Naked RNA immunization with replicons derived from poliovirus and Semliki Forest virus genomes for the generation of a cytotoxic T cell response against the influenza A virus nucleoprotein

Marco Vignuzzi, Sylvie Gerbaud, Sylvie van der Werf and Nicolas Escriou

Unité de Génétique Moléculaire des Virus Respiratoires, URA 1966 CNRS, Institut Pasteur, 25 rue du Dr Roux, F-75724 Paris cedex 15, France

The potential of RNA-based vaccines was evaluated for the generation of a protective immune response in the mouse model of influenza type A virus infection using the internal nucleoprotein (NP) as antigen. This antigen is of particular interest, since it has the potential to elicit protective cytotoxic T lymphocytes (CTL) against heterologous strains of influenza A virus. In view of the short half-life of RNA, self-replicating RNAs or replicons of the positive-stranded genomes of Semliki Forest virus (SFV) and poliovirus were engineered to synthesize the influenza A virus NP in place of their structural proteins. NP expression was demonstrated by immunoprecipitation after transfection of cells with RNA from the SFV (rSFV-NP) and poliovirus (rΔP1-E-NP) genome-derived replicons transcribed in vitro. C57BL/6 mice were injected intramuscularly with these synthetic RNAs in naked form. Both replicons, rSFV-NP and rΔP1-E-NP, induced antibodies against the influenza virus NP, but only mice immunized with the rSFV-NP replicon developed a CTL response against the immunodominant H-2Dβ epitope NP366. Finally, the protective potential of the CTL response induced by immunization of mice with rSFV-NP RNA was demonstrated by the reduction of virus load in the lungs after challenge infection with mouse-adapted influenza A/PR/8/34 virus and was comparable to the protective potential of the response induced by plasmid DNA immunization. These results demonstrate that naked RNA immunization with self-replicating molecules can effectively induce both humoral and cellular immune responses and constitutes an alternative strategy to DNA immunization.

Introduction

The ongoing antigenic variability of influenza virus surface antigens remains a problem for the production of current commercial vaccines, which principally protect the host by inducing neutralizing antibodies to these antigens and are unable to induce strong mucosal or cellular immune responses. In particular, the cytotoxic T lymphocyte (CTL) response, which is directed against the nucleoprotein (NP) (Kees & Krammer, 1984; Parker & Gould, 1996; Yewdell et al., 1985) and other relatively conserved internal viral proteins implicated in virus replication and expressed early in infected cells, is essentially absent. In humans, the importance of this CTL response is not yet clearly defined, but studies suggest that it plays at least a co-operative role in virus clearance. In the absence of pre-existing strain-specific neutralizing antibody, protection against influenza virus infection in humans mediated predominantly by CTLs has been inferred (McMichael et al., 1983) and in immunosuppressed patients, cases of persistent influenza virus infections have been reported (McMinn et al., 1999; Rocha et al., 1991). Moreover, the importance of a CTL response is supported by the existence of CTL escape mutants (Voeten et al., 2000), indicating that during an epidemic season, a CTL response in humans is operating and applying selective pressure on circulating influenza virus strains. The role of the CTL response is more clearly demonstrated in the mouse model. For instance, it has been shown that reduction of virus load in the lungs and histological recovery correlate with specific CD8+ CTL responses (Allan et al., 1990; Topham & Doherty, 1998) and that adoptive transfer of CTL clones activated in vitro can provide protection against influenza virus in mice (Graham & Braciale, 1997; Taylor & Askonas, 1986).
Efforts to develop approaches that would fortify the immune response by inducing CTLs against the influenza virus NP, using systems that express whole NP, have received particular attention. Immunization of mice with influenza virus NP expressed by recombinant vaccinia virus (Endo et al., 1991; Lawson et al., 1994; Stitz et al., 1990), fowlpox virus (Webster et al., 1991), Semliki Forest virus (SFV) (Zhou et al., 1995) or Sindbis virus (SIN) (Tsui et al., 1998) has been shown to induce immune responses that, in some cases, confer at least partial protection. An alternative to these approaches, gene immunization, is based on the inoculation of DNA expression vectors that contain gene sequences encoding a foreign protein. Immunization with naked DNA vectors encoding the influenza virus NP has been shown to induce antibodies, cellular responses and protection against both homologous and cross-strain challenge infection by influenza A virus variants (Bot et al., 1996; Ulmer et al., 1993, 1998). The advantages of DNA immunization include ease of production, purification and administration of the vaccine and a long-lasting immunity. This long-lived immunity lasts for more than 1 year in the mouse model and is probably due to the long-term persistence and expression of the injected DNA (Wolff et al., 1992). For this very reason, some questions remain from a clinical standpoint as to the potential risk of DNA sequence integration into the host genome, although preliminary studies in animals have not shown integration events to lead to insertional mutagenesis (Nichols et al., 1995).

To avoid these potential hazards, RNA has been proposed as the expression vector; however, development of this approach faces new problems posed by the short intracellular half-life of RNA and its degradation by ubiquitous RNases. Initial attempts used mRNA to induce immune responses; the RNA was administered either intramuscularly (Conry et al., 1996; Ulmer et al., 1993, 1998). The advantages of DNA immunization include ease of production, purification and administration of the vaccine and a long-lasting immunity. This long-lived immunity lasts for more than 1 year in the mouse model and is probably due to the long-term persistence and expression of the injected DNA (Wolff et al., 1992). For this very reason, some questions remain from a clinical standpoint as to the potential risk of DNA sequence integration into the host genome, although preliminary studies in animals have not shown integration events to lead to insertional mutagenesis (Nichols et al., 1995).

Methods

- **Cells and viruses.** HeLa Young and BHK-21 cells were grown at 37 °C in complete Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mM sodium pyruvate, 4.5 mg/ml glucose, 100 U/ml penicillin and 100 µg/ml streptomycin and supplemented with 5% heat-inactivated foetal calf serum (FCS) (TechGen).

- **EL4 (mouse lymphoma, H-2d) and P815 (mice mastocytoma, H-2b) cells were maintained in complete RPMI 1640 medium containing 10 mM HEPES, 50 µM β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin and supplemented with 10% FCS.**

- Mouse-adapted influenza A/PR/8/34(ma) virus (H1N1) was derived from serial passage of pulmonary homogenates of infected mice to naive mice, as described previously (Oukka et al., 1996). Subsequent virus stocks were produced by a single allantoic passage in 11-day-old embryonated hen’s eggs, which did not affect virus pathogenicity for mice.

- **Construction of the pCI-NP expression vector.** Viral genomic RNA was extracted from lung homogenates of influenza A/PR/8/34(ma) virus-infected mice using 5 M guanidium isothiocyanate and phenol and reverse-transcribed into cDNA. Next, the sequences encoding influenza virus NP, including a Sall site before the initiation codon, were amplified by PCR using Pwo DNA polymerase (Roche). The resulting DNA fragment was cloned between the Sall and the Klenow-treated SalI sites of plasmid pTG186 (Kiery et al., 1986). Based on the consensus sequence, which can be obtained from the authors upon request, plasmid pTG-NP82 was reconstructed. A silent mutation was introduced at codon 107 (E, GAG → GAA), which destroyed an EcoRI site for the purpose of the subsequent cloning steps. An additional mutation at codon 277 (Pro → Ser) was present in all NP sequences used in this study; this mutation does not directly affect the major histocompatibility complex (MHC) class I-restricted immunodominant epitope of interest. NP366 (aa 366–374). The Sall–Sall fragment of pTG-NP82 was then inserted into the expression vector pCI-NP82 (Kieny et al., 1994; Stitz et al., 1993, 1998). The advantages of DNA immunization include ease of production, purification and administration of the vaccine and a long-lasting immunity. This long-lived immunity lasts for more than 1 year in the mouse model and is probably due to the long-term persistence and expression of the injected DNA (Wolff et al., 1992). For this very reason, some questions remain from a clinical standpoint as to the potential risk of DNA sequence integration into the host genome, although preliminary studies in animals have not shown integration events to lead to insertional mutagenesis (Nichols et al., 1995).

To avoid these potential hazards, RNA has been proposed as the expression vector; however, development of this approach faces new problems posed by the short intracellular half-life of RNA and its degradation by ubiquitous RNases. Initial attempts used mRNA to induce immune responses; the RNA was administered either intramuscularly (Conry et al., 1996) or by liposome-encapsulated injection to protect the RNA during administration (Martinon et al., 1993). To further improve delivery of these molecules, encapsidated self-replicating RNAs or replicons derived from the genomes of positive-stranded RNA viruses have been developed. In such systems, the structural protein sequences of the RNA genome are substituted for heterologous sequences that express a foreign protein, while the non-structural protein genes are retained; these replicons are capable of undergoing one round of replication.

The genomes of the alphaviruses SFV and SIN have been manipulated in this manner to allow the expression of foreign proteins (Frolov et al., 1996). Packaging of such replicons stabilizes the RNA molecules and injection of the resulting virus-like particles induces an array of immune responses against the given protein. Similarly, the capsid coding sequences of poliovirus positive-sense RNA have been deleted to permit the expression of foreign proteins (Choi et al., 1991; Percy et al., 1992) and, when packaged into virus-like particles, this replicon can induce immune responses after injection into mice transgenic for the poliovirus receptor (Moldoveanu et al., 1995; Porter et al., 1997).

In this study, we evaluated the ability of recombinant replicons to induce both humoral and cellular immune responses when injected in the form of naked RNA, arguing that packaging these vectors is unnecessary, since their replicative nature alleviates the need for large quantities of input RNA. In the case of recombinant SFV vectors encoding the haemagglutinin and NP molecules of influenza A virus, the injection of naked RNA has been found to induce specific antibodies (Dalemans et al., 1995; Zhou et al., 1994). We showed here that two recombinant replicons, one derived from the SFV genome and the other from the poliovirus genome, which both encode the internal influenza A virus NP protein in place of the structural proteins, can elicit humoral responses after injection of naked RNA. Moreover, injection of naked rSFV-NP replicon RNA was found to induce a CTL response that was specific for the immunodominant epitope of the influenza virus NP and to reduce virus load in the lungs of infected mice challenged with a mouse-adapted influenza virus to the same extent as that seen using the DNA immunization technique.
between the SalI and SmaI sites of expression plasmid pCI (Promega) to yield plasmid pCI-NP.

### Construction of plasmids for the in vitro transcription of recombinant replicons.

Plasmid pSFV-NP was constructed by the insertion of the NP gene, derived from pT7-PV1-52, into the SalI site of pSFV1, which contains a subgenomic cDNA of SFV downstream from the SP6 RNA polymerase promoter (Liljestrom & Garoff, 1990).

Plasmids containing poliovirus cDNAs with P1 deletions and substitutions were derived from plasmid pT7-PV1-52, which contains the full-length poliovirus type I (Mahoney) infectious cDNA downstream of the phage T7 promoter (Marc et al., 1989). Plasmid pΔP1-E contains a subgenomic poliovirus cDNA in which nt 746–3366 were replaced by a SacI–XhoI–SalI polylinker (GAGCCTGAGCTGTCGAC). In addition, the SalI site of pBR322 vector backbone of pΔP1-E was removed by digestion with EcoRI/EcoNI, Klenow-filling and ligation.

Plasmid pΔP1-E-NP was constructed by the in-frame insertion of sequences encoding the influenza A/PR/8/34 (ma) virus NP, derived from pT7-PV2 at digestion with Ncol/Mfel and treatment with the Klenow fragment, into the Klenow-filled Xhol site of pΔP1-E.

### In vitro transcription of plasmid DNA.

The poliovirus and SFV genome-derived plasmids were linearized with EcoRI and SphI, respectively, and transcribed using the RibomAX Large Scale RNA Production systems (Promega) (T7 polymerase for the former, SP6 polymerase for the latter), according to the manufacturer’s instructions. SFV transcripts were capped during transcription using 3 mM of cap analogue (Epigenic Technologies). For in vivo studies, reaction mixtures were treated with RQ1 DNase (15 U/µg DNA) (Promega) for 20 min at 37°C, extracted with phenol–chloroform, precipitated first in ammonium acetate–isopropanol alcohol, then in sodium acetate–isopropanol alcohol and resuspended in endotoxin-free PBS (Life Sciences). For in vitro studies, reaction mixtures were processed in the same way, but precipitated once with sodium acetate–isopropanol alcohol and resuspended in DH₂O.

### Rabbit reticulocyte lystate in vitro translation.

RNA transcribed in vitro was translated using the in vitro Flexi Reticulocyte Lysate system (Promega) supplemented with 0.8 mg/ml [35S]methionine (1000 Ci/mmole; Amersham), 80 mM KCl and 20% HeLa cell S10 extract (a kind gift from Lisette Cohen, Institut Pasteur, Paris, France). Reaction mixtures were incubated for 3 h at 30°C, treated with 100 µg/ml RNAse A in 10 mM EDTA for 15 min at 30°C and finally analysed by 12% SDS–PAGE and autoradiography on Kodak X-OMAT film.

### RNA transfection.

Transfection of RNA into HeLa or BHK-21 cells was performed by electroporation using an Easyjet plus electroporator (Equibio). Briefly, 5 × 10⁶ cells were trypsORIZED, washed twice, resuspended in 400 µl ice-cold PBS and electroporated in the presence of 16 µg RNA or DNA using a single pulse (240 V, 900 µF, maximum resistance) for HeLa cells or a double pulse (1200 V, 25 µF, 150 Ω then 150 V, 2100 µF, 99 Ω) for BHK-21 cells in 0.4 cm electrode gap cuvettes. Cells were immediately transferred into DMEM supplemented with 2% FCS and distributed into four 35 mm diameter tissue culture dishes.

### Analysis of RNA replication.

At different time intervals post-transfection, cytoplasmic RNA was prepared using standard procedures (Sambrook et al., 1989). After denaturation, RNA samples were spotted onto a nylon membrane (Hybond-N, Amersham), hybridized with a [32P]-labelled RNA probe complementary to nt 3471–4830 of poliovirus RNA, essentially as described previously (Marc et al., 1989), and exposed on a Storm phosphorimager (Molecular Dynamics).

### Analysis of influenza virus NP expression in RNA-transfected cells.

Influenza A/PR/8/34 virus-infected or RNA/DNA-transfected cells were metabolically labelled with [35S]methionine (50 µCi/ml) (1000 Ci/mmol; Amersham) for 2 h at times of peak expression. Next, cells were washed in PBS and lysed with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 0.5% protease inhibitor cocktail (Sigma). Cell extracts were then immunoprecipitated overnight at 4°C in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 0.1% deoxycholate, 0.1% SDS, 0.5% NP40 and 0.5% Protease Inhibitor cocktail) in the presence of protein A–Sepharose beads (Amersham) with rabbit antibodies raised against influenza A/PR/8/34 virus. The immunoprecipitates, washed in RIPA buffer and eluted in Laemmli sample buffer at 65°C, were analysed by SDS–PAGE and visualized by autoradiography on Kodak X-OMAT film.

### Immunizations.

Male C57/Bl6 mice (IFLA CREDO) 7 to 8 weeks of age were injected intramuscularly with 100 µl PBS (50 µl in each tibialis anterior muscle) containing 50 µg of plasmid DNA, 25 µg of poliovirus replicon RNA or 10 µg of capped SFV replicon RNA. Booster injections were administered at three or four week intervals. DNA used for injection was prepared using the NucleoBond Megaprep kit, followed by extraction steps with Triton X-114 and phenol–chloroform and tested for the absence of endotoxin (<100 U/mg), as measured with the QCL-1000 Endotoxin kit (BioWhittaker). RNA preparations were analysed before and after injection by agarose gel electrophoresis to verify the absence of degradation.

### Antibody titre.

Blood from mice was collected 1 week before immunization and 3 weeks after each injection. Serial dilutions of pooled serum samples were used to determine NP-specific antibody titres by ELISA using 0.5 µg of detergent-disrupted influenza A/PR/8/34 virus per well as antigen.

### Cytotoxicity assay.

Spleen cells were collected 3 weeks after the last immunization and seeded into upright T75 flasks at 2 × 10⁶ cells/ml in complete RPMI 1640 supplemented with 10% FCS, non-essential amino acids, 1 mM sodium pyruvate and 2.5% concanavalin A. They were then re-stimulated for 7 days with 10⁶ syngeneic spleen cells/ml, which had been pulsed for 3 h with 10 µM NP366 peptide (ASNENMETM, Neosystem), washed and irradiated (2500 rads). Cytotoxic activity of the re-stimulated effector cells was measured using a standard ⁵¹Cr-release cytotoxicity assay, essentially as described previously (Escricou et al., 1995). EL4 and P815 target cells were pulsed or not with NP366 peptide (10 µM) during ⁵¹Cr-labelling. Spontaneous and maximal release of radioactivity were determined by incubating cells in medium alone or in 1% Triton X-100, respectively. The percentage of specific ⁵¹Cr release was calculated as (experimental release — spontaneous release)/maximal release — spontaneous release) × 100.

### Challenge infection of mice with influenza A/PR/8/34 (ma) virus.

At 1 or 3 weeks after the third immunization, mice were lightly anaesthetized with 100 mg/kg ketamine (Merial) and challenged intranasally with 100 p.f.u. (0.1 LD₅₀) influenza A/PR/8/34 (ma) virus in 40 µl PBS. Mice were sacrificed 7 days after challenge infection and lung homogenates were prepared and titred for virus on MDCK cell monolayers in a standard plaque assay. Statistical analyses were performed on the log of the virus titres, measured for individual mice using the Student t-test with the assumptions used for small samples (normal distribution of the variable, same variance for the populations to be compared).

### Results

**Production of recombinant replicons derived from the SFV and poliovirus genomes**

For the production of SFV genome-derived replicons, the sequences encoding the mouse-adapted influenza A/PR/8/34
virus NP were inserted into the polylinker of the SFV expression vector pSFV1 (Liljestrom & Garoff, 1991) downstream of the 26S subgenomic promoter in place of sequences encoding the SFV structural proteins, as described in Methods (Fig. 1a). SP6 RNA polymerase transcription of pSFV-NP linearized with SpeI allowed the synthesis of capped positive-sense recombinant RNAs capable of initiating a replication cycle when transfected into cells (data not shown).

For the production of poliovirus genome-derived replicons, the plasmid vector pΔP1-E was first constructed, in which the capsid protein-coding sequences of P1 were substituted with a polylinker, such that sequences corresponding to an optimal cleavage site for the 2A protease, consisting of the nine C-terminal amino acids of VP1 and the first amino acid of 2A (as defined by Hellen et al., 1992), were maintained (Fig. 1b). The sequences coding for the influenza virus NP were then inserted
Naked RNA vaccines against influenza A virus

Fig. 2. In vitro translation and processing of the recombinant poliovirus polyprotein. Synthetic RNAs corresponding to the full-length poliovirus genome rT7-PV1-52 (lane 1), the subgenomic replicon rΔP1-E (lane 2) and the recombinant replicon rΔP1-E-NP (lane 3) were translated in vitro in rabbit reticulocyte lysates supplemented with HeLa S10 cell extract (see Methods). Translation products were analysed by SDS–PAGE and visualized by autoradiography. Poliovirus protein precursors as well as some of their major cleavage products are indicated on the left. Molecular masses are indicated on the right next to the molecular mass marker (lane 4). The influenza virus NP encoded by the recombinant replicon is indicated by an arrow (lane 3).

upstream of this reconstituted cleavage site and in-frame with the rest of the sequences encoding the poliovirus polyprotein to yield plasmid pΔP1-E-NP (Fig. 1b).

The recombinant RNAs, rΔP1-E and rΔP1-E-NP, derived from in vitro transcription with T7 RNA polymerase of pΔP1-E and pΔP1-E-NP-linearized DNAs were translated in vitro in rabbit reticulocyte lysates. As shown in Fig. 2, the replicon-encoded polyproteins were properly cleaved to express the non-structural proteins necessary for RNA amplification (as shown by the end products of cleavage), such as the 2A, 3CD, 2BC and 2C proteins. In particular, correct in cis cleavage of the reconstituted VP1–2A site by the 2A protease was observed. For the subgenomic replicon rΔP1-E, however, this cleaved product migrated as a doublet, which could be explained as a mixture of properly cleaved product and uncleaved VP1*–2A fusion protein containing the 13 amino acid residues of the reconstituted cleavage site and polylinker. It could be argued that the very short stretch of amino acids before the cleavage site was too short to permit 100% cleavage. For the recombinant replicon rΔP1-E-NP, such uncleaved product, which would have appeared as a 72 kDa NP–VP1*–2A fusion protein, was barely visible, suggesting that the addition of the NP sequences alleviated the spatial restriction seen in the previous case. Expression of the properly cleaved NP–VP1* fusion protein was therefore revealed by the presence of a band with the expected molecular mass of 56 kDa (Fig. 2).

Replicative characteristics of poliovirus genome-derived replicons rΔP1-E and rΔP1-E-NP

Since the influenza virus NP has been shown to associate non-specifically with RNAs (Kingsbury et al., 1987; Yamanaka et al., 1990), an interaction with the poliovirus RNA could hypothetically affect overall replication efficiency. Therefore, synthetic RNA transcripts of rΔP1-E and rΔP1-E-NP, as well as parental rT7-PV1-52, were transfected into HeLa cells and total cytoplasmic RNA was extracted at various times post-transfection. Hybridization after slot blotting using a radiolabelled riboprobe complementary to nt 3417–4830 of poliovirus RNA revealed efficient replication of all RNAs (Fig. 3). Similar results were obtained after transfection of L929 murine cells (data not shown). In our current studies, cells were transfected by electroporation, which was more efficient than the classic DEAE-dextran technique (> 90% transfection efficiency). Under these conditions, all three RNA species induced cytopathic effects, regardless of the presence or absence of capsid proteins, and resulted in the general destruction of the cell monolayer (data not shown). Taken together, these results illustrated that the insertion of sequences encoding the influenza virus NP had no negative effects on RNA replication.
Expression of influenza virus NP by recombinant SFV and poliovirus genome-derived replicons

Nucleoprotein expression was analysed by immunoprecipitation (using antibodies against influenza A/PR/8/34 virus) of cytoplasmic extracts from cells transfected with SFV or poliovirus genome-derived replicons or plasmid DNA, or infected with influenza A/PR/8/34 virus, as described in Methods. Cells were pulse-labelled with $^{35}$S-methionine for 2 h prior to harvest at times of peak expression, as determined in preliminary experiments. As shown in Fig. 4(a), a protein with an apparent molecular mass of 54 kDa was specifically immunoprecipitated from extracts of cells transfected with rSFV-NP (Fig. 4a, lane 2) and co-migrated with the NP produced by 293 cells infected with influenza A/PR/8/34 virus (Fig. 4a, lane 1). No immunoreactive proteins could be detected from the mock-transfected cells or from cells transfected with replicon RNA derived from the empty vector pSFV1.

Similarly, transfection of HeLa cells with the recombinant replicon rΔP1-E-NP (Fig. 4b, lane 5) resulted in high levels of NP-related protein expression. The majority of NP expressed by the poliovirus genome-derived replicon migrated slightly slower than the native form of NP expressed by pCI-NP DNA-transfected cells (Fig. 4b, lane 3). This difference in molecular mass accounted for the additional amino acid residues of the NP–VP1* fusion protein and was consistent with the fact that proteolytic processing of the poliovirus polyprotein occurred at the reconstituted VP1*–2A cleavage site. Interestingly, the presence of bands of a smaller molecular mass (Fig. 4b, lane 5) suggested that the NP produced may have been cleaved, in part, by one of the poliovirus proteases (2A or 3C). Examination of the NP sequences for such cleavage sites (Y–G for 2A protease or Q–G for 3C protease) confirmed this possibility; nevertheless, these potential cleavage by-products remained a minority. Thus, both SFV and poliovirus genome-derived replicon replicons were shown to direct efficient expression of the influenza virus NP in transfected cells.

Induction of NP-specific antibodies after immunization with recombinant rSFV-NP and rΔP1-E-NP replicons

In order to establish the feasibility of using naked replicon injection to elicit a heterospecific CTL response, we first determined the conditions of RNA injection required to obtain an antibody response comparable to that observed after one injection of plasmid DNA, which in published literature was shown to be sufficient to induce a CTL response (Ulmer et al., 1993). To this end, naked RNA or DNA was administered
intramuscularly at monthly intervals (one, two or three times) to C57BL/6 mice and the specific anti-NP antibody response was examined by ELISA, as described in Methods.

As shown in Fig. 5, one injection of 10 µg of naked rSFV-NP RNA induced serum antibodies against influenza virus NP, although the specific ELISA titres were reproducibly lower than those obtained after one injection of 50 µg of pCI-NP DNA. At 3 weeks after a booster injection, a strong increase in the anti-NP antibodies in the sera of rSFV-NP-injected mice was observed, reaching a level comparable to that obtained after two injections of pCI-NP DNA. Three injections of 25 µg of naked rAP1-E-NP RNA proved necessary to raise NP-specific antibodies to levels equal to or slightly higher than those achieved by one injection of plasmid pCI-NP DNA. Thus, these findings showed that poliovirus replicons were immunogenic when injected as naked RNA, although less so when compared to the SFV replicons.

**Induction of an NP-specific CTL response after injection of recombinant SFV replicon as naked RNA**

In order to evaluate whether recombinant rSFV-NP and rAP1-E-NP injected as naked RNA were able to induce specific CTLs directed against the dominant H-2D^d^-restricted epitope NP366, C57BL/6 mice were injected intramuscularly either once or twice with 10 µg of rSFV-NP RNA, three times with 25 µg of rAP1-E-NP RNA or once with 50 µg of pCI-NP DNA as a positive control. This immunization schedule was defined according to the previously determined anti-NP antibody response and based on the observation that one injection of plasmid DNA was sufficient to induce a detectable NP-specific CTL response at levels just below those of mice having recovered from sub-lethal influenza A/PR/8/34 (ma) virus infection (data not shown). Splenocytes from immunized mice were harvested 3 weeks after the last injection, stimulated in vitro with peptide NP366 and tested for cytolysis activity 7 days later using a classic chromium-release assay against syngeneic EL4 target cells either loaded with peptide NP366 or not (see Methods for details). The percentage of specific lysis is shown at various effector:target ratios. Solid lines indicate DNA or RNA encoding the influenza virus NP, dashed lines indicate DNA or RNA not encoding the influenza virus NP. Data from one experiment representative of three are shown.

---

**Fig. 5.** Anti-NP antibody response to immunization with naked RNA. C57BL/6 mice were immunized two or three times at four week intervals with 50 µg of plasmid DNA (pCI or pCI-NP), 10 µg of SFV genome-derived replicon RNA (rSFV or rSFV-NP) or 25 µg of poliovirus genome-derived replicon RNA (rAP1-E or rAP1-E-NP). Sera were collected before (0) the first (1), second (2) or third (3) injection or after. Titres are represented as the reciprocal of the highest dilution of pooled sera for a given group of five or six mice giving an optical density measurement at 450 nm equal to three times that of background levels in a direct ELISA test using purified split influenza A/PR/8/34 virions as antigen. Data from one experiment representative of two are shown.

**Fig. 6.** Induction of NP-specific CTL response by naked RNA immunization. Groups of four C57BL/6 mice were immunized at three week intervals with the following vaccination protocols: one injection of 50 µg of pCI (○) or pCI-NP (●) DNA, one injection of 10 µg of rSFV (□) or rSFV-NP (■) RNA, two injections of 10 µg of rSFV (●) or rSFV-NP (■) RNA, or three injections of 25 µg of rAP1-E (△) or rAP1-E-NP (▲) RNA. Splenocytes were collected 3 weeks after the last immunization and stimulated in vitro for 1 week with irradiated syngeneic splenocytes loaded with peptide NP366. These cells were used as effector cells in a classic chromium-release assay against syngeneic EL4 target cells either loaded with peptide NP366 (a) or not (b) (see Methods for details). The percentage of specific lysis is shown at various effector:target ratios. Solid lines indicate DNA or RNA encoding the influenza virus NP, dashed lines indicate DNA or RNA not encoding the influenza virus NP. Data from one experiment representative of three are shown.
sequences (Fig. 6, hatched lines), nor was any lysis detected on syngeneic targets not charged with peptide (Fig. 6b). Finally, for all effector populations, lysis of allogenic P815 target cells (H-2b) remained at background levels regardless of whether or not they were incubated with peptide (data not shown), indicating that the cytolytic activity was H-2-restricted and was thus likely to derive from MHC-I-restricted CD8+ T cell effector cells. In contrast, splenocytes from rAP1-E-NP-injected mice failed to lyse either of the EL4 targets, despite maintaining the same immunization schedule (i.e. three injections of 25 µg of RNA) that was found to induce NP-specific antibodies. Three injections of 100 µg of rAP1-E-NP RNA also failed to induce a detectable CTL response (data not shown).

**Protective immunity in vivo**

Previous studies have found that CTLs directed against the influenza virus NP and induced by naked DNA immunization were protective against a challenge infection with influenza virus (Bot et al., 1996; Ulmer et al., 1993). To determine whether the CTL responses induced by naked RNA immunization with SFV genome-derived replicons could also contribute to protection by reducing virus titre in the lungs, C57BL/6 mice were injected three times with either rSFV-NP RNA or pCI-NP DNA and challenged with a sub-lethal dose of the homologous influenza A/PR/8/34 (ma) virus (10^2 p.f.u.) 1 or 3 weeks thereafter. Virus load in the lungs was evaluated 7 days after challenge by plaque assay on MDCK cells. In all six studies, such as the one shown in Fig. 7, immunization with either naked SFV RNA or naked DNA encoding the influenza virus NP lowered virus lung titres by one to two logs. The observed reduction of virus loads after injection of each NP-coding molecule as compared to the respective blank vector was found to be significant by the Student t-test (P < 0.001). It is worth noting that although the drop in virus titre was moderate, which would correlate with the high virulence of our mouse-adapted virus strain (LD50 was 10^2 p.f.u. for C57BL/6 mice), the reduction in titre achieved with naked RNA immunization was as efficient as that obtained with naked DNA immunization.

**Discussion**

The principal aim of this study was to explore and evaluate the feasibility of adapting standard naked DNA immunization protocols for naked RNA immunization using positive-stranded RNA virus genomes that have structural protein-coding sequence deletions and are capable of a single round of replication, as vaccine vectors against influenza virus.

The genome of poliovirus is a good candidate for naked RNA immunization because encapsidated subgenomic poliovirus replicons expressing viral, bacterial and tumour antigen peptides are being developed as prototypes for an AIDS vaccine that elicits humoral and cellular immune responses in non-human primates (Crotty et al., 1999). We showed here that when administered as naked RNA, a recombinant poliovirus replicon, rAP1-E-NP, engineered to express the influenza virus NP was able to induce NP-specific antibodies in mice after three injections, which suggested that the replicon did replicate at least to some extent in vivo. This was further supported by the fact that a neutralizing antibody response against the poliovirus capsid proteins was elicited after injection with the full-length poliovirus genome in the form of naked RNA into C57BL/6 mice, which do not express the poliovirus receptor (data not shown). However, no CTL response was detected towards the immunodominant epitope of the influenza virus NP in C57BL/6 mice injected with rAP1-E-NP.

Firstly, this lack of CTL response could be due to the possibility that the poliovirus genome replicates poorly in...
murine cells in vivo, particularly in myocytes, if these are indeed the site of protein expression, after intramuscular naked nucleic acid immunization (Corr et al., 1999). Secondly, there is question as to whether or not the MHC-I-restricted NP366 peptide was properly presented by r∆P1-E-NP-transfected cells. It was shown that the protein secretion pathway is altered by poliovirus proteins 2B and 3A and recent evidence by Kirkegaard and colleagues has shown that 3A protein expression was responsible for strong inhibition of MHC-I-dependent antigen presentation in poliovirus-infected cells (Deitz et al., 2000; Doedens & Kirkegaard, 1995).

However, Mandl et al. (1998) have demonstrated that a CTL response can be induced in mice transgenic for the poliovirus receptor by a recombinant poliovirus containing sequences that encode the H-23-restricted CTL epitope of chicken ovalbumin. In this situation, the genome of the virus enters cells by infection rather than by uptake of injected naked RNA and multiple cycles of replication and re-infection of neighbouring cells were possible. Therefore, the inability to detect a CTL response may be due to differences in the number or type of cells receiving replicon RNA, levels of expression or route of immunization. In vitro antigen presentation assays are being developed to determine if NP peptide presentation does indeed occur in cells replicating the r∆P1-E-NP recombinant replicon.

Alternatively, the CTL response induced by the poliovirus recombinant replicon might be skewed towards another peptide, e.g. one of the poliovirus non-structural proteins that could be hypothetically dominant to the NP peptide, in murine cells. Indeed occur in cells replicating the rSFV-NP recombinant replicon.

Finally, it is well accepted that the strong immunogenicity of DNA vaccines is a consequence of long-lasting antigen expression together with the adjuvant effect of short non-coding immunostimulatory sequences centered around unmethylated Cpg motifs in the plasmid DNA backbone itself (Sato et al., 1996). In the case of the highly effective naked RNA immunization with SFV replicons, it has been suggested that virus-like RNA replication could provide an adjuvant effect by triggering a series of ‘danger signals’ which in turn would activate the innate immune system of the host (Leitner et al., 1999). It is tempting to speculate that poliovirus could block, or at least inhibit, this process by an as yet unknown mechanism, thus resulting in a poorly immunogenic replicon that is able to avoid detection by the host immune system.

Injection of naked RNA from the recombinant rSFV-NP replicon designed to express the influenza virus NP proved to be effective at inducing anti-NP antibodies after two injections; antibody titres were at levels comparable to those obtained by one injection of NP-encoding plasmid DNA. Induction of antibodies specific for an influenza virus protein by this type of vector when administered as naked RNA has already been described (Dalemans et al., 1995; Zhou et al., 1994). Recently, using LacZ as a model tumour antigen for cancer therapy, Ying et al. (1999) have shown that injection of naked recombinant SFV RNA can elicit an antibody response, activate CD8+ T cells to release interferon-γ and protect from tumour challenge. Here, we showed that the injection of naked recombinant rSFV-NP RNA could induce, in addition to antibodies, CTLs that target the NP366 epitope in a response that was found to be comparable to that induced by plasmid DNA. Furthermore, we showed that in a virus challenge model using a mouse-adapted strain of influenza virus, RNA immunization worked as well as DNA immunization in the degree of protection conferred, as measured by reduction of virus load in the lungs. It will be interesting to determine whether even greater protection is conferred after challenge with less virulent strains of mouse-adapted influenza virus and to evaluate the role of anti-NP CTLs induced by naked RNA immunization against a heterologous influenza virus of the same or a different subtype.

This work was supported in part by the Ministère de l’Education Nationale, de la Recherche et de la Technologie (EA 302). We would like to thank Ida Rijks for the production of influenza A/PR/8/34(ma) virus, Annette Martin for helpful suggestions and discussions, Lisette Cohen and Katherine Kean for valuable advice on in vitro translations and Nadia Naffakh for critical reading of the paper.

References


Naked RNA vaccines against influenza A virus


Received 10 January 2001; Accepted 12 March 2001