Experimental infection of macaques with human metapneumovirus induces transient protective immunity

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Human metapneumovirus (hMPV), a member of the family Paramyxoviridae, is a causative agent of acute respiratory-tract illness. Two main hMPV lineages circulate worldwide and reinfections occur frequently. It is unclear what level of protection is induced by natural hMPV infection, what the durability of this protection is and whether it differs for reinfection with homologous or heterologous viruses. Here, protective immunity in cynomolgus macaques at different time points after inoculation with molecularly cloned prototype viruses of the two main lineages of hMPV has been addressed. Animals received a homologous challenge at 4, 6 or 12 weeks after the primary infection. In addition, animals that had been inoculated three times within 10 weeks were challenged with homologous or heterologous virus 8 months later. Primary infection with $10^7$ TCID$_{50}$ resulted in virus shedding and induction of virus-neutralizing antibody responses, with higher titres against the homologous than the heterologous virus. Infections associated with virus shedding and seroconversion protected completely from homologous reinfection within 6 weeks, and partly at 12 weeks, after primary infection. Eight months later, protection had waned to virtually undetectable levels. This study demonstrates that experimental hMPV infection induces transient protective immunity.

INTRODUCTION

Human metapneumovirus (hMPV), a member of the family Paramyxoviridae, is a causative agent of acute respiratory-tract illness in humans (van den Hoogen et al., 2001). Clinical studies have identified young infants, immunocompromised patients and the elderly as risk groups for hMPV-associated severe disease (van den Hoogen et al., 2003; Williams et al., 2005, 2006). hMPV causes clinical symptoms similar to those associated with the closely related Human respiratory syncytial virus (RSV) (Morrow et al., 2006; van den Hoogen et al., 2003; Wilkesmann et al., 2005; Williams et al., 2005, 2006). Phylogenetic studies have identified two major genetic lineages of hMPV that circulate worldwide (A and B), with each lineage divided into at least two sublineages (1 and 2). A cross-neutralization study with ferret sera collected early after infection indicated that the two main lineages represent two serotypes (van den Hoogen et al., 2004). However, the existence of serotypes has not been confirmed with sera from other animal species and the relevance for humans is still under debate (Biacchesi et al., 2005; Skiadopoulos et al., 2004).

Experimental hMPV infections of Syrian golden hamsters, chimpanzees and African green monkeys indicated that primary infections protected from subsequent reinfections with homologous and heterologous viruses if the animals were challenged after 4–6 weeks (Biacchesi et al., 2005; MacPhail et al., 2004; Skiadopoulos et al., 2004). In the human population, reinfections with viruses from homologous and heterologous lineages have been reported to occur throughout life (Ebihara et al., 2004b; Henderson et al., 1979; Leung et al., 2005; van den Hoogen et al., 2001), suggesting that protective immunity may be transient.

Systemic acute virus infections, such as those caused by measles, mumps and rubella viruses, induce lifelong protective immunity (Griffin, 2001), whereas most viruses that are restricted to replication in the respiratory tract, such as parainfluenza virus and RSV, induce transient immunity (Chanock et al., 2001; Collins et al., 1996; Durbin & Durbin, 2004). Presumably as the result of waning antibody levels, reinfections with respiratory viruses can occur throughout life and can cause severe disease in adults with underlying disease (Hall et al., 1991; Henderson et al., 1979). To date, little is known about the clinical impact of reinfections with hMPV; one heterologous reinfection within 1 month of the primary infection has been reported (Ebihara et al., 2004a). Information on the longevity of the humoral immune response induced by prior hMPV infections is also still limited.
Because of the clinical impact of hMPV, serious efforts to develop vaccines have been initiated. Promising live-attenuated vaccines have been obtained with reverse-genetics techniques, using recombinant viruses based on parainfluenzavirus types 1 and 3 and hMPV (Biacchesi et al., 2004a; Herfst et al., 2004a; MacPhail et al., 2004; Tang et al., 2005). In animal models, these candidate vaccines induced protective immunity against subsequent infection with either homologous or heterologous hMPV strains 4–6 weeks after immunization (Biacchesi et al., 2004b). However, in most cases, these vaccines are aimed at inducing antibodies against the conserved fusion protein of hMPV, thus circumventing antibody responses against the more variable hMPV surface glycoproteins G and SH. Protective immunity induced by these immunizations for periods of longer than 6 weeks has not been reported (Buchholz et al., 2005; MacPhail et al., 2004; Tang et al., 2005).

Previously, we have demonstrated that cynomolgus macaques are a suitable non-human primate model for hMPV infections (Kuiken et al., 2004) by using a serially passaged wild-type virus isolate (NL/1/00). Because molecularly cloned viruses representing both main hMPV lineages (NL/1/00 and NL/1/99) have become available, this model needed re-evaluation. The present study was designed to evaluate the duration and specificity of antibody responses upon repeated infections with molecularly cloned prototype viruses representing the two main lineages.

**METHODS**

**Viruses.** The construction and rescue of recombinant viruses NL/1/00 (prototype for lineage A) and NL/1/99 (prototype for lineage B) have been described elsewhere (Herfst et al., 2004). After virus rescue from 293-T cells, the virus was passaged twice for 5 days on Vero-118 cells in infection medium [Isocoe’s modified Dulbecco’s medium (IMDM) supplemented with 0.02 % trypsin and 3 % bovine albumin fraction V]. Vero-118 is a subclone of Vero-WHO cells selected for equal permissiveness to all genetic lineages of hMPV (Kuiken et al., 2004).

After freeze–thawing, the supernatants were stored in 25 % sucrose (w/v) at an intramuscular dose of 0.4 ml kg⁻¹. The total virus dose was thawed immediately before inoculation and diluted in 6 ml PBS. Each animal was inoculated intratracheally with 5 ml virus solution and the remaining 1 ml was divided between the two nostrils and the conjunctivae. In the first experiment, four animals were inoculated with hMPV NL/1/00 and four with NL/1/99, at two different doses (10⁶ and 10⁸ TCID₅₀). In all subsequent experiments, a dose of 10⁷ TCID₅₀ was used. An overview of the experimental design is shown in Table 1.

**Sample collection.** To monitor virus replication, animals were anaesthetized lightly with ketamine (10 %, w/v); intramuscular dose 0.15 ml kg⁻¹) every 1–2 days after inoculation, and pharyngeal and nasopharyngeal swabs were collected in 1.5 ml transport medium consisting of Hanks’ balanced salt solution supplemented with 10 % glycerol, 200 U penicillin ml⁻¹, 200 μg streptomycin ml⁻¹, 100 U polymyxin B sulfate ml⁻¹, 250 μg gentamicin ml⁻¹ and 50 U nystatin ml⁻¹ (all from ICN). Samples were mixed vigorously and aliquots of 250 μl were stored at −70 °C until processing. To test for serum antibody responses, blood samples were collected at regular time intervals and sera were stored frozen at −20 °C.

**Real-time RT-PCR.** RNA was isolated from 200 μl throat- or nose-swab material or from 5 × 10⁵ BAL cells in 400 μl lysis buffer by using a High Pure viral RNA kit (Roche Diagnostic) for RT-PCR and stored at −70 °C until processing. On day 3 after each inoculation, bronchoalveolar lavage (BAL) samples were collected by intratracheal infusion and subsequent recovery of 10 ml PBS with a flexible catheter. BAL cells were centrifuged (10 min, 400 g), resuspended in PBS, counted and 5 × 10⁵ cells were added to 400 μl RNA isolation buffer (provided with the High Pure viral RNA kit; Roche Diagnostic) for RT-PCR and stored at −70 °C until processing. To test for serum antibody responses, blood samples were collected at regular time intervals and sera were stored frozen at −20 °C.

**Study design.** Ten cynomolgus macaques (Macaca fascicularis) were inoculated with recombinant hMPV. At the start of the study, the animals were 18–20 months old and had a body weight of 1.9–3.2 kg. All animals were seronegative for both hMPV lineages, as tested by ELISA, immunofluorescence and virus-neutralization assays. From the first inoculation until 3 weeks after the third, when virus shedding had stopped, the animals were housed in negatively pressurized HEPA (high-efficiency particulate air)-filtered isolator cages. The animals were subsequently housed as one group in a standard cage and were moved to isolator cages again from inoculation 4 until 3 weeks after inoculation 5. The animal studies were approved by the Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with national animal-experimentation guidelines.

Before inoculation, animals were anaesthetized by using a cocktail of ketamine (2.5 %, w/v), xylazine (0.25 %, w/v) and atropine (0.025 %, w/v) at an intramuscular dose of 0.4 ml kg⁻¹. The total virus dose was thawed immediately before inoculation and diluted in 6 ml PBS. Each animal was inoculated intratracheally with 5 ml virus solution and the remaining 1 ml was divided between the two nostrils and the conjunctivae. In the first experiment, four animals were inoculated with hMPV NL/1/00 and four with NL/1/99, at two different doses (10⁶ and 10⁸ TCID₅₀). In all subsequent experiments, a dose of 10⁷ TCID₅₀ was used. An overview of the experimental design is shown in Table 1.

**Table 1. Study design**

Virus (dose) inoculated at the indicated time points is shown.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Inoculation 1 (week 0)</th>
<th>Inoculation 2 (week 6)</th>
<th>Inoculation 3 (week 10)</th>
<th>Inoculation 4 (week 58)</th>
<th>Inoculation 5 (week 74)</th>
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<tbody>
<tr>
<td>1, 2</td>
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<td>NL/1/00 (10⁷)</td>
<td>NL/1/00 (10⁵)</td>
<td>NL/1/00 (10⁷)</td>
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<td>NL/1/00 (10⁶)</td>
<td>NL/1/00 (10⁷)</td>
<td>NL/1/00 (10⁵)</td>
<td>NL/1/00 (10⁷)</td>
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<tr>
<td>5, 6</td>
<td>NL/1/99 (10⁶)</td>
<td>NL/1/99 (10⁷)</td>
<td>NL/1/99 (10⁵)</td>
<td>NL/1/99 (10⁷)</td>
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<tr>
<td>7, 8</td>
<td>NL/1/99 (10⁶)</td>
<td>NL/1/99 (10⁷)</td>
<td>NL/1/99 (10⁵)</td>
<td>NL/1/00 (10⁷)</td>
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<td>9, 10</td>
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<td>–</td>
<td>NL/1/00 (10⁷)</td>
<td>NL/1/00 (10⁷)</td>
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the detection of NL/1/99 virus. Each sample was tested three times by RT-PCR and genome copy numbers were quantified by using a standard curve generated by RNA runoff transcripts of a PCR product, as described previously (Maertzdorf et al., 2004). Data are shown as the geometric means of the genome copy numbers in samples from two animals with the same infection history.

**Virus isolation.** Virus isolation was performed on Vero-118 cells as described previously (Kuiken et al., 2004). Briefly, Vero-118 cells were plated in 96-well plates in IMDM supplemented with 10% fetal calf serum (Greiner Bio-One). The next day, cells were washed with IMDM without additions, and 100 μl of threefold serial dilutions of the collected swab samples was applied to the cells by spinoculation (O’Doherty et al., 2000). After 1 h incubation at 37°C, the sample was removed, the cells were washed and subsequently incubated with infection medium. After 7 days culture, an immunofluorescence assay was used to identify infected cultures as described previously (van den Hoogen et al., 2004).

**Virus neutralization.** Virus-neutralizing (VN) antibody titres were determined by a plaque-reduction virus-neutralization assay. Heat-inactivated (30 min, 56°C) serum samples, diluted by 2⁻³, 2⁻⁴, 2⁻⁵, and 2⁻⁶, were incubated for 1 h at 37°C with 50 p.f.u. NL/1/00 or NL/1/99 expressing the enhanced green fluorescent protein (EGFP) at position 3 of the genome. Subsequently, the serum/virus mixtures were added to Vero-118 cells in 24-well plates and incubated at 37°C on a rocking platform. After 2 h, the supernatants were removed and 1 ml IMDM containing 2% bovine albumin fraction V, 1% methylcellulose (MSD) and 0.02% trypsin was added to the wells. Six days later, fluorescent plaques were counted by using a Typhoon 9410 variable mode imager (GE Healthcare). VN titres are expressed as the dilution resulting in 50% reduction of the number of plaques in the control wells on the same plate, calculated according to the method of Reed & Muench (1938). Per assay, each serum was tested in duplicate, and per serum, the assay was repeated three times against both hMPV NL/1/00 and NL/1/99. Results are shown as the geometric mean titre (GMT) calculated from these three assays.

## RESULTS

### Infectious dose

In order to find the optimal infectious dose for the molecularly cloned viruses NL/1/00 and NL/1/99, macaques were initially inoculated with two different doses (10⁴ and 10⁶ TCID₅₀). Viral genomes were only detected in swabs collected from animals inoculated with 10⁶ TCID₅₀ virus (Fig. 1b, d). For NL/1/00-injected animals, viral genomes were detected between days 1 and 10 in the nose and throat. For the NL/1/99-injected animals, viral genomes were detected between days 6 and 10 in the nose and between days 3 and 10 in the throat. Besides the longer shedding period for NL/1/00-inoculated animals, the numbers of viral genomes at peak days of shedding were approximately 1000-fold higher than those in NL/1/99-inoculated animals. Viral genomes were also detected in BAL samples collected 3 days after inoculation with 10⁶ TCID₅₀, with little difference between NL/1/00- and NL/1/99-inoculated animals (data not shown).

For the NL/1/00-inoculated animals, infectious virus was recovered from samples containing >5 × 10⁵ genome copies in 100 μl at peak days of virus shedding (data not shown). In the samples collected from NL/1/99-inoculated animals, the genome copy numbers remained below 1 × 10⁵ genomes and no infectious virus could be detected. The virological data were confirmed by serology: in contrast to the 10⁶ TCID₅₀-inoculated animals (Fig. 2a, c), the 10⁶ TCID₅₀-inoculated animals developed a virus-specific VN antibody response (Fig. 2b, d).

**HMPV infection protects from early reinfection**

The animals receiving 10⁴ TCID₅₀ were subsequently regarded as naïve animals, because no virus could be detected upon inoculation and serum antibodies were absent, as measured by VN (Fig. 2a, c), ELISA and immunofluorescence assays (data not shown). We also concluded that 10⁶ TCID₅₀ might have been a suboptimal infectious virus dose to achieve a robust infection, in particular for NL/1/99. Thus, we used a higher dose (10⁷ TCID₅₀) for all subsequent inoculations.

Six weeks after the first inoculation, a second inoculation was performed with 10⁷ TCID₅₀ of the homologous viruses. In the four animals that had previously received 10⁴ TCID₅₀ but had remained seronegative, virus shedding and specific VN antibody responses were detected and reached levels similar to those in the four animals receiving 10⁶ TCID₅₀ in the first part of the experiment (Figs 1e, g, 2a, c). For the 10⁷ TCID₅₀ NL/1/00-inoculated animals, virus shedding from the throat was similar to that observed for the 10⁶ TCID₅₀-inoculated animals in the first inoculation. Virus shedding from the nose appeared to be higher this time, and viral genomes were detected from day 1 post-inoculation onwards (Fig. 1e). For the NL/1/99-inoculated animals, the higher dose resulted in higher genome copy numbers in the respiratory tract and an earlier peak of shedding, day 5 versus day 8 post-inoculation (Fig. 1g versus Fig. 1d). Mean copy numbers of viral genomes for the NL/1/99-inoculated animals were still 10–50-fold lower than those seen in NL/1/00-inoculated animals. Similar to inoculation 1, no infectious virus could be detected in NL/1/99-inoculated animals during this second part of the experiment, despite detection of viral genomes by RT-PCR. Inoculation with 10⁷ TCID₅₀ of both viruses resulted in the induction of virus-specific antibody responses (Fig. 2a, c).

The animals that had previously been inoculated with 10⁶ TCID₅₀ were largely protected from subsequent challenge 6 weeks later with 10⁷ TCID₅₀ (Fig. 1f, h). No viral genomes were detected in nose or throat swabs, although the 5 × 10⁵ BAL cells collected on day 3 post-inoculation contained 0.06–8 × 10⁴ (GMT, 5.6 × 10⁴) genome copies, which was similar to the 0.02–5 × 10⁵ (GMT, 3.3 × 10⁴) genome copies in the control animals. In addition, this second inoculation resulted in boosting of the serum VN antibody levels (Fig. 2b, d).

A third inoculation was undertaken 4 weeks after the last challenge (10 weeks after the first inoculation). This
Fig. 1. Detection of hMPV by real-time RT-PCR in nose and throat samples obtained from cynomolgus macaques inoculated with NL/1/00 (a, b, e, i, j, m, n, o, p) or NL/1/99 (c, d, g, h, k, l). (a, c) Primary inoculation with 10⁴ TCID₅₀; (b, d) primary inoculation with 10⁶ TCID₅₀; (e–p): inoculation with 10⁷ TCID₅₀; (e–h) second inoculation with homologous virus at 6 weeks post-primary inoculation; (i–l) third inoculation with homologous virus at 4 weeks post-second inoculation; (m, n) fourth inoculation with homologous virus at 48 weeks post-third inoculation; (o, p) fourth inoculation with heterologous virus at 48 weeks post-third inoculation. Each bar represents the geometric mean titre (GMT) ± SD for two animals with the same inoculation history. Time points, virus type and dose used for the different inoculations are indicated. Sampling days are indicated below the x-axis.
inoculation served two goals: first, to study the possibility of reinfection with the homologous virus in the presence of high antibody titres, and second, to study whether antibody titres could be boosted to even higher levels. All animals were fully protected from infection: no virus genomes could be detected in nose, throat or BAL samples (Fig. 1i–l). In the animals that had received the low virus dose ($10^4$ TCID$_{50}$) during the first inoculation (Fig. 1a, c), a minor boost in serum VN antibody levels was detected, despite the absence of detectable virus shedding (Fig. 2a, c).

**Fig. 2.** hMPV NL/1/00- and NL/1/99-specific VN antibody titres. Symbols and error bars represent the GMT of two animals per group. Animals were inoculated with $10^4$ (a, c) or $10^6$ (b, d) TCID$_{50}$ NL/1/00 or NL/1/99, respectively. The animals were challenged with $10^7$ TCID$_{50}$ NL/1/00 at weeks 6, 10 and 58 post-primary inoculation (a, b) or with $10^7$ TCID$_{50}$ NL/1/99 at weeks 6 and 10 and $10^7$ TCID$_{50}$ NL/1/00 at week 58 post-primary inoculation (c, d). Sampling weeks are indicated below the x-axis.
At the end of inoculation 3, all animals had high levels of virus-specific VN antibody titres. Within 8 weeks of this third inoculation, antibody titres declined to levels comparable to those seen 6 weeks after the primary high-dose inoculation (Fig. 2, week 18).

Clinical signs were not observed after any of the experimental infections. In the 8 months following the third inoculation, two of the animals (1 and 8) died of causes unrelated to the hMPV inoculations.

**Waning immunity 3–11 months after infection**

Eleven months after the last virus inoculation, specific VN antibody levels were still detectable, although at low levels (Fig. 2, week 58). At this stage, all animals were challenged with $10^7$ TCID$_{50}$ NL/1/00, which was the homologous virus for three animals (Fig. 1m, n) and the heterologous virus for the other three (Fig. 1o, p). Two naive animals were used as controls (Fig. 3a).

None of the heterologous-challenged animals displayed any sign of protection: viral genomes were detected in nose, throat and BAL samples at levels comparable to those in the control animals (Figs 1o, p, 3a). Both control animals and the three heterologously-challenged animals demonstrated peak genome copy numbers between days 3 and 5 post-inoculation of around $10^7$ genomes per 100 µl. On days 3 and 5 post-inoculation, infectious virus was recovered from the throat samples of all animals. Of the three homologously-challenged animals, one animal (2) was partially protected, as demonstrated by lower viral genome copy numbers in the throat samples and absence of viral genomes in the nose and BAL samples (Fig. 1m). The other two homologous-challenged animals displayed a peak of viral shedding between days 3 and 5 post-inoculation, similar to the control animals, and peak genome copy numbers were as high as those detected in the control animals (Figs 1n, 3a). From all three homologously-challenged animals, infectious virus was recovered from the throat samples at peak days of virus shedding. All animals responded with a boost in VN antibody titles with peak levels comparable to those recorded shortly after inoculation 3 (Fig. 2).

In order to address protective immunity later than 6 weeks but earlier than 11 months after hMPV infection, the control animals from inoculation 4 ($n=2$) were challenged after 12 weeks with $10^7$ TCID$_{50}$ homologous virus. These animals had an intermediate level of protection: virus replication could be detected, but peak genome copy numbers and viral shedding kinetics were not as high as those seen after inoculation 4 (Fig. 3b). Whilst after inoculation 4 (primary inoculation for these animals), virus shedding was detected between days 1 and 9 post-inoculation (Fig. 3a), after inoculation 5, virus shedding occurred between days 3 and 8 for animal 9 and only at day 3 (throat only) for animal 10. In addition, peak viral genome copy numbers were 100-fold higher after inoculation 4 than those detected after inoculation 5 (Fig. 3). Finally, the BAL samples contained 1000-fold fewer viral genomes at 3 days post-inoculation 5 than the samples collected after inoculation 4 (data not shown).

**Antigenic differences between hMPV NL/1/00 and NL/1/99**

All sera were tested in VN assays against NL/1/99 and NL/1/00. In general, homologous titres were higher than heterologous titres (Fig. 2). Homologous NL/1/00-inoculated animals had, on average, 16-fold higher homologous than heterologous VN antibody titres, whereas NL/1/99-inoculated animals had on average sixfold higher homologous VN titres. After heterologous challenge (animals 5, 6 and 7 at inoculation 4), antibody levels to NL/1/00 and NL/1/99 reached similar levels (Fig. 2).

**DISCUSSION**

Previously, we established a cynomolgus macaque model for hMPV infection, using $10^5$ TCID$_{50}$ NL/1/00 (a prototype, uncloned virus isolate for lineage A). In the present study, inoculation with $10^5$ TCID$_{50}$ of molecularly cloned NL/1/00 resulted in infection of the respiratory tract and seroconversion. Inoculation with cloned NL/1/99 virus did not result in robust infection, as indicated by lower genome copy numbers and the fact that virus could not be reisolated from nose or throat samples. Therefore, we chose $10^7$ TCID$_{50}$ for inoculation with cloned low-passage stocks of NL/1/00 and NL/1/99 as a standard dose for all subsequent inoculations. However, even after inoculations...
with $10^7$ TCID$_{50}$ NL/1/99, infectious virus was not recovered. In Syrian golden hamsters, the NL/1/99 virus also replicated less efficiently than the NL/1/00 virus (data not shown). Both recombinant viruses were sequenced completely and no mutations were detected compared with the wild-type viruses. In studies with African green monkeys inoculated with prototype viruses of lineage A and B other than NL/1/00 and NL/1/99, the lineage B virus also replicated less efficiently (MacPhail et al., 2004; Skiadopoulos et al., 2004). Thus, it is possible that lineage B viruses in general replicate less efficiently in animal models. In humans, lineage B viruses are detected as frequently as lineage A viruses and insufficient data are available on the possible differences in clinical impact between the two viruses.

We evaluated protective immunity in cynomolgus macaques against homologous and heterologous reinfection with hMPV at different time points after primary infection. As shown for African green monkeys and chimpanzees (Biacchesi et al. 2005; Skiadopoulos et al., 2004), the macaques in our study were protected from homologous challenge at 4–6 weeks after a successful primary infection. However, challenge at 11 months after the last infection resulted in a robust infection, despite two or three previous infections. In addition, a homologous challenge 12 weeks after the primary infection resulted in detection of viral genomes in the respiratory tract, albeit with lower titres than in those macaques challenged 11 months after the last infection. These data suggest that, as hMPV epidemics occur every winter, a proportion of the population may be susceptible to hMPV in each winter season, irrespective of infection history.

Our data on homologous reinfection 11 months after primary infection demonstrate that immunity against hMPV wanes over time. Although we have only shown this for NL/1/00 infections, the low VN antibody titres in the NL/1/99-infected animals 11 months after primary infection suggest that homologous reinfection with NL/1/99 is also possible. The heterologous challenge at 11 months does not provide information on waning immunity. This heterologous challenge was included because homologous protection could have been expected, and the heterologous challenge would provide additional information on cross-protection. These data also show that vaccination studies should not only test for protection 4–6 weeks after vaccination, but should also aim at inducing protection for a longer period of time.

It is interesting to note that VN antibody titres measured 8 months after the last inoculation were in the same range as those measured 4–6 weeks after the primary infections, with the animals protected 4–6 weeks after the primary infection and not at 11 months. For RSV, VN antibodies have been implicated in protection from lower respiratory-tract infection. In our study, we could detect hMPV genomes in the BAL samples of the macaques challenged 6 weeks or 8 months after the previous infections, while VN antibody titres were still present. This indicates that the animals need a certain threshold of VN antibody titre or that other correlates of protection must exist. However, these data could also indicate the existence of other correlates of protection, such as mucosal or cellular immunity.

We have previously described the two major genetic lineages of hMPV as separate serotypes, but the authenticity and relevance of this hypothesis have since been under debate (Biacchesi et al., 2005; Skiadopoulos et al., 2004). The present study shows that, also in non-human primates, homologous titres are, in general, higher than heterologous titres. This may, for instance, have consequences for seroprevalence studies, as seen in the study of Skiadopoulos et al. (2004) where captive-bred chimpanzees were positive for hMPV serotype A and negative for serotype B VN antibodies. The full impact of homologous and heterologous protection is still not resolved, although it seemed that the heterologous challenge at 8 months was more successful than the homologous challenge. In animal models, both homologous and heterologous protection at 4–6 weeks after infection has been described. However, in humans, a heterologous reinfection associated with severe disease within 1 month of the primary infection in an otherwise healthy infant has been reported (Ebihara et al., 2004a) and we have demonstrated homologous reinfection in cynomolgus macaques 12 weeks after the primary infection. Our data demonstrate that only after infection with both types of virus are antibody titres equally high to both lineages of hMPV. The presented data would argue for including both types of virus in future vaccines, in particular if such vaccines are based in part on the variable genes G and SH.

This study confirms the usefulness of macaques as a non-human primate model for hMPV infection. However, the required dose for robust infection is rather high, as was also seen for RSV infections in these animals (De Swart et al., 2002). Two alternative primate species may be considered that may be more susceptible for hMPV infection. African green monkeys have been used previously for RSV and hMPV (Kakuk et al., 1993; MacPhail et al., 2004; Tang et al., 2004, 2005), but have as a disadvantage the limited availability of reagents to study specific immune responses. Chimpanzees are probably the most susceptible species, but studies in this species are restricted for clear ethical and practical reasons.

In conclusion, this study demonstrates that hMPV infections induce transient protective immunity in cynomolgus macaques and confirms that lineage A and B viruses represent different serotypes. These data should be taken into account for seroprevalence studies, as well as studies on vaccines and other intervention strategies.

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