JOURNAL OF VIROLOGY, Dec. 2004, p. 13037–13045 Vol. 78, No. 23
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.23.13037–13045.2004

Immunogenicity and Protection Efficacy of Replication-Deficient Influenza A Viruses with Altered NS1 Genes

Boris Ferko,*† Jana Stasakova,† Julia Romanova, Christian Kittel, Sabine Sereinig, Hermann Katinger, and Andrej Egorov

Institute of Applied Microbiology, Vienna, Austria

Received 10 May 2004/Accepted 14 July 2004

We explored the immunogenic properties of influenza A viruses with altered NS1 genes (NS1 mutant viruses). NS1 mutant viruses expressing NS1 proteins with an impaired RNA-binding function or insertion of a longer foreign sequence did not replicate in murine lungs but still were capable of inducing a Th1-type immune response resulting in significant titers of virus-specific serum and mucosal immunoglobulin G2 (IgG2) and IgA, but with lower titers of IgG1. In contrast, replicating viruses elicited high titers of serum and mucosal IgG1 but less serum IgA. Replication-deficient NS1 mutant viruses induced a rapid local release of proinflammatory cytokines such as interleukin-1β (IL-1β) and IL-6. Moreover, these viruses also elicited markedly higher levels of IFN-α/β in serum than the wild-type virus. Comparable numbers of virus-specific primary $CD8^+$ T cells were determined in all of the groups of immunized mice. The most rapid onset of the recall $CD8^+$-T-cell response upon the wild-type virus challenge was detected in mice primed with NS1 mutant viruses eliciting high levels of cytokines. It is noteworthy that there was one NS1 mutant virus encoding NS1 protein with a deletion of 40 amino acids predominantly in the RNA-binding domain that induced the highest levels of IFN-α/β, IL-6 and IL-1β after infection. Mice that were immunized with this virus were completely protected from the challenge infection. These findings indicate that a targeted modification of the RNA-binding domain of the NS1 protein is a valuable technique to generate replication-deficient, but immunogenic influenza virus vaccines.

Human influenza, caused by influenza A and B viruses, is a highly infectious acute respiratory disease spreading around the world in seasonal epidemics resulting in high morbidity and significant mortality. Influenza viruses have a segmented negative-strand RNA genome that encodes 10 or 11 proteins depending on the strain. The exchange of individual genome segments between different virus subtypes during a mixed infection (genetic reassortment) and the relatively rapid accumulation of point mutations in virus surface glycoproteins due to the high mutation rate of the RNA genome are the main reasons for antigenic "shift" and "drift" variations of emerging viruses escaping the preexisting immunity of the human population (53–55). Attempts to develop a vaccine inducing a long-lasting protection against influenza have thus far been unsuccessful. In order to protect humans against circulating epidemic influenza virus strains, vaccine producers have to generate vaccines containing actualized influenza A (H1N1 and H3N2) and B virus components almost annually (19).

The vaccination at present is accomplished with the commercially available chemically inactivated (killed) or live cold-adapted (ca) attenuated influenza virus vaccines (10, 28, 36). The vaccine efficacy for both types of vaccines has been reported to be comparable in adults. However, live vaccines, apart from the easy and painless nasal administration induce not only the homotypic serum antibodies but also mucosal antibodies and cross-reactive cell mediated immune responses, resulting in a more protective, broader, and longer-lasting immunity than those induced by an inactivated vaccine (3, 4, 6, 11, 20).

* Corresponding author. Mailing address: Institute of Applied Microbiology, Muthgasse 18B, A-1190 Vienna, Austria. Phone: 43/1-36006-6594. Fax: 43/1-36006-1249. E-mail: b.ferko@iam.boku.ac.at.

† B.F. and J.S. contributed equally to this study.

The concept of a live attenuated vaccine includes the generation of a genetically stable "master strain" by an adaptation of a suitable wild-type (w.t.) influenza virus strain (usually of the H2N2 subtype) to grow at 25°C (cold adaptation) in embryonated chicken eggs. Vaccine strains are virus strains selected in embryonated eggs upon mixed infection with the master strain providing six internal genes (containing several point mutations responsible for attenuation and cold-adapted phenotype) and the epidemic virus strain donating the two remaining genes coding for viral surface antigens: hemagglutinin (HA) and neuraminidase (NA) (27, 34).

Although the cold-adapted live influenza virus vaccines are considered sufficiently safe, the exact genetic and molecular mechanisms of attenuation are not completely understood. It is claimed that the nature of the safety of cold-adapted influenza vaccines is based on a large number of point mutations distributed across the internal gene segments. However, only a small number of mapped mutations localized in the polymerase genes are responsible for the attenuation of cold-adapted virus strains that are unable to replicate at normal body temperature (21, 22). In fact, the genetic stability of live cold-adapted vaccine strains or other promising temperature-sensitive (*ts*) vaccine candidate strains are often questioned since viruses reisolated from vaccinated hosts reveal additional point mutations that might eventually function as "suppressor" mutations even causing enhanced replication properties and a possible loss of the *ts* phenotype of the revertant virus (22, 39, 47, 48). Moreover, the safety of cold-adapted vaccines has never been proven when it is reassorted with a new highly virulent influenza virus subtype such as H5N1 viruses.

Reflecting the potential risks of the cold-adapted live attenuated influenza virus vaccines, an alternative approach to the

design of live virus vaccines based on the reverse genetics system with Vero cells in order to obtain influenza viruses containing modifications in the NS1 gene has been developed (14). Importantly, viruses encoding altered NS1 proteins (NS1 mutant viruses) were attenuated in mice due to the impairment of the NS1 protein-mediated alpha/beta interferon (IFN-α/β) antagonist function (14, 17). In contrast to live cold-adapted strains containing several point mutations responsible for attenuation, NS1 mutant viruses are genetically stable. Obviously, a large deletion or lack of the entire NS1 cistron (delNS1 virus) cannot be compensated for by any suppressor mutation. A targeted engineering of the NS1 gene could, theoretically, provide a completely attenuated phenotype to any epidemic strain in IFN-competent hosts.

Previously, few NS1 mutant viruses have been shown to induce significant humoral and cellular immune responses in mice, and this approach is considered to be a powerful tool for the development of a new generation of live attenuated influenza virus vaccines (45). In the present study, we address the question of how the immunogenicity of the NS1 mutant viruses is influenced by the specific genetic alteration of the NS1 gene. For this purpose, we investigated the ability of a set of influenza viruses encoding various modifications (including deletions or insertion of foreign sequences) in the functional domains of the NS1 protein to stimulate an early release of proinflammatory cytokines and the generation of adaptive immune responses in an in vivo mouse model. Here, we demonstrate that mice immunized with replication-deficient influenza NS1 mutant viruses that induced high levels of IFN-α/β, interleukin-1β (IL-1β), and IL-6 also elicited significant humoral and cellular immune responses protecting immunized mice from the homotypic pathogenic w.t. virus challenge. Our results indicate that targeted alterations, especially those of the RNA-binding and dimerization domain of the NS1 protein, are a valuable tool for generating influenza virus vaccine strains with unique properties in terms of attenuation, genetic stability, immunogenicity, and safety.

MATERIALS AND METHODS

Cells. Vero cells (ATCC CCL-81) were used for transfection experiments, for selection and plaque purification of the rescued transfectant viruses, and for virus titrations. Vero cells were adapted to and further cultivated in Dulbecco modified Eagle medium (DMEM)–Ham F-12 (Biochrom F4815) with 4 mM L-glutamine and protein-free supplement (proprietary formulation; Polymun Scientific GmbH, Vienna, Austria). In a series of experiments, 30 U of human IFN-α (human leukocyte-derived NIBSC First International Standard 1999)/ml were added to the cell culture medium.

Generation of NS1 mutant viruses. NS1 mutant viruses were generated by ribonucleoprotein (RNP) transfection (33). The RNP complexes were formed by mixing the purified influenza virus polymerase preparations, and the RNA was transcribed by the T3 RNA polymerase from linearized NS1 plasmids containing the appropriate mutation, e.g., the introduction of stop codons at different positions or insertion of the nucleotides 210 to 618 of the Nef gene of human immunodeficiency virus type 1/NL4-3 between nucleotides 400 and 401 of the PR8 w.t. NS gene (14, 16). RNP complexes were transfected into Vero cells previously infected with an influenza helper virus, followed by incubation for 18 h at 37°C, and the supernatants containing transfectant viruses were passaged and then plaque purified in Vero cells. The isolated viruses were analyzed by reverse transcription-PCR (RT-PCR) utilizing two primers, one complementary to positions 1 to 21 at the 3′ noncoding end and the other containing the last 38 nucleotides of the 5′ noncoding end of the A/PR/8/33 (PR8 w.t.) NS gene. The PR/NS1del40-80 virus is a spontaneous transfectant virus encoding an in-frame deletion of the amino acid residues 40 to 80 of the NS1 protein, as confirmed by the RT-PCR during the selection process of the NS1 mutant viruses. Virus infectious titers were determined in Vero cells by plaque assay in medium containing DMEM–Ham F-12, 4 mM L-glutamine, 5 μg of trypsin/ml, 0.01% DEAE-dextran (Pharmacia), and 0.6% agar (Sigma) and expressed in PFU/ml.

Mice and immunizations. Female B2D6F1 mice that were at least 8 weeks old (Charles River Laboratories, Wiga, Germany) were kept under conventional conditions and were provided with a standard diet and water ad libitum. Groups of randomly divided mice were immunized intranasally (i.n.) in the absence of ether anesthesia with the NS1 mutant viruses (10^6 PFU/mouse) and PR8 w.t. virus (2.5×10^3 to 3×10^3 PFU/mouse) or intraperitoneally (i.p.) with 0.5 ml of viral suspensions (4×10^6 to 8×10^6 PFU/mouse). The control groups of mice were immunized with phosphate-buffered saline (PBS) (naive mice) or UV light-induced inactivated undiluted stock of the PR8 w.t. virus (8×10^7 PFU/ml). UV virus inactivation was performed by exposing the viral fluid (0.5 ml/6-cm petri dish) to 15-W UV light at a distance of 25 cm for a duration of 90 s. Several mice primed with NS1 mutant viruses were challenged i.n. 4 months later with 1.2×10^6 PFU/mouse (this dose approximately corresponds to a 1,000 50% lethal dose [LD_{50}] dose).

Viral replication in murine respiratory tracts. To determine viral replication in mouse respiratory tracts, mice (three mice/group) were immunized in the presence of ether anesthesia (NS1 mutant viruses,10^6 PFU/mouse; PR8 w.t. virus, 10^3 PFU/mouse) and were sacrificed at days 2, 4, and 6 after i.n. inoculation of the viral stocks. Lungs were aseptically removed, pooled, and 10% (wt/vol) tissue suspension was prepared. The suspensions were centrifuged at $3,000 \times g$ for 5 min, and the supernatants were assayed for infectious viral particles in plaque assays with Vero cells (15).

Colorimetric detection of a dsRNA-NS1 protein complex. A total of 1 μg of biotinylated double-stranded RNA (dsRNA; 400 bp, kindly donated by G. Fabini, Glycobiology Division, Institut fur Chemie, Universität fur Bodenkultur, Vienna, Austria) per ml of 5× saline sodium citrate buffer (750 mM NaCl, 75 mM sodium citrate) containing 0.05% (vol/vol) Tween 20 (100 μl/well) was added overnight to streptavidin immobilizer plates (Exiqon). Vero cells were infected with viruses at a multiplicity of infection of 2 and lysed at 12 h postinfection (p.i.) with cell extraction solution (Pierce). Since the quantities of NS1 proteins in cell lysates were found to be comparable (Western blot analysis with mouse polyclonal serum specifically recognizing N-terminal 25 amino acid residues of the NS1 proteins), the cell extracts were serially diluted in dilution buffer (10 mM Tris-HCl [pH 7.9], 150 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol). The NS1 protein bound to the biotinylated dsRNA was detected by mouse polyclonal anti-NS1 specific serum (1:1,000) and then with goat anti-mouse IgG-conjugated with horseradish peroxidase-antibody conjugate (Sigma). −3,3,5,5′-tetramethylbenzidine (TMB ONE; Fermentas) was used as a substrate. The reaction was stopped with 2.5 M H_2SO_4, and the absorbance was measured (wavelength, 450 nm).

Specimen collection. Blood was collected from the murine retroorbital venous plexus 3 weeks after the immunization, and the sera were prepared, eventually pooled prior to assay, or stored at −20°C. To obtain murine nasal secretions, salivation was induced by the i.p. injection of 0.1 mg of pilocarpine-HCl (Sigma). Concurrent with the salivation, small amounts of nasal secretions in the nasal openings were observed and immediately absorbed with the aid of sterile wicks (Spectrum Laboratories, Inc., Houston, Tex.), which were then placed into 50 μl of ice-cold sterile PBS supplemented with protease inhibitors (Roche) for 4 h. After this elution step, group-specific pools were prepared, vigorously vortexed, and centrifuged at $15,000 \times g$ for 5 min (15). In order to exclude the effect of residual virus and other cytokines on cells utilized in the IFN-α/β bioassay, mucosal samples were adjusted to pH 2 with 0.5 N HCl, followed by incubation for 30 min at 4°C, and the neutral pH was again restored with 0.5 N NaOH prior to assay or storage at −20°C.

ELISA. A modified enzyme-linked immunosorbent assay (ELISA) protocol was performed (15). Briefly, sucrose purified and UV-inactivated PR8 w.t. virus (adjusted to 200 hemagglutination units/well in carbonate buffer [pH 9.6]) was used as a coating antigen. Serial dilutions of pooled sera in PBS containing 1% skim milk (Serva) were added to the coated plates, and the mixtures incubated for 1.5 h at room temperature. Bound antibodies were detected with goat anti-mouse IgG1, IgG2a, or IgA conjugated with horseradish peroxidase (Zymed). Plates were stained with TMB as a substrate, and the absorbance was measured (wavelength, 450 nm). The cutoff value was defined as the mean value of absorption of negative control sera plus two standard deviations. The results of a representative assay from a multiple series of experiments are shown.

Cytokine ELISAs. The amounts of IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in mouse sera and mucosal secretions were determined by means of specific quantitative sandwich ELISA kits according to the manufacturer's manuals (R&D). The results of a representative experiment from a multiple series of experiments are shown.

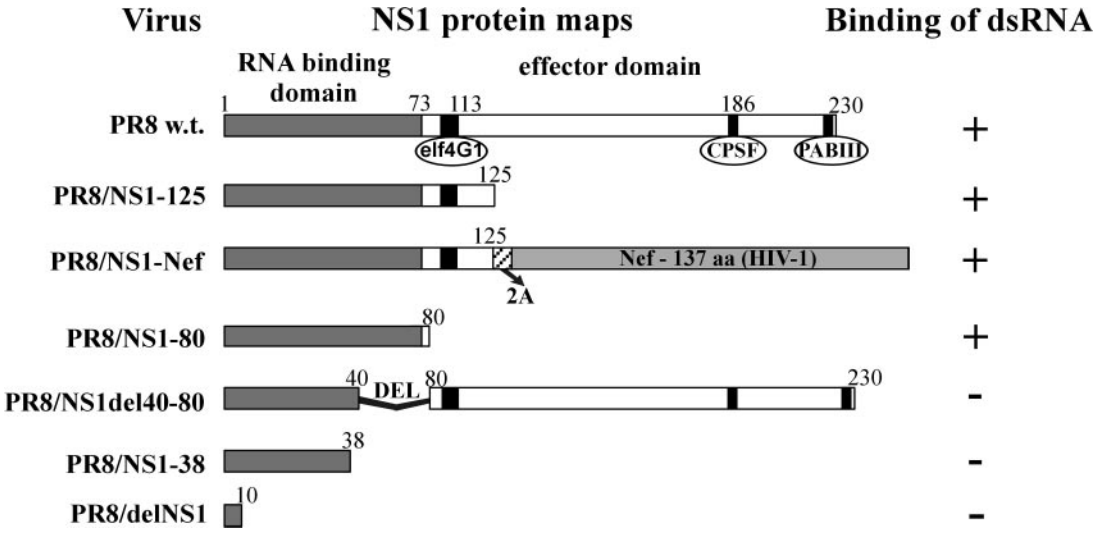

FIG. 1. Schematic maps of modified NS1 proteins and their ability to bind dsRNA. NS1 proteins expressed by the PR8 w.t. virus and NS1 mutant viruses are outlined schematically. The amino acid residue numbers are denoted for better orientation. The RBD (gray) and the effector domain (white) of the NS1 protein are indicated. The positions of sites involved in interactions with cellular proteins (black bars), e.g., the eukaryotic initiation factor 4GI (eIF4GI), the cleavage and polyadenylation specificity factor (CPSF), and the poly(A)-binding protein II are shown (PABIII) (1, 30). The self-cleaving 2A sequence (2A; hatched bar) and the Nef derived sequence (light gray bar) are depicted in the recombinant NS1-Nef protein (16). The deletion of 40 amino acids in the NS1del40-80 protein is outlined as beveled line. The capacity of the NS1 proteins to bind dsRNA is summarized to the right. NS1 proteins (present in lysates of infected cells) marked as "+" were capable of binding dsRNA in the same range even at high lysate dilutions (1:1,000; optical density of >0.2), whereas the "−" marks mean that NS1 proteins were unable to bind dsRNA, since only the background absorbance signals (optical density of <0.05) were determined at initial 1:2 lysate dilutions by the dsRNA-NS1 binding colorimetric assay described in Materials and Methods.

Isolation of lymphocyte cell populations. Spleens and lymph nodes draining the murine respiratory tracts (mediastinal, tracheobronchial lymph nodes) of immunized mice (three mice per group) were collected at day 10 after the immunization (spleens) or 60 h after challenge with the pathogenic PR8 w.t. virus (lymph nodes). Spleens and draining lymph nodes were mechanically dissociated into single cell suspensions by means of cell strainers (Falcon). The erythrocytes present in the cell suspensions were lysed with Tris-buffered ammonium chloride, washed several times, and finally resuspended in DMEM containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), IL-2 (30 U/ml), and 50 μM 2-mercaptoethanol.

Cell separation. Single cell suspensions were depleted in some experiments of $CD8^+$ cells utilizing saturating concentrations of biotinylated rat anti-mouse CD8 MAb (5H10-1; BD Pharmingen). Cells were then incubated with magnetic microbeads conjugated to streptavidin by a magnetic cell separation technique (Miltenyi Biotech, Bergisch Gladbach, Germany). The efficiency of the cell separation was controlled by flow cytometry. Cell suspensions were always depleted up to 95% of the labeled cells (data not shown).

ELISPOT assay. A protocol for an immediate ex vivo $CD8^+$ IFN-γ enzyme-linked immunospot (ELISPOT) assay was adapted utilizing the synthetic peptide TYQRTRALV NP147-155 peptide (NP peptide), an *H-2K*^{d}-restricted immunodominant cytotoxic-T-lymphocyte (CTL) epitope of the influenza A virus nucleoprotein (16, 37). Briefly, threefold serial dilutions of cell populations derived from murine spleens and draining lymph nodes were transferred to wells coated with anti-IFN-γ monoclonal antibody (MAb; R4-6A2; BD Pharmingen). Cells were incubated for 22 h at 37°C and in 5% CO_2 in DMEM containing 10% fetal calf serum, IL-2 (30 U/ml), penicillin, streptomycin, and 50 μM 2-mercaptoethanol in the presence of the synthetic peptide. A biotinylated anti-IFN-γ MAb (XMG1.2; BD Pharmingen) was utilized as a conjugate antibody, and then the plates were incubated with streptavidin peroxidase (Roche). Spots representing IFN-γ-secreting $CD8^+$ cells were developed utilizing the substrate 3-amino-9-ethylcarbazole (Sigma) containing hydrogen peroxide in 0.1 M sodium acetate (pH 5.0). The spots were counted with the aid of a dissecting microscope, and the results were expressed as the mean number of IFN-γ-secreting cells with ± the standard error of the mean of triplicate cultures. Cells incubated in the absence of the synthetic peptide developed <20 spots/10^6 cells. Since depletion of $CD8^+$ cells usually resulted in a >90% reduction of spot formation, cell separation was omitted in most assays (data not shown).

Determination of IFN-α/β in a functional luciferase bioassay. The principle of this bioassay, as described elsewhere (2, 41), is based on the function of the Mx protein, which is an IFN-inducible intracellular protein mediating resistance against negative-strand RNA viruses in many vertebrates. In brief, appropriate serial dilutions of pooled mouse sera and nasal secretions, as well as mouse α and β fibroblast IFN used as a standard (Sigma I 1258), were diluted in medium 199 (Gibco) containing penicillin, streptomycin, L-glutamine, and 10% fecal calf serum and then added to wells of a 96-well plate containing L-M MxL B10-D5 cells (kindly provided by G. R. Adolf [Boehringer Ingelheim Austria, Vienna]) stably transfected with a plasmid containing IFN-inducible mouse Mx promoter upstream of a luciferase reporter gene. After an incubation period of 6 h in a 5% CO_2 incubator at 37°C, the supernatants were removed and the cells were washed with PBS containing 2 mM EDTA and then lysed in a lysis buffer (100 μl/well; 8 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 15% glycerol, 25 mM Tris-phosphate buffer [pH 7.8]). The cell lysates were supplemented with 100 μl/well with the lysis buffer containing in addition 2 mM ATP and 100 μM D-luciferin sodium salt (assay buffer). The luciferase activity of the wells was then measured in 96-well luminometer (Mediators, Inc., Vienna, Austria), and the results were expressed in units per milliliter. Representative results from multiple experiments are shown.

RESULTS

Several transfectant viruses that originated from the w.t. A/PR8/33 virus encoding altered NS1 proteins containing deletions or insertions of foreign sequences have been generated in our laboratory (14, 16). The structure of mutated NS1 proteins is outlined in Fig. 1. NS1 mutant viruses could be distributed into two groups according to their capacity to bind dsRNA. Based on previous data showing that first N-terminally 73 amino acid residues of the NS1 protein comprising the RNA-binding domain (RBD) are responsible for RNA binding (49), we found, as expected, that only NS1 proteins expressed by PR8/NS1-80, PR8/NS1-125, PR8/NS1-Nef, and PR8 w.t. viruses (Fig. 1) are capable of binding the dsRNA efficiently.

TABLE 1. Virus replication in Vero cells and murine respiratory tissues

Cell line	Virus replication in:		
	Vero cells[a] (log PFU/ml)	Vero cells + IFN-α[a] (log PFU/ml)	Murine lungs[b] (log PFU/g of tissue)
PR8delNS1	6.9	<1	<1
PR81NS1del40-80	6.8	<1	<1
PR8/NS1-38	6.2	<1	<1
PR81NS1-80	6.5	6.4	3.8
PR81NS1-125	7.4	7.2	5.2
PR81NS1-Nef	7.3	7	<1
PR8 w.t.	7.7	7.5	6.8

[a] Vero cells were infected at multiplicity of infection of 0.1 in the absence or presence of IFN-α (30 U/ml) in the culture medium as indicated in the table. The virus yield was then determined in a plaque assay on Vero cells at 48 h p.i. as described in Materials and Methods.

[b] Mice were immunized with 10^6 PFU/mouse (NS1 mutant viruses) or 1.2×10^3 PFU/mouse (PR8 w.t. virus). The maximal viral load in lungs was detected 4 days p.i. Results are presented as the log PFU per mililiter of 10% (wt/vol) tissue extracts.

NS1 mutant viruses with an impaired RBD are sensitive to IFN-α/β and are replication deficient in IFN-competent substrates. NS1 mutant viruses encoding NS1 proteins with impaired RBD are unable to replicate in IFN-competent cell substrates, e.g., MDCK cells and cells of an allantoic cavity of 10-day-embryonated chicken eggs or mouse respiratory tracts. However, these viruses are capable of replicating to high titers in IFN-incompetent cell substrates, e.g., Vero cells (14). We have found that viruses with inhibited NS1 function of binding dsRNA are highly sensitive to IFN-α/β since no replication was observed in Vero cells when IFN-α was added to the culture medium (Table 1). These NS1 mutant viruses were also replication deficient in the respiratory tracts of i.n.-infected mice.

The expressed recombinant NS1-Nef protein of the PR8/NS1-Nef virus was able to bind the dsRNA (Fig. 1). Interestingly, the PR8/NS1-Nef virus was resistant against the IFN action in Vero cells but was completely attenuated in mice (Table 1). In comparison, the PR8/NS1-125 virus encoding just the N-terminal 125 amino acid residues of the NS1 protein without the insertion of the foreign sequences replicated to high titers in mouse lungs. We concluded that the Nef insert provided another attenuation mechanism eventually not associated with the NS1 protein functions (Fig. 1).

Replication-deficient NS1 mutant viruses induce a Th1 type of immune response characterized by significant titers of virus-specific serum and mucosal IgG2a and IgA but with low titers of IgG1. Subsequently, we sought to determine whether replication-deficient NS1 mutant viruses (viruses encoding NS1 proteins with impaired RBD and the PR8/NS1-Nef virus) are able to induce a relevant humoral immune response in an in vivo mouse model. Mice immunized with replication-deficient NS1 mutant viruses developed markedly lower titers of virus specific serum IgG1 in contrast to the PR8 w.t. virus and the replicating NS1 mutant viruses. Interestingly, all replication-deficient NS1 mutant viruses induced serum titers of IgG2a and IgA similar or exceeding those induced by the

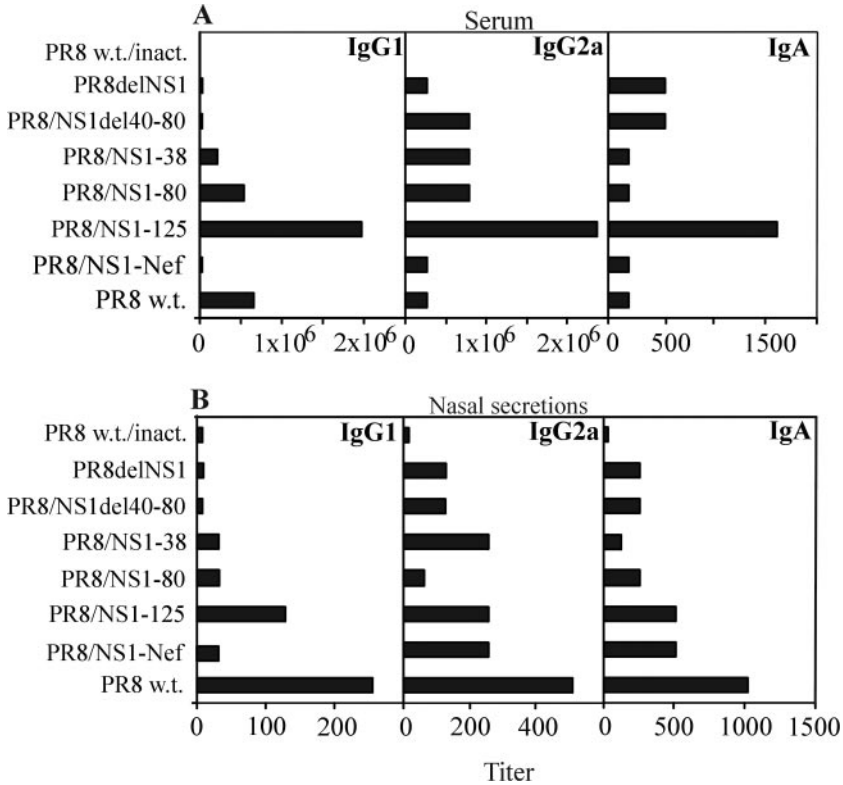

FIG. 2. Detection of virus specific IgG1, IgG2a, and IgA in serum and nasal secretions of primed mice. Mice were immunized i.n. with viruses as indicated in the figure. Serum samples and mouse nasal secretions were obtained 3 weeks after immunization. Virus-specific IgG1, IgG2a, and IgA titers were determined by ELISA in pooled mouse serum samples (A) and nasal secretions (B) as described in Materials and Methods.

TABLE 2. Peak cytokine levels in serum and mucosal samples of immunized mice[a]

Cell line	Cytokine level in:			
	Serum (IFN-α/β) (U/ml) at 12 h p.i.[b]		Nasal secretions (pg/ml) at 6 h p.i.	
	i.p.	i.n.	IL-1β	IL-6
PR8delNS1	16,197	1,387	1,285	502
PR8/NS1del40-80	19,460[c]	7,322[c]	2,246	2,882
PR8/NS1-38	8,548	999	1,715	388
PR8/NS1-80	3,309	<300	1,118	305
PR8/NS1-125	24,568	1,642	1,583	401
PR8/NS1-Nef	25,101	2,955	1,510	320
PR8 w.t.	1,691	<300	1,299	522

[a] Mice were immunized with NS1 mutant and the PR8 w.t. viruses. Serum and nasal secretions were collected 6, 12, and 24 h p.i. and then pooled and assessed for the presence of IFN-α/β, IL- 1β, and IL-6 as described in Materials and Methods.

[b] Shown are the peak values of i.p.-immunized mice and i.n.-immunized mice as indicated.

[c] Maximal values were determined already at 6 h p.i.

replicating PR8 w.t. virus (Fig. 2A) Virus-specific IgA and IG2a in nasal secretions were the predominant antibody subtypes detected in all of the groups of mice that were immunized. The highest IgG1 titers were detected in the nasal secretions of mice immunized with the replicating PR8 w.t. and PR8/NS1-125 virus, whereas mice that were immunized with the replication-deficient NS1 mutant viruses developed markedly lower titers of virus specific IgG1 (Fig. 2B).

Replication-deficient NS1 viruses induce antiviral and proinflammatory cytokines early after infection. Since several cytokines released early after viral infection are known to positively influence the development of adoptive immune response, influenza NS1 mutant viruses were investigated for their ability to induce IFN-α/β and major proinflammatory cytokines in mice. To minimize the replication advantage of some NS1 mutant viruses and the PR8 w.t. virus, mice were injected i.p. with 0.5 ml of viral suspensions (4×10^6 to 8×10^6 PFU/mouse). Surprisingly, not only the NS1 mutant viruses with the impaired RBD but also both viruses encoding the 125 amino acids of the NS1 protein (PR8/NS-125 and PR8/NS1-Nef) potently induced markedly higher levels of IFN-α/β in mouse serum compared to those induced by the PR8 w.t. virus. Table 2 shows the peak levels measured 12 h p.i. in the sera of mice. The levels of IFN-α/β in the sera of mice immunized with replication-deficient NS1 mutant viruses decreased rapidly and were under the detection limit of 36 h p.i. When mice were immunized by the i.n. route (1×10^6 to 2×10^6 PFU/mouse) high levels of IFN-α/β were detected 5 to 6 h p.i. in mouse sera. However, the maximal levels of IFN-α/β were obtained in mouse sera at 12 h p.i. except the PR8/NS1del40-80 virus inducing the peak level already at 6 h p.i. In this experimental setting, we failed to directly detect IFN-α/β in the eluted mouse nasal secretions.

In addition, we were able to detect high levels of IL-1β in nasal secretions but not in the sera of mice immunized with NS1 mutant viruses 5 to 6 h p.i., exceeding levels induced by the PR8 w.t. virus. The mucosal IL-6 levels in mice primed with replication-deficient NS1 mutant viruses were similar to those induced by the w.t. virus. Cytokine levels were lower (PR8 w.t. virus) or undetectable (NS1 mutant viruses) 24 h p.i. (data not shown). Interestingly, the PR8/NS1del40-80 induced the highest levels of IFN-α/β virus in serum, as well as the highest levels of Il-1β and IL-6 at 6 h p.i. in mucosa. In most cases the levels of TNF-α in serum or mucosa were undetectable or oscillating in the range of the detection limit of the assay. UV-inactivated PR8 w.t. virus when measured did not induce detectable levels of cytokines.

Replication-deficient NS1 mutant viruses induce primary CD8$^+$-T-cell responses. We then evaluated the potential of NS1 mutant viruses to induce virus specific cellular immune responses. The highest number (ca. 400 IFN-γ-secreting cells/million cells) of NP peptide-specific CD8$^+$ T cells was detected in single cell populations derived from spleens of mice immunized with the replicating PR8 w.t. and PR8/NS1-125 viruses (Fig. 3A). However, considerably high numbers of NP-specific CD8$^+$ cells were detected in spleens of mice immunized with replication-deficient NS1 mutant viruses ranging from ca. 180 IFN-γ spot-forming cells per million cells (PR8delNS1 virus immunized mice) up to 320 spots per million cells detected in splenic single cell populations of PR8/NS1-Nef virus-immunized mice (Fig. 3A).

Replication-deficient NS1 mutant viruses induce a rapid onset of the recall CD8$^+$-T-cell responses upon challenge. Since it has been proven many times that systemic and mucosal antibodies can protect mice from reinfections, we focused rather on the recall CD8$^+$-T-cell responses upon pathogenic w.t. virus challenge. Mice primed by the i.n. route with the NS1 mutant viruses and the PR8 w.t. virus were i.n. challenged 4 months later with an ~1,000 LD_{50} of the homotypic pathogenic PR8 w.t. virus. A rapid onset of the recall CD8$^+$ response was observed 60 h postchallenge, especially in the lymph nodes draining the respiratory tracts of mice primed with the replication-deficient PR8/NS1-Nef, PR8/NS1del40-80, and PR8delNS1 mutant viruses surpassing the numbers of CD8$^+$ T cells detected in the groups of mice immunized with the replicating NS1mutant and PR8 w.t. viruses (Fig. 3B).

Immunized mice are protected against lethal virus challenge. Mice immunized with replication-deficient NS1 mutant viruses were significantly protected against the i.n. challenge with a 1,000 LD_{50} of the pathogenic PR8 w.t. virus in the absence of anesthesia (Table 3). Complete protection against the homotypic challenge stimulus was observed in mice primed with the replicating PR8/NS1-80, PR8/NS1-125, and the PR8 w.t. viruses. It is noteworthy that the replication-deficient PR8/NS1del40-80 and PR8/NS1-Nef viruses that induced the highest levels of IFN-α/β in mice (Table 2) also completely protected mice against the challenge virus (Table 3). Although mice immunized with PR8/NS1-38 and PR8/delNS1 demonstrated a high survival rate, not all mice within these groups could completely clear the viral infection in their respiratory tracts (Table 3).

DISCUSSION

In the few last years several innovative strategies for the development of a new generation of live influenza vaccines have appeared, including those generating live replication-deficient vaccines with partially or completely deleted NS1 or NS2 genes (14, 45, 51). Such vaccines are able to perform just an abortive infection and are devoid of possible complications

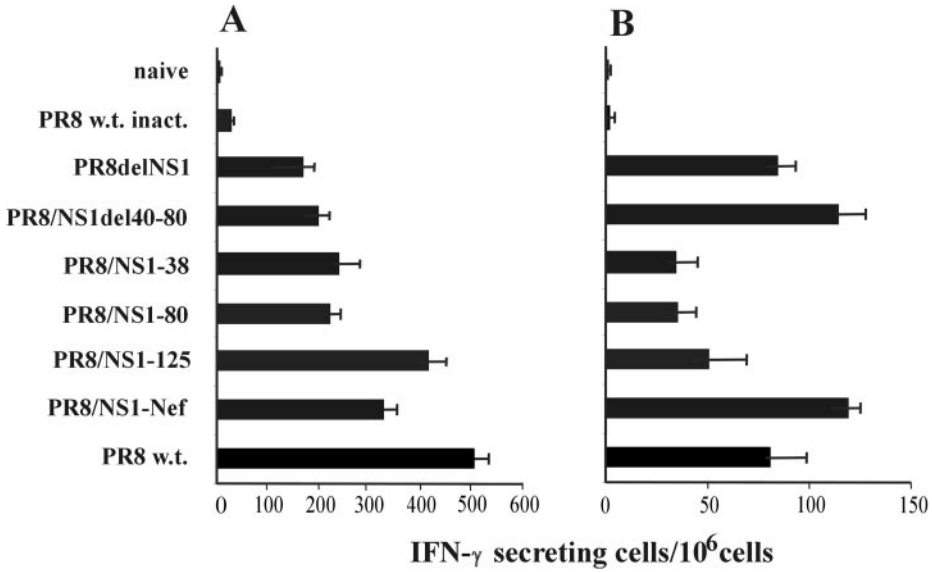

FIG. 3. Quantification of NP peptide-specific IFN-γ-secreting cells. Mice were immunized i.n. with NS1 mutant viruses and the PR8 w.t. virus. (A) The primary response was determined in single spleen cell suspensions obtained 10 days after the immunization of the mice. (B) Recall responses were determined in single cell populations obtained from mediastinal lymph nodes draining the respiratory tracts of mice challenged with the PR8 w.t. virus 4 months after the primary immunization. Single cell populations were assessed for NP peptide-specific IFN-γ-secreting $CD8^+$ T cells in an ELISPOT assay. The mean numbers of antigen-specific IFN-γ-secreting cells plus the standard error of the mean of triplicate cultures are shown.

associated with replicating cold-adapted influenza vaccines, e.g., the residual virulence and viral genome stability. However, the putative low immunogenicity of replication-deficient vaccines after i.n. administration is considered to be an obstacle for their utilization. In the present study we compared the immunogenic potential of replicating and nonreplicating in vivo influenza A NS1 mutant viruses.

The NS1 protein plays a key role in the inhibition of the IFN-α/β activation pathways in the early antiviral (innate) response of host cells and is thus responsible for viral pathogenesis (5, 14, 17, 43, 44). The ~230-amino-acid protein consists of two principal domains—the RBD and the effector domain that contains sites for the binding of diverse proteins required, e.g., for processing and cellular transport of cellular mRNAs and translation of viral mRNAs (1, 30). The RBD located within the first 73 N-terminal amino acids of the NS1 protein exhibits a dimeric six-helical chain fold determining a protein dimer formation (49) which is probably further stabilized by a putative dimerization site present in the adjacent effector domain (50). Alterations performed at different positions within the NS1 gene have been reported to be associated with loss of various protein functions, resulting in a partial or complete virus attenuation (12, 14, 17, 30, 35, 50).

TABLE 3. Protection and viral load in immunized mice after a lethal virus challenge[a]

Cell line	No. of survivors/ no. tested[b]	Viral load in murine lungs (PFU/g of tissue)[c]
Uninfected	0/8	5.4×10^5
PR8delNS1	6/8	3.4×10^3
PR81NS1del40-80	8/8	<1
PR8/NS1-38	7/8	1.8×10^3
PR81NS1-80	8/8	<1
PR81NS1-125	8/8	<1
PR81NS1-Nef	8/8	<1
PR8 w.t.	8/8	<1

[a] Mice were i.n. challenged in the absence of anesthesia with 1,000 LD_{50} of the PR8 w.t. virus 4 months after priming.
[b] Mice were monitored for 15 days after challenge.
[c] Shown are virus titers of pooled lung extracts prepared 3 days after challenge (five mice/group).

The NS1 mutant viruses utilized in our experiments could be divided in two groups: those encoding NS1 proteins with retained RBD-mediated functions and those with inhibited RNA-binding activity. NS1 mutant viruses with impaired or missing RBDs replicated efficiently in the IFN-deficient Vero cells but did not replicate in mice (Table 1) (14). In addition, these viruses were sensitive to exogenous IFN-α, since no virus yield could be obtained from infected Vero cells cultivated in the presence of IFN-α (Table 1).

In contrast, the NS1 mutant viruses expressing NS1 proteins with intact RBD capable of binding dsRNA replicated efficiently in mice and IFN-competent cells (Table 1). It is noteworthy that the introduction of about 150 amino acid residues derived from the HIV-1 Nef protein into the NS1 open reading frame of the PR8/NS1-125 virus completely abolished the replication potential of the PR8/NS1-Nef virus in mouse lungs, although this virus replicates in MDCK cells and embryonated eggs alike the NS1-125 virus (data not shown). It might be possible that the replication deficiency of the PR8/NS1-Nef virus could be attributed to a mechanism not associated with the function of the NS1 protein, since we observed structural alterations of the PR8/NS1-Nef virions by electron microscopy (data not shown). Several data also indicate that Nef molecules expressed in the infected cells interact with cytoskeleton components and might interfere with virion formation (16). However, this hypothesis remains to be further investigated.

Previously, it was shown that deletion of the viral NS1 gene increased the number and magnitude of expression of cellular genes implicated in the major antiviral pathways, demonstrating the importance of the NS1 protein in the regulation of the host cell response triggered by virus infection (18). It is very likely that the immune responses detected in mice immunized with NS1 mutant viruses may be determined by the immunomodulatory effects of virus-induced antiviral and proinflammatory cytokines. For instance, IFN-α/βs are induced rapidly to viral infection and, in addition to their direct antiviral properties, they stimulate innate and adaptive immune responses in several ways, thus serving as a signal that links innate and adaptive immunity (13, 31). By upregulating chemokine gene expression, IFN-α/β contributes to the further recruitment of monocytes, macrophages, and other Th1-type immune cells to the site of infection. The antigen presentation of macrophages and dendritic cells improves due to upregulation of HLA gene expression. Strikingly, IFN-α/βs are able to augment the antibody response and significantly contribute to the development of a Th1 immune response and to the T-cell survival, upregulation of IL-12 receptor expression, and IFN-γ gene expression in NK and T cells (13, 24, 25, 31).

All NS1 mutant viruses encoding an incomplete or missing RBD of the NS1 protein rapidly induced markedly higher levels of IFN-α/β in serum upon i.n. and i.p. immunization of mice than did the PR8 w.t. virus. Moreover, it is obvious that not only the intact RBD but also a substantial part of the effector domain of the NS1 protein is required to efficiently combat the IFN-α/β-induced cellular antiviral response since the slightly attenuated NS1-125 mutant virus and the replication-deficient NS1-Nef virus induced high amounts of IFN-α/βs in mice (Table 2). In keeping with our results, a similar NS1-126 mutant virus was shown to induce high amounts of IFN-β-mRNA (18).

In addition, replication-deficient NS1 mutant viruses with impaired RBDs were capable of inducing high levels of the proinflammatory cytokines IL-1β and IL-6 but not of TNF-α in mouse nasal secretions. IL-1β and IL-6 genes have been reported to be constitutively expressed in respiratory tract cells (9). It was shown recently that, despite a detectable TNF-α gene expression, TNF-α could not be detected after an influenza virus infection in epithelial cell culture supernatants (46). In our experiments, peak cytokine levels were detected by about 6 h p.i. and, except for the PR8.w.t. virus, no cytokines could be detected later than 24 h p.i. in mucosal secretions. These findings might be explained by the poor growth of replication-competent NS1 mutant viruses in murine nasal tissues at later time points. Since these cytokines have been detected in local secretions but not in the systemic compartment, we believe that fever and disease symptom manifestation mediated by IL-1β and IL-6 are probably of minor importance (26). IL-1β and IL-6 are pleiotropic cytokines that act on a wide variety of cell types. They both have important regulatory functions in the immune system and mediate a wide variety of effects, including the host defense responses to local and systemic disease and injury (29, 42, 52). An important biological activity of IL-1β is the stimulation of T-helper cells, but IL-1β also directly acts on B cells, promoting their proliferation and the synthesis of immunoglobulins (7). IL-6 is a B-cell differentiation factor and an activation factor for T cells. In the presence of IL-2, IL-6 induces the differentiation of mature and immature T cells into cytotoxic T cells. IL-6 can induce the final maturation of B cells into immunoglobulin-secreting plasma cells (23).

Antiviral IFN-α/β and the proinflammatory cytokines IL-1β and IL-6 have been utilized as adjuvants for different types of vaccines. IFN-α/βs, as strong inducers of Th1 type immune response, also exhibit a powerful exogenous adjuvant effect when administered with the human influenza vaccine inducing specific IgG2a and IgA conferring protection from a virus challenge (38). IL-1 has been reported, for example, as a potent adjuvant enhancing mucosal immune response for coadministered antigens, whereas the coadministration of IL-6 gene with the DNA vaccine plasmids encoding influenza virus HA and NP enhanced influenza virus-specific CTL responses and completely protected mice from a lethal challenge with a w.t. influenza virus (8, 32).

In fact, replication-deficient NS1 mutant viruses induced a Th1-type immune response displaying high magnitudes of virus-specific IgG2a and IgA but weak IgG1 in serum, whereas replicating NS1 mutant viruses and the w.t. virus induced high titers of virus-specific IgG1 and IgG2a but lower IgA serum titers. The highest titers of antibodies were detected for the replicating PR8/NS1-125 virus. This can be explained by the ability of the PR8/NS1-125 virus to induce high levels of immunomodulating IFN-α/β in serum and its significant replication in mouse respiratory tracts (Tables 1 and 2). Moreover, mice immunized with the PR8/NS1-125 virus recovered rapidly from the disease and remained healthy in contrast to slowly recovering moribund mice immunized with the noninterferonogenic PR8 w.t. virus. It has to be noted that mice immunized with nonreplicating NS1 mutant viruses developed antibodies detectable in the nasal mucosal secretions. Virus-specific IgA, followed by IgG2a, has been the most prominent isotype detected in murine mucosal secretions. Higher levels of virus-specific IgG1 were detected in nasal secretions of mice immunized with replicating NS1 mutant viruses or w.t. virus.

Remarkably, replication-deficient NS1 mutant viruses induced comparable numbers of virus-specific $CD8^+$-T-cell responses if compared to those induced by the replicating NS1 mutant viruses or the PR8 w.t. virus. In addition, replication-deficient NS-1 mutant viruses induced a memory response, since primed mice developed rapid recall responses upon challenge with a high pathogenic dose of the homologous PR8 w.t. virus. Increased numbers of $CD8^+$ T cells in the lymph nodes draining the murine respiratory tracts 60 h postchallenge were determined in mice primed with NS1 mutant viruses that induced the highest levels of IFN-α/β and/or the proinflammatory cytokines. This observation might by supported by previous investigations indicating that some proinflammatory cytokines and especially IFN-α/β. might be involved in the induction of surface markers typical for memory cells (40). All NS1 mutant viruses showed a high degree of protection against the challenge PR8 w.t. virus. However, the PR8/NS1del40-80 virus, which induced the highest amounts of IFN-α/β, IL-1β, and IL-6 in mice, appeared to be the most promising vaccine candidate since it completely protected mice from the challenge virus. Although the PR8/NSdel40-80 virus encodes an NS1 protein with impaired RBD but almost complete effector domain sequence, the NS1 dimer formation is probably im-

paired and the folding of NS1 monomers is unpredictable. The functions of such modified NS1 proteins are therefore not fully elucidated and are currently being investigated. We assume that the increased early peak levels of IL-1β detected in mucosal secretions of mice immunized with this virus may indicate a certain role of the NS1 protein in the regulation of influenza virus-induced IL-1β release. However, additional in vitro experiments are necessary to prove this hypothesis.

The replication-deficient NS1 mutant viruses encoding impaired RBD of the NS1 are capable of stimulating an efficient virus-specific adaptive immunity on the systemic, mucosal, and cellular levels, thus possessing almost an optimal balance of safety and immunogenicity. The generation of influenza viruses with targeted manipulations in the NS1 gene opens novel and challenging possibilities in influenza virus vaccine development.

ACKNOWLEDGMENTS

This study was supported in part by the Austrian Science Fund project (P13715) and by Polymun Scientific GmbH, Vienna, Austria.

We thank R. Fleischhacker for the determination of IFN-α/β in the functional bioassay.

REFERENCES

1. **Aragon, T., S. de la Luna, I. Novoa, L. Carrasco, J. Ortin, and A. Nieto.** 2000. Eukaryotic translation initiation factor 4GI is a cellular target for NS1 protein, a translational activator of influenza virus. Mol. Cell. Biol. **20:**6259–6268.
2. **Asano, A., H. K. Jin, and T. Watanabe.** 2003. Mouse Mx2 gene: organization, mRNA expression and the role of the interferon-response promoter in its regulation. Gene **306:**105–113.
3. **Belshe, R. B., W. C. Gruber, P. M. Mendelman, I. Cho, K. Reisinger, S. L. Block, J. Wittes, D. Iacuzio, P. Piedra, J. Treanor, J. King, K. Kotloff, D. I. Bernstein, F. G. Hayden, K. Zangwill, L. Yan, and M. Wolff.** 2000. Efficacy of vaccination with live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine against a variant (A/Sydney) not contained in the vaccine. J. Pediatr. **136:**168–175.
4. **Belshe, R. B., W. C. Gruber, P. M. Mendelman, H. B. Mehta, K. Mahmood, K. Reisinger, J. Treanor, K. Zangwill, F. G. Hayden, D. I. Bernstein, K. Kotloff, J. King, P. A. Piedra, S. L. Block, L. Yan, and M. Wolff.** 2000. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J. Infect. Dis. **181:**1133–1137.
5. **Bergmann, M., A. Garcia-Sastre, E. Carnero, H. Pehamberger, K. Wolff, P. Palese, and T. Muster.** 2000. Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J. Virol. **74:**6203–6206.
6. **Beyer, W. E., A. M. Palache, J. C. de Jong, and A. D. Osterhaus.** 2002. Cold-adapted live influenza vaccine versus inactivated vaccine: systemic vaccine reactions, local and systemic antibody response, and vaccine efficacy: a meta-analysis. Vaccine **20:**1340–1353.
7. **Boraschi, D., P. Bossu, G. Macchia, P. Ruggiero, and A. Tagliabue.** 1996. Structure-function relationship in the IL-1 family. Front. Biosci. **1:**d270–d308.
8. **Boyaka, P. N., A. Tafaro, R. Fischer, K. Fujihashi, E. Jirillo, and J. R. McGhee.** 2003. Therapeutic manipulation of the immune system: enhancement of innate and adaptive mucosal immunity. Curr. Pharm. Des. **9:**1965–1972.
9. **Brydon, E. W., H. Smith, and C. Sweet.** 2003. Influenza A virus-induced apoptosis in bronchiolar epithelial (NCI-H292) cells limits pro-inflammatory cytokine release. J. Gen. Virol. **84:**2389–2400.
10. **Cha, T. A., K. Kao, J. Zhao, P. E. Fast, P. M. Mendelman, and A. Arvin.** 2000. Genotypic stability of cold-adapted influenza virus vaccine in an efficacy clinical trial. J. Clin. Microbiol. **38:**839–845.
11. **Cox, R. J., K. A. Brokstad, and P. Ogra.** 2004. Influenza virus: immunity and vaccination strategies: comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand. J. Immunol. **59:**1–15.
12. **Donelan, N. R., C. F. Basler, and A. Garcia-Sastre.** 2003. A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J. Virol. **77:**13257–13266.
13. **Durbin, J. E., A. Fernandez-Sesma, C. K. Lee, T. D. Rao, A. B. Frey, T. M. Moran, S. Vukmanovic, A. Garcia-Sastre, and D. E. Levy.** 2000. Type I IFN modulates innate and specific antiviral immunity. J. Immunol. **164:**4220–4228.
14. **Egorov, A., S. Brandt, S. Sereinig, J. Romanova, B. Ferko, D. Katinger, A. Grassauer, G. Alexandrova, H. Katinger, and T. Muster.** 1998. Transfectant influenza A viruses with long deletions in the NS1 protein grow efficiently in Vero cells. J. Virol. **72:**6437–6441.
15. **Ferko, B., D. Katinger, A. Grassauer, A. Egorov, J. Romanova, B. Niebler, H. Katinger, and T. Muster.** 1998. Chimeric influenza virus replicating predominantly in the murine upper respiratory tract induces local immune responses against human immunodeficiency virus type 1 in the genital tract. J. Infect. Dis. **178:**1359–1368.
16. **Ferko, B., J. Stasakova, S. Sereinig, J. Romanova, D. Katinger, B. Niebler, H. Katinger, and A. Egorov.** 2001. Hyperattenuated recombinant influenza a virus nonstructural-protein-encoding vectors induce human immunodeficiency virus type 1 nef-specific systemic and mucosal immune responses in mice. J. Virol. **75:**8899–8908.
17. **Garcia-Sastre, A., A. Egorov, D. Matassov, S. Brandt, D. E. Levy, J. E. Durbin, P. Palese, and T. Muster.** 1998. Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology **252:**324–330.
18. **Geiss, G. K., M. Salvatore, T. M. Tumpey, V. S. Carter, X. Wang, C. F. Basler, J. K. Taubenberger, R. E. Bumgarner, P. Palese, M. G. Katze, and A. Garcia-Sastre.** 2002. Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc. Natl. Acad. Sci. USA **99:**10736–10741.
19. **Gerdil, C.** 2003. The annual production cycle for influenza vaccine. Vaccine **21:**1776–1779.
20. **Gorse, G. J., E. E. Otto, D. C. Powers, G. W. Chambers, C. S. Eickhoff, and F. K. Newman.** 1996. Induction of mucosal antibodies by live attenuated and inactivated influenza virus vaccines in the chronically ill elderly. J. Infect. Dis. **173:**285–290.
21. **Herlocher, M. L., A. C. Clavo, and H. F. Maassab.** 1996. Sequence comparisons of A/AA/6/60 influenza viruses: mutations which may contribute to attenuation. Virus Res. **42:**11–25.
22. **Herlocher, M. L., H. F. Maassab, and R. G. Webster.** 1993. Molecular and biological changes in the cold-adapted "master strain" A/AA/6/60 (H2N2) influenza virus. Proc. Natl. Acad. Sci. USA **90:**6032–6036.
23. **Horn, F., C. Henze, and K. Heidrich.** 2000. Interleukin-6 signal transduction and lymphocyte function. Immunobiology **202:**151–167.
24. **Julkunen, I., K. Melen, M. Nyqvist, J. Pirhonen, T. Sareneva, and S. Matikainen.** 2000. Inflammatory responses in influenza A virus infection. Vaccine **19:**S32–S37.
25. **Julkunen, I., T. Sareneva, J. Pirhonen, T. Ronni, K. Melen, and S. Matikainen.** 2001. Molecular pathogenesis of influenza A virus infection and virus-induced regulation of cytokine gene expression. Cytokine Growth Factor Rev. **12:**171–180.
26. **Kaiser, L., R. S. Fritz, S. E. Straus, L. Gubareva, and F. G. Hayden.** 2001. Symptom pathogenesis during acute influenza: interleukin-6 and other cytokine responses. J. Med. Virol. **64:**262–268.
27. **Kendal, A. P.** 1997. Cold-adapted live attenuated influenza vaccines developed in Russia: can they contribute to meeting the needs for influenza control in other countries? Eur. J. Epidemiol. **13:**591–609.
28. **Kendal, A. P., H. F. Maassab, G. I. Alexandrova, and Y. Z. Ghendon.** 1981. Development of cold-adapted recombinant live, attenuated influenza A vaccines in the USA and USSR. Antivir. Res. **1:**339–365.
29. **Kroemer, G., I. Moreno de Alboran, J. A. Gonzalo, and C. Martinez.** 1993. Immunoregulation by cytokines. Crit. Rev. Immunol. **13:**163–191.
30. **Krug, R. M., W. Yuan, D. L. Noah, and A. G. Latham.** 2003. Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology **309:**181–189.
31. **Le Bon, A., and D. F. Tough.** 2002. Links between innate and adaptive immunity via type I interferon. Curr. Opin. Immunol. **14:**432–436.
32. **Lee, S. W., J. W. Youn, B. L. Seong, and Y. C. Sung.** 1999. IL-6 induces long-term protective immunity against a lethal challenge of influenza virus. Vaccine **17:**490–496.
33. **Luytjes, W., M. Krystal, M. Enami, J. D. Pavin, and P. Palese.** 1989. Amplification, expression, and packaging of foreign gene by influenza virus. Cell **59:**1107–1113.
34. **Maassab, H. F., and M. L. Bryant.** 1999. The development of live attenuated cold-adapted influenza virus vaccine for humans. Rev. Med. Virol. **9:**237–244.
35. **Noah, D. L., K. Y. Twu, and R. M. Krug.** 2003. Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3′ end processing of cellular pre-mRNAS. Virology **307:**386–395.
36. **Palese, P., and A. Garcia-Sastre.** 2002. Influenza vaccines: present and future. J. Clin. Investig. **110:**9–13.
37. **Power, C. A., C. L. Grand, N. Ismail, N. C. Peters, D. P. Yurkowski, and P. A. Bretscher.** 1999. A valid ELISPOT assay for enumeration of ex vivo, antigen-specific, IFNγ-producing T cells. J. Immunol. Methods **227:**99–107.
38. **Proietti, E., L. Bracci, S. Puzelli, T. Di Pucchio, P. Sestili, E. De Vincenzi, M. Venditti, I. Capone, I. Seif, E. De Maeyer, D. Tough, I. Donatelli, and F. Belardelli.** 2002. Type I IFN as a natural adjuvant for a protective immune response: lessons from the influenza vaccine model. J. Immunol. **169:**375–383.

39. **Romanov Iu, R., A. Egorov, K. V. Lisovskaia, T. E. Medvedeva, G. N. Nevedomskaia, G. I. Aleksandrova, and A. I. Klimov.** 1989. The effect of amplifying reproduction of influenza virus in mouse lungs during simultaneous infection with two cold-adapted strains. Vopr. Virusol. **34:**547–553. (In Russian.)
40. **Schlueter, A. J., A. M. Krieg, P. de Vries, and X. Li.** 2001. Type I interferon is the primary regulator of inducible Ly-6C expression on T cells. J. Interferon Cytokine Res. **21:**621–629.
41. **Schumacher, B., D. Bernasconi, U. Schultz, and P. Staeheli.** 1994. The chicken Mx promoter contains an ISRE motif and confers interferon inducibility to a reporter gene in chick and monkey cells. Virology **203:**144–148.
42. **Slifka, M. K., and J. L. Whitton.** 2000. Clinical implications of dysregulated cytokine production. J. Mol. Med. **78:**74–80.
43. **Smith, E. J., I. Marie, A. Prakash, A. Garcia-Sastre, and D. E. Levy.** 2001. IRF3 and IRF7 phosphorylation in virus-infected cells does not require double-stranded RNA-dependent protein kinase R or IκB kinase but is blocked by Vaccinia virus E3L protein J. Biol. Chem. **276:**8951–8957.
44. **Talon, J., C. M. Horvath, R. Polley, C. F. Basler, T. Muster, P. Palese, and A. Garcia-Sastre.** 2000. Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein J. Virol. **74:**7989–7996.
45. **Talon, J., M. Salvatore, O. N. Re, Y. Nakaya, H. Zheng, T. Muster, A. Garcia-Sastre, and P. Palese.** 2000. Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. Proc. Natl. Acad. Sci. USA **97:**4309–4314.
46. **Tong, H. H., J. P. Long, P. A. Shannon, and T. F. DeMaria.** 2003. Expression of cytokine and chemokine genes by human middle ear epithelial cells induced by influenza A virus and *Streptococcus pneumoniae* opacity variants. Infect. Immun. **71:**4289–4296.
47. **Treanor, J., M. Perkins, R. Battaglia, and B. R. Murphy.** 1994. Evaluation of the genetic stability of the temperature-sensitive PB2 gene mutation of the influenza A/Ann Arbor/6/60 cold-adapted vaccine virus. J. Virol. **68:**7684–7688.
48. **Treanor, J. J., R. Buja, and B. R. Murphy.** 1991. Intragenic suppression of a deletion mutation of the nonstructural gene of an influenza A virus. J. Virol. **65:**4204–4210.
49. **Wang, W., K. Riedel, P. Lynch, C. Y. Chien, G. T. Montelione, and R. M. Krug.** 1999. RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. RNA **5:**195–205.
50. **Wang, X., C. F. Basler, B. R. Williams, R. H. Silverman, P. Palese, and A. Garcia-Sastre.** 2002. Functional replacement of the carboxy-terminal two-thirds of the influenza A virus NS1 protein with short heterologous dimerization domains. J. Virol. **76:**12951–12962.
51. **Watanabe, T., S. Watanabe, G. Neumann, H. Kida, and Y. Kawaoka.** 2002. Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. J. Virol. **76:**767–773.
52. **Watkins, L. R., M. K. Hansen, K. T. Nguyen, J. E. Lee, and S. F. Maier.** 1999. Dynamic regulation of the proinflammatory cytokine, interleukin-1beta: molecular biology for non-molecular biologists. Life Sci. **65:**449–481.
53. **Webster, R. G.** 2000. Immunity to influenza in the elderly. Vaccine **18:**1686–1689.
54. **Yewdell, J., and A. Garcia-Sastre.** 2002. Influenza virus still surprises. Curr. Opin. Microbiol. **5:**414–418.
55. **Zambon, M. C.** 1999. Epidemiology and pathogenesis of influenza. J. Antimicrob. Chemother. **44**(Suppl. B)**:**3–9.