Evaluation of a modified vaccinia virus Ankara (MVA)-based candidate pandemic influenza A/H1N1 vaccine in the ferret model

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The zoonotic transmissions of highly pathogenic avian influenza viruses of the H5N1 subtype that have occurred since 1997 have sparked the development of novel influenza vaccines. The advent of reverse genetics technology, cell-culture production techniques and novel adjuvants has improved the vaccine strain preparation, production process and immunogenicity of the vaccines, respectively, and has accelerated the availability of pandemic influenza vaccines. However, there is still room for improvement, and alternative vaccine preparations can be explored, such as viral vectors. Modified vaccinia virus Ankara (MVA), originally developed as a safe smallpox vaccine, can be exploited as a viral vector and has many favourable properties. Recently, we have demonstrated that an MVA-based vaccine could protect mice and macaques against infection with highly pathogenic influenza viruses of the H5N1 subtype. In the present study, recombinant MVA expressing the haemagglutinin (HA) gene of pandemic influenza A/H1N1 virus was evaluated in the ferret model. A single immunization induced modest antibody responses and afforded only modest protection against the development of severe disease upon infection with a 2009(H1N1) strain. In contrast, two immunizations induced robust antibody responses and protected ferrets from developing severe disease, confirming that MVA is an attractive influenza vaccine production platform.

INTRODUCTION

The development of (pre)pandemic influenza vaccines has become a priority during the last 10 years since the first human cases of highly pathogenic avian influenza A/H5N1 virus infection in 1997 (WHO, 2010a, b). In addition to viruses of the H5N1 subtype that continue to circulate and cause infections of humans, avian influenza viruses of other subtypes including H7N7 and H9N2 have been transmitted to humans and caused fatal disease occasionally (de Wit et al., 2008; Fouchier et al., 2004; Koopmans et al., 2004). The need for rapid availability of vaccines against such zoonotic viruses was further exemplified after the worldwide spread of a new influenza A/H1N1 virus causing the first pandemic of the 21st century (WHO, 2009).

Major efforts have been made to develop new influenza vaccines. However, the relatively long response times and limited production capacity, poor immunogenicity and the antigenic variation of the zoonotic viruses complicate vaccine development. To address these issues, the production of efficacious vaccines has been improved with the use of reverse genetics to produce vaccine strains more rapidly and adjuvants to enhance immunogenicity and broaden the immune response (Kreijtz et al., 2009c). In addition to these developments, novel vaccine production platforms have been explored such as the use of viral vectors for the delivery of influenza virus proteins [e.g. haemagglutinin (HA)] (Stephenson et al., 2010). Modified vaccinia virus Ankara (MVA) is a replication-deficient poxvirus that was originally developed as a safe smallpox vaccine and has proven to be safe in humans (Mayr & Danner, 1978). It has been demonstrated that recombinant MVA driving the expression of foreign genes can be used as a viral vector. Favourable properties of MVA vector vaccines include

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high-level clinical safety, the availability of technologies for large-scale manufacturing, the ability to deliver substantial amounts of recombinant antigen and a particular immunostimulatory capacity due to adjuvant host responses such as type I interferon and chemokine synthesis (Lehmann et al., 2009; Waiblinger et al., 2007). Thus, recombinant MVA is an interesting candidate vector to serve as a high-yield production platform for pandemic influenza vaccines (Drexler et al., 2004; Rimmelzwaan & Sutter, 2009).

The potential of MVA as an influenza vector vaccine in mice was already recognized more than 16 years ago (Sutter et al., 1994). Recently, we demonstrated that a candidate H5N1 vaccine based on the MVA technology was highly immunogenic in mice and macaques and afforded protection against a lethal challenge infection in both species (Kreijtz et al., 2007, 2009b). Protective immunity was not only induced to the homologous challenge virus but also against infection with a heterologous virus from an antigenically distinct clade of H5N1 viruses. Moreover, protective immunity could be induced with low vaccine doses and even after a single immunization of mice (Kreijtz et al., 2009a).

Effective H1N1 vaccines have become available during the course of the 2009 pandemic caused by swine A/H1N1 viruses. However, they became available at a relatively slow rate and in insufficient numbers. The production of sufficient numbers of pandemic vaccine doses for a global vaccination campaign would take an estimated 1.5 to 4 years (Collin et al., 2009; IFPMA, 2009). Furthermore, up front, it was not known whether the use of an adjuvant was required for the pandemic vaccine to be immunogenic. Therefore, there is still a demand for alternative pandemic vaccine production technologies that would facilitate the rapid availability of efficacious vaccines.

For this reason, we wished to evaluate a 2009 H1N1 candidate MVA-based vaccine in ferrets, since this species constitutes an accepted model for the evaluation of influenza vaccines (Bodewes et al., 2010; Maher & DeStefano, 2004), including the currently used new H1N1 vaccines. Ferrets were immunized once or twice with an MVA expressing the HA gene of influenza H1N1 virus A/California/04/2009 (MVA-HA-Ca/09) to test vaccine immunogenicity. Subsequently the immunized animals were challenged with new H1N1 virus A/Netherlands/602/2009 (A/NL/602/09). Two immunizations with MVA-HA-CA/09 protected ferrets against severe disease caused by infection with new influenza A/H1N1 virus.

**RESULTS**

**Antibody responses after immunization**

After a single immunization, 82 and 91 % of the animals that received MVA-HA-CA/09 developed antibody titres against influenza virus A/NL/602/09 as detected in the haemagglutination inhibition (HI) and virus neutralization (VN) assays, respectively (Table 1). The geometrical mean titres (GMT) in the group receiving one immunization were 40.4 (SD=2.0) and 66.2 (SD=3.1) in the HI and VN assays, respectively (Table 1). Upon a second immunization, HI and VN antibody titres increased to 121.3 (SD=1.9) and 485.0 (SD=2.5) on the day of challenge infection, and all animals developed detectable virus-specific antibody titres, which remained the same 4 days post-infection (p.i.) (data not shown). No virus-specific antibodies were detected in sera of the PBS- or wild-type (wt) MVA-immunized control animals.

**Clinical signs after infection**

Upon challenge infection, all animals that received a single immunization developed clinical signs. They became lethargic, developed dyspnoea and lost their appetite. Animals that received two immunizations also showed clinical signs, although the animals that received two doses of MVA-HA-Ca/09 showed only mild symptoms and were more alert than the other animals. After infection, animals suffered from weight loss, with mean percentages of 18.1 (SD=1.5), 16.5 (SD=2.9) and 14.1 (SD=4.2) for the ferrets that received a single dose of PBS, wtMVA and MVA-HA-Ca/09, respectively (Table 2). Reduction of weight loss was more pronounced in the animals that received two immunizations with MVA-HA-CA/09 [6.6 % (SD=4.4)]. The mean weight loss in this group was significantly lower than that of PBS control animals (P=0.018). Animals that received a single immunization developed fever around day 1–2 after challenge infection that lasted for approximately 1–2 days (data not shown). A similar rise in body temperature was observed after infection of MVA-HA-Ca/09-immunized animals.

**Virus replication in the respiratory tract**

Upper (nasal and pharyngeal) and lower (lungs) respiratory tract samples were tested for the presence of infectious virus. Animals in all groups had detectable virus titres in their nose on day 2 p.i., which increased on day 4 p.i. (Fig. 1a, c). Virus was detectable in the pharyngeal swabs of all animals on days 2 and 4. The mean titres in the animals that received a single immunization were $10^{6.3}$ (SD=$10^{6.4}$),

| Table 1. Antibody titres 28 days after immunization with MVA-HA-Ca/09 |
|-----------------|-----------------|-----------------|
| **Assay**       | **Single**      | **Two**         |
|                 | immunization    | immunizations   |
| HI – A/Neth/602/09 | 40.4 (2.0)      | 121.3 (1.9)     |
| VN – A/Neth/602/09 | 66.2 (3.1)      | 485.0 (2.5)     |

Values for the single immunization are mean titres from 11 MVA-HA-Ca/09-immunized animals. Values for the double immunization are mean titres of five MVA-HA-Ca/09 immunized animals. Single animals for each experiment were unavailable due to unrelated complications. Values in parentheses are standard deviations.

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Table 2. Clinical symptoms and lung condition after infection with influenza A/Netherlands/602/09

Values for fever show the peak in body temperature; normal body temperature in the ferrets was 38.2 °C (sd=0.5). Relative lung weight was calculated from the lung weight relative to the body weight at day 4 p.i. Values in the parentheses are standard deviations.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Weight loss (%)</th>
<th>Fever (°C)</th>
<th>Affected lung area (%)</th>
<th>Relative lung weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single immunization</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PBS</td>
<td>18.1 (1.5)</td>
<td>40.4 (0.6)</td>
<td>62.0 (26.6)</td>
<td>2.0 (0.3)</td>
</tr>
<tr>
<td>wtMVA</td>
<td>16.5 (2.9)</td>
<td>40.5 (0.6)</td>
<td>62.0 (22.0)</td>
<td>1.7 (0.3)</td>
</tr>
<tr>
<td>MVA-HA-Ca/09</td>
<td>14.1 (4.2)</td>
<td>40.0 (0.7)</td>
<td>38.3 (24.2)</td>
<td>1.5 (0.5)</td>
</tr>
<tr>
<td><strong>Two immunizations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>16.1 (4.2)</td>
<td>40.9 (0.5)</td>
<td>61.2 (17.0)</td>
<td>1.8 (0.4)</td>
</tr>
<tr>
<td>wtMVA</td>
<td>15.6 (8.4)</td>
<td>41.1 (0.4)</td>
<td>45.0 (12.7)</td>
<td>1.7 (0.1)</td>
</tr>
<tr>
<td>MVA-HA-Ca/09</td>
<td>6.6 (4.4)*</td>
<td>40.3 (0.2)</td>
<td>15.5 (11.5)†</td>
<td>1.1 (0.2)†</td>
</tr>
</tbody>
</table>

*Significant difference in comparison with PBS-immunized group (P<0.05).
†Significant difference in comparison with PBS- and wtMVA-immunized groups (P<0.05).

10^6.3 (sd=10^0.6) and 10^6.1 (sd=10^0.4) for the PBS, wtMVA and MVA-HA-Ca/09 groups, respectively (Fig. 1b), on day 4 p.i. Virus titres in pharyngeal swabs on day 2 p.i. of the groups that received two immunizations were 10^5.5 (sd=10^0.5), 10^5.5 (sd=10^0.8) and 10^4.8 (sd=10^0.7). Virtually no reduction in titres was seen on day 4 p.i. in these animals (Fig. 1d).

In the lungs, the mean virus titres on day 4 p.i. for the animals that were immunized once were 10^7.2 (sd=10^0.8), 10^7.1 (sd=10^0.9) and 10^6.3 (sd=10^0.4) for the PBS, wtMVA and MVA-HA-Ca/09 groups, respectively (Fig. 2a). The lung virus titres of MVA-HA-Ca/09-vaccinated animals were significantly lower than those of the animals administered PBS (P=0.010). The animals that received two immunizations with PBS or wtMVA had similar lung virus titres (Fig. 2b), whereas animals that were immunized twice with MVA-HA-Ca/09 had a mean lung virus titre of 10^4.0 (sd=10^2.8) that again was statistically significantly lower than that of the PBS (P=0.006) and wtMVA (P=0.047) control groups.

Lung pathology after infection

Four days after infection, the ferrets were euthanized and the lungs were examined for gross pathological and histopathological changes. Macroscopic lesions were visible in all ferrets on day 4, with multifocal to coalescing consolidation characterized by a dark red colour and increased firmness of the tissue. In animals that received a single immunization with PBS or wtMVA, these areas covered 62% (sd=27) and 62% (sd=22) of the lung whereas, in MVA-HA-Ca/09-immunized animals, only 38% (sd=24) of the lung tissue was affected (Table 2). For the PBS and wtMVA groups that received two immunizations, 61% (sd=17) and 45% (sd=13), respectively, of the lung area was affected. MVA-HA-Ca/09-immunized animals displayed a mean area of 15.5%
smaller than the areas observed in the PBS (SD 11.5) of lung consolidation, which was significantly smaller than the areas observed in the PBS (P=0.006) and wtMVA (P=0.012) groups. These differences coincided with differences in relative lung weight of 1.1 % (SD=0.2) in MVA-HA-Ca/09-vaccinated ferrets, the lung weight being significantly lower than that of the PBS (P=0.018) and wtMVA (P=0.009) control groups (Table 2).

PBS- and wtMVA-immunized animals and those that received a single immunization with MVA-HA-Ca/09 developed severe bronchointerstitial pneumonia after infection. This was characterized by multifocal to coalescing lesions, tending towards the latter, corresponding with the macroscopic observations of the lungs. The pneumonia was characterized by extensive damage to the different epithelial structures and marked cellular infiltrate throughout the lesions. The epithelial structures affected were predominantly bronchiolar walls and the alveoli (Fig. 3). Extensive loss of bronchiolar epithelium resulted in disruption of the bronchiolar wall, and the bronchiolar lumina were filled with cellular debris. Furthermore, bronchioles were surrounded by a cuff of inflammatory cells consisting of lymphocytes and some neutrophils. The alveoli were filled with cellular infiltrate, mostly alveolar macrophages, lymphocytes and neutrophils, and the epithelial lining was lost due to necrosis. In contrast to the severe histopathological changes seen in the PBS- and wtMVA-immunized animals and those that received a single dose of MVA-HA-Ca/09, the animals that were immunized twice with MVA-HA-Ca/09 developed only mild pathological changes in their lungs (Fig. 3). Mainly bronchioles were affected, with moderate epithelial necrosis in the wall and marked lymphoid cuffing surrounding the affected bronchioles. Alveoli were only mildly affected with mild cellular infiltrate and mild necrosis; however, this concerned mainly the alveoli in peribronchiolar regions of affected bronchioles.

**Virus-infected cells detected by immunohistochemistry**

On day 4 p.i., antigen (nucleoprotein)-expressing cells were detected in the lungs of animals of all groups. In the unprotected PBS and wtMVA control animals and those that received a single dose of MVA-HA-Ca/09, infected cells were widespread and were located in the bronchiolar walls and mainly in the alveolar walls. The predominant infected cells were type II-like pneumocytes (Fig. 3), with fewer type I-like pneumocytes. Antigen-expressing cells were also present in animals that received two immunizations with MVA-HA-Ca/09; however, the number was generally smaller than in the other groups, and the infected cells were mainly located in the peribronchiolar region (Fig. 3). Bronchiolar epithelial cells were the abundant infected cell type, although some alveolar pneumocytes were positive as well. The sites of infection co-localized to a great extent with the histopathological changes.

**DISCUSSION**

In the present study, the immunogenicity and effectiveness of an MVA-based candidate 2009 H1N1 influenza vaccine was assessed in ferrets. One immunization with MVA-HA-Ca/09 induced low virus-specific antibody responses in the majority of the animals and afforded only modest protection against virus replication in the respiratory tract and the development of histopathological changes in the lungs upon challenge infection with 2009 H1N1 influenza strain A/NL/602/09. There was no clear correlation between antibody titres induced after one immunization and a reduction of lung virus titres. In contrast, two immunizations with MVA-HA-Ca/09 induced robust antibody titres in all animals and were associated with lower virus replication in the upper and lower respiratory tract upon challenge infection. Furthermore, animals that were immunized twice with MVA-HA-Ca/09 had reduced clinical signs and were protected against development of severe histopathological lesions upon inoculation with influenza virus A/NL/602/09. The observed protection was virus-specific and was probably mediated by virus-neutralizing antibodies and not by non-specific immune responses induced by the vector, since immunization with
wtMVA failed to afford any protection. In agreement with findings in mice and macaques (Kreijtz et al., 2007, 2009b; Stittelaar et al., 2001) and results from administration in humans (Mayr & Danner, 1978), MVA vaccination was tolerated well by the ferrets, although a small and transient rise in body temperature was observed after vaccination.

Although significant protection was achieved by vaccination with MVA-HA-Ca/09, residual virus replication was observed in ferrets. In contrast, macaques immunized with a recombinant MVA expressing the HA gene of H5N1 influenza virus A/Vietnam/1194/04 were fully protected against infection with the homologous strain or a

Fig. 3. Histopathological changes and antigen-expressing cells in the lungs of ferrets on day 4 p.i. with influenza virus A/Netherlands/602/09 (H1N1). Representative slides to show histopathological changes and virus replication were selected for unprotected animals that were unprotected as they were immunized with PBS, wtMVA or a single dose of MVA-HA-Ca/09. Animals that received two immunizations with MVA-HA-Ca/09 were protected from severe disease. Magnification, ×100. H&E, Haematoxylin and eosin; IHC, immunohistochemistry.
heterologous strain from an antigenically distinct clade of H5N1 viruses (Kreijtz et al., 2009b). It could be argued that this discrepancy may be related to difference in species and, for example, a lower susceptibility of ferrets to poxvirus infection. However, recombinant vaccinia viruses expressing different foreign viral antigens, including the HA of influenza virus, have been shown to induce protective immunity in ferrets (Jakeman et al., 1989). A recombinant MVA expressing the spike or nucleocapsid protein of severe acute respiratory syndrome (SARS) coronavirus also proved to be immunogenic (Czub et al., 2005; Weingartl et al., 2004). It is therefore unlikely that the differences in protective effectiveness in monkeys and ferrets were related to the species that was used.

Alternatively, the lack of full protection may be explained by differences in expression levels between the H1 protein and H5 protein or lower immunogenicity of the HA of influenza virus A/California/4/2009 compared with that of A/Vietnam/1194/04. However, the H1 and VN antibody titres induced by one and two administrations of the MVA-HA-Ca/09 vaccine were comparable to those obtained with two immunizations with the MVA expressing the H5 of influenza virus A/Vietnam/1194/04 in macaques (Kreijtz et al., 2009b). Thus, it is unlikely that the incomplete protection was related to the vaccine used or the species.

It is more likely that the relatively high dose of $10^6$ TCID$_{50}$ of influenza virus A/NL/602/09 used to inoculate the ferrets intratracheally prevented full protection from being achieved. This challenge dose was chosen based on a dose-escalation study that demonstrated that intratracheal inoculation of ferrets with this dose resulted in a robust challenge infection that caused extensive virus replication in the lungs and resulted in the development of severe pneumonia (van den Brand et al., 2010). The challenge dose is probably disproportionate compared with the human infectious dose in the field (Whitley, 2010). For the same reason, a single immunization with MVA-HA-Ca/09 could not be expected to afford full protection, as was also observed for other influenza A/H1N1 vaccines (Kobinger et al., 2010).

Although immunization with MVA-H1 achieved a reduction of virus replication in the lower respiratory tract, it did not reduce replication in the upper respiratory tract. In contrast, vaccination with MVA-H5 reduced virus replication in both the lower and upper respiratory tract. However, since it is well-established that H5N1 viruses replicate poorly in the upper respiratory tract because of absence of specific receptors (SA-z-2,3-Gal) used by avian influenza virus, it is likely that the virus detected in the upper respiratory tract of H5N1-infected macaques was derived from the lungs (van Riel et al., 2006).

It is possible that, for efficient protection against H1N1 virus replication in the upper respiratory tract, other arms of the immune system need to be activated, such as the production of mucosal antibodies, which may contribute to reducing transmission.

Immunization with MVA-HA-Ca/09 induced HI and VN virus-specific antibody titres that are considered to be protective and that fulfil EMA (European Medicines Agency) criteria for influenza vaccine potency. Interestingly, it has been shown that, in the absence of detectable virus-specific antibodies, protection can be achieved after vaccination with MVA-based influenza vaccines expressing HA (Kreijtz et al., 2007, 2009b). It is most likely that vaccination with MVA-based vaccines not only induces protective antibody responses but can also activate other arms of the immune system that contribute to protection against infection, such as the induction of virus-specific T cells.

In conclusion, immunization with a recombinant MVA vector expressing the HA gene of 2009 (H1N1) influenza virus A/California/4/2009 induced protective antibody titres and protected ferrets against the development of severe disease upon infection with a high dose of a new H1N1 influenza virus. Although a recombinant MVA was used for the production of influenza viral antigen in vitro (Schmeisser et al., 2010), to our knowledge the present study reports for the first time the evaluation of recombinant MVA for the delivery of the HA of 2009 H1N1 influenza virus in ferrets.

Our findings confirm that the MVA technology is a versatile vaccine production platform and that its further development is warranted as a basis for future-generation (pre)pandemic influenza vaccines.

METHODS

Generation of recombinant virus. The HA gene of influenza virus A/California/4/2009 (GenBank accession no. FJ960821) was synthesized by GENEART AG (Regensburg, Germany) without sequence optimization, flanked by Hin1I and Kpnl restriction sites and inserted into the vector plasmid pLW-9 (Wyatt et al., 1996). The resulting plasmid was designated pIII-HA-H1N1. The gpt-lacZ selection cassette was amplified from the vector plasmid pIInewLacZgptdel (Staib et al., 2003) by oligonucleotides 5'-GCCAATTGTTATTTTGACACCAGAACCCCCCA-3' and 5'-GCCAATTGTAGCGACCGGAGATTGGCG-3' (sites for the restriction enzyme MfeI are underlined) and cloned into the EcoRI site of pIII-HA-H1N1, resulting in the vector plasmid pIII-HA-H1N1-gpt-LacZ. Recombinant MVA expressing the HA gene (MVA-HA-CA/09) was generated by transfection of MVA-infected chicken embryo fibroblasts (CEF) with pIII-HA-H1N1-gpt-LacZ plasmid DNA and clonally isolated following the transient dominant selection method as described previously (Falkner & Moss, 1990). To generate vaccine preparations, MVA-HA-CA/09 was amplified in CEF, purified by ultracentrifugation through sucrose, reconstituted and diluted in PBS. Expression of the HA protein was confirmed in cell lysate from CEF cells infected with the recombinant MVA (data not shown).

Challenge virus. Influenza virus A/Netherlands/602/2009 (A/NL/602/09) (pH 1N1) was isolated from a 3-year-old child and was the first laboratory-confirmed case of the pandemic influenza virus in the Netherlands (Munster et al., 2009). The virus was isolated and cultured in Madin–Darby canine kidney (MDCK) cells and the infectious virus titre was determined as described previously (Rimmelzwaan et al., 1998). The sequence identity of the HA protein of A/NL/602/09 compared with that of the A/California/4/2009 strain is 99.4%.
**Ferrets.** Young adult purpose-bred female ferrets (*Mustela putorius furo*, between 6 and 12 months old) were used. Ferrets that were seronegative for antibodies against circulating influenza A/H1N1 and A/H3N2 viruses and the new influenza A/H1N1 virus (2009) and Aleutian disease virus were used in this experiment. An independent animal ethics committee (DEC consult) approved the experimental protocol before the start of the experiment.

**Immunizations and infection.** Animals were immunized once or twice intramuscularly with 10⁶⁵ p.f.u. MVA-HA-CA/09 (n=6) with an interval of 4 weeks. Only five animals were left for the two-immunization group for a reason unrelated to the experiment. PBS (n=6) and wtMVA (n=6) were used as negative control immunizations. Before each immunization and before the challenge, blood was drawn from the animals to determine influenza-specific antibodies. Four weeks after the second immunization, the animals were inoculated intratracheally under anaesthesia [ketamine/medetomidine (reversed with atipamezole)] with influenza virus A/RL/602/09 (H1N1) at a dose of 10⁶ TCID₅₀ as described previously (van den Brand et al., 2010). On day 0, 2 and 4 p.i., nasal and pharyngeal swabs were taken from the animals under anaesthesia and, 4 days p.i., the animals were euthanized and necropsies were performed. From the time of infection onwards, the animals were housed in Bio-safety Level 3 containment facilities.

**Serology.** Serum samples obtained from immunized animals were tested for the presence of virus-specific antibodies. To this end, they were treated with cholera filtrate and heat-inactivated at 56 °C and tested in an HI assay following a standard protocol using 1% turkey erythrocytes and four HA units of influenza virus A/RL/602/09 (Palmer et al., 1975). For this purpose, reverse genetics virus was constructed with the HA and NA of the 2009 H1N1 virus and the remaining six gene segments of influenza virus A/Puerto Rico/8/34 (A/PR/8/34). The antibody titres obtained with this virus were comparable to those obtained with the wild-type strains (not shown).

Serum samples obtained from immunized animals were tested for the presence of virus-neutralizing antibodies using a micro-VN assay with the reverse genetics virus described above (Rimmelzwaan et al., 1999). In brief, 50 µl serially diluted serum samples were incubated with 100 TCID₅₀ virus for 1 h at 37 °C and then the mixture was added to MDCK cells. After 1 h, the cells were washed and subsequently cultured in Eagle’s minimal essential medium containing 4 µg trypsin ml⁻¹, 2 mM l-glutamine, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 0.15% NaHCO₃, 20 mM HEPES and 0.1 mM non-essential amino acids. After 5 days, residual virus replication was assessed by measuring HA activity in the culture supernatants. Serum samples obtained from ferrets infected with a non-lethal dose of influenza virus A/Netherlands/602/09 were used as positive controls. For calculation purposes, serum samples with an antibody titre of <10 were arbitrarily assigned a titre of 5.

**Virus replication in the respiratory tract.** To detect virus in the upper respiratory tract, nasal and pharyngeal swabs were taken from the animals on days 0, 2 and 4 p.i. and stored in transport medium [Hanks’ medium (MEM) with lactalbumin, glycerol, penicillin, streptomycin, polymyxin B, nystatin and gentamicin] at −70 °C until use. For the lower respiratory tract, lobes of the right lung were sampled, including the accessory lobe. Before sampling, the lung weights were determined and expressed as a percentage of the total body weight (relative lung weight). Lung samples were snap frozen on dry ice with ethanol and stored at −70 °C. Subsequently, they were homogenized in transport medium with a FastPrep-24 (MP Biomedicals) and the homogenate was centrifuged briefly before dilution in the assay. Quintuplicate tenfold serial dilutions of these samples and quadruplicate tenfold serial dilutions of the swab samples were used to determine the virus titres on confluent layers of MDCK cells, as described previously (Rimmelzwaan et al., 1999).

**Histopathology and immunohistochemistry.** Formalin-inflated left lungs were fixed in 10% neutral-buffered formalin and subsequently cross-sections were made, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method with a monoclonal antibody (clone HB65 IgG2a; American Type Culture Collection) against the nucleoprotein of influenza A virus. Goat-anti-mouse IgG2a–horse-radish peroxidase (Southern Biotech) was used as a secondary antibody. The peroxidase was revealed using diaminobenzidine as a substrate, resulting in a deep-red precipitate in the nuclei of influenza A virus-infected cells and a less intense red staining of their cytoplasm. The sections were counterstained with haematoxylin.

**Statistical analysis.** Data were analysed using the Mann–Whitney test and differences were considered significant at P<0.05.

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