Influenza B mutant viruses with truncated NS1 proteins grow efficiently in Vero cells and are immunogenic in mice

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Contemporary influenza B virus strains were generated encoding C-terminally truncated NS1 proteins. Viable viruses containing the N-terminal 14, 38, 57 or 80 aa of the NS1 protein were rescued in Vero cells. The influenza B virus NS1-truncated mutants were impaired in their ability to counteract interferon (IFN) production, induce antiviral pro-inflammatory cytokines early after infection and show attenuated or restricted growth in IFN-competent hosts. In Vero cells, all of the mutant viruses replicated to high titres comparable to the wild-type influenza B virus. Mice that received a single, intranasal immunization of the NS1-truncated mutants elicited an antibody response and protection against wild-type virus challenge. Therefore, these NS1-truncated mutants should prove useful as potential candidates for live-attenuated influenza virus vaccines.

INTRODUCTION

Influenza viruses belong to the family Orthomyxoviridae and are enveloped, negative-sense RNA viruses with a segmented, single-stranded genome (Wright & Webster, 2001). Whereas influenza A virus has a broad host reservoir in many avian and mammalian species, influenza B virus is thought to be almost exclusively restricted to humans (Wright & Webster, 2001). Most of the proteins expressed by influenza A and B viruses are believed to have similar functions, despite the biological and epidemiological differences between these two viruses. Clear exceptions are the pro-apoptotic PB1-F2 protein found uniquely in most influenza A virus strains (Chen et al., 2001) and the NB protein expressed solely by influenza B virus, which contributes to virulence (Imai et al., 2008; Shaw et al., 1983).

Various hosts counteract viral invasion by inducing an antiviral response activated by their innate immune system. This response involves three major steps: (i) detection of viral infection and type I interferon (IFN) secretion by the infected cell and (ii) binding of IFN to extracellular receptors and transcriptional induction of IFN-stimulated genes, leading to (iii) the induction of an ‘antiviral state’ of infected and neighbouring cells. In detail, the activation of the host type I IFN system is mediated predominantly by recognition of influenza virus-derived RNA bearing 5’ triphosphates by the cytoplasmic RNA helicase RIG-I (Pichlmair et al., 2006; Yoneyama et al., 2004). Interaction of RIG-I with MAVS (also known as IPS-1, VISA or Cardif) triggers a signalling cascade resulting in activation of the kinases Traf family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK-1) and IkB kinase ε (IKKe), leading to phosphorylation of the transcription factor IFN-regulating factor 3 (IRF-3) (Fitzgerald et al., 2003; Kawai et al., 2005; McWhirter et al., 2004; Meylan et al., 2005; Seth et al., 2005; Sharma et al., 2003; Xu et al., 2005). Activated IRF-3 translocates and accumulates in the nucleus. Together with the nuclear co-activator CBP/p300, nuclear factor κB (NF-κB) and activating transcription factor 2 (ATF2/c-Jun) induce transcription of IFN-α/β (Du & Maniatis, 1992; Juang et al., 1998; Lin et al., 1998; Peters et al., 2002; Sato et al., 1990; Wathnelet et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998). IFN-α and -β are secreted from the infected cell and bind to the IFN receptor on the cell surface. This triggers a signal cascade through the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway leading to
the formation of the IFN-stimulated gene factor 3 (ISGF-3) transcription complex, which in turn is responsible for the expression of more than 100 IFN-stimulated genes, some of which are associated with antiviral activity, e.g. Mx proteins; ISG-15, -54 and -56; 2',5'-oligoadenylate synthetase (2',5'-OAS) and protein kinase R (PKR) (Garcia-Sastre & Biron, 2006; Holzinger et al., 2007; Samuel, 2001). This establishment of an antiviral state is associated with the secretion of such pro-inflammatory cytokines and chemokines as interleukin-6 (IL-6), tumour necrosis factor-

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in TNF-\(\alpha\), IL-18, CCL3, CCL4, CCL5 (RANTES), IFN-\(\gamma\) and many more (Julkunen et al., 2001; Kaufmann et al., 2001; Matikainen et al., 2000).

During its evolution, viruses have developed a mechanism to overcome this defence strategy of the innate immune system. Early on, it was demonstrated that the non-structural protein 1 of influenza A virus (A/NS1) plays this role by antagonizing the IFN response of the infected cell (Egorov et al., 1998). Thus, A/NS1 has been associated with a number of regulatory functions during virus infection: (i) inhibition of host mRNA polyadenylation and pre-mRNA splicing, which contributes to the virus-induced shut-off of host protein synthesis (Lu et al., 1994; Nemeroff et al., 1998); (ii) binding to the poly(A) tails of mRNAs, thus inhibiting their nuclear export (Qiu & Krug, 1994); (iii) regulation of viral RNA polymerase activity (Marion et al., 1997; Shimizu et al., 1994); (iv) interaction with the subunit of the cleavage and polyadenylation specificity factor (CPSF), poly(A)-binding protein II (PABII) and NS1-BP, leading to the selective inhibition of nuclear export of cellular mRNAs and the inhibition of splicing (Nemeroff et al., 1998; Wolff et al., 1996, 1998); (v) blocking the activation of transcription factors such as IRF-3, IRF-7, NF-\(\kappa\)B and ATF-2/c-Jun, thereby inhibiting the induction of IFN-\(\alpha/\beta\) (Levy et al., 2002; Ludwig et al., 2002, 2006; Talon et al., 2000a; Wang et al., 2000); (vi) sequesteration of dsRNA, preventing activation of the IFN cascade; and (vii) binding to Myx protein, 2',5'-OAS and PKR, the latter being one of the major antiviral proteins responsible for inhibiting translational expression of cellular proteins (Dittmann et al., 2008; Lu et al., 1995; Min & Krug, 2006; Samuel, 2001).

Although the influenza B virus NS1 protein (B/NS1) only has about 20% sequence identity with A/NS1, many of the functions are shared by B/NS1, with some distinct exceptions. In contrast to influenza A virus, no NS1-mediated nuclear retention of mRNAs has been observed during influenza B infection (Wang & Krug, 1996). Only B/NS1 inhibits conjugation of ISG15 protein to its downstream binding partners and selectively induces the onset of early response genes in infected cells such as ISG56 (Lenschow et al., 2007; Yuan et al., 2002; Yuan & Krug, 2001), a function that is not shared with influenza A viruses (Kim et al., 2002; Talon et al., 2000b; Wang & Krug, 1996). The N-terminal RNA-binding domain (RBD) of B/NS1 suffices to inhibit PKR activation and has IFN antagonistic potential (Donelan et al., 2004). In the context of a recombinant virus, the C-terminal part of B/NS1 is required to inhibit IFN-\(\alpha/\beta\) induction in epithelial cells (Dauber et al., 2006). Some other functions of NS1 are common to both influenza types, e.g. binding to RNA via an N-terminal RBD.

It has been shown that influenza A viruses lacking a functional NS1 protein (ANS1) are attenuated in IFN-competent hosts such as MDCK cells and wild-type (wt) mice, but replicate in IFN-deficient Vero cells and STAT1-knockout (STAT1-KO) or PKR-KO mice (Egorov et al., 1998; Garcia-Sastre et al., 1998; Palese et al., 1999). Despite replication deficiency in normal hosts, animals immunized with ANS1 viruses elicit a strong immune response, demonstrated by increased antibody titres, elevated cytokine expression, activation of T-helper cells and protection against wt virus challenge (Donelan et al., 2003; Ferko et al., 2004; Talon et al., 2000b).

Using a reverse genetics system in Vero cells, we describe here the construction and properties of isogenic influenza B mutant viruses encoding C-terminally truncated NS1 proteins differing in length from 80 to 14 aa. Analogous to the data found for influenza A virus NS1 mutants (Egorov et al., 1998), these viruses were found to be attenuated in IFN-competent hosts but replicated to high titres in Vero cells. All of the constructed mutants were able to induce an antibody immune response upon intranasal (i.n.) immunization of mice and protected animals after homologous influenza virus challenge.

METHODS

Cells, viruses and viral infections. Vero cells (ATCC CCL-81) were used for transfection experiments, propagating viruses and virus titrations. Vero cells were adapted to and cultivated in serum-free OptiPro medium (Invitrogen) with 4 mM l-glutamine (Gibco). A549 cells (ATCC CCL-185) were cultivated in Dulbecco’s modified Eagle’s medium (Gibco) and used for growth curves of rescued viruses. Immortalized PKR-KO and wt mouse embryonic fibroblasts (MEFs) were obtained from H. Unger (University of Veterinary Medicine, Vienna, Austria), cultivated in minimal essential medium (Gibco) with 10% fetal calf serum (FCS; Gibco) and used for growth curves of rescued viruses. Primary human nasal epithelial cells (HNECs; Proviro) were cultivated in airway-epithelial cell growth medium (Proviro). Influenza virus B/Vienna/33/06 (B/Malaysia/2506/04-like) was provided by M. Redlberger (Institute for Virology, Vienna, Austria). Influenza B/Thuringen/02/06 (B/Jiangsu/10/03-like) virus was obtained from B. Schweiger (German National Reference Center for Influenza, Robert Koch Institute, Berlin, Germany). Both viruses were adapted to grow in serum-free Vero cells. For determination of viral replication levels, the indicated cells were washed with PBS and incu...
Generation of NS1-truncated viruses. The haemagglutinin (HA) and neuraminidase (NA) genes of B/Thuringen/02/06 virus and the remaining six genes of the B/Vienna/33/06 master strain including the non-coding regions were sequenced and each was cloned into pHW2006, a synthetically produced vector similar to pHW2000 (Hoffmann et al., 2002) generated by GE Healthcare. CD14-positive cells were purified by standard gradient centrifugation with Ficoll-Paque (GE Healthcare). CD14-positive cells were separated by immunomagnetic sorting using the VARIOMACS technique (Miltenyi Biotec) according to the manufacturer’s instructions. Isolated CD14-positive cells obtained from leukocyte-rich buffy coats of healthy donors were cultured in polystyrene six-well plates with a hydrophobic surface (Greiner Bio-One). Cells (2 x 10^6 per well) were cultivated in RPMI 1640 containing 10% FCS. On day 7, 1 ml of the supernatants analysed for viral yield by determining TCID_{50} ml^{-1} (PBS/Tween/I-Block). Serial dilutions of sera from immunized and, as a control, naive mice in PBS/Tween/I-Block were applied to the plates (50 µl per well) and incubated for 1.5 h at room temperature. After washing, secondary rabbit anti-mouse IgG1 or IgG2a antibodies conjugated to horseradish peroxidase (Invitrogen) were added. After an additional washing step, plates were stained with Ultra-TMB substrate (Thermo Scientific). The reaction was stopped with 4 M H_2SO_4 and absorbance was measured at 450 nm. The cut-off value was defined as the mean value of absorption of the blank +3 SD and is shown as one representative result from two independent experiments.

RESULTS

Rescue of influenza B mutant viruses encoding C-terminally truncated NS1 proteins in Vero cells

Eight plasmids expressing HA and NA from B/Thuringen/02/06 and the remaining six genes from B/Vienna/33/06 master strain were used to generate influenza B virus NS1-truncated mutants by reverse genetics. We found that this 6:2 gene composition led to high rescue efficiency in Vero cells (data not shown). Translation of NS1 was terminated by two consecutive in-frame stop codons at aa 14, 38, 57 and 80. The non-translated part downstream of the stop codons up to the splicing signal of NS2/NEP was deleted to prevent reversion to wt NS1. A schematic representation of the constructs is shown in Fig. 1(a). The NS1 truncation mutants were rescued in Vero cells, as was the recombinant wt virus. The resulting rescued truncation viruses containing the N-terminal NS1-specific 14, 38, 57 and 80 aa, respectively, were designated NS1-14, NS1-38, NS1-57, NS1-80 and NS1-wt. The different sizes of the NS1 gene of the generated mutant viruses, analysed by RT-PCR, confirmed the specific truncations as shown in Fig. 1(b). Despite several attempts, we did not succeed in rescuing a NS1-B virus, in which the ORF of NS1 is completely deleted and NS2/NEP is expressed as monocistronic mRNA.
reaching titres of $10^7$–$10^8$ TCID$_{50}$ ml$^{-1}$, which were comparable to those found with the wt virus. Replication of NS1-truncated viruses was severely attenuated in IFN-competent A549 cells compared with Vero cells. Whilst NS1-wt virus replicated to high titres of $4.4 \times 10^7$ TCID$_{50}$ ml$^{-1}$, the growth of NS1–80 virus was significantly impaired with a difference of approximately 4 orders of magnitude. The NS1 mutants expressing an NS1 protein of less than 80 aa were even more attenuated, showing almost complete growth restriction in A549 cells, with titres close to or below the detection limit of $2 \times 10^2$ TCID$_{50}$ ml$^{-1}$. Similar results were observed in human macrophages where only wt virus was able to replicate to 6 logs and none of the NS1 mutants replicated (data not shown).

In the next step, we investigated whether knocking out PKR, an antiviral protein known to be counteracted by the N-terminal domain of B/NS1 was sufficient to restore viral growth in IFN-competent cells. We compared the ability of viruses with the truncated proteins to grow in mouse embryonic fibroblasts derived from PKR-KO mice (Fig. 2c). This cell line supported the growth of wt virus up to greater than 7 logs. The NS1-80 virus replicated almost to wt levels, whereas the NS1-truncated mutants expressing an NS1 protein of less than 80 aa showed growth properties that were reduced by approximately 3 logs. None of the influenza B virus NS1-truncated mutants was able to replicate in MEFs derived from wt mice, indicating that the truncated NS1 protein was not able to counteract the antiviral action of PKR (data not shown).

**Fig. 1.** Generation of recombinant wt influenza B virus and NS1-truncated mutants. (a) Schematic representations of the NS genes and NS-specific mRNAs of the wt and NS1–14, NS1–38, NS1–57 and NS1–80 truncation viruses. The asterisks indicate two consecutive in-frame stop codons. The region downstream of the stop codons up to the splicing signal of NEP/NS2 was deleted. (b) RT-PCR analysis of viral NS segments. RNA was isolated from the wt influenza B virus and the NS1-truncated viruses, and the NS segments were reverse transcribed and amplified by PCR. The resulting products were separated on a 2 % agarose gel and stained with ethidium bromide. Fragment sizes are indicated.

**Fig. 2.** Growth properties of wt influenza B virus and the NS1-truncated mutants in different cell lines. Confluent monolayers of A549 cells (a), Vero cells (b) and PKR-KO MEFs (c) were infected with the indicated viruses at an m.o.i. of 0.01 and incubated at 33°C. At the indicated time points, the supernatants were harvested and the infectious titre was determined as TCID$_{50}$ ml$^{-1}$. All values below the detection limit of $1\times 10^2$ TCID$_{50}$ ml$^{-1}$ were considered to be $10^0$. 

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Influenza B virus NS1-truncated mutants induce antiviral and pro-inflammatory cytokines in macrophages and human nasal epithelial cells

It is well known that wt influenza viruses are able to antagonize the type 1 IFN response, as well as cytokine release from infected cells in various cell types (Dauber et al., 2004, 2006; Egorov et al., 1998; García-Sastre et al., 1998; Stasakova et al., 2005). In order to demonstrate the influence of B/NS1 on cytokine regulation, we evaluated the potential of the truncated mutants NS1-14, NS1-38 and NS1-80 and the NS1-wt virus to induce IFN-α/β and the major pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in 7-day-old human macrophages and primary nasal epithelial cells, respectively. The NS1-wt virus was fully competent to inhibit the release of IFN-α/β, TNF-α, IL-1β and IL-6, whereas all of the NS1-truncated viruses tested induced markedly higher levels of these cytokines in both macrophages and nasal epithelial cells (Fig. 3). Although the NS1-80 mutant virus showed intermediate growth capacity in IFN-competent cells, it appeared that the first 80 aa of NS1 were not sufficient to block the activation of IFN and other pro-inflammatory cytokines. Our data implied that C-terminal deletions of the B/NS1 are associated with the loss of functions responsible for inhibiting pro-inflammatory and antiviral cytokine production in human macrophages and nasal epithelial cells.

Influenza B virus NS1-truncated mutants are replication deficient in mice

Due to the attenuated replication pattern in A549 cells, we used a mouse model to examine whether the same attenuating effect was observable in vivo. Female BALB/c mice (6–8 weeks old) were infected i.n. with $5 \times 10^5$ TCID$_{50}$ NS1-truncated or wt virus per mouse. Viral titres in the lungs and nasal turbinates of mice were determined as TCID$_{50}$ (ml 10% tissue homogenate)$^{-1}$ in Vero cells at 3 days p.i. The geometric mean titres are shown in Table 1. The influenza B NS1-wt virus replicated to a titre of $3.83 \times 10^4$ TCID$_{50}$ ml$^{-1}$ in lung tissue and $6.29 \times 10^3$ TCID$_{50}$ ml$^{-1}$ in nasal turbinates. None of the NS1-truncated viruses could be reisolated from the lung and nasal tissue, indicating a replication-deficient phenotype.

Influenza B virus NS1-truncated mutants induce a virus-specific IgG response in mice

Next, we investigated whether the replication-deficient NS1-truncated mutants induced a humoral immune response in mice. Animals immunized with any of the NS1 mutant viruses showed substantial virus-specific serum antibody levels, even after a single i.n. immunization, as determined by serum ELISA 29 days post-immunization (Fig. 4). In contrast to wt influenza B virus,
a tendency to polarize the immune response towards Th1 was detected for the NS1-truncated mutants, reflected by a predominance of IgG2a antibodies over IgG1. The serum of non-immunized control mice did not yield a significant titre and did not show any polarization effect. Haemagglutination inhibition titres of immunized mice were detectable in up to 50% of responders with titres ranging from 8 to 16.

Mice are protected against wt virus challenge after a single immunization with NS1-truncated mutants

To investigate whether a single i.n. immunization of the replication-deficient NS1-truncated mutants induced protective immunity, mice were challenged with $5 \times 10^5$ TCID$_{50}$ homologous influenza NS1-wt per mouse at 32 days post-immunization. This NS1-wt challenge virus was found to reach the highest titres in mouse lungs among several contemporary strains. The challenge virus was derived from a human isolate and therefore did not induce symptoms such as weight loss or lethality in mice (data not shown). Mice were sacrificed at 3 days post-challenge and viral titres were determined in lungs and reported as geometrical mean titres of TCID$_{50}$ (ml 10% tissue homogenate)$^{-1}$. Following challenge with NS1-wt virus, none of the naïve mice was protected against infection, as indicated by viral loads of $2.47 \times 10^4$ TCID$_{50}$ ml$^{-1}$ in lung tissue. In contrast, all mice immunized with NS1-wt virus or with any of the NS1-truncated viruses were completely protected, as demonstrated by the absence of detectable challenge virus in their lungs (Table 2).

**DISCUSSION**

Seasonal influenza vaccines need to protect against circulating type A and type B influenza viruses. The purpose of this study was to develop contemporary, genetically defined and attenuated influenza B vaccine strains for production in certified Vero cells, to show that rescued strains may be suitable for i.n. immunization of humans. It has previously been shown that influenza A viruses with C-terminally truncated NS1 proteins of different lengths are attenuated in IFN-competent systems such as MDCK cells and mice. Their attenuation was dependent on the length of their NS1 protein, but they were able to replicate to high titres in IFN-incompetent Vero cells (Egorov et al., 1998), directly indicating the role of A/NS1 in IFN antagonism. In a similar approach, we developed a reverse genetics system employing contemporary influenza B virus adapted to high growth in Vero cells and generated a set of recombinant influenza B viruses with C-terminal deletions in their NS1 protein. The resulting viruses had NS1 proteins ranging from 14 to 80 aa, resulting in loss of the complete functional RBD (Wang & Krug, 1996).

Our data showed that the generated influenza B mutant viruses lacking a functional NS1 protein were capable of growth in IFN-deficient Vero cells and were highly attenuated in IFN-competent A549 cells, which is in

### Table 1. Replication of wt influenza B virus and the NS1-truncated mutants in mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Lung TCID$_{50}$ ml$^{-1}$</th>
<th>Nasal turbinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1-14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NS1-38</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NS1-57</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NS1-80</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NS1-wt</td>
<td>$3.83 \times 10^4$</td>
<td>$6.29 \times 10^3$</td>
</tr>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not detectable.

### Table 2. Protection of mice immunized with influenza B virus NS1-truncated mutants against wt influenza B virus challenge

<table>
<thead>
<tr>
<th>Virus used for immunization</th>
<th>TCID$_{50}$ ml$^{-1}$</th>
<th>No. protected/total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1-14</td>
<td>ND</td>
<td>4/4</td>
</tr>
<tr>
<td>NS1-38</td>
<td>ND</td>
<td>4/4</td>
</tr>
<tr>
<td>NS1-57</td>
<td>ND</td>
<td>4/4</td>
</tr>
<tr>
<td>NS1-80</td>
<td>ND</td>
<td>4/4</td>
</tr>
<tr>
<td>NS1-wt</td>
<td>ND</td>
<td>3/3</td>
</tr>
<tr>
<td>Control</td>
<td>$2.47 \times 10^4$</td>
<td>0/4</td>
</tr>
</tbody>
</table>

*Mice with a titre below the detection limit of $1 \times 10^2$ TCID$_{50}$ ml$^{-1}$ were considered to be protected.

ND, Not detectable.
accompanying with data found for influenza A virus. This demonstrated that, although influenza A and B virus NS1 proteins share only limited sequence homology and differ considerably in their regulatory functions, their major function of antagonizing the host’s innate immune system is similar.

We showed that the deletion mutant NS1-80 replicated to high titres in PKR-KO MEFs but was attenuated in A549 cells. This indicated that PKR is a major virus growth-restricting factor. This finding is in accordance with the data published by Dauber et al. (2006), who demonstrated that influenza B virus mutants with impaired RNA-binding capacity could not block the activation of PKR and were therefore highly attenuated in IFN-competent cells. Hence, the genetic inactivation of PKR is sufficient to largely rescue efficient replication of the NS1-80 virus. In contrast, deletion mutants shorter than 80 aa were highly attenuated in PKR-KO MEFs. We hypothesize that antiviral mechanisms other than PKR may be involved and that the N-terminal 80 aa of NS1 are sufficient to antagonize these. Mx and ISG15 could be excluded from the other IFN-induced antiviral proteins known to interact with influenza virus replication, because the former is not expressed in cells derived from most laboratory mice strains and the latter is not expected to be recognized by the B/NS1 protein of 80 aa (Yuan & Krug, 2001). Whether activation of 2′,5′-OAS or other antiviral proteins can be blocked by the partial function of the N-terminal 80 aa of B/NS1 remains to be investigated.

We were able to rescue an influenza B virus expressing an NS1 protein as short as 14 aa, which we assume to have no function. However, we failed to generate an influenza B virus completely lacking the NS1 open reading frame, in which NS2/NEP is expressed from a monocistronic and not from a spliced mRNA. Dauber et al. (2004) generated a ΔNS1 influenza B/Lee/40 virus (ΔNS1-B), which did not grow in 11-day-old embryonated eggs but could be propagated in 6-day-old eggs, which have an immature immune system (Dauber et al., 2004). It is not yet clear why ΔNS1-B does not replicate in IFN-deficient Vero cells, whilst the analogous influenza A ΔNS1 virus does. One reason why the influenza B NS1-14 virus but not ΔNS1-B is able to replicate in Vero cells may be the lack of a natural splice site in ΔNS1-B, potentially leading to overexpression of NS2/NEP mRNA and resulting in disrupted regulation of nuclear export function or in a disparity in the cellular processing of spliced to unspliced protein (Reed & Cheng, 2005). We speculate that this change in the expression strategy of NS2/NEP could be compensated for by other viral genes and that it might be possible to adapt a ΔNS1-B virus to replicate in Vero cells.

It has been shown previously that influenza A mutant viruses lacking the NS1 gene or expressing an NS1 protein with an impaired RBD and dimerization domain induce 10–50 times higher levels of pro-inflammatory cytokines in infected macrophages compared with wt virus (Stasakova et al., 2005). For type B viruses, the amino acids downstream of NS1 aa 104 were found to be essential for the control of hyperinduction of IFN-α/β in a continuous epithelial cell line (Dauber et al., 2006). The current study highlights the general importance of the B/NS1 protein in downregulating pro-inflammatory and antiviral cytokines, as shown by higher levels of IFN-α/β, TNF-α, IL-6 and IL-1β of the influenza B NS1-truncated mutants compared with wt virus in macrophages and primary HNECs.

This report is the first description of a targeted approach to generate live influenza B virus vaccine strains by reverse genetics whose attenuation mechanism is based on partial deletion of the NS1 protein. The generated B/NS1 mutant viruses can be used for further clinical development of an i.n.-administered live-attenuated vaccine. They offer the advantage of being completely replication-deficient and not provoking any shedding. Immunized animals were completely protected from wt virus challenge in mouse lungs, irrespective of the length of the remaining NS1.

We could show that mice receiving a single dose of replication-deficient virus induced anti-influenza IgG titres and had a tendency to polarize the immune system towards Th1, which is considered the best correlate of protection against viral infection (Proietti et al., 2002; Tovey et al., 2008). Due to the lack of a functional NS1 protein, an increased local production of cytokines and chemokines potentially stimulates the humoral and cellular arms of the immune system. As most inactivated vaccines are dependent on an adjuvant for stimulating such a response, we propose calling this phenomenon the ‘self-adjuvanting effect’.

Our data emphasize that IFN-sensitive influenza B viruses with truncations in their NS1 gene can be produced efficiently in Vero cells and are immunogenic in animals. This presents a new perspective for further vaccine development strategies.

ACKNOWLEDGEMENTS

This work was supported financially by EU grant FLUVACC contract no. 518281. We would like to thank Brunhilde Schweiger (Robert Koch Institute) and Monika Redlberger (Institute of Virology) for clinical influenza B virus isolates and Andrea Zöhner (Robert Koch Institute) for excellent technical support. Furthermore, we would like to thank Daniela Ribarits for support in cloning, Markus Wolschek for providing plasmid phW2006 and technical expertise, Elisabeth Maurer for support in cell culture work, Boris Ferko for help with animal experiments and Julia Romanova for a critical review of the manuscript (all from Avir Greenhills Biotechnology).

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