. Chakraverty, M. *et al.* Update: Influenza activity-United States and worldwide, and composition of the 1993–94 influenza vaccine. *Morbid. Mortal. Weekly Rep.* **42**, 177–180 (1993).
8. Shu, L.L., Bean, W.J. & Webster, R.G. Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. *J. Virol.* **67**, 2723–2729 (1993).
9. Ito, T. *et al.* Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. *J. Virol.* **65**, 5491–5498 (1991).
10. Rocha, E.P. *et al.* Comparison of 10 influenza A (H1N1 and H3N2) haemagglutinin sequences obtained directly from clinical specimens to those of MDCK cell- and egg-grown viruses. *J. gen. Virol.* **74**, 2513–2518, (1993).
11. Nichol, K.L. *et al.* The efficacy and cost effectiveness of vaccination against influenza among elderly persons living in the community. *New Engl. J. Med.* **331**, 778–784 (1994).
12. Clements, M.L. *et al.* Use of single-gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of wild-type human influenza A virus for squirrel monkeys and adult human volunteers. *J. clin. Microbiol.* **30**, 655–662 (1992).
13. Murphy, B.R. *et al.* Evaluation of three strains of influenza A virus in humans and in owl, cebus, and squirrel monkeys. *Infect. Immun.* **28**, 688–691 (1980).
14. Berendt, R.F. & Scott, G.H. Evaluation of commercially prepared vaccines for experimentally induced type A/New Jersey/76 influenza virus infections in mice and squirrel monkeys. *J. infect. Dis.* **136**, S712–S717 (1977).
15. Berendt, R.F. & Hall, W.C. Reaction of squirrel monkeys to intratracheal inoculation with influenza A/New Jersey/76 (swine) virus. *Infect. Immun.* **16**, 476–479 (1977).
16. Yetter, R.A., Barber, W.H. & Small, P.A. Heterotypic immunity to influenza in ferrets. *Infect. Immun.* **29**, 650–653 (1980).
17. Smith, H. & Sweet, C. Lessons for human influenza from pathogenicity studies with ferrets. *Rev. infect. Dis.* **10**, 56–75 (1988).
18. Sears, S.D. *et al.* Comparison of live, attenuated H1N1 and H3N2 cold-adapted and avian-human influenza A reassortant viruses and inactivated virus vaccine in adults. *J. infect. Dis.* **158**, 1209–1219 (1988).
19. Couch, R.B. *et al.* Influenza: Its control in persons and populations. *J. infect. Dis.* **153**, 431–440 (1986).
20. Murphy, B.R. *et al.* Temperature-sensitive mutants of influenza virus. III. Further characterization of the *ts*-1[E] influenza A recombinant (H3N2) virus in man. *J. infect. Dis.* **128**, 479–487 (1973).
21. Katz, J.M. & Webster, R.G. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. *J. infect. Dis.* **160**, 191–198 (1989).
22. Newman, R.S. *et al.* Immune response of human volunteers and animals to vaccination with egg-grown influenza A (H1N1) virus is influenced by three amino acid substitutions in the haemagglutinin molecule. *Vaccine* **11**, 400–406 (1993).
23. Sawyer, L.S.W. *et al.* Neutralization sensitivity of human immunodeficiency virus type 1 is determined in part by the cell in which the virus is propagated. *J. Virol.* **68**, 1342–1349 (1994).
24. Mahr, A. & Payne, L.G. Vaccinia recombinants as vaccine vectors. *Immunobiology* **184**, 126–146 (1992).
25. Graham, F.L. & Prevec, L. Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology* **20**, 363–390 (1992).
26. Asano, Y. *et al.* Experience and reason: 20-year follow-up of protective immunity of the Oka strain live varicella vaccine. *Pediatrics* **94**, 524–526 (1994).
27. Wolff, J.A. *et al.* Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. molec. Genet.* **1**, 363–369 (1992).
28. Manthorpe, M. *et al.* Gene therapy by intramuscular injection of plasmid DNA: Studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* **4**, 419–431 (1993).
29. Clements, M.L. *et al.* Evaluation of the infectivity, immunogenicity, and efficacy of live cold-adapted influenza B/Ann Arbor/1/86 reassortant virus vaccine in adult volunteers. *J. infect. Dis.* **161**, 869–877 (1990).
30. Powers, D.C. *et al.* In elderly persons live attenuated influenza A virus vaccines do not offer an advantage over inactivated virus vaccine in inducing serum or secretory antibodies or local immunologic memory. *J. clin. Microbiol.* **29**, 498–505 (1991).
31. Yang, Y.P. *et al.* Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. natn. Acad. Sci. .U.S.A.* **91**, 4407–4411 (1994).
32. Cooney, E.L. *et al.* Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. *Lancet* **337**, 567–572 (1991).
33. Redfield, R.R. *et al.* Disseminated vaccinia in a military recruit with human immunodeficiency virus (HIV) disease. *New Engl. J. Med.* **316**, 673–676 (1987).
34. Baba, T.W. *et al.* Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**, 1820–1825 (1995).
35. Kuwano, K. *et al.* Active immunization against virus infections due to antigenic drift by induction of cross-reactive cytotoxic T lymphocytes. *J. exp. Med.* **169**, 1361–71 (1989).
36. Mbawuike, I.N. *et al.* Influenza A subtype cross-protection after immunization of outbred mice with a purified chimeric NS1/HA2 influenza virus protein. *Vaccine* **12**, 1431–48 (1994).
37. Fries, L.F. *et al.* Safety and immunogenicity of a recombinant protein influenza A vaccine in adult human volunteers and protective efficacy against wild-type H1N1 virus challenge. *J. infect. Dis.* **167**, 593–601.