

Plasmid DNA encoding influenza virus haemagglutinin induces Th1 cells and protection against respiratory infection despite its limited ability to generate antibody responses

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Direct intramuscular injection of plasmid DNA can generate immune responses against encoded antigens. However, the relative ability of DNA vaccines to induce cellular and humoral immunity after a single or booster immunization and the persistence of this response have not been fully elucidated. In this study, induction and maintenance of antibody and T cell subtypes with different doses of naked DNA encoding the haemagglutinin (HA) gene of influenza virus were examined and compared to the immune responses and protection induced by respiratory tract infection and immunization with a killed virus vaccine. Like natural infection, immunization with HA DNA induced potent Th1 responses. Spleen cells from mice immunized once with HA DNA in the dose range 10 ng to 100 µg secreted significant levels of IFN- γ , but low or undetectable IL-5, in response to influenza virus *in vitro*. Furthermore, CD4⁺ HA-specific Th1 clones were generated from spleens of immunized mice. Although T cell responses waned 12 weeks after a single immunization, antigen-specific Th1 cells persisted in the spleen for at least 6 months after two booster immunizations. In contrast, influenza virus-specific ELISA IgG titres were low after a single immunization and required two booster immunizations to reach significant levels. Furthermore, haemagglutination inhibition (HI) antibodies were weak or undetectable after two immunizations. Nevertheless, two doses of HA DNA conferred almost complete protection against respiratory challenge with live virus. Thus, despite the limited ability to induce antibodies, DNA vaccines confer protective immunity against influenza virus infection, which appears to be mediated by Th1 cells.

Introduction

An alternative approach to conventional whole virus or protein subunit vaccines, which may overcome many of the difficulties associated with current vaccines, involves the direct injection of naked plasmid DNA encoding protective viral proteins (Liu, 1995; Donnelly, 1997; Webster, 1999). Significantly, this method of immunization appears to mimic natural virus infections in that the antigens are produced in their native conformation for the generation of functionally relevant antibody responses. Furthermore, the expressed proteins can be processed and presented by APC in association with MHC class I and class II molecules to elicit cytotoxic and helper T

(Th) cell responses (Donnelly, 1997; Liu, 1995). Therefore, DNA vaccines could not only serve as potentially safer alternatives to immunization with attenuated viruses, but may also provide increased protective efficacy relative to inactivated or protein subunit vaccines.

Immunization with plasmid DNA encoding influenza virus haemagglutinin (HA) is capable of inducing cell-mediated and humoral immunity and in protecting against intranasal challenge with homologous influenza virus (Fynan *et al.*, 1993; Ulmer *et al.*, 1994; Webster *et al.*, 1994; Bot *et al.*, 1997; Deck *et al.*, 1997; Feltquate *et al.*, 1997). Furthermore, induction of class I-restricted CTL and protection of mice against heterologous virus challenge has been demonstrated with plasmid DNA encoding the nucleoprotein of influenza virus (Ulmer *et al.*, 1993). It appears that these vaccines selectively induce type 1 T cells, whereas protein vaccines induce Th2 or

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mixed Th1/Th2 responses. Although antibody responses have been reported after a single immunization with a DNA vaccine (Deck *et al.*, 1997), most studies with HA DNA have reported antibody responses only after a booster immunization (Fynan *et al.*, 1993; Ulmer *et al.*, 1994; Bot *et al.*, 1997; Feltquate *et al.*, 1997). Furthermore, the antibody response seen after a single immunization does not reach that observed with natural infection or with conventional influenza virus vaccines which are normally administered as a single dose (Bot *et al.*, 1997; Feltquate *et al.*, 1997). Therefore, the relative ability of DNA vaccines to generate cell-mediated and humoral immunity remains controversial, and the persistence of the immunity and the optimum dose and schedule to selectively prime distinct arms of the immune response remain to be defined.

In this study, we have examined the effect of the immunizing dose and schedule on the induction of cellular and humoral immune responses and the persistence of this response using DNA encoding the HA gene from influenza virus A/Sichuan/2/87 (H3N2). We demonstrate that HA DNA, like natural infection, selectively induces influenza virus-specific type 1 T cell responses, but the persistence of these responses and the induction of antibodies, is dependent on booster immunizations. Nevertheless, despite its limited ability to induce humoral immunity, the DNA vaccine could protect against intranasal challenge with live influenza virus.

Methods

■ **Mice.** Female BALB/c mice were purchased from Harlan. Mice were maintained according to the guidelines of the Irish Department of Health and were 6–8 weeks old at the initiation of experiments.

■ **Plasmid construction and purification.** A/Sichuan/2/87 (H3N2) full-length HA was amplified by PCR and cloned into plasmid pL17. pL17 is a pUC-based plasmid carrying a bacterial origin of replication and ampicillin-resistance gene for growth and selection in *Escherichia coli* K12. It also carries a truncated enhancer region, full promoter and full Intron A gene from human CMV and a CMV terminator sequence. Plasmid DNA was purified using Qiagen endofree mega kits.

■ **Immunization and infection of mice.** Mice were infected with approximately 5×10^5 50% tissue culture infective dose (TCID₅₀) per ml of A/Sichuan/2/87 influenza virus (infectious allantoic fluid) by intranasal administration under light anaesthesia. Intramuscular (i.m.) injections of HA DNA in 125 µl PBS per mouse were given at four sites (both quadriceps and biceps). β-Propiolactone inactivated A/Sichuan/2/87 influenza virus, had equivalent efficacy to commercially produced homologous influenza virus vaccines in animal and laboratory assays, and was used as the whole virus vaccine (15 µg HA per dose). This vaccine was also administered by the i.m. route. At the indicated time-points, mice were sacrificed by cervical dislocation and spleens, lymph nodes and blood samples were removed.

■ **Generation of influenza virus-specific T cell clones.** T cell clones were generated from the spleens of individual BALB/c mice as described previously (Mills *et al.*, 1986). Spleens were taken 14 days after

i.m. immunization with 50 µg HA DNA. T cell lines were first established by culturing spleen cells (2×10^6 per ml) for 4–5 days with 0.8 µg/ml purified A/Sichuan/2/87 influenza virus in RPMI 1640 supplemented with 2% normal mouse serum at 37 °C with 5% CO₂. After this period, 5 U/ml recombinant IL-2 (rIL-2) was added to cultures and cells were cultured for a further 7 days. Surviving T cells were then washed once and recultured at 1×10^5 cells per ml for 7 days with syngeneic irradiated spleen cells (2×10^6 per ml) as feeder cells. T cell lines were established by maintaining these cultures at 1×10^5 cells per ml in a 4 day feed/7 day starve cycle, by alternately culturing with 0.8 µg/ml purified influenza virus and 2×10^6 per ml APC (irradiated syngeneic spleen cells) or with 5 U/ml rIL-2.

T cell lines were cloned by limiting dilution at 1 cell per well in 200 µl volumes in 96-well plates in the presence of APC (2×10^6 per ml), virus (0.8 µg/ml) and rIL-2 (5 U/ml). A further 5 U/ml rIL-2 was added to cultures 5 days later. The clones were restimulated after a further 7 days incubation in 1 or 2 ml volumes in 24-well plates, and progressively expanded to 25 ml tissue culture flasks. T cell clones were maintained by restimulation at the initial concentration of 1×10^5 cells per ml with virus and irradiated APC every 10 days. The specificity of the T cell clones was tested against A/Sichuan/2/87 influenza virus using proliferation and cytokine assays.

■ **Cytokine assays.** T cell cytokine production was assessed by culturing spleen cells or lymph node cells (2×10^6 per ml) or T cell clones (10^5 per ml) and APC (2×10^6 per ml) with purified inactivated virus at concentrations of 0.4, 4.0 or 12 µg/ml. Negative and positive control stimuli included medium alone and anti-CD3 (2.0 µg/ml) plus phorbol myristate acetate (PMA; 25 ng/ml), respectively. Concentrations of IFN-γ, IL-4 and IL-5 were measured by immunoassay using pairs of commercially available monoclonal antibodies (PharMingen) as described previously (Mills *et al.*, 1998).

■ **Antibody assays.** The levels of serum antibody against influenza virus were determined by ELISA. Purified influenza virus (12.5 µg/ml) was used to coat plates. Bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG (Sigma). Antibody levels are expressed as the mean endpoint titre ($\pm$ SD).

Haemagglutination inhibition (HI) antibodies were determined by assessing the ability of sera to inhibit agglutination of turkey red blood cells. Sera were incubated overnight at 37 °C with 4 volumes of receptor-destroying enzyme, prepared from *Vibrio cholerae*, in order to remove non-specific inhibitors. After inactivation of the receptor-destroying enzyme at 56 °C for 30 min, twofold dilutions of sera were incubated with approximately 10^6 TCID₅₀ of A/Sichuan/2/87 virus. The assay was developed by adding 0.7% (v/v) turkey red blood cells and the HI titres read as the reciprocal of the highest dilution causing complete inhibition of agglutination.

■ **Influenza virus challenge and virus titrations.** Mice were infected with 50 MID₅₀ (50% mouse infectious dose) live influenza virus in PBS with 2% (w/v) BSA (approximately 10^5 TCID₅₀). The virus was administered to non-anaesthetized mice in 50 µl volumes bilaterally by intranasal instillation. At intervals after challenge, nasal washes were performed using 0.5 ml PBS with 2% (w/v) BSA per mouse. Serial tenfold dilutions of nasal washes, prepared in serum-free Eagle's minimal essential medium with TPCK-treated trypsin, were incubated for 4 days at 35 °C on confluent monolayers of MDCK cells in 96-well tissue culture plates. The presence of virus in each well was determined by incubation of 50 µl supernatant with an equal volume of 0.7% (v/v) turkey red blood cells. The virus titre of each specimen, expressed as TCID₅₀ per ml, was calculated using the Karber equation (Hawkes, 1979).

Results

Natural infection with influenza virus induces a polarized Th1 response

Since respiratory infection with influenza virus induces immune responses that protect against subsequent infection with homologous virus, the rationale of our vaccine strategy was to generate immune responses that mimic natural infection. To determine the type and persistence of the local and systemic immune response generated by natural infection, BALB/c mice were respiratory tract-infected with approximately 5×10^5 TCID₅₀ influenza virus and T cell responses were assessed in the spleen and draining lymph nodes 2, 12 and 24 weeks later. Spleen cells from infected mice secreted high concentrations of IFN- γ with undetectable IL-5 in response to *in vitro* stimulation with a range of doses of influenza virus and this response persisted for at least 6 months after virus challenge (Fig. 1A). The generation of a panel of influenza virus-specific CD4⁺ T cell clones from the spleens of infected mice, which secreted IFN- γ but not IL-5 in response to *in vitro* stimulation with inactivated influenza virus, confirmed the selective induction of Th1 cells (data not shown). A polarized Th1 response was also detected in the cervical lymph nodes at

2 and 12 weeks post-infection (Fig. 1B). At 24 weeks, cervical lymph node cells secreted both IFN- γ and IL-5 in response to *in vitro* restimulation with influenza virus, suggesting that the local immune response had broadened to a mixed Th1/Th2 type in the draining lymph nodes.

DNA immunization induces a polarized Th1 response

In order to compare the immune responses generated following immunization with HA DNA to that observed with a natural infection, BALB/c mice were immunized i.m. and T cell responses were assessed in the spleen and in local and distant lymph nodes. Two weeks after immunization with a single dose of 100 μ g HA DNA, spleen cells from immunized mice secreted significant amounts of IFN- γ , but undetectable IL-5, following *in vitro* restimulation with a range of concentrations of influenza virus (Fig. 2A). Spleen cells from immunized mice were found to be capable of producing IL-5, as determined by the response to the polyclonal activator PMA and anti-CD3 (Fig. 2A). The selective induction of Th1 cells with the HA DNA *in vivo* was confirmed through the generation of Th1 clones from mice immunized with HA DNA. Four distinct influenza virus-specific CD4⁺ T cell clones successfully established from individual mice secreted signifi-

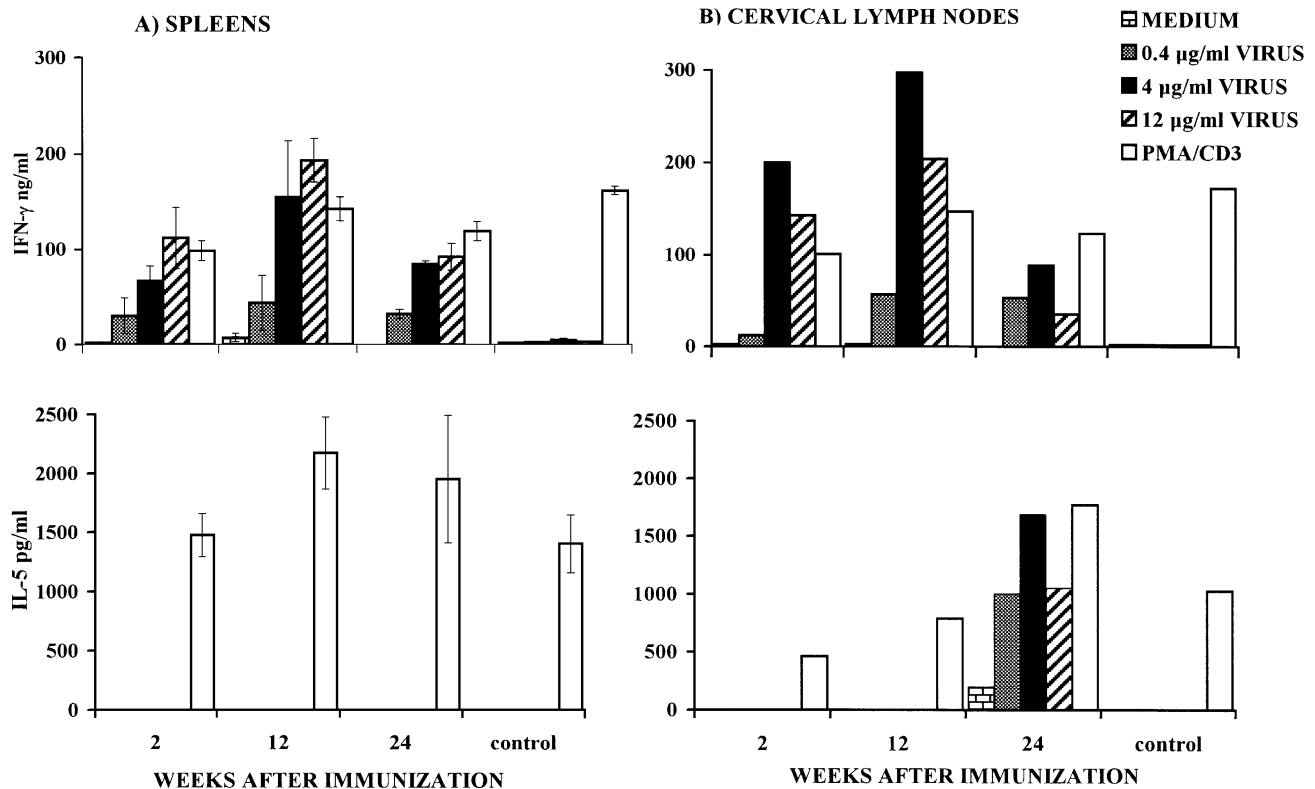


Fig. 1. Natural infection with influenza virus induces a persistent systemic Th1 response. Mice were intranasally infected with approximately 5×10^5 TCID₅₀ influenza virus. Spleen cells (A) or cervical lymph node cells (B) were restimulated *in vitro* with purified inactivated influenza virus (0.4, 4.0 or 12 μ g/ml), PMA and anti-CD3, or medium alone. IFN- γ and IL-5 concentrations in 72 h culture supernatants were measured by immunoassay. Results are mean cytokine concentrations ($\pm$ SD) for four to five mice per group performed on individual spleen cell preparations or pooled lymph node cells.

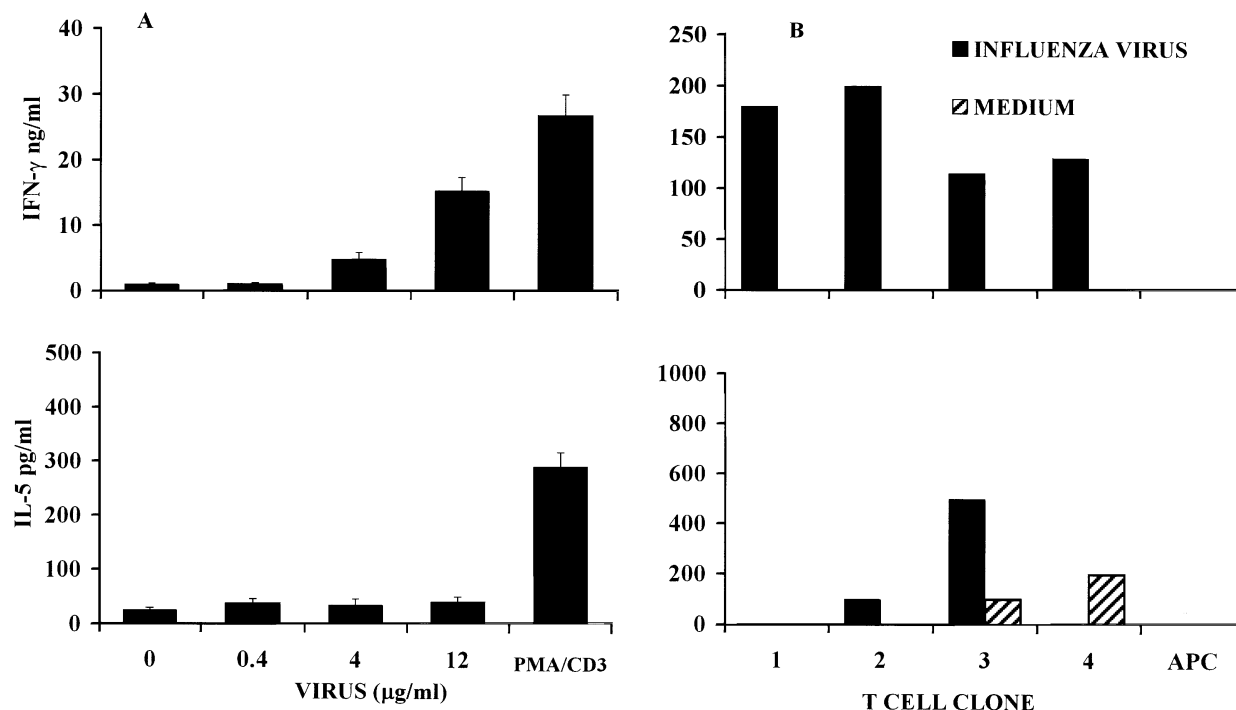


Fig. 2. Intramuscular immunization with HA DNA induces CD4⁺ Th1 cells in the spleen. (A) Mice were immunized with a single dose of HA DNA and, 2 weeks later, spleen cells were restimulated *in vitro* with either purified inactivated influenza virus (0.4, 4.0 or 12 µg/ml), PMA and anti-CD3, or medium alone. IFN-γ and IL-5 concentrations in 72 h culture supernatants were measured by immunoassay. Results are mean cytokine concentrations (±SD) for four to five mice per group. (B) CD4⁺ T cell clones, established from the spleens of mice immunized with HA DNA, were stimulated *in vitro* with influenza virus (4 µg/ml) and syngeneic APC (2×10^6 per ml) and cytokine production was assessed in culture supernatants after 3 days.

cant amounts of IFN-γ with undetectable or low levels of IL-5 (Fig. 2B) or IL-4 (not shown) in response to stimulation with influenza virus.

In order to examine the effect of DNA dose on the T cell response, BALB/c mice were immunized with HA DNA in the dose range 0.01–100 µg. High concentrations of IFN-γ were detected in supernatants of spleen cells stimulated *in vitro* with influenza virus. Reduced levels of IFN-γ production were detected from spleen cells isolated from mice that had received lower doses of DNA. However, an antigen-specific response was still detectable at doses as low as 0.1 and 0.01 µg per mouse (Fig. 3A). In contrast, IL-5 production was undetectable in antigen-stimulated spleen cells, regardless of the immunizing dose (data not shown).

Multiple immunizations with HA DNA induces a persistent polarized Th1 response in the spleen, but not in the draining lymph nodes

Despite the strong Th1 cell response at 2 weeks post-immunization (Fig. 2; Fig. 3A), a single immunization with HA DNA did not induce a persistent T cell response. Influenza virus-specific IFN-γ production was weak or undetectable 12 weeks (Fig. 3B) and 24 weeks (Fig. 3C) after a single immunization with HA DNA in the dose range 0.01–100 µg per mouse. In contrast, spleen cells obtained from mice

immunized with three doses of 100 µg HA DNA secreted significant amounts of IFN-γ and little or no IL-5 in response to inactivated virus *in vitro* and this response was detected at 2, 12 and 24 weeks after immunization (Fig. 4). This suggests that multiple immunizations are required for a persistent T cell response, which remains polarized to the Th1 subtype. However, the response was not as persistent following multiple immunizations with lower doses of DNA (1.0 or 10 µg), with a weak antigen-specific response detected at 12 and 24 weeks (Fig. 4). Unlike the draining lymph node cells from the site of influenza virus infection (Fig. 1B), influenza virus-specific T cell responses could not be detected in any of the lymph nodes examined (cervical, thoracic, inguinal, axillary, brachial and popliteal) after single or multiple immunizations with HA DNA (data not shown). These results suggest that the lymph nodes are not a major site of T cell activation following i.m. immunization with HA DNA.

Natural infection induces a significantly stronger antibody response than immunization with DNA

Serum samples taken from mice following natural infection or single or multiple immunizations with HA DNA were examined for influenza virus-specific IgG by ELISA. DNA immunization generated weak influenza virus-specific serum IgG titres. A single immunization with 100 µg HA DNA

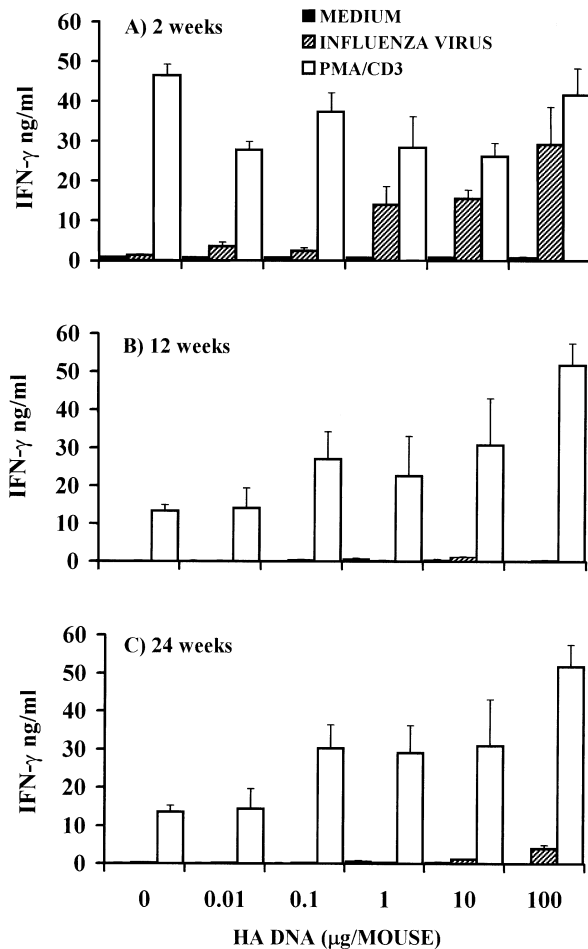


Fig. 3. Persistence of the T cell response after a single immunization with a range of doses of HA DNA. Mice were immunized once with 0.01–100 µg HA DNA and T cell responses were assessed in the spleen 2 (A), 12 (B) and 24 (C) weeks later. Spleen cells were restimulated *in vitro* with either purified inactivated influenza virus (12 µg/ml), PMA and anti-CD3, or medium alone. Results are mean cytokine concentrations ($\pm$ SD) in supernatants for spleen cells for four to five mice per group. Antigen-specific IL-5 was undetectable in all samples.

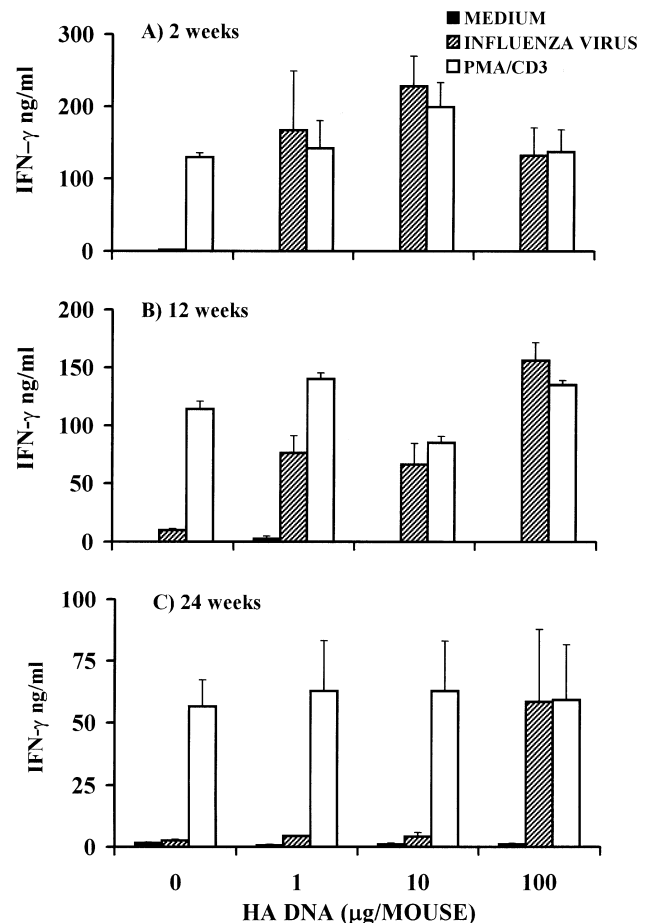


Fig. 4. Multiple immunizations with HA DNA induce a persistent influenza virus-specific Th1 response in the spleen which is dose dependent. Mice were immunized three times at 4-week intervals and T cell responses were assessed 2 (A), 12 (B) and 24 (C) weeks after the final immunization. Spleen cells were restimulated *in vitro* with either inactivated influenza virus (12 µg/ml), PMA and anti-CD3, or medium alone. Results are mean cytokine concentrations ($\pm$ SD) in supernatants for spleen cells for four to five mice per group. Antigen-specific IL-5 was undetectable in all samples.

induced a log₁₀ antibody titre of 2.6, which is significantly below the titre of 4.0 observed following natural infection with influenza virus (Fig. 5). Booster immunization with HA DNA enhanced antibody production, but the titre remained below that induced by natural infection (Fig. 5). Furthermore, the responses waned significantly after 6 months, whereas the IgG titres were tenfold higher 6 months after infection with influenza virus.

In an attempt to assess the functional capacity of the antibody responses, HI antibody titres were determined. A single dose of 100 µg HA DNA failed to generate a detectable HI antibody response. After two immunizations with HA DNA, a HI titre of 1/20 was detected in one of three experiments, but remained below the level of detection in two experiments (Table 1 and data not shown). In contrast, low but significant levels of HI antibodies were detectable after

infection or immunization with a single dose of an influenza virus vaccine (Table 1).

HA DNA vaccine confers protection against challenge with live influenza virus

Although immunization with HA DNA induced weak antibody responses, it generated potent cell-mediated immunity and since it has been suggested that both arms of the immune response can contribute to protection against infection, it was important to establish if the HA DNA vaccine could confer protection against influenza virus infection. When mice were challenged with influenza virus 2 weeks after a single immunization with 100 µg HA DNA, the virus titres were lower in the lungs of immunized compared with control mice 2 and 3 days after challenge. Although HI antibodies

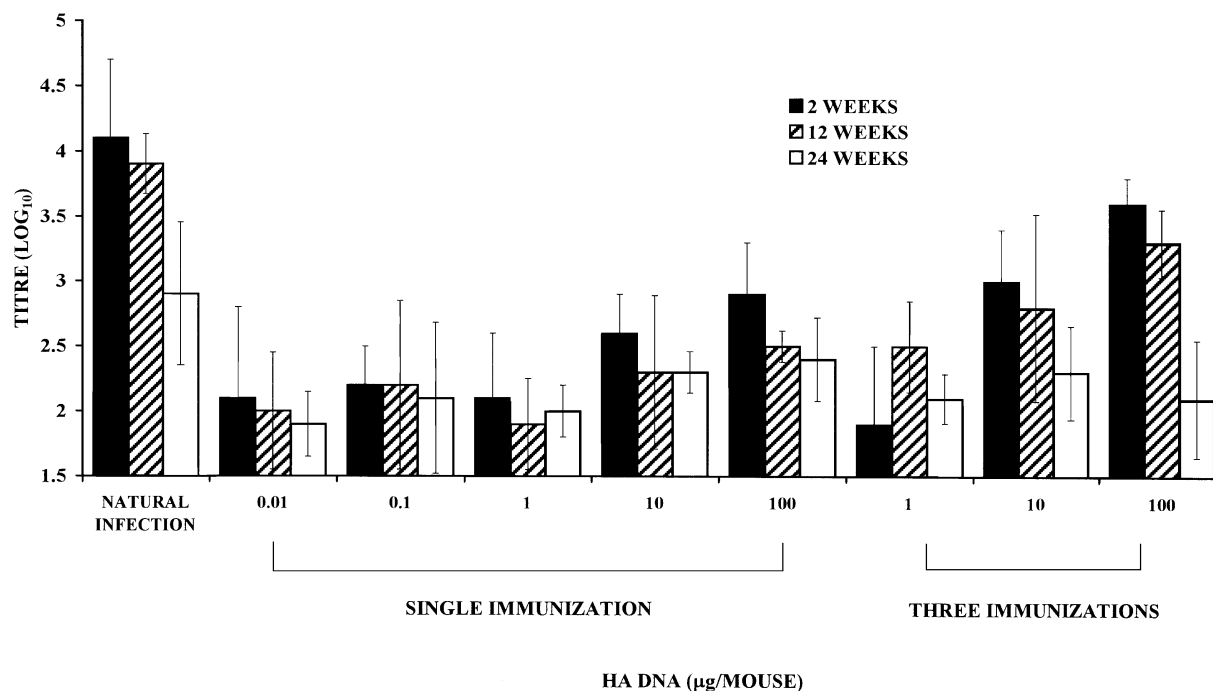


Fig. 5. Natural infection induces higher levels of anti-influenza virus-specific IgG than three doses of 100 µg HA DNA. Mice were immunized as described in Figs 3 and 4 or were infected once intranasally with influenza virus. Serum antibody titres were determined by ELISA 2, 12 and 24 weeks after immunization or infection. Data are the means of antibody titres ($\pm$ SD) for four to five mice per group.

Table 1. HI antibodies in the sera of immunized mice before and after intranasal infection

Mice were immunized i.m. with one or two doses of 100 µg HA DNA, whole virus vaccine or PBS only as a control, and were challenged with homologous influenza virus 2 weeks after the last immunization. Results are mean HI titres for five to ten mice per group assessed 2 weeks after the last immunization and 2 weeks after intranasal challenge with influenza virus.

Experiment	Immunogen*	HI antibody titre	
		Post-immunization	Post-challenge
1	Control	†	40
	HA DNA (1)	†	320
	HA DNA (2)	20	160
2	Control	†	40
	HA DNA (2)	†	80
	Whole virus vaccine (1)	40	320

* Number of immunizations is given in parentheses.

† Undetectable HI antibody.

were undetectable on the day of challenge in mice immunized with a single dose, compared with the control mice, they increased significantly to a titre of 1/320 after challenge. In a

second challenge experiment, because of the weak antibody responses we used two doses of HA DNA and included a conventional influenza virus vaccine for comparison, administered as a single dose. Our rationale for choosing one dose of the whole virus vaccine was based on the fact that this type of influenza virus vaccine is always administered as a single dose in humans. Furthermore, the whole virus vaccine generated potent antibody responses after a single immunization, whereas the antibody responses were comparatively weak with a single dose of the DNA vaccine. Immunization with two doses of HA DNA conferred almost complete protection against influenza virus with little or no virus detectable in nasal washes at any time-point post-challenge. In contrast, mice immunized with the whole virus vaccine had significantly higher virus shedding at 3 and 4 days post-challenge (Fig. 6). HI antibodies were low or undetectable on the day of challenge in mice immunized once or twice with HA DNA, but were detectable at higher levels than in non-immunized mice after challenge. In contrast, low but significant levels of HI antibodies were detectable after a single dose of an influenza virus vaccine, and this increased to 1/320 after challenge.

Discussion

The results of this study demonstrate that immunization with naked plasmid DNA encoding the HA gene of influenza virus induces potent systemic Th1 responses, which mimics

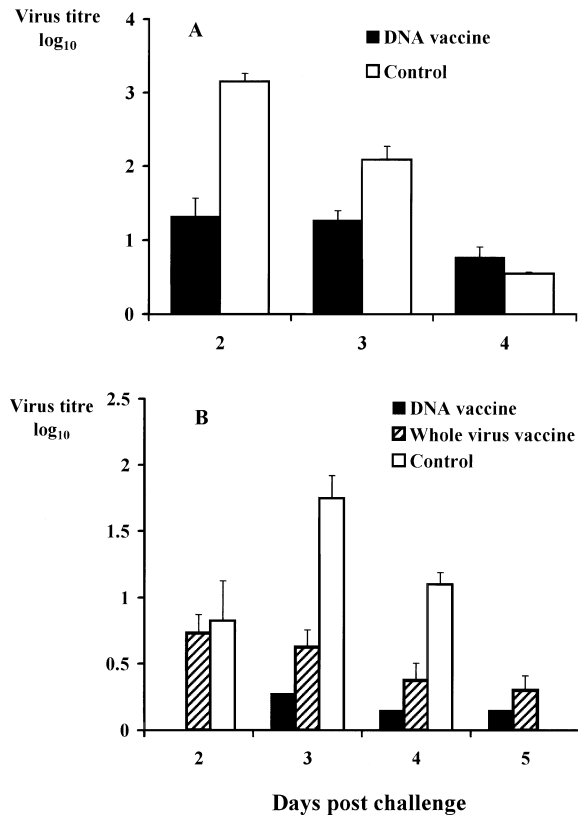


Fig. 6. Immunization with HA DNA confers protection against challenge with live influenza virus. Mice were immunized i.m. with one (A) or two (B) doses of 100 µg HA DNA, one dose of whole virus vaccine or PBS only as a control, and were challenged with homologous influenza virus 2 weeks after the last immunization. Results are mean virus titres ($\pm$ SD) in nasal washes for five to ten mice per group at each time-point.

that observed following respiratory infection with influenza virus. However, multiple immunizations with HA DNA are required for persistence of the response and for the generation of relatively modest antibody levels. Nevertheless, the HA DNA vaccine conferred a high level of protection against challenge with live influenza virus. These findings demonstrate that an influenza virus HA DNA vaccine is a potent inducer of systemic type 1 T cells, which are capable of mediating protection in the respiratory tract.

One approach to vaccine design is to attempt to achieve a response that mimics natural infection, without the risk associated with a live virus. We therefore investigated the type and persistence of the immune response generated with the DNA vaccine and compared these with the responses induced by natural infection with influenza virus. Our results suggest that a single intranasal challenge with influenza virus generates antigen-specific IFN- γ -secreting T cells in the spleen and this confirms earlier reports that natural infection selectively induces Th1 responses in BALB/c mice (Feltquate *et al.*, 1997; Graham *et al.*, 1998). However, a more heterogeneous cytokine profile has been reported for T cell clones generated from spleens of influenza virus-infected C57BL/6 and CBA/Ca

mice suggesting that the induction of both Th1 and Th2 cells is influenced by genetic background (Graham *et al.*, 1998). We observed a broadening to a mixed Th1/Th2 response in the draining lymph nodes 6 months after infection of BALB/c mice. Consistent with previous reports (Feltquate *et al.*, 1997; Bot *et al.*, 1997), we also demonstrated that immunization of BALB/c mice with HA DNA selectively primed systemic Th1 cells. Our studies extended these early reports by demonstrating that IFN- γ -secreting influenza virus-specific T cells were detected in the spleen with a single dose as low as 0.01 µg DNA. In addition, CD4⁺ T cell clones generated from the spleens of immunized mice also secreted significant amounts of IFN- γ in response to influenza virus, providing direct evidence for the generation of Th1 cells following immunization with HA DNA.

The potent induction of cell-mediated immunity may reflect endogenous expression of the antigenic protein either in muscle cells or professional APC after i.m. immunization. This may explain the induction of potent class I-restricted CTL with DNA vaccines (Donnelly, 1997), but not the selective induction of Th1 cells. Although there is no direct evidence of distinct processing pathways for Th2 cells, we have observed that vaccine delivery systems that allow protein antigens to enter the endogenous route of antigen processing also favour the induction of CD4⁺ Th1 cells (Moore *et al.*, 1995, 1999). It is possible that the IFN- γ produced by CD8⁺ T cells may exert a positive influence on Th1 induction, while suppressing the development of Th2 cells. In addition, certain bacterial immunostimulatory sequences, DNA sequences containing unmethylated CpG motifs, stimulate APC to express co-stimulatory molecules and, more importantly, to produce IL-12, which promotes the development of Th1 cells (Brazolot Millan *et al.*, 1998; Davis, 1998; Klinman, 1998; Krieg *et al.*, 1998; Chu *et al.*, 1997; Klinman *et al.*, 1997).

Despite a strong influenza virus-specific T cell response in the spleens 2 weeks following a single dose of HA DNA, multiple immunizations were required for persistence of the response. Furthermore, local T cell responses could not be detected in the local lymph nodes of mice immunized with HA DNA, whereas potent influenza virus-specific T cell responses were detected in the cervical lymph nodes from respiratory tract-infected mice. Recent studies have demonstrated a specific response in the draining lymph nodes following i.m. immunization with DNA (Torres *et al.*, 1997; Casares *et al.*, 1997). However, it has also been suggested that following i.m. injection, the functional DNA appears to move as free DNA through the blood to the spleen where professional APC initiate responses (Robinson & Torres, 1997). In a separate investigation, we found little or no response in the draining lymph nodes following immunization with HA DNA by four different routes (data not shown). However, a DNA construct encoding human immunodeficiency virus gp120 did elicit T cell responses in lymph nodes that drain the site of injection (J. Daly, P. A. Johnson and K. H. G. Mills, unpublished obser-

vations). It appears that, following i.m. immunization, the HA DNA preferentially migrates from the muscle to the spleen either as naked plasmid or within transfected APC. In support of this hypothesis, we detected plasmid DNA in the circulation and transfected APC in the spleen within 1 h of i.m. immunization with HA DNA (P. A. Johnson, M. A. Conway and K. H. G. Mills, unpublished observations). This novel finding that the site of immune activation may vary with different plasmid constructs has implications for the future design of vaccines based on this technology.

In contrast to the potent systemic cellular immune responses, only low concentrations of circulating anti-influenza virus antibodies were detected following a single immunization of HA DNA over a wide dose range. However, booster immunization with high doses of DNA did enhance serum antibody titres, and this was further augmented after a third immunization, but the levels remained below that observed following infection with influenza virus. Functional antibodies detected by HI assays were at or below the threshold of detection following two immunizations with 100 µg HA DNA, but were enhanced, compared with non-immunized mice, after influenza virus respiratory challenge. Although these data appear to contradict the conclusions on antibody induction in other reports on DNA vaccines encoding influenza virus HA, this can in part be explained on the experimental approach employed in the different studies. Previous studies have reported antibody responses after at least two and, in most cases, three immunizations. Our study, as well as that of Feltquate *et al.* (1997), showed that conventional influenza virus vaccines, which are administered in a single immunization and a single exposure to the live virus induced antibody levels equal to or greater than that observed with three doses of 100 µg HA DNA.

It has already been reported that immunization of mice with HA DNA can confer protective immunity (Fynan *et al.*, 1993; Ulmer *et al.*, 1994; Bot *et al.*, 1997; Deck *et al.*, 1997) and it has been concluded that protection is related to HI antibody induction (Deck *et al.*, 1997). However, it has also been suggested that memory B cells may be important in protection (Bot *et al.*, 1997) and our investigation is consistent with this conclusion. We observed that two doses of HA DNA vaccine provided excellent protection against challenge with live virus. Although HI antibodies were undetectable prior to challenge, the titres were higher in immunized mice compared with control mice after challenge, suggesting that the DNA vaccine had primed antigen-specific B cells *in vivo* which produced protective antibodies after challenge. Alternatively, the protection observed with the HA DNA vaccine may be mediated solely through the induction of cell-mediated immunity. This conclusion is consistent with reports which have demonstrated that mice that lack mature B cells and do not secrete immunoglobulin can clear an influenza virus infection from the respiratory tract (Topham *et al.*, 1996; Graham & Braciale, 1997).

The present investigation has demonstrated that the humoral immunity induced with a DNA vaccine based on the HA molecule of influenza virus does not approach that observed either by influenza virus respiratory infection or immunization with a whole influenza virus vaccine. Nevertheless, HA DNA was capable of conferring high levels of protective immunity against respiratory infection with A/Sichuan/2/87 influenza virus. Although the protection induced surpassed that conferred with a whole virus vaccine, booster immunization was necessary with the DNA vaccine. Our demonstration that HA DNA was capable of selectively inducing Th1 cells, which mimics that generated following virus infection, point to a role for cellular immunity in protection.

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