Towards a Human Metapneumovirus Vaccine

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Naar een humaan metapneumovirus vaccin

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General introduction and outline
of this thesis

HUMAN METAPNEUMOVIRUS

Human metapneumovirus (HMPV) is a member of the family *Paramyxoviridae*, subfamily *Pneumovirinae*, genus *Metapneumovirus*, and was first described in 2001 (Fig. 1) (117,227). This study was based on twenty-eight unidentifiable viruses isolated from patients with respiratory tract infection (RTI) over the preceding 20 years. By using a random PCR amplification strategy known as RAP-PCR (186, 238), sequences were derived which resembled those of avian metapneumovirus (AMPV). Based on sequence homology, gene constellation and virological data, the virus was identified as a new member of the *Metapneumovirus* genus (117) and was named human metapneumovirus (HMPV) (227). AMPV, previously known as avian pneumovirus (APV) or turkey rhinotracheitis virus (TRTV), is the type species of the metapneumovirus genus and the etiologic agent of upper RTI in turkeys (125, 241).
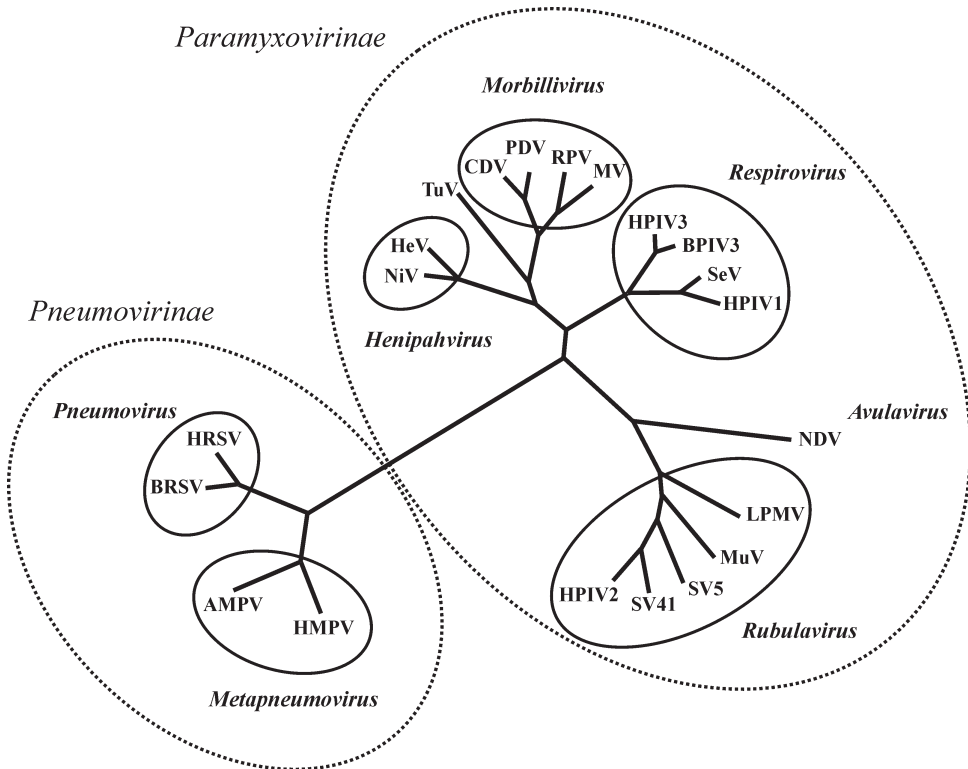
Figure 1. Schematic diagram of known members of the *Paramyxoviridae* family based on phylogenetic analysis of the nucleotide sequences for the polymerase gene of these viruses. Abbreviations: NiV: Nipah virus, HeV: Hendra virus, TuV: Tupaia virus, CDV: canine distemper virus, PDV: phocine distemper virus, RPV: rinderpest virus, MV: measles virus, HPIV: human parainfluenza virus, BPIV: bovine parainfluenza virus, SeV: sendai virus, NDV: newcastle disease virus, LPMV: La Piedad Michoacan paramyxovirus, MuV: mumps virus, SV5: simian virus 5, SV41: simian virus 41, HMPV: human metapneumovirus, AMPV: avian metapneumovirus, BRSV: bovine respiratory syncytial virus, HRSV: human respiratory syncytial virus.

HMPV is closely related to human respiratory syncytial virus (HRSV), the type species of the *Pneumovirus* genus, an important cause of lower RTIs during infancy and early childhood (99, 202). In most cases HRSV infection remains restricted to the upper respiratory tract (URT), and causes relatively mild disease (94). However, in some cases the infection also involves the lower respiratory tract (LRT), leading to severe disease. Patients may need mechanical ventilation and in some cases outcome can be fatal (75). Two different subgroups, HRSV-A and HRSV-B, can be distinguished on basis of sequence homology or their reaction patterns with monoclonal antibody panels (3, 159). HRSV has a worldwide distribution and causes yearly epidemics in the winter season of moderate climate zones and in the rainy season of tropical climate zones (37). The risk groups for severe HRSV-induced disease include premature infants, young children, elderly people, patients with underlying pulmonary or cardiac disease, and immune-compromised patients (58, 68, 95, 115).

CLINICAL IMPACT OF HMPV

The seasonality of HMPV infections resembles that of HRSV and influenza virus infections, with recurrent epidemics during the winter months (67, 104, 151, 231). Many studies have demonstrated that HMPV is responsible for a substantial proportion of lower RTI in infants and young children and is second only to HRSV as a cause of bronchiolitis in early childhood (18, 78, 82, 160, 231, 233, 244). Around 5-15% of children experience HMPV infection during their first two years of life (7, 18, 57, 66, 146, 151, 177, 230, 231, 234, 244, 247), while approximately 70% acquire HRSV infection during their first year of life (86). Other studies confirmed that primary infections with HMPV on average occur somewhat later than those with HRSV (21, 63). A wide spectrum of clinical symptoms associated with HMPV infection has been reported in patients of all ages. In the general community, HMPV-infected adults usually experience relatively mild common cold-like respiratory symptoms such as cough, rhinorrhea, hoarseness, sore throat and sometimes fever (67, 212, 225). In hospitalized children, patients with underlying disease, immune-compromised individuals and fragile elderly, HMPV disease tends to be more severe (19, 48, 116, 123, 147). A spectrum of symptoms involving both the lower and upper respiratory tract including cough, rhinorrhea, wheeze and dyspnea has been reported for this group of patients, (19, 123). Resulting diagnoses may range from rhinopharyngitis to bronchitis and pneumonia. Some patients may be admitted to intensive care units. In addition diarrhea, vomiting, rash, febrile seizures, feeding difficulties, conjunctivitis and otitis media have been reported (17, 65, 174, 231). Several studies have documented HMPV infections that were acquired nosocomially (19, 29, 31). For this reason, it was suggested to consider or implement isolation procedures when HMPV infection is suspected, especially in pediatric units with severely compromised patients and in intensive care units (29).

Primary infection with HMPV does not induce lifelong immunity (246). Actually, re-infection with either homologous or heterologous strains can occur, even during the same winter season (61). Despite the fact that re-infection is difficult to prevent, HMPV infections do result in protection from subsequent infection of the LRT. As a consequence, clinical signs related to re-infection in adults

are generally mild, except in immune-compromised individuals who may be at risk of developing severe disease upon re-infection (71, 176).

Asthma or wheezing exacerbation have been associated with HMPV infection in children (78, 152, 160, 244). Moreover, in a case-control study, a significant association between HMPV infection and wheezing among children younger than 3 years was found (243). Therefore, it is of interest to study the potential link between HMPV and asthma more closely.

VIRUS STRUCTURE AND GENOME ORGANIZATION

Although our knowledge about the structure of HMPV is limited, there is much that we can learn from other paramyxoviruses and especially from HRSV and AMPV. HMPV virions are pleiomorphic particles in the size range of 150-600 nm, with short envelope projections in the range of 13-17 nm (227). In analogy to its subfamily members, HMPV virions are surrounded by a lipid membrane envelope containing the three surface glycoproteins fusion (F), attachment (G) and small hydrophobic (SH) proteins. Inside the envelope of pneumoviruses lies a helical ribonucleocapsid complex (RNP), which consists of nucleoproteins (N), phosphoproteins (P), large polymerase proteins (L) and the nonsegmented single-stranded negative sense RNA genome (37). The HMPV genomic RNA is approximately 13.3 kb in length and consists of at least eight genes which potentially encode at least nine proteins (226). The pneumovirus matrix (M) protein resides between the envelope and the RNP and is important for the virion architecture. In addition, members of the *Pneumovirinae* subfamily express the M2 gene, encoding two partially overlapping open reading frames (ORFs), which give rise to two proteins M2.1 and M2.2 (226). For HRSV it was shown that the M2.1 protein is an essential transcriptional elongation factor, whereas the M2.2 protein is involved in regulating transcription and RNA replication (40, 101). In comparison with pneumoviruses, metapneumoviruses lack the non-structural proteins NS1 and NS2 and the gene order is also different (HMPV/AMPV: '3-N-P-M-F-M2-SH-G-L-5'; HRSV: '3-NS1-NS2-N-P-M-SH-G-F-M2-L-5') (Fig. 2) (226, 254).
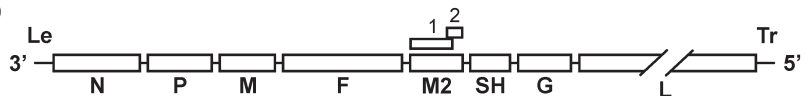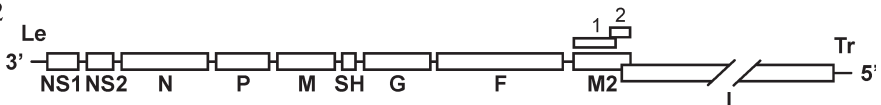
Figure 2. Genomic maps of HMPV isolate NL/1/00 and HRSV isolate A2 (approximately to scale) showing the important differences between HMPV and HRSV: HRSV expresses two extra proteins NS1 and NS2, the positions of the SH and G proteins differ, and for HRSV there is an overlapping reading frame for M2 and L. The double lines crossing the L ORF indicate the shortened representation of the L gene. Le: leader, N: nucleoprotein, P: phospoprotein, M: matrix protein, F: fusion protein, SH: small hydrophobic protein, G: attachment protein, L: large polymerase protein, Tr: trailer, NS1 and NS2: non-structural proteins 1 and 2.

VIRUS ENTRY AND REPLICATION

Paramyxoviruses replicate entirely in the cytoplasm, and this process begins with adsorption of the virus to the cellular receptor on the host cell, directed by the viral attachment protein, variously called G, H (hemagglutinin) or HN (hemagglutinin-neuraminidase) (Fig. 3) (143). Subsequently, the F protein initiates fusion of the viral and cellular membranes.
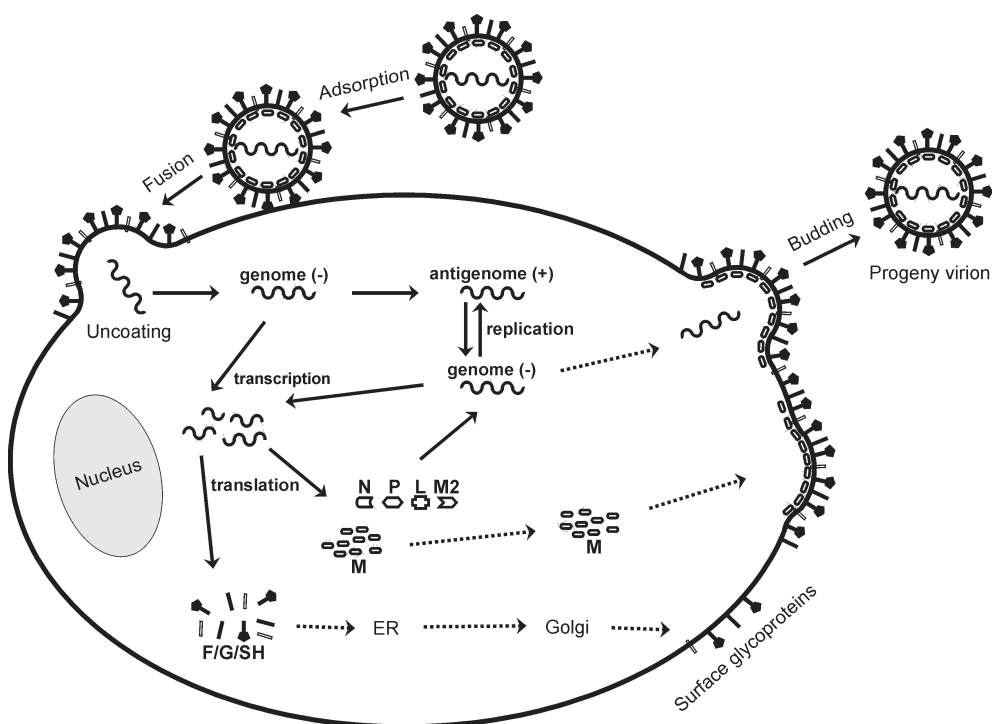
Figure 3. Schematic representation of the HMPV life cycle. After attachment of the virion to the plasma membrane, the viral and plasma membrane fuse, resulting in uncoating of the virion and release of the RNP (containing the negative sense viral RNA) in the cytoplasm. After primary transcription, the genome is replicated to produce the antigenome. This antigenome is used to synthesize genomic RNA, which is used to produce additional antigenomes, for incorporation into progeny virions or as template for secondary transcription. After translation, M proteins and RNPs are transported intracellularly (indicated by dotted lines) to the plasma membrane and the viral glycoproteins F, G and SH from the ER to Golgi to plasma membrane. Finally, new virions are assembled which are subsequently released from the plasma membrane by a budding process.

The F proteins of paramyxoviruses are class I viral fusion proteins that are synthesized as inactive precursor proteins, referred to as F0, that must be cleaved by host endoproteases to yield the disulfide-linked F1 and F2 polypeptides (139, 190). HMPV replication in cell culture was found to be dependent on trypsin (227). Mutagenesis of the putative cleavage site of the HMPV F protein resulted in viruses that were less dependent on trypsin *in vitro* (194). In contrast to HMPV (and all

other members of the *Paramyxoviridae* family), it was shown for HRSV (and BRSV) that processing of the F0 protein depends on the independent cleavage of two different cleavage sites, and both cleavages are required for the F protein to be active in membrane fusion as judged by syncytia formation (87). Within the F1 subunit there are two conserved heptad repeat regions (HRA and HRB) that play an important role in membrane fusion (Fig. 4) (175, 191). At the C-terminal end of the molecule is another conserved domain, called the transmembrane domain (TM), which serves as a membrane anchor in the viral membrane (156). The F protein is present on the viral membrane as a trimer. In the pre-fusion structure, the HRA is present in a meta-stable conformation, followed by an early fusion intermediate in which the HRB strands open up (Fig. 4). Next, a pre-hairpin intermediate is formed, in which HRA forms a triple-stranded coiled coil and subsequently, the fusion peptide is inserted into the target cell membrane (148). Finally, a huge conformational change, in which the HRB region folds towards the HRA region, brings the target membranes into close proximity of each other and overcomes the energy barrier to fuse the membranes (138).
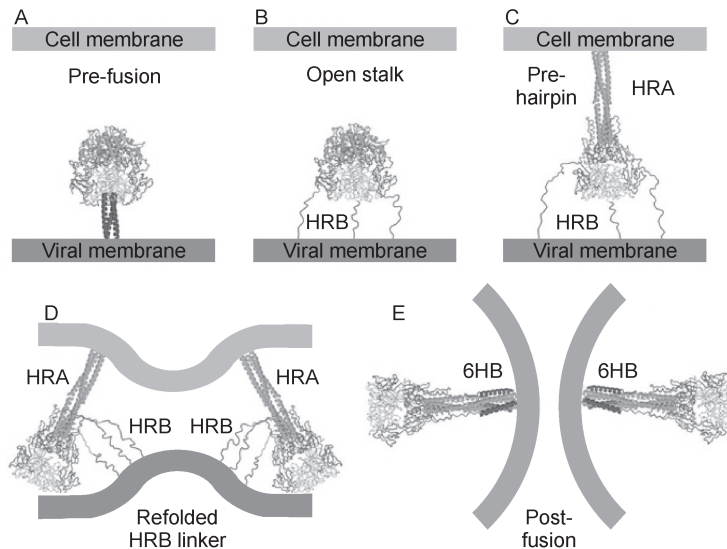
Figure 4. Model of paramyxovirus F-mediated membrane fusion. The F protein is thought to adopt five conformations during membrane fusion. The F protein is first present in an inactive pre-fusion conformation (A), followed by a metastable open stalk conformation (B). Next, a pre-hairpin intermediate is formed, in which HRA forms a triple-stranded coiled coil and the fusion peptide is inserted into the target membrane (C). The fusogenic hairpin structure actively brings together the viral and cellular membranes (D), followed by the assembly of the final 6HB that completes the conformational change (E). (Adapted from ref. 14).

Upon fusion, the viral RNP containing the negative sense genome is released into the cytoplasm. At early times of infection, this genome directs the synthesis of viral mRNA. After translation of the primary transcripts and accumulation of the viral proteins, the negative sense genome is replicated to produce a full-length complementary copy, called the antigenome. After synthesis, the

antigenomic RNA is used as a template to direct synthesis of genomic RNA. As indicated in Figure 3, these progeny negative sense genomes can serve three subsequent functions: as a template to produce additional antigenomes, for incorporation into progeny virions during the budding process, or as additional templates for transcription to produce much higher levels of mRNA transcripts.

The viral gene junctions of paramyxoviruses that modulate transcription can be divided into three segments: a gene end (GE) region at the 3' end of the upstream gene, the intergenic region (IG) between the two genes that is normally not transcribed, and a gene start (GS) region for the downstream 5' gene (Fig. 5) (38, 56). The GE region contains a signal directing the viral polymerase to terminate transcription, whereas reinitiation of transcription is directed by sequences at the downstream GS site (56). The frequency of reinitiation is not perfect, resulting in a gradient of mRNA that decreases according to distance from the 3' genome end, with N mRNA being found in higher abundance than L mRNA (Fig. 6). For HRSV, it was shown that differences in the efficiency of the various GE signals also affect the relative levels of gene expression (102, 158).
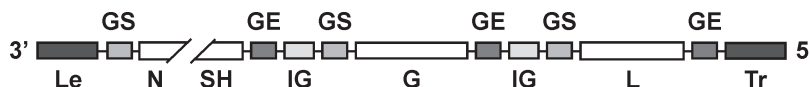
Figure 5. Viral transcription modules of HMPV. Note that part of the genome between N and SH is omitted. The 3' terminal 15 nucleotides of the leader act as an efficient promoter for RNA replication. All genes have a gene end (GE) region at the 3' end of the upstream gene, followed by the intergenic region (IG) and a gene start (GS) region for the downstream 5' gene. Synthesis of each mRNA starts at the first nucleotide of the GS signal, and each gene ends with a GE signal that directs polyadenylation and termination of the completed mRNA.

After transcription and subsequent translation, the M protein is transported to the plasma membrane, whereas the viral envelope proteins F, G and SH travel through the endoplasmic reticulum (ER) and the Golgi apparatus to be inserted the plasma membrane. Progeny virions are then formed by a budding process, which takes place at sites of the plasma membranes where viral components like the RNP, the viral glycoproteins and the M protein are assembled (189). The assembly requires coordinated localization of the viral components, which appears to be accomplished through protein-protein and protein-lipid interactions, many of which involve the M protein.

REVERSE GENETICS

Reverse genetics has become an important tool that facilitates the genetic manipulation of viruses using recombinant DNA technologies. The first RNA virus that was recovered using these techniques was the positive-sense poliovirus (184). Simply transfecting plasmid, or RNA transcribed from the plasmids containing the poliovirus genome, into susceptible cells resulted in the recovery of infectious poliovirus (130, 184). For the recovery of negative-sense RNA viruses, difficult obstacles had to be overcome. Precise 5' and 3' ends are required for replication and packaging of the genomic
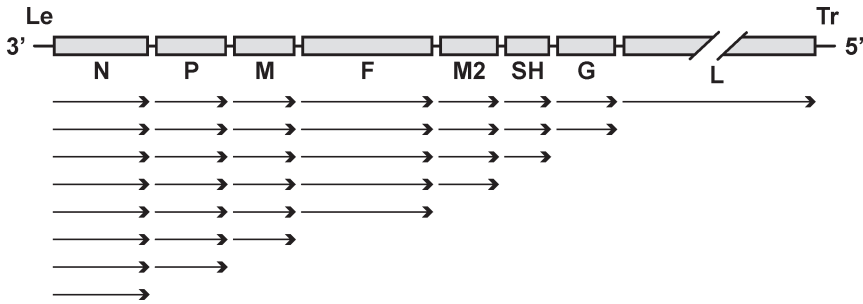
Figure 6. Sequential transcription has a polar gradient due to polymerase fall-off. The arrows indicate the decreasing gradient of mRNA as a result of an imperfect transcription reinitiation. Note that N mRNA is present in higher abundance than L mRNA.

RNA, and the viral RNA polymerase is essential for transcribing both mRNA and complementary, positive-sense antigenome RNA. The systems for the recovery of recombinant paramyxovirus use a cDNA plasmid encoding the full-length viral RNA in sense or antisense orientation flanked by sequences to ensure the transcription of genome-length RNA such as a T7 RNA polymerase promoter at the 5' end, and a self-cleaving ribozyme followed by a T7 terminator at the 3' end (196). The T7 RNA polymerase can be expressed either from plasmids, modified vaccinia virus Ankara (MVA) (239), or recombinant fowl pox virus (22). Alternatively, cells constitutively expressing the T7 RNA polymerase, for example BSR-T7 cells (baby hamster kidney cells stably expressing T7 polymerase), can be used (24). Before using plasmids encoding the full-length viral genome, so-called minigenomes are generated and used to optimize rescue conditions. Minigenomes consist of a reporter gene, for example green fluorescent protein (GFP) or chloramphenicol acetyl transferase (CAT), flanked by the viral leader and GS of N at one side and the GE of L and trailer at the other side, which are enclosed by a T7 promotor/terminator cassette and a self cleaving ribozyme as described above (92). When plasmids encoding the viral N protein and the polymerase proteins L and P, also under the control of T7-promotors, are co-transfected with the minigenome, expression of the reporter gene can be measured. With this rather straightforward minigenome system, transfection conditions and plasmid ratios can be optimized. When the minigenome system is operating satisfactorily, the reporter gene can be replaced by the full-length viral genome. After transcription of RNAs from the T7 promotors and translation of the encoded proteins, N proteins assemble around the antigenomic RNAs and form RNPs. The polymerase proteins then replicate these RNPs to form RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and subsequent translation, infectious virus is assembled as described above.

Since reverse genetics systems facilitate the specific manipulation of the genetic properties of the virus, it can provide a helpful tool in fundamental and applied virology, for example to design and develop recombinant live-attenuated vaccines. With this system, chimeric viruses, deletion mutants and even viruses expressing additional (foreign) genes can be generated.

VACCINATION AND TREATMENT

Treatment options for paramyxoviruses are still limited. Ribavirin, a nucleotide analogue, is the only antiviral drug approved by the Federal Drug Administration (FDA) for treatment of HRSV disease. Ribavirin was found to reduce morbidity in high risk young children and bone marrow transplants and decreased the duration of mechanical ventilation, oxygen treatment, and hospitalization (91, 208, 209). However, the safety and clinical efficacy remain controversial.

Palivizumab (Synagis, MedImmune Inc.), a virus neutralizing monoclonal antibody preparation against the HRSV F protein, has been demonstrated to substantially reduce hospitalization for severe HRSV-induced lower RTI in large clinical trials involving preterm infants (173). Premature infants are at risk for severe HRSV as a result of reduced levels of maternal antibodies, immaturity of the immune system, and underdeveloped lungs with smaller airways and less pulmonary reserve compared with full-term infants (153). Enhancement of the binding of palivizumab to the HRSV F protein resulted in a second-generation monoclonal antibody, motavizumab (Numax, MedImmune Inc.), which is currently under study in phase III clinical trials (250). In cotton rats, at equivalent concentrations, treatment with motavizumab resulted in up to 100-fold reduction of pulmonary HRSV titers compared to palivizumab (249).

The lack of effective antivirals for paramyxoviruses and the limited cost-effectiveness of monoclonal antibodies in full-term infants leads to the conclusion that immunization remains the most effective means of reducing morbidity and mortality.

For members of the *Paramyxovirinae* subfamily, very young infants are the main target group for immunization. Since these children have not yet developed a mature immune system, a multidose vaccine strategy may be needed, starting in the first weeks of life. The vaccination efficacy in these children may also be hampered by the presence of maternally derived antibodies, which may provide partial protection to wild-type virus infections but also suppress the primary immune response on immunization (43). Other risk groups for HRSV and HMPV-mediated severe disease are the frail elderly and immune-compromised individuals. In general, protection against infection is not a realistic goal, because mucosal immunity to respiratory viruses is short lived and incomplete. Protection against severe disease, and especially lower respiratory tract disease, might be a more feasible goal (44).

For HRSV many different strategies have been explored to date, but have still not resulted in a vaccine licensed for human use. The current options for development of viral vaccines against HRSV or HMPV are described below.

Inactivated vaccines
A classical inactivated vaccine basically contains a virus that is rendered non-infectious by inactivation. Inactivated vaccines are usually administered by the intramuscular (i.m.) or subcutaneous route. Most inactivated viruses or proteins have a poor intrinsic immunogenicity. Therefore,

formulation with an adjuvant is required to give an adequate immune response. An adjuvant is an agent that stimulates the immune system and increase the response to a vaccine, without having any specific antigenic effect by itself (211). For example, the antigen can be adsorbed on particles of the adjuvant (such as Alum), made particulate by emulsification in mineral oils (such as Specol), or incorporated into the colloidal particles of for istance iscoms (immune-stimulating complexes) (211). At this moment, alum and MF59 (an oil-in-water emulsion) are the only adjuvants licensed for human use.

Live attenuated vaccines

A live attenuated vaccine is generally composed of a virus of reduced virulence through attenuation, but is still able to multiply in the host and induce a virus-specific immune response. Viral attenuation has often been accomplished by *in vitro* or *in vivo* passage of the virus.

For HRSV, a number of live-attenuated viruses have been developed by such conventional methods. The most promising set of candidates was developed by serial passage of HRSV at increasingly suboptimal temperature (cold-passage, cp), followed by chemical mutagenesis and screening to identify temperature-sensitive (ts) mutants (45, 46). Unfortunately, preclinical and clinical analysis of a cpHRSV derivative and a number of cptsHRSV derivatives showed that they were attenuated, but retained an unacceptable level of residual virulence (248). On the other hand, some vaccine candidates were too attenuated and failed to induce adequate immunity: the difficulty for this approach has historically been to achieve an optimal balance between attenuation and immunogenicity.

Subunit vaccines

Subunit vaccines consist of one or a few specific proteins or glycoproteins, which have been associated with the induction of protective immune responses. The proteins can be purified from the pathogen, or be produced in prokaryotic or eukaryotic expression systems. For HRSV, subunit vaccines have been evaluated clinically for use in HRSV-experienced individuals, in whom they appeared to be safe. One candidate HRSV vaccine consists of purified F protein (PFP), isolated from HRSV-infected cell culture (235). This vaccine has been evaluated extensively in adults, children with and without underlying disease, and in the elderly, and was found to be well tolerated and moderately immunogenic (70). However, a large multicenter study that was performed in children of 1 to 12 years with cystic fibrosis did not provide evidence of significant protection against HRSV infection or disease (179). Another candidate subunit HRSV vaccine, called BBG2Na, consists of a recombinant bacterially expressed fragment of the G protein. This vaccine was reported to be moderately immunogenic in adults and the elderly (182). However, clinical development of this vaccine was interrupted due to the appearance of unexpected side effects (purpura) in a few immunized volunteers (11, 84). In addition, studies in non-human primates suggested that the vaccine had a slight propensity to induce hypersensitivity responses in immunologically naïve animals (53) (see also below).

Heterologous vaccines

Heterologous vaccines are based on the Jennerian approach, in which closely related agents share epitopes that are involved in either inducing complete protection or priming the immune response such that there is a rapid immune response after exposure to the pathogen. This was first described by Edward Jenner, who discovered that milkmaids who had developed cowpox from contact with cow udders were protected from the smallpox. Closely related viruses of a single species as well as closely related viruses from other species can induce cross-protection. Bovine RSV (BRSV) has been evaluated as a live intranasal vaccine against HRSV because it shares significant antigenic cross-reactivity in its F protein and is attenuated in primates due to host range restriction. Unfortunately, BRSV was over-attenuated and insufficiently immunogenic in chimpanzees (25).

Recombinant vaccines

Live vaccines have often been considered to be an interesting approach for immunization, since all of the relevant antigens would be present in the vaccine. Unfortunately, it has not always been possible to attenuate every pathogen satisfactorily by conventional methods. Furthermore, even if they were attenuated, the molecular basis for the attenuation was in most cases unknown, making it difficult to exclude that reversion could occur. The development of reverse genetics systems has made it possible to overcome these disadvantages. Furthermore, attenuation of viruses has since then also been achieved by deleting specific virulence genes. In addition, foreign genes can also be introduced into the location of the gene deletion, or as an extra gene, thereby providing protection to multiple agents in one vaccine (multivalent vaccine). Reverse genetics was recently used to design a HRSV vaccine in which mutations from previously cpts viruses were combined with the deletion of the SH gene. This virus appeared to be satisfactorily attenuated and moderately immunogenic in HRSV-naïve infants, but there was some evidence of genetic instability (132). Recombinant bovine/human parainfluenza virus (PIV) type 3 (rB/HPIV3), a recombinant bovine PIV3 (rBPIV3) in which the F and HN genes were replaced with their HPIV3 counterparts, was already evaluated in clinical studies in 1- and 2-month-old infants and was found to be safe, immunogenic, and non-transmissible in a day care setting (131, 140). Immunization of hamsters with this rB/HPIV3, in which the F or G proteins of HRSV were introduced, resulted in a high level of resistance to challenge with HRSV or HPIV type 3, 28 days later (195).

Modified vaccinia virus Ankara (MVA) is a replication-deficient poxvirus, which can be used as a recombinant vector. In mice, MVA expressing the HRSV F or G protein induced higher HRSV antibody titers than those achieved by wt HRSV infection. After challenge infection, replication of HRSV was restricted in both the upper and lower respiratory tracts (251). In another study in mice, immunization with MVA expressing F or G promoted a balanced humoral immune responses and production of Th1 cytokines. However, upon HRSV challenge, mice immunized with MVA-F or MVA-G lost weight as compared to control wtMVA-immunized animals, indicating enhanced disease (168). Two immunizations with MVA-F or MVA-G in infant macaques did not predispose for immunopathology upon challenge after 4 months. However, these immunizations primed for secondary immune responses upon challenge, but none of the animals were protected (54).

DNA vaccines

DNA vaccines are made up of plasmids that express a specific viral antigen. Immunization with plasmid DNA may be a safe approach since a single gene induces the immune response, which ensures that no infection occurs, and there is no risk for transmission. A second advantage of DNA immunization is that immune responses can be induced in the presence of maternal antibodies. However, a point of concern is the possibility that plasmid DNA integrates into the genomic DNA of the host and therefore the safety of DNA vaccines is still uncertain. Besides that, the feasibility of this approach for use in neonates (or young infants) is still unclear at this time. For HRSV, plasmids expressing the complete or partial G protein have been used in immunization experiments in mice and cotton rats (4, 144, 154). Immunization with these plasmids induced a balanced Th1/Th2 response, comparable to that induced by live HRSV. Furthermore, HRSV viral replication was significantly inhibited in the lungs of animals. Plasmids encoding the F gene of BRSV were studied for their immunogenicity and protective efficacy in Rhesus macaques. Immunized animals had decreased HRSV in their lungs after challenge infection, and there was a qualitative difference in histopathology observed between immunized and non-immunized animals (232). Recently, optimization of codon usage of the HRSV F protein resulted in enhancement of F expression levels. Immunization with codon optimized F expressing plasmids resulted in induced antibody levels and a 13- to 170-fold reduced viral load in mice (220).

OBSTACLES IN THE DEVELOPMENT OF PARAMYXOVIRUS VACCINES

Efforts to develop a vaccine against HRSV began over 40 years ago, and there is still no licensed vaccine. Vaccine development aiming at protecting naïve individuals from disease caused by HRSV or the closely related HMPV, are hampered by the experience with a formalin-inactivated (FI-) HRSV vaccine. In 1966 and 1967, studies using FI-HRSV were conducted, following the success of other chemically inactivated viral vaccines. Vaccines were administered to infants and children aged 2 months to 9 years in two or three intramuscular doses separated by 1 to 3 months (32). After natural HRSV exposure during the next winter season, vaccinated children not only proved to be unprotected but actually developed enhanced disease as compared to children that had received a control PIV vaccine. Most remarkably, 80% of HRSV vaccinees needed hospitalization, whereas only 5% of HRSV-infected children in the control group required hospital admission. The enhanced disease was mainly characterized by pneumonia, bronchiolitis, rhinitis, or bronchitis, and two of the immunized children died (134). In 1967 the same problem arose for measles (MV), when the use of FI-MV adjuvanted with alum predisposed infants for developing atypical measles upon later infection with wild-type MV (79). These events were later reproduced in animal models, and proved to be associated with immunopathological responses related to the priming of unbalanced cellular immune responses (51, 169, 181, 183). These experiences from the past should be considered carefully when developing vaccines to protect against respiratory disease induced by HMPV, and different vaccine strategies may be needed for different risk groups for infection.

OUTLINE OF THIS THESIS

Since these studies started only one year after the first description of HMPV in 2001, no information was available with respect to the possible existence of multiple genotypes of HMPV in humans. In chapter 2, we analyzed the genetic diversity of HMPV by phylogenetic analysis of partial sequences of a large number of F proteins and complete sequences of a large number of G proteins. In chapter 3, we describe the development of a reverse genetics system for the recovery of HMPV lineages A and B identified in chapter II. This system provided us with an important tool for applied and fundamental research, and allowed us to generate recombinant virus that contain exactly defined genetic properties. In chapter 4, recombinant HMPV A and B viruses generated in this system were used in an experimental cynomolgus macaque infection model. Animals previously inoculated with HMPV were tested for possible protection from homologous reinfection within 6 weeks and eight months. In chapter 5, we established an experimental infection model in Syrian golden hamsters, in which we evaluated the immunogenicity and protective efficacy of an adjuvanted F subunit vaccine. Animals that were immunized twice with an iscom-matrix adjuvanted F subunit vaccine were tested for their immunity against homologous and heterologous challenge infection. In chapter 6 we describe the development of two live attenuated vaccine (LAV) candidates. The first LAV was generated by introducing mutations that were found after passaging HMPV at slowly decreasing temperatures. The second LAV was generated after the introduction of HRSV mutations in the HMPV genome that are known to be responsible for a ts-phenotype in HRSV. Both viruses which indeed turned out to be temperature-sensitive in vitro were tested for attenuation and protective immunogenicity in vivo in hamsters. In chapter 7, the immunogenicity of the F subunit vaccine and the live-attenuated HMPV containing the HMPV ts-mutations, as described in chapter 5 and 6 respectively, was evaluated in cynomolgus macaques. In chapter 8, we assayed the low pH dependency for HMPV membrane fusion, promoted by the F protein. Finally, in chapter 9, all results from the previous chapters are evaluated in a summarizing discussion.

Antigenic and genetic variability of human metapneumoviruses

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and Vigi Virus, São Paulo, Brazil [3]

ABSTRACT

Human metapneumovirus (hMPV) is a member of the subfamily *Pneumovirinae* within the family *Paramyxoviridae*. Other members of this subfamily, respiratory syncytial virus and avian pneumovirus, can be divided in subgroups on the basis of genetic or antigenic differences or both. For HMPV, the existence of different genetic lineages has been described on the basis of variation in a limited set of available sequences. We address the antigenic relationship between genetic lineages in virus neutralization assays. In addition, we analyzed the genetic diversity of HMPV by phylogenetic analysis of sequences obtained for part of the fusion protein (n = 84) and the complete attachment protein open reading frames (n = 35). On the basis of sequence diversity between attachment protein genes and the differences in virus neutralization titers, two HMPV serotypes were defined. Each serotype could be divided in two genetic lineages, but these did not reflect major antigenic differences.

INTRODUCTION

The human metapneumovirus (HMPV) has recently been identified as a causative agent of respiratory tract illnesses (RTI) in humans world-wide (67, 78, 170) and is a member of the Pneumovirinae subfamily within the Paramyxoviridae family (226). The Pneumovirinae subfamily consists of two genera: the pneumoviruses and the metapneumoviruses. Human respiratory syncytial virus (HRSV) the major viral cause of severe respiratory tract illnesses in children, is the type species of the pneumoviruses (37). Avian pneumovirus (APV), the causative agent of respiratory tract illnesses in turkeys and chickens (85), was the sole member of the Metapneumovirus genus until the discovery of HMPV (137).

For most pneumoviruses, different subgroups or subtypes have been identified. For HRSV, two subgroups have been identified on the basis of differences in nucleotide sequences, reactivity patterns with monoclonal antibodies, and in vitro neutralization assays with subgroup-specific antisera (3, 34, 83, 159). Additional genotypes have been identified within subgroups, largely on the basis of the high variability of the attachment protein gene (27, 213). The fusion (F) and the attachment (G) proteins are the main targets for the neutralizing and protective antibody response (2, 121, 167), with F being one of the most conserved proteins and G the most variable (120, 122, 126, 164). For APV, two different subgroups (A and B) have been defined on the basis of nucleotide sequences of the G protein and neutralization tests by using monoclonal antibodies that also recognize the G protein, but these subgroups belonged to one serotype (42). APV type C, a possible second serotype, was identified based on the lack of cross-reactivity with antisera specific for groups A and B, and the nucleotide sequences also proved to be significantly different from strains belonging to group A or B (198, 199). In addition, subgroup D may exist, which contains isolates from France that are not neutralized by monoclonal antibodies raised against viruses belonging to either subgroup A, B or C (8).

For HMPV, two major genetic lineages have been identified worldwide based on analysis of a limited set of sequences (177, 212, 227). One feature of HMPV that poses a challenge in developing a future vaccine is that infections may occur in the presence of preexisting immunity. Very young children (<1 year) have been infected by the virus, and re-infections have also been demonstrated (176). HMPV might cause repeated infections throughout life, similar to HRSV, which could be due either to incomplete immunity or to genetic heterogeneity of the virus.

To develop vaccines, the extent of genetic and antigenic variability of the different HMPV transmembrane glycoproteins must be understood. We analyzed the genetic diversity of HMPV by phylogenetic analyses of sequences obtained for part of the F (n = 84) and the complete G open reading frames (ORFs) (n = 35). In addition, we addressed the antigenic relationship between the different lineages with virus neutralization assays using lineage-specific antisera raised in ferrets. Virologic studies have used a definition of a homologous-to-heterologous virus neutralization titer ratio of >16 as a definition for serotypes (76). On the basis of our results and the described definition, we now define the two major lineages of HMPV as serotype A and B. In accordance with

the definition and our results, the sublineages within each serotype are not identified as different serotypes. At least two serotypes of HMPV are present in the human population, a finding that has implications for developing intervention strategies, such as immunization and vaccination.

MATERIALS AND METHODS

Sample collection, RNA isolation, RT-PCR assays and sequencing
HMPV-positive nasopharyngeal aspirate samples were obtained from different cohort studies: 61 samples from the Netherlands, 11 samples from Finland, 8 samples from England, 1 from Hong-Kong, and 2 from Brazil. Clinical samples had been obtained from 1981 to 2002. Samples were obtained from young children, infants, adults, the elderly and immunocompromised persons, who had mild to severe respiratory tract illnesses. Epidemiologic and clinical data for most isolates have been described elsewhere (28, 119, 231).

Similar to the influenza nomenclature, sequences are identified by the country of origin, an identification number, and year of isolation. RNA isolation was performed as described previously (227). cDNA was synthesized at 42°C for 60 min with random hexamer primers (Promega, Leiden, The Netherlands) and superscript II RNase H-reverse transcriptase (Invitrogen, Merelbeke, Belgium). An aliquot of cDNA was used in a polymerase chain reaction (PCR) assay to amplify the full-length G ORF or a fragment of the F ORF. Primers: SH7: 5'- TACAAAACAAGAACATGGGACAAG-3' and SH-8 5'-GAGATAGACATTAACAGTG-GATT-3' (G ORF), BF100 5'-CAATGCAGGTATAACACC AGCAATATC-3' and BF101 5'-GCAACAATTGAACTGATCTTCAGGAAAC-3' (F ORF). Thermo-cycling was performed under the following conditions: 94 °C for 1 min., 40 °C for 2 min., 72 °C 3 min. (40 cycles). When necessary, a nested PCR was performed using 5 µL of PCR product with primers SH7 and SH8 for the G ORF or primers BF 103 5'-ACATGCCAACATCTGCAGGACAAA TAAAAC-3' and BF104 5'-ACATGCTGTTCACCTTCAACTTTGC-3' for the F ORF. PCR products were sequenced directly on both strands with multiple primers as described previously (227). When identical sequences were obtained (suspicious of laboratory contamination) and to confirm sequence uncertainties such as frame shifts, we repeated the RNA isolation, RT-PCR, and subsequent sequencing with the original materials.

Phylogenetic analysis
Nucleotide sequences were aligned using the Clustal W program running within the Bioedit software package, version 5.0.9. Maximum likelihood trees were generated with the Seqboot and Dnaml packages of Phylip version 3.6 by using 100 bootstraps and 3 jumbles. The consensus tree was calculated by using the Consense package of Phylip 3.6 and was subsequently used as usertree in Dnaml to recalculate the branch lengths from the nucleotide sequences. Finally, the trees were rerooted at midpoint using the Retree software of Phylip 3.6. Trees were visualized with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9 (73). Sequences are available from Genbank under accession numbers AY295930 to AY296012 (F partial) and AY304360 to AY304362 (complete F for NL/17/00, NL/1/99 and NL/1/94 respectively), AF371337 (complete

genome NL/1/00) and AY296014 to AY296047 (complete G regions).

Virus preparations and titrations

Viruses were isolated on tertiary monkey kidney (tMK) cells as previously described (227). For each genetic lineage a prototype virus isolate was chosen on the basis of its ability to grow to high titers on tMK cells, and to reflect the specific genotype for the lineage. Virus titrations were cultured for 7 days, and infected wells were identified by immune fluorescence assays (IFA) with HMPV-specific polyclonal antiserum raised in guinea pigs. Titers were expressed in 50% tissue culture infectious dose ($TCID_{50}$).

Antisera

Lineage-specific polyclonal HMPV antisera were raised by inoculating ferrets with 1 mL of virus-infected tMK supernatants containing approximately 10^4-10^5 $TCID_{50}$ virus. All inoculations were performed in duplo, and the animals with the highest antibody responses are shown. Sera were collected at days 0 and 28 postinfection (ferret 1 and 2) or at days 0 and 21 (ferret 3 to 6). Infections were performed as follows: ferrets 1 and 3: HMPV NL/1/00, prototype virus for lineage A1. Ferrets 2 and 5: HMPV NL/1/99, prototype virus for lineage B1. Ferret 4: HMPV NL/17/00, prototype virus for lineage A2 and ferret 6: HMPV UK/5/01 a virus from lineage B2. Ferrets were housed in isolator cages to avoid cross-infections.

HMPV-specific polyclonal antisera were raised in guinea pigs as previously described (227). Antisera raised in separate guinea pigs against viruses from the two main genetic lineages (A and B) were mixed 1:1, and this mixture tested positive against all HMPV isolates in IFA.

Virus neutralization assays

Virus neutralization assays of heat-inactivated (30 min 56 °C) ferret serum samples were performed as previously described (227). Briefly, twofold serial serum dilutions starting at 1:8 were incubated with approximately 30 $TCID_{50}$ virus. Seven days after infection of tMK cells with the antibody and virus mixture, IFA was performed with the guinea pig antiserum. The virus neutralization titer was defined as the reciprocal of the highest serum dilution at which no positive IFA signal was obtained (depicted as means of duplicate measurements). Each experiment included virus titrations of the working solution of the virus, using twofold dilutions, and 10-100 $TCID_{50}$ per well was considered acceptable.

RESULTS

Variation in the fusion protein gene

Partial F gene sequences (nucleotide [nt] 780-1,221 in the F ORF) were obtained from clinical samples collected from 84 HMPV-infected patients. Phylogenetic analysis of these sequences confirmed two main genetic lineages, A and B. Each of these lineages appeared to consist of two sublineages, which were tentatively named A1, A2, B1 and B2 (Figure 1A).

A

NL/14/00
NL/2/00
NL/4/02
NL/8/01
UK/1/00
NL/13/00
NL/25/01
NL/5/02
NL/27/01
NL/6/02
NL/2/02
NL/30/01
NL/20/01
NL/28/01
NL/26/01
NL/9/02
NL/10/01
UK/9/01
NL/6/01
UK/7/01
FL/10/01
UK/1/01
FL/3/01
FL/8/01
FL/4/01
FL/1/02
NL/1/00
BR/3/01
NL/7/02
BR/2/01

A1

74

NL/4/93
NL/1/93
NL/2/93
NL/2/96
NL/29/01
NL/3/02
NL/17/00
NL/1/98
NL/17/01
NL/24/01
NL/22/01
NL/23/01
NL/1/95
NL/3/96
NL/1/81

A2

80

FL/2/01
FL/9/01
FL/1/01
FL/5/01
NL/19/01
NL/1/01
UK/11/01
UK/10/01
NL/3/99
NL/2/99
NL/1/99
NL/11/00
FL/7/01
NL/9/01
NL/3/98
NL/13/01
NL/12/00
NL/21/01
NL/1/02
NL/12/01
NL/5/01

B1

78

NL/28/00
NL/11/01
NL/15/01
NL/12/02
NL/16/01
NL/18/01
UK/5/01
NL/9/00
FL/6/01
HK/1/02
NL/4/01
NL/3/01
NL/7/00
UK/8/01
NL/1/94
NL/1/82
NL/6/97
NL/1/96

B2

66

100

0.1

B

FL/4/01
FL/3/01
FL/8/01
NL/1/00
BR/2/01
NL/2/02
NL/10/01
FL/10/01

A1

100

NL/29/01
NL/3/02
NL/17/00
NL/2/96
NL/22/01
NL/23/01
NL/24/01
NL/1/95
NL/3/96
NL/3/93
NL/2/93
NL/1/93
NL/1/81

A2

98

100

NL/5/01
NL/9/01
NL/12/00
NL/21/01
NL/1/99
NL/11/00

B1

100

NL/9/01
UK/5/01
NL/4/01
NL/3/01
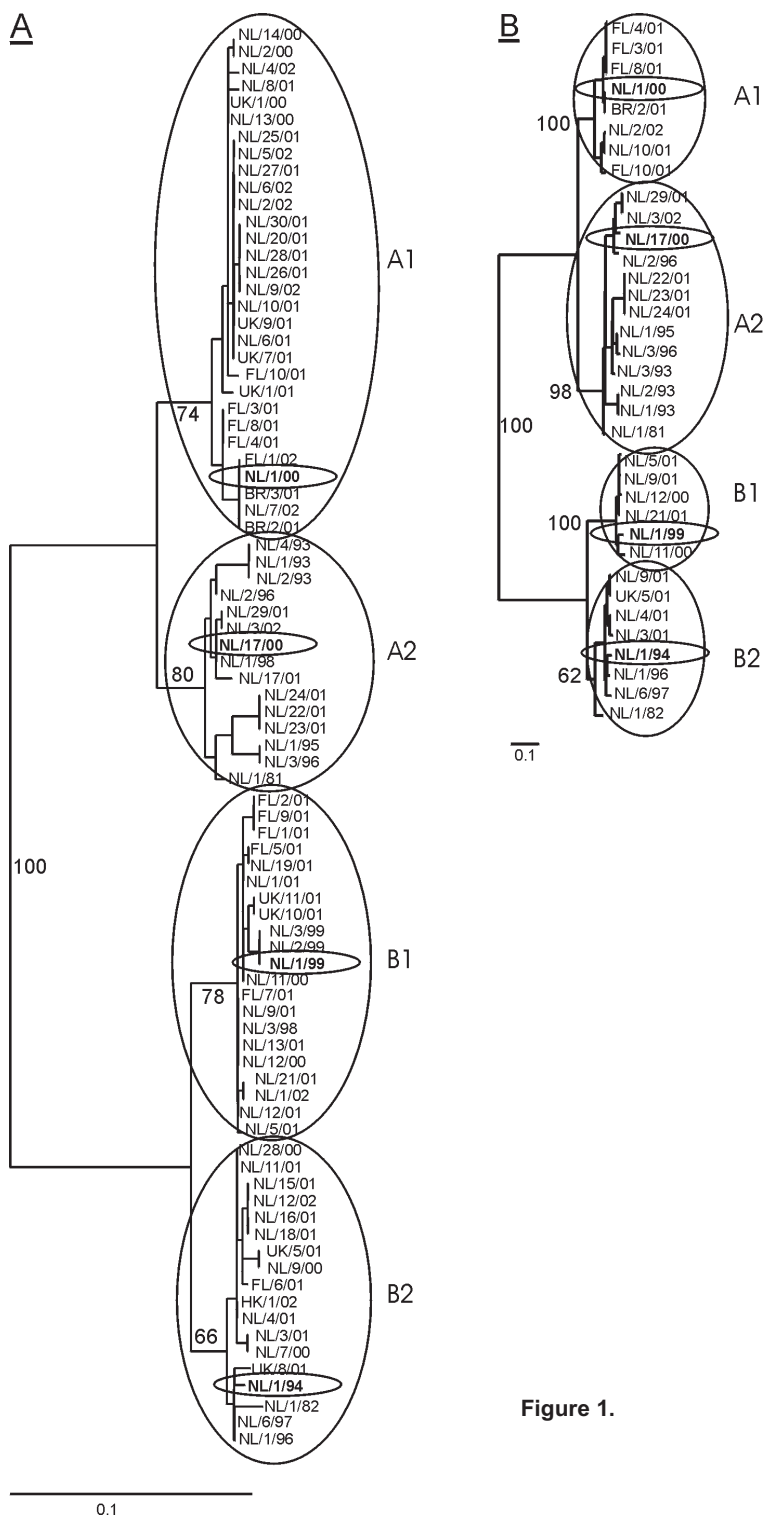**NL/1/94**
NL/1/96
NL/6/97
NL/1/82

B2

62

0.1

Figure 1.

Comparison of the sequences showed high percentage identities between members of the same sublineage (nt: 97%-100%, amin acids [aa]: 99%-100%), members of the two different sublineages within each main lineage (nt: 94%-96%, aa: 97%-99%), and between members of the two different main lineages A and B (nt: 84%-86%, aa: 94%-97%). Whereas no specific amino acid residue substitutions could be found between sequences from subgroups A1 and A2, there were 5 specific aa substitutions between sequences from genotypes A and B, and one substitution between B1 and B2 (Table 1). The low variability was also observed when complete F protein genes from prototype viruses for each sublineage were sequenced (Figure 2).

Table 1. Lineage-specific amino acid substitutions between the four sublineages in the fusion open reading frame between position 260 and 407.

Sublineage	aa_{286}	aa_{296}	aa_{312}	aa_{348}	aa_{404}
A1	V	K	Q	K	N
A2	V	K	Q	K	N
B1	I	N	K	R	P
B2	I	D	K	R	P

Variation in the attachment protein gene

Nucleotide sequences of the region between the start codons of the G and the polymerase (L) reading frame were obtained for 35 samples. Phylogenetic analysis showed the same clustering of the sequences over the four sublineages as seen for the F protein gene (Figure 1B). The G region showed some variation in length, from860 nt to 908 nt. The first 657-708 nt have been described as the putative primary G ORF (226). Alignment of the primary G ORFs showed a variation in length, even for members of the same sublineage, due to single nucleotide substitutions that resulted in premature termination codons (Figure 3). For two samples a change in ORF was observed as a result of an addition (BR/2/01: G at position 519) or a deletion (NL/2/93: C at position 243) of a single nucleotide. These mutations resulted in relatively short G ORFs (NL/2/93: 110 aa; BR/2/01: 193 aa) because of premature termination and in drastic changes in the deduced amino acid sequences of the carboxy-terminus of the G proteins.

Comparison of the primary G ORF sequences, excluding sequences of NL/2/93 and BR/2/01 because of the putative frameshifts, revealed a relatively high percentage identity between members of the same sublineage (nt: 93%-100%, aa: 75%-99.5%), less identity between members of the two

Figure 1. Phylogenetic trees constructed based on the (A) partial F gene (Open reading frame [ORF] position 780-1,221, n = 84) or (B) the complete G coding region (start G ORF to start L ORF, n = 35). Trees were generated by maximum likelihood analysis using 100 bootstraps and 3 jumbles. The scale representing the percentage of nucleotide changes is shown for each tree. Bootstrap values are based on the consensus trees, and relevant numbers are shown in the tree. The four prototype viruses are shown in boldface, with ovals drawn around them. NL. viruses from the Netherlands; FN, viruses obtained from Finland; UK, viruses obtained from the United Kingdom; HK, viruses obtained from Hong Kong; BR, viruses obtained from Brazil.

```
                      Signal peptide                                                      80
A1: NL/1/00  MSWKVVIIFSLLITPQHGLKESYLEESCSTITEGYLSVLRTGWYTNVFTLEVGDVENLTCADGPSLIKTELDLTKSALRE
A2: NL/17/00 ...........................................................S....................
B1: NL/1/99  .....M..I..................................................T....................
B2: NL/1/94  .....M..I..................................................T....................

                         Fusion domain                                                   160
A1: NL/1/00  LRTVSADQLAREEQIENPRQSRFVLGAIALGVATAAAVTAGVAIAKTIRLESEVTAIKNALKKTNEAVSTLGNGVRVLAT
A2: NL/17/00 .K..............RQSR.......................................T...................
B1: NL/1/99  .K...............................I...........N...G...Q...............
B2: NL/1/94  .K...............................I...........N...G...T...............

                                                                                         240
A1: NL/1/00  AVRELKDFVSKNLTRAINKNKCDIADLKMAVSFSQFNRRFLNVVRQFSDNAGITPAISLDLMTDAELARAVSNMPTSAGQ
A2: NL/17/00 .......................D........................................................
B1: NL/1/99  ......E.......S...R.........................................Y.......
B2: NL/1/94  ......E.......S.............................................Y.......

                                                                                         320
A1: NL/1/00  IKLMLENRAMVRRKGFGILIGVYGSSVIYMVQLPIFGVIDTPCWIVKAAPSCSGKKGNYACLLREDQGWYCQNAGSTVYY
A2: NL/17/00 ...........................T.......................E...........................
B1: NL/1/99  .................................I.......E.N...............K.......
B2: NL/1/94  .................................I.......E.D...............K.......

                                                                                         400
A1: NL/1/00  PNEKDCETRGDHVFCDTAAGINVAEQSKECNINISTTNYPCKVSTGRHPISMVALSPLGALVACYKGVSCSIGSNRVGII
A2: NL/17/00 ................................................................................
B1: NL/1/99  .........................R...................................W....
B2: NL/1/94  .........................R...........................

                                                                                         480
A1: NL/1/00  KQLNKGCSYITNQDADTVTIDNTVYQLSKVEGEQHVIKGRPVSSSFDPVKFPEDQFNVALDQVFESIENSQALVDQSNRI
A2: NL/17/00 ...................................................I...............N...........
B1: NL/1/99  ...P...............................................I...............K.
B2: NL/1/94  ...P...............................................IR...........K.

                      Membrane anchor                                 540
A1: NL/1/00  LSSAEKGNTGFIIVIILIAVLGSTMILVSVFIIIKKTKKPTGAPPELSGVTNNGFIPHNL
A2: NL/17/00 .......................S.....I...............S.
B1: NL/1/99  .N...............V..V....L...S..II......R.........N....G.....S.
B2: NL/1/94  .N...................L...S..II......R.........N....G.....S.
```

Figure 2. Amino acid sequence comparison of the fusion protein genes of prototype human metapneumovirus isolates of each sublineage. The predicted signal peptide, fusion domain, and membrane anchor are shown in italics in boldface type, the cleavage sites are boxed, and the region sequenced for 84 samples is underlined in boldface type. Periods indicate the position of identical amino acid residues relative to isolate NL/1/00.

different sublineages within each main lineage (nt: 76%-83%; aa: 60%-75%), and low sequence identity between members of the two different main lineages A and B (nt: 50%-57%, aa: 30%-37%).

The position of the hydrophobic domain, a high percentage of proline, serine, and threonine residues and a cysteine residue at position 27 are features shared by all HMPVs. Whereas the cytoplasmic tail was conserved among all members (58%-70% aa identity), the proposed ectodomains (start aa 51) were quite variable (18%-25% aa identity between lineage A and B). The number and position of potential sites for *N*-linked glycosylation sites varied even within each sublineage, ranging from two to six potential sites, with one located at the proposed cytoplasmic tail conserved among all lineages.

Geographical and temporal distribution
Analysis of HMPV sequences obtained from samples received from different countries indicated that sequences from Finland, the United Kingdom, and the limited sequences from Asia and

South America, were found on branches between the Dutch sequences in the F tree, and not as a separate lineage. The variation between sequences obtained from samples from a single country was found in the same range as the variation found between samples obtained from different countries. In agreement with the genetic lineages of HMPV observed worldwide, which usually includes sequences similar to those of isolate NL/1/00 or NL/1/99, geographic clustering does not appear to apply to HMPV. The different HMPV samples were obtained during the last 20 years with most from 2000 to 2002 and 14 in the 1990s. As indicator for possible fixation of amino acid variation over time, we analyzed the G ORF amino acid sequence of members in sublineage A2 and B1 (containing samples from 1981 to 2002) in more detail. The amino acid sequence variation between the viruses from 1981 and 2001 was in the same range as the variation found between viruses from 2001, and alignments of the sequences did not reveal fixation of amino acid changes between 1981 and 2002. Thus, antigenic drift, as observed for influenza A and B viruses, does not appear to be an important phenomenon for HMPV.

Antigenic variation

To address the antigenic variation between the genetic lineages A and B, we raised antisera in ferrets against isolate NL/1/00, the prototype virus for lineage A1, and against isolate NL/1/99, the prototype virus for lineage B1. The serum samples were collected 28 days postinfection and tested in virus neutralization assays against the homologous and heterologous viruses. In three independent experiments, the virus titer used per well varied from 10 to 50 $TCID_{50}$: this variation did not affect the measured virus neutralization titers (Table 2). Ferret 1, infected with the lineage A prototype virus (NL/1/00), showed a 48- to 128-fold higher virus neutralization titer against the homologous virus NL/1/00 than to the heterologous virus NL/1/99. Similarly, ferret 2, infected with the lineage B prototype virus NL/1/99, had a 16- to 96-fold higher homologous than heterologous virus neutralization titer.

Table 2. Homologous and heterologous virus neutralizing antibody titers of serum samples obtained from ferrets infected with HMPV viruses belonging to different genetic sublineages[a,b]

Experiment	Virus used in virus neutralization	$TCID_{50}$/well	Ferret 1 NL/1/00 [A1]	Ferret 2 NL/1/99 [B1]
1	NL/1/00	12	**1,024**	32
	NL/1/99	9	16	**512**
2	NL/1/00	30	**1,024**	8
	NL/1/99	20	8	**768**
3	NL/1/00	40	**768**	24
	NL/1/99	25	16	**768**
Ratio A-B			48-128	
Ratio B-A				16-96

a Virus neutralizing antibody titers obtained in three independent experiments for serum samples collected 28 days postinfection from ferrets 1 and ferret 2 (infected with NL/1/00 and NL/1/99 respectively). TCID50, 50% tissue culture infectious dose; HMPV, human metapneumovirus.
b Homologous virus neutralization titers are bold. Values are average of duplicate measurements. Ratios are given as the homologous virus neutralization titers divided by the heterologous virus neutralization titers.

```
A1                                                                              80
NL/1/00   MEVKVENIRTIDMLKARVKNRVARSKCFKNASLVLIGITTLSIALNIYLIINYKMQKNTSESEHHTSSSPMESSRETPTV
FI/4/01   ................................................................................
NL/10/01  ...........................................I...............T..E.....
NL/2/02   ...........................................I...............T..E.....
BR/2/01   ............S.................................

                                                                                160
NL/1/00   PTDNSDTNSSPQHPTQQSTEGSTLYFAASASSPETEPTSTPDTTNRPPFVDTHTTPPSASRTKTSPAVHTKNNPRTSSRT
FI/4/01   ............................................N...........................I.....
NL/10/01  .M......PGS.Y..........H...............S...........L.I.P..
NL/2/02   .M......PGS.Y..........H...............S....IR....L.I.P..
BR/2/01   ......................

                                                                                236
NL/1/00   HSPPRATTRTARRTTTLRTSSTRKRPSTASVQPDISATTHKNEEASPASPQTSASTTRIQRKSVEANTSTTYNQTS
FI/4/01   ....W...........................A....................T........ . ....
NL/10/01  ....W.M...V.G.........I.........S......H.....V...A....A.P...GM..S.......
NL/2/02   ....W.M...V.G.........I.........S......H.....V...A....A.P...GM..S.......
BR/2/01   ............NHHSPHKQHK.ETVHSISPT

A2                                                                              80
NL/17/00  MEVKVENIRAIDMLKARVKNRVARSKCFKNASLILIGITTLSIALNIYLIINYTIQKTTSESEHHTSSPPTEPNKEASTI
NL/1/81   .............................................S...T...
NL/1/93   ........V.........V........V........S..S..GT...
NL/1/95   ...........................................S...T...
NL/3/96   ...........................................S...T...
NL/22/01  ...........................................S...T...
NL/3/02   ...........................................S.....
NL/2/93   ........V.................V.........V........S..S..GT...

                                                                                160
NL/17/00  STDNPDINPSSQHPTQQSTENPTLNPAASASPSETEPASTPDTTNRLSSVDRSTAQPSESRTKTKPTVHTINNPNTASST
NL/1/81   PI......N........S....V.................T..............K..S.V.R.
NL/1/93   P.......N........S....T...V.............A....T........L....K..LS...R.
NL/1/95   .I......N........SL..S.T..V.........S...S.......T.....AR......KK.I.S.V.R.
NL/3/96   .I..S....N........SL..S.T..V.........S...S.......T.....AR......KK.I.S.V.R.
NL/22/01  PI......N........SL..Y.TS.V.S........GI..H..F.....T........NR....KK.ISS.V.R.
NL/3/02   ........N........ ...........S.............V....N....L....R..LS.....
NL/2/93   PQTTQTSIQIHNIQLNSPQKA.HST.QPR

                                                                            220
NL/17/00  QSPPRTTTKAIRRATTFRMSSTGKRPTTTLVQSDSSTTTQNHEETGSANPQASASTMQN
NL/1/81   ....L.A....VL...A..T...R.......S.................SQHTNNIKPN
NL/1/93   ....A....VL.D.A.HT.........S...G.........S.S.........DQDTNNTKQN
NL/1/95   ...L.A...VL...A..T...EG...S.........................
NL/3/96   ...L.A...VL.....A.....EG...S...................QHTNIAKPN
NL/22/01  .......A..VP...AL.T....E......P..P.............QHTNIARPN
NL/3/02   .......A........L.....R...................QHTNNIKPN
NL/2/93   

B1                                                                              80
NL/1/99   MEVRVENIRAIDMFKAKIKNRIRSSRCYRNATLILIGLTALSMALNIFLIIDHATLRNMIKTENCANMPSAEPSKKTPMT
NL/11/00  ................................................................................
NL/5/01   ............................................................P....R.....
NL/21/01  ............................................................P.........

                                                                                160
NL/1/99   STAGPNTKPNPQQATQWTTENSTSPVATPEGHPYTGTTQTSDTTAPQQTTDKHTAPLKSTNEQITQTTTEKKTIRATTQK
NL/11/00  .....S.E......... ...A..L.S.....P.I..........LP..............T.....
NL/5/01   .................  ...A......LH......P........LP......A......T.E...R
NL/21/01  .................  ...A......LH......P........LP..............T....R

                                                                        224
NL/1/99   REKGKENTNQTTSTAATQTTNTTNQIRNASETITTSDRPRTDTTTQSSEQTTRATDPSSPPHHA
NL/11/00  ...E..... .................T..............I......D.............QSGAKPK
NL/5/01   ....... ...... ....... ...........S.........Q......A...QGSAKPK
NL/21/01  ....... ...............I..............S.............H...QGSAKPK

B2                                                                              80
NL/1/94   MEVRVENIRAIDMFKAKMKNRIRSSKCYRNATLILIGLTALSMALNIFLIIDYAMLKNMTKVEHCVNMPPVEPSKKTPMT
NL/1/82   .........T....... ...............TF....... ...A...............
NL/1/96   ................. ...........................T. ...............
NL/6/97   ................. ...........................T. ...............
NL/9/00   ................. ...........................T. ...............
NL/3/01   ................. ...........................KS...R..............
UK/5/01   ................. ...........................T. ...............

                                                                                160
NL/1/94   SAVDLNTKPNPQQATQLAAEDSTSLAATSEDHLHTGTTPTPDATVSQQTTDEYTTLLRSTNRQTTQTTTEKKPTGATTKK
NL/1/82   .T..SS.G.....T..WTT....................L........P.KH..P.....G.............R.IA..
NL/1/96   ........L......TT.............L..........H.........................
NL/6/97   ........L......TT.........G.P...........H........A........
NL/9/00   ....S........TT.......L..P...............H.........A........
NL/3/01   ........R......TT.......L.G.............V.....H.........AA...RV..N.
UK/5/01   ...............TT.......L..P.............H.........A........

                                                                                236
NL/1/94   ETTTRTTSTAATQTLNTTNQTSYVREATTTSARSRNSATTQSSDQTTQAADPSSQPHHTQKSTTTTYNTDTSSPSS
NL/1/82   ....NQ.........F......RNG..T.I.......D......E..N.TT.........A.........
NL/1/96   .................NG.......T....N.....
NL/6/97   ...............P......NG.........G....N...I.......S...........A......F...
NL/9/00   ...... ......NG.......N...............E...SQ.............L..
NL/3/01   ..I.........NNG.......N.................SQ.....I.........
UK/5/01   ...... ......NG.......N...............E.N..SQ...............L..
```

Figure 3.

In a second experiment, ferret antisera were raised to viruses from all four sublineages. To measure the most specific serologic response, serum samples were collected 21 days postinfection, after which homologous and heterologous virus neutralization titers were measured (Table 3).

Table 3. Homologous and heterologous virus neutralizing antibody titers of sera obtained from ferrets infected with HMPV viruses belonging to different genetic sublineages[a,b]

Virus used in virus neutralization	Ferret 3 NL/1/00 [A1]	Ferret 4 NL/17/00 [A2]	Ferret 5 NL/1/99 [B1]	Ferret 6 UK/5/01 [B2]
NL/1/00	**256**	256	6	32
NL/17/00	512	**768**	12	24
NL/1/99	16	32	**256**	384
UK/5/01	12	64	256	**512**
Ratio A-B	16-21	12-24		
Ratio B-A			21-43	16-21

[a] Virus neutralizing antibody titers obtained for sera collected 21 days post infection from ferrets 3 to 6 (infected with NL/1/00, NL/17/00, NL/1/99 and UK/5/01, respectively). HMPV, human metapneumovirus.
[b] Homologous virus neutralization titers are bold. Values are average of duplicate measurements. Ratios are given as the homologous virus neutralization titers divided by the heterologous virus neutralization titers.

Within each main genetic lineage, a high degree of cross-neutralization was observed between viruses from the two sublineages (e.g., A1 vs. A2 and B1 vs. B2), which is reflected in the low ratio between homologous to heterologous virus neutralization titers (0.5 to 3.0). Although serum samples from ferrets 3 to 6 had slightly lower homologous virus neutralization titers than those of ferrets 1 and 2, serum samples raised against viruses from the main lineage A still showed a 12- to 24-fold higher virus neutralization titer against the lineage A viruses than to lineage B viruses. Similarly, serum samples raised against viruses from lineage B had a 16- to 43-fold higher virus neutralization titer against the lineage B viruses than to lineage A viruses.

DISCUSSION

In this study, the genetic heterogeneity of HMPV was addressed by analysis of the nucleotide and predicted amino acid sequences of part of the F (n = 84), complete F (n = 4), and the complete G (n = 35) protein genes. Phylogenetic analysis of these sequences showed two main lineages

Figure 3. Amino acid sequence comparison of the putative attachment (G) protein of human metapneumovirus strains per genetic sublineage; A1 (A), A2 (B), B1 (C), and B2 (D). For each lineage only representative samples are depicted, resulting in 24 sequences. Representative sequences were chosen, so that form each year in which samples were obtained at least one sequence is depicted, and sequences with only a few amino acid substitutions were omitted. Potential N-linked glycosylation sites are underlined, and periods indicate the position of identical amino acid residues relative to the first sequence in each subgroup. Numbers indicate the nucleotide position in the primary G Open reading frames.

(A and B) with each divided into two sublineages (1 and 2). As was described for HRSV and APV, the F protein was highly conserved, which is in agreement with F proteins of pneumoviruses having structural and functional constraints for amino acid mutations (210). On the basis of the high percentage sequence identity for the complete F proteins of the prototype viruses of the four lineages, sequences for the complete F proteins of all 84 samples would probably demonstrate similar low variability. In contrast to the F protein, the nucleotide and predicted amino acid sequences of the complete G coding regions showed high sequence diversity (as low as 30%-37% aa identity). Besides the high amino acid sequence variation, we observed variation in length of the different G proteins. Where most of the length variation was due to nucleotide substitutions, two of the samples revealed a change in reading frame due to deletion or addition of single nucleotides. Frameshift mutations and use of alternative reading frames have been described for HRSV (26, 80, 214). As described for isolate NL/1/00, the G coding region of the 35 samples sequenced in the present work revealed long alternative ORFs. However these secondary ORFs varied in length and position compared to the ones described (226). Whether premature stopcodons, the incidence of frame shift mutations, and possible use of alternative reading frames influence the antigenic properties of the viruses needs to be examined in more detail.

Phylogenetic analyses showed that the HMPV samples obtained from different years and from different countries were randomly distributed over all four sublineages. For HRSV it has also been reported that very similar viruses were isolated at different times and from geographically distant sites (26). Different lineages within HRSV subgroup A and B have been found on the basis of the variation in the G protein. Within each subgroup, progressive accumulation of amino acid changes was noted, suggesting that the G protein of HRSV might be susceptible to immune pressure (26). Analysis of the amino acid sequences of the HMPV samples described in this study did not indicate such accumulation over time. However the following observations indicate that the variation of the HMPV G protein might occur as a result of immunogenic pressure in a same manner as was postulated for the RSV G protein: 1) most of the amino acid sequence variation was found in the extracellular domain of the G protein, 2) the variation found at the amino acid sequence level was higher than that at the nucleotide sequence level, 3) the number and position of potential glycosylation sites were not conserved, and 4) deletions, additions and substitutions of single nucleotides resulted in premature stopcodons and drastic changes of the carboxy terminal of the protein (26, 122). Until a larger number of more chronologically diverse HMPV samples have been examined, this issue remains inconclusive.

To address the antigenic relationship between members of the different HMPV lineages, we tested ferret sera raised against viruses from the four sublineages in virus neutrlization assays. Serological responses upon infections tend to broaden over time. On the basis of the relatively close genetic relationship between sublineages A1 and A2 or B1 and B2, we decided to collect serum samples at an early time point, to obtain large antigenic differences between the four sublineages. The low homologous virus neutralization titers in serum samples collected 21 days postinfection may explain the lower ratio between homologous and heterologous virus neutralization titers as

compared to sera collected 28 days postinfection. The studies with serum samples collected at 21 days postinfection showed that viruses within one main lineage (e.g., A1 and A2 or B1 and B2) were antigenically closely related. The difference in homologous and heterologous virus neutralization titers between members of the two different lineages A and B (12- to 128-fold higher homologous titer than heterologous titer) indicate a difference in antigenicity between lineage A and B. Classic virology studies have used a definition of a homologous-to-heterologous virus neutralization titer ratio of >16 for defining serotypes. This same definition notes that if neutralization shows a certain degree of cross-reaction between two viruses in either or both directions (homologous-to-heterologous titer ratio of 8 or 16), distinctiveness of serotype is assumed if substantial differences in sequences are observed (76). On the basis of the presented results, and based on the described definition, we propose defining the two main lineages of HMPV as serotypes A and B. The HMPV samples were obtained from different study populations, from different countries, and from patients with a wide spectrum of clinical signs. So far, we have no indication for an association between infection with either of the serotypes and a specific study group or with severity of disease. More epidemiological studies are needed to address this issue.

The circulation of two serotypes of HMPV might have implications for the development of vaccines. Studies in cynomolgous macaques showed that reinfection is suppressed by high titers of virus neutralization antibodies against the homologous virus and far less by heterologous virus neutralization antibodies (data not shown). So far, one heterologous reinfection has been reported in humans (176). However, children approximately ≥5 years of age have higher virus neutralization antibody titers than those 1-2 years of age (227), which suggests that reinfections may occur frequently, most likely with the viruses from the heterologous serotype. For RSV, the importance of difference in antigenicity between the two subgroups regarding protective immunity and vaccine development is still a subject of discussion. However, in animals and humans, the neutralizing capacity against homologous viruses is higher than that against heterologous viruses, and in animals high homologous virus neutralization titers protect against reinfection. In humans, reinfection often occurs with a strain from the heterologous group, and high homologous virus neutralization antibody titers protect against severe infection (213). The two serotypes of HMPV might resemble the two subgroups of HRSV in immunogenic properties, although more extensive epidemiologic and immunologic studies have to prove this. The cross-reactive immunity provided by the F protein may be sufficient to overcome the effects of changes in the G protein. For HRSV the immune response against the F protein is cross-reactive between subgroup A and B, whereas the response against the G protein is subgroup (and sometimes even genotype) specific (121, 124, 167). The prophylactic use of a virus neutralization monoclonal antibody preparation directed against the HRSV-F protein has been shown to decrease the severity of lower respiratory tract diseases caused by both subgroups of HRSV (90, 105, 201). In a similar way, the conserved F protein of HMPV could be a target for the development of monoclonal antibodies for treatment of HMPV-infected individuals.

Our data support a technical description of two serotypes of HMPV in experimentally infected

ferrets. The existence and relevance of these serotypes in other animal species, including humans, has yet to be determined.

Our results in combination with data published by others (17, 177, 212) demonstrate that HMPV clusters in two globally distributed serotypes. However, the identification of two serotypes does not exclude the possible existence of more serotypes or sublineages. The described viruses were all identified by using primers against conserved regions in the genome of the four prototype viruses, but in order to allow identification of more diverse HMPV strains, virus isolation of original materials is a standard procedure in our laboratory.

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Recovery of human metapneumovirus genetic lineages A and B from cloned cDNA

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ABSTRACT

Human metapneumovirus (hMPV) is a newly discovered pathogen associated with respiratory tract illness, primarily in young children, immunocompromized individuals, and the elderly. The genomic sequence of the prototype hMPV isolate NL/1/00 without the terminal leader and trailer sequences has been reported previously. Here we describe the leader and trailer sequences of two hMPV isolates, NL/1/00 and NL/1/99, representing the two main genetic lineages of hMPV. Minigenome constructs in which the green fluorescent protein or chloramphenicol acetyl transferase genes are flanked by the viral genomic ends derived from both hMPV lineages and transcribed using a T7 RNA polymerase promoter-terminator cassette were generated. Cotransfection of minigenome constructs with plasmids expressing the polymerase complex components L, P, N and M2.1 in 293T or baby hamster kidney cells resulted in expression of the reporter genes. When the minigenome was replaced by a sense or antisense full-length cDNA copy of the NL/1/00 or NL/1/99 viral genomes, recombinant virus was recovered from transfected cells. Viral titers up to $10^{7.2}$ and $10^{5.7}$ 50% tissue culture infective dose/ml were achieved with the sense and antisense plasmids, respectively. The recombinant viruses replicated with similar kinetics to the parental viruses in Vero cells. This reverse genetics system provides an important new tool for applied and fundamental research.

INTRODUCTION

The human metapneumovirus (hMPV) was recently identified in respiratory specimens obtained from children in The Netherlands suffering from respiratory tract illness (RTI) (227). Subsequently hMPV has been detected around the world, in clinical samples collected from children, immunocompromized individuals and elderly individuals suffering from RTI (7, 18, 33, 64, 65, 67, 151, 174, 177, 216, 221, 233). Clinical signs and symptoms associated with hMPV infection are similar to those caused by respiratory syncytial virus (RSV), ranging from mild respiratory problems to severe cough, bronchiolitis and pneumonia, often accompanied by high fever, myalgia and vomiting (17, 65, 67, 170, 174, 176, 216, 229, 231). The genomic organization of hMPV resembles that of avian pneumovirus (APV), and hMPV has therefore been classified as the first mammalian member of the family Paramyxoviridae, subfamily Pneumovirinae, genus Metapneumovirus (226). Two main genetic lineages of hMPV were first described for the Dutch isolates (227) and the presence of these genotypes has been confirmed worldwide (6, 14, 18, 174, 177, 233). Recently, the two main lineages were found to represent two serotypes of hMPV, A and B, each of which can be further divided in two genetic sublineages A1, A2, B1, and B2 (229).

For APV, the other member of the metapneumovirus genus, a minigenome system has been established (187), but a recombinant virus rescue system is not yet available. Such reverse genetics systems have been reported for other paramyxoviruses, including RSV, measles virus, parainfluenza viruses, rinderpest virus and canine distemper virus (5, 39, 81, 114, 185). The systems for the recovery of recombinant paramyxovirus use a cDNA plasmid encoding the full-length viral RNA in sense or antisense orientation flanked by sequences to ensure the transcription of genome-length RNA such as a T7 RNA polymerase promoter-terminator cassette and a hepatitis delta virus (HDV) ribozyme sequence. Upon transcription of the full-length hMPV cDNA by T7 RNA polymerase expressed either from plasmids, modified vaccinia virus Ankara or recombinant fowl pox virus and coexpression of the viral polymerase protein complex (N, P, L and M2.1), a recombinant virus can be produced.

Reverse genetics systems provide a powerful tool for fundamental virus research and for the generation of vaccine candidates, including live-attenuated vaccines, because point mutations, deletions and insertions can be engineered to suit specific needs. Foreign genes can be introduced in order to attenuate viruses or to create chimeric vaccines. By using reverse genetics, one can envisage creating chimeric viruses between APV and hMPV as potential vaccine candidates in humans and poultry or chimeric vaccines between hMPV and other human respiratory pathogens (RSV, parainfluenza virus) to combat RTI in humans.

Here we describe the sequence analysis of the genomic termini of NL/1/00 and NL/1/99, representative strains for hMPV serotypes A (lineage A1) and B (lineage B1), respectively. We successfully used this information for the design of minigenome replication systems using chloramphenicol acetyl transferase (CAT) or green fluorescent protein (GFP) as reporter genes. Subsequently, the reporter genes of the minireplicons were replaced with full-length cDNA copies of the NL/1/00 (serotype A) or NL/1/99 (serotype B) genomes, and recombinant hMPVs were recovered.

MATERIALS AND METHODS

Cells and viruses

Virus isolates hMPV NL/1/00 and NL/1/99 were propagated in tertiary monkey kidney cells as described previously (227). 293T cells were grown in Dulbecco's modified eagle medium (BioWhittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (FCS), nonessential amino acids, 100 IU of penicillin/ml, 100 µg of streptomycin/ml and 2mM glutamine. BSR-T7 cells, a kind gift of Dr. K.K. Conzelmann, were grown in the same media without non-essential amino acids and supplemented with 0.5 mg of G418 (Life Technologies, Breda, The Netherlands)/ml. Vero cells were grown in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 10% FCS, 100 IU penicillin/ml, 100 µg streptomycin/ml and 2mM glutamine. For hMPV replication, Vero cells were grown in Dulbecco's modified eagle medium supplemented with 3% FCS, 100 IU penicillin/ml, 100 µg streptomycin/ml, 2mM glutamine and 0.25 mg trypsin/ml. For virus titration or plaque assays, Vero cells were grown in Iscove's modified Dulbecco's medium supplemented with 4% bovine serum albumin fraction V (Invitrogen, Breda, The Netherlands), 100 IU penicillin/ml, 100 µg streptomycin/ml, 2mM glutamine and 3.75 µg trypsin/ml.

293T cells were transfected using the $CaPO_4$ precipitation method (172) and BSR-T7 cells were transfected using Lipofectamine 2000 (Invitrogen), according to the instructions of the manufacturer.

Identification of leader and trailer sequences of hMPV

Viral RNA was isolated from concentrated NL/1/00 and NL/1/99 virus stocks using RNAzolB according to instructions from the manufacturer (Campro Scientific, Veenendaal, The Netherlands). The viral RNA was circularized using T4 RNA ligase (New England Biolabs, Frankfurt am Main, Germany) for 1 h at 37°C and cDNA was synthesized using random hexamer primers (Promega, Leiden, The Netherlands) and RNase H-free superscript II reverse transcriptase (Invitrogen) for 50 min. at 42°C. The ligated junction of the leader and trailer was PCR amplified using primers in the 5' end of the N open reading frame (ORF) and the 3' end of the L ORF. The PCR products were sequenced upon cloning in pCR2.1 (Invitrogen).

A second approach was used to identify the terminal nucleotides of the leader of NL/1/00. Viral RNA was isolated using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif.), according to the instructions from the manufacturer. This viral RNA was polyadenylated by incubation with poly (A) polymerase (Ambion, Austin, Tex.) at 37°C for 1 h and purified using a NucAway spin column (Ambion). The viral RNA was reverse transcribed using a primer complementary to the poly(A) tail region and Superscript I reverse transcriptase (Invitrogen). PCR reactions were carried out and sequenced upon cloning in plasmid pCR2.1 (Invitrogen).

Plasmid construction

(i) Minigenome constructs. An NdeI (nucleotide [nt] 2379)-to-HpaI (nt 136) fragment was removed

from plasmid pSP72 (Promega) and replaced by a synthetic T7 promoter extended with two or three G residues, two BsmBI sites, the HDV ribozyme, and a T7 terminator to yield pSP72-P_{T7}-δ-T_{T7}. The hMPV genomic leader sequence with the gene start of N, and the genomic trailer sequence with the gene end of L were amplified by PCR and ligated and were separated by two BsmBI sites. This fragment was ligated in the BsmBI site of pSP72-P_{T7}-δ-T_{T7} to yield pSP72-P_{T7}-Tr-Le-δ-T_{T7}. The ORFs of GFP or CAT were amplified by PCR using primers with type II restriction sites (BbsI, BsaI or BsmBI) and cloned in the BsmBI sites between the N gene start and L gene end signals of hMPV to yield pSP72-P_{T7}-Tr-CAT-Le-δ-T_{T7} and pSP72-P_{T7}-Tr-GFP-Le-δ-T_{T7}. The junctions between the elements of the minigenome plasmids are shown in Fig. 1.

```
                            pSP72
        1         2                      3       4
        ↓         ↓                      ↓       ↓
    ┌──────┬──┬──┬──────────────────┬──┬──┬─────┐
    │ T-T7 │δ │Le│GS   CAT or GFP   │GE│Tr│ P-T7│
    └──────┴──┴──┴──────────────────┴──┴──┴─────┘

  1  GCTGGGACCATGCCGGCC|ACGCGAAAAAAACGCGTA

                               MetGluLysLysIleThr
  2  AATATGGGACAAGTGAAA|ATGGAGAAAAAAATCACT

     TrpGlnGlyGlyAlaEnd
  3  TGGCAGGGCGGGGCGTAA|AAAATGATAAAAATGATA

  4  ATACGGTTTTTTTGCCGT|CCTATAGTGAGTCGTATT
                        <-----------------
```
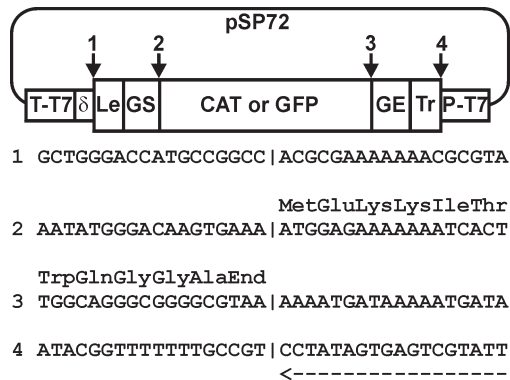
Figure 1. Schematic representation of a minigenome construct. A CAT or GFP reporter gene is flanked by the viral leader sequence (Le) and gene start signal (GS) of the nucleocapsid gene on one end, and the gene end signal (GE) of the polymerase gene and the viral trailer (Tr) on the other end. This minigenome is cloned in the context of a T7 RNA polymerase promoter-terminator (P-T7, T-T7) cassette. The authenticity of the transcribed ends of the negative-sense minigenome are determined by the position of the T7 promoter sequence and a HDV ribozyme sequence (δ). The important junctions, depicted with numbers 1-4 are shown in detail below the map, with the translated sequence of the CAT gene as the example.

(ii) Polymerase constructs. The N, P and M2.1 ORFs of NL/1/00 and NL/1/99 were amplified by PCR using primers spanning the start and stop codons and flanked by NcoI and XhoI sites, respectively, and were cloned in the multiple cloning site of pCITE (Novagen) to yield plasmids pCITE-N, pCITE-P and pCITE-M2.1. Constructs encoding the L gene of NL/1/00 or NL/1/99 were assembled from overlapping PCR fragments using restriction sites in the L gene (Fig. 2) and were cloned in pCITE. The restriction sites used were NcoI (introduced at nt 7180 before the start codon of L), SwaI (nt 8806), EcoRI (nt 10998), and SmaI (introduced after the PacI site at nt 13283 in the trailer) for NL/1/00, and BamH1 (nt 7127), NsiI (nt 9565), KpnI (nt 11753) and AccI (nt 13276 in trailer) for NL/1/99.

(iii) Full-length cDNA vectors. The minigenome plasmids (pSP72-P_{T7}-Tr-Le-δ-T_{T7}) of NL/1/00 and NL/1/99 were used to construct full-length cDNA vectors. The cDNA encoding the genome of

NL/1/00 was assembled from overlapping PCR fragments using restriction sites MluI (nt 12), ApaLI (nt 3293), StuI (4480), SalI (6649), ClaI (8678), EcoRI (nt 10998), and PacI (nt 13283) (Fig. 2). The cDNA encoding the genome of NL/1/99 was assembled from overlapping PCR fragments using restriction sites MluI (nt 12), SacI (nt 2034), XbaI (nt 2979), BamHI (nt 5500 and 7127), NsiI (nt 9565), KpnI (nt 11753), and AccI (nt 13276) (Fig. 2). In the genome of both NL/1/00 and NL/1/99, a genetic marker, an AflII restriction enzyme site, was introduced at amino acid (aa) position 8 in the N ORF of the hMPV genome using a QuickChange site-directed mutagenesis kit (Stratagene). Also, for NL/1/99, a negative-sense full-length clone was generated with the extra AflII restriction enzyme site, to compare the functionality of rescue with positive- or negative-sense plasmids.

All plasmid inserts were sequenced to ensure the absence of mutations. All primer sequences used for plasmid construction are available upon request.
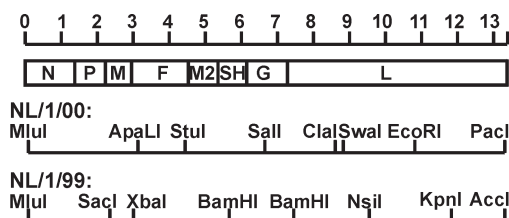
Figure 2. Restriction maps of hMPV NL/1/00 and NL/1/99. The genomic organization of hMPV is shown, roughly drawn to scale. The restriction sites that were used to generate the full-length cDNA constructs and the polymerase expression plasmids are shown for both virus isolates.

Minigenome assays

Minigenome plasmids were cotransfected with plasmid pAR3126, expressing a T7 RNA polymerase, and pCITE-L, pCITE-N, pCITE-P and pCITE-M2.1 in six-well plates containing 3×10^5 to 5×10^5 293T cells per well (172). Transfections were done overnight and medium was refreshed the next day. In a second approach transfections were done using BSR-T7 cells, a baby hamster kidney cell line stably expressing T7 RNA polymerase (24). Minigenomes were cotransfected with pCITE-L, pCITE-N, pCITE-P and pCITE-M2.1 in six-well plates when cell monolayers were 80 to 95 % confluent. Cells were analyzed 3 days after transfection using a CAT enzyme-linked immunosorbent assay (Roche Diagnostics, Almere, The Netherlands) according to the instructions from the manufacturer or a flow cytometer equipped with an argon laser emitting at 488 nm (Becton Dickinson, Erembodegem, Belgium).

Recovery of recombinant hMPV

Both 293T cells and BSR-T7 cells were used for rescue of recombinant hMPV. 293T cells were transfected overnight using the CaPO$_4$ method with the four pCITE polymerase complex plasmids, pAR3126 and the full-length hMPV cDNA plasmid. BSR-T7 cells were transfected for 5 hours with Lipofectamine 2000 (Invitrogen) and the same plasmids without pAR3126. After transfection, the media was replaced with fresh media supplemented with trypsin and incubated for 2 to 4 days. Cells

were either used to prepare lysates or for direct cocultivation with Vero cells. To prepare lysates, cells were scraped with a rubber cell scraper, pooled with the supernatant, and subjected to one -70°C freeze-thaw cycle. The virus preparation was cleared from cellular debris by centrifugation and used to inoculate 60 to 70 % confluent Vero cells in 100-mm dishes. After 3 or 4 days, half of the medium was replaced with fresh medium, and after 6 or 7 days, the supernatant was collected and the infected Vero cells were immunostained using a guinea pig polyclonal antiserum raised against hMPV and a fluorescein isothiocyanate-labeled rabbit anti-guinea pig serum (DakoCytomation, Heverlee, Belgium). The cells were analyzed using a flow cytometer, and the supernatant was used for virus titrations, reverse transcription (RT)-PCR and restriction digests to confirm rescue of recombinant virus.

RT-PCR to identify genetic marker
In both NL/1/00 and NL/1/99, a silent mutation was introduced in the codon that encodes leucine at aa position 8 in the N ORF of the full-length genome. At this position, CCTAAG (NL/1/99) or CCTGAG (NL/1/00) were changed to CTTAAG in order to introduce an AflII restriction site. After harvesting the rescued virus and RNA isolation with the High Pure RNA Isolation Kit (Roche Diagnostics), RT-PCR was performed with specific primers to amplify the region in which the mutation was introduced. After amplification, a control digestion was done with AflII to confirm that the recovered virus was recombinant in nature.

Virus titration
Titrations were performed with 10-fold serial dilutions in 96-well plates. Briefly, confluent monolayers of Vero cells were spin-inoculated (15 min, 2000 x g) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37°C. Fresh medium (100µl) was added to each well after 3 days. Seven days after inoculation, infected wells were identified by immunofluorescence assays with hMPV specific polyclonal antiserum raised in guinea pigs. Titers expressed as 50% tissue culture infectious dose (TCID$_{50}$) were calculated as described by Reed and Muench (188).

Plaque assay
Twenty-four-well plates containing 95% confluent monolayers of Vero cells were inoculated with 10-fold serial virus dilutions for 1 h at 37°C, after which the media was replaced by 0.5 ml of fresh media and 0.5 ml 2% methyl cellulose (MSD, Haarlem, The Netherlands) and cells were incubated at 37°C for 4 days. Methyl cellulose overlays were removed and cells were fixed with 100% methanol. Blocking was performed for 1 h at 37°C with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline, and cells were subsequently incubated with hMPV specific polyclonal antiserum for 1 h at 37°C, followed by incubation with horseradish peroxidase-labeled rabbit anti-guinea pig antibodies (DakoCytomation). Positive plaques were counted after incubation with AEC substrate chromogen (DakoCytomation) to determine viral titers.

Growth curve
To generate growth curves, 25-cm^2 flasks containing confluent Vero cells were inoculated at 37°C

for 2 h with wild type and recombinant hMPV virus strains at a multiplicity of infection of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed 2 times with media before addition of 7 ml of fresh media and incubation at 37°C. Every 2 days, 1ml of the supernatant was collected and replaced by fresh media. Plaque assays or end-point titrations were performed to determine viral titers in PFU per milliliter or $TCID_{50}$ per milliliter, respectively. There is a general correspondence between viral titers determined by these two methods, but the end-point titrations yielded approximately10-fold higher titers; thus, 10 $TCID_{50}$/ml corresponds with approximately 1 PFU/ml.

Nucleotide sequence accession numbers
The updated and new sequences discussed here were submitted to GenBank under accession no. AF371337 and AY525843, respectively.

RESULTS

Identification of leader and trailer sequences
In order to determine the authentic nucleotide sequences of the hMPV genomic RNA leader and trailer, viral RNA isolated from concentrated virus stocks was circularized using T4 RNA ligase. cDNA was synthesized from this RNA, and the region containing the ligated junction of the genomic termini was amplified by PCR and cloned in plasmid pCR2.1. For NL/1/00 and NL/1/99, 26 and 35 clones were sequenced ,respectively, and the position of the junction was determined after comparing all sequences. All but 2 clones showed the same trailer cDNA sequence 5'-TACGGTTTTTTTGCCGT-3'. In the 2 deviant clones, the terminal T nucleotide was missing. The majority of the leader cDNA sequences was 5'-GCGAAAAAAACGCGTAT-3', two nucleotides shorter than we had anticipated based on the leader sequences published for APV and RSV (155, 187). All other clones displayed three, four, five, or even longer nucleotide deletions at the leader end (Table 1). We next performed 3' rapid amplification of cDNA ends (RACE) analysis as an alternative strategy to determine the 3' end of the hMPV genome. To this end, fresh viral genomic RNA of NL/1/00 was isolated, which

Table 1. Determination of leader sequences of hMPV NL/1/99 and NL/1/00 obtained by RT-PCR following ligation of viral RNA and 3' RACE[a].

Leader sequence positive strand	NL/1/99 RT-PCR circles (n=26)	NL/1/00 RT-PCR circles (n=35)	NL/1/00 poly(A) tailing and 3'RACE (n=79)
ACGCGAAAAAAACGCG	0 (0)	0 (0)	15 (19)
CGCGAAAAAAACGCG	0 (0)	0 (0)	6 (8)
GCGAAAAAAACGCG	15 (58)	30 (58)	5 (6)
CGAAAAAAACGCG	6 (23)	2 (6)	23 (29)
GAAAAAAACGCG	2 (8)	1 (3)	0 (0)
AAAAAAACGCG	2(8)	0 (0)	0 (0)
Larger deletion	1 (4)	2 (6)	30 (38)

a All data given as number (%)

was polyadenylated and subsequently reverse transcribed using a primer complementary to the poly(A) tail region. The cDNA was amplified by PCR and cloned in plasmid pCR2.1. Sequence analysis revealed that 15 out of 79 clones (19%) contained the following hMPV leader sequences 5' ACGCGAAAAAAACGCGTAT 3', which included the AC terminal dinucleotide observed for other paramyxovirus leader termini, but were lacking in the constructs derived upon RT-PCR of ligated viral RNA. The other 81% of the sequences revealed the absence of 3'- proximal nucleotides. From these data, we conclude that the correct leader cDNA sequence starts with AC and that the shorter leader sequences resulted from nuclease activity present in the virus preparations. It is of interest to note that the hMPV leader and trailer sequences are less complementary than the termini of related paramyxoviruses (Fig. 3).

```
hMPV Le: 3' UGCGCUUUUUUUGCG
            |||  ||||||||
hMPV Tr: 5' ACGGCAAAAAAACCG

APV  Le: 3' UGCUCUUUUUUUGCG
            |||||||||||||
APV  Tr: 5' ACGAGAAAAAAACCG

RSV  Le: 3' UGCGCUUUUUUACGC
            |||  |||||||| | |
RSV  Tr: 5' ACGAGAAAAAAAGUG
```

Figure 3. Complementarity of leader and trailer sequences of hMPV, APV, and RSV. Viral leader (Le) and trailer (Tr) sequences were aligned, and the complementarity is indicated with connection lines, showing that the terminal nucleotides of hMPV were less complementary than those of RSV and APV. The 3' terminal U residue in the APV genome has not been detected experimentally, but was added in analogy to other paramyxoviruses (187).

HMPV minigenome assays

Minigenome assays were performed to test whether the sequences of the genomic termini were correct and the plasmids encoding the viral polymerase complex components were functional. For this purpose, we used minigenome reporter constructs containing GFP or CAT ORFs in antisense orientation and flanked by the genomic termini of hMPV. A T7 phage RNA polymerase promoter was added upstream of the last genomic hMPV nucleotide of the trailer, and a HDV ribozyme sequence was linked to the hMPV leader to ensure authentic ends of the viral RNAs (Fig. 1). The minigenome constructs were cotransfected overnight in 293T-cells with pAR3126 expressing T7 RNA polymerase and pCITE plasmids containing the N, P, L and M2.1 ORFs. In a first experiment 1.5 µg of minigenome, 1.5 µg of T7 RNA polymerase and 1.8 µg of polymerase expression plasmids (0.6 µg of N, 0.6 µg of P, 0.3 µg of L, 0.3 µg of M2.1) were cotransfected, yielding 1.3 ng of CAT/ml after 24 hours and 11 ng of CAT/ml after 48 hours (data not shown). Constructs with and without the terminal AC dinucleotide in the leader were both functional, but the construct without the terminal AC nucleotides gave rise to lower (32 % reduced) levels of CAT expression (data not shown). Subsequently, we optimized the amounts of total plasmid DNA for transfection and the relative

amounts of each individual plasmid using CAT-expression as the readout. The optimal amounts of plasmids were 1μg of minigenome, 1.5μg of pAR3126, 0.8μg of pCITE-N, 0.4μg of pCITE-P, 0.4μg of pCITE-M2.1 and 0.4μg of pCITE-L. By using these optimized amounts, we achieved levels of CAT expression between 20 and 80 ng/ml for both the NL/1/00 and NL/1/99 minigenome systems. When the vector expressing the M2.1 protein is excluded from the experiments, the reporter gene is expressed at lower levels, indicating that the M2.1 protein is dispensable for minireplicon replication, whereas each of the other components is indispensable (Fig. 4). In BSR-T7 cells transfected with 1.5μg of minigenome, 1.2μg of pCITE-N, 0.6μg of pCITE-P, 0.6μg of pCITE-M2.1, and 0.6μg of pCITE-L, CAT expression levels up to 140 ng/ml were obtained 48 hours after transfection (data not shown).
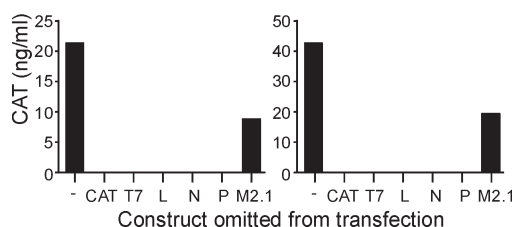
Figure 4. CAT expression in minigenome replication assays in 293T cells. 293T cells were transfected with a T7 RNA polymerase expressing plasmid, plasmids pCITE-L, pCITE-N, pCITE-P, and pCITE-M2.1 and the minigenome vector of either NL/1/00 (left panel) or NL/1/99 (right panel). Each of the individual plasmids was omitted from the transfection mixture to test the requirement of each individual component. Cell lysates were harvested 48 h after transfection and levels of CAT were determined by enzyme-linked immunosorbent assay.

We next tested the GFP minigenome constructs in 293T cells using the optimal amounts of plasmids determined with the CAT minigenome constructs. Forty-eight hours after cotransfection, up to 13 % of the cells revealed high fluorescence by flow cytometry when all constructs were transfected, whereas such GFP expression was absent when the pCITE expression vectors were omitted (data not shown).

In BSR-T7 cells transfected with the GFP minigenome and pCITE expression vectors, 67.4% of the cells were positive by flow cytometry and 1.1% by fluorescence microscopy. This difference in the proportion of GFP-positive cells likely reflected the detection limits of the two assays; only the proportion of cells expressing very high levels of GFP as determined by flow cytometry can be detected under a normal fluorescence microscope.

Assembly of hMPV cDNA and recovery of recombinant hMPV
Full-length hMPV cDNA clones were generated for both NL/1/00 and NL/1/99 by assembling DNA fragments of up to 3 kb that were generated by RT-PCR. This was done by using unique restriction enzyme sites present in the viral genome (Fig. 2). The full-length genomes were cloned in the minigenome vector (Fig. 1), by using restriction sites present in the leader and trailer. In order to demonstrate that the virus recovered by reverse genetics was derived from plasmid

DNA, a restriction enzyme site (AflII) was added at aa position 8 in the N ORF of the full-length cDNA construct. The NL/1/00 cDNA construct contained three nucleotide substitutions and a five nucleotide insertion compared to the GenBank sequence (AF371337). Upon subsequent sequence analysis of the original virus stock, the three nucleotide substitutions were found to be incorrect in the Genbank sequence entry (nt positions 3838, 8350, and 12218 in the GenBank sequence, were changed to A, T and T, respectively). A five-nucleotide insertion, TAAAA, was found in the intergenic region between F and M at position 4709 as compared to the GenBank sequence. This nucleotide insertion was absent in the virus stock from which the Genbank sequence was derived, but present in all other passages of virus stocks of NL/1/00. The plasmid encoding the full-length genome of NL/1/99 did not show any mutations compared to the wild type sequence (accession no. AY525843).

Various approaches were followed for virus rescue from plasmids and were found to be successful. Initially, recombinant virus was rescued from 293T cells transfected overnight with 5 µg of the full-length cDNA plasmid, 1.5 µg of pAR3126, 0.8 µg of pCITE-N, 0.4 µg of pCITE-L, 0.4 µg of pCITE-P and 0.4 µg of pCITE-M2.1. Recombinant virus was also rescued from 293T cells infected with fowlpox-T7 and subsequently transfected with plasmids. However, the most efficient virus rescue was observed in BSR-T7 cells transfected for 5 h with 3 µg of the full length cDNA plasmid, 0.3 µg of pCITE-N, 0.3 µg of pCITE-P, 0.15 µg of pCITE-L and 0.24 µg of pCITE-M2. The cells and supernatant were harvested 48 hours after transfection, subjected to one freeze-thaw cycle, and used to inoculate Vero cells. Cytopathic effects were observed after 6 days in culture, at which time the majority of cells expressed hMPV proteins as determined by staining with an hMPV polyclonal antiserum and flow cytometry (data not shown). Virus titers in the supernatants collected at day 6 ranged from $10^{5.7}$ to $10^{7.2}$ $TCID_{50}$/ml.

The replication kinetics of the wild-type and recombinant hMPV strains are shown in Fig. 5A, revealing that they were indistinguishable. Both wildtype and recombinant NL/1/00 appeared to replicate less efficiently than NL/1/99 in Vero cells. This difference in replication kinetics was observed in all assays that we performed with the NL/1/00 and NL/1/99 viruses. Probably the Vero cell line used in these experiments is more permissive for NL/1/99 than for NL/1/00.

For NL/1/99, recombinant viruses were rescued using plasmids in which the cDNA genome was either cloned in the plus or minus sense orientation. Virus rescue from positive-sense cDNA resulted in virus titers higher than those for negative-sense cDNA (10^7 and $10^{5.7}$ $TCID_{50}$, respectively). Virus rescue in the presence and absence of the pCITE vector expressing M2.1 was equally efficient (data not shown).

The AflII restriction site in the NL/1/99 genome did not affect virus replication in Vero cells (Fig. 5A) or the efficiency of virus rescue (cDNA constructs with and without AflII sites gave rise to virus titers of 10^7 and $10^{7.2}$, respectively). From these virus stocks, RNA was isolated and subjected to RT-PCR analysis and AflII restriction digestion, confirming that both NL/1/00 and NL/1/99 were recovered from plasmid DNA (Fig. 5B).

DISCUSSION

Here we describe the recovery of recombinant hMPV solely from cloned cDNAs. We selected two virus isolates, NL/1/00 and NL/1/99 representing each of the two main genetic lineages or serotypes of hMPV (227). These isolates were selected because they were found to replicate efficiently in tertiary monkey kidney cells and Vero cells.

The extreme termini of the viral genomes of NL/1/00 and NL/1/99 were determined by circularization of viral RNA with T4 RNA ligase, RT-PCR amplification and sequencing. Whereas the trailer sequences were of the expected length, the leader sequences were two or more nucleotides shorter than expected from the leader sequence of RSV (155) according to this approach. A 3'RACE method was used as an alternative strategy to determine the cDNA leader sequence of hMPV, revealing the presence of an additional AC dinucleotide in a proportion of the sequenced clones.
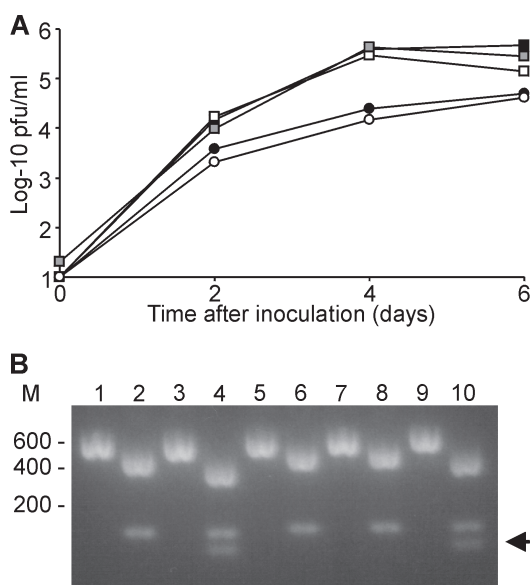
Figure 5. Replication kinetics of wild-type and recombinant hMPV in Vero cells and verification of the presence of recombinant genomes. Vero cells, infected at a multiplicity of infection of 0.1 with wild-type and recombinant hMPV NL/1/00 (circles) and NL/1/99 (squares), were washed and incubated for 6 days. Samples were collected every two days, and virus titers were determined by plaque assay (A). Closed symbols represent wild-type virus, open symbols recombinant virus with an additional AflII site and the gray symbol a NL/1/99 recombinant without the additional AflII site. The presence of the AflII site was confirmed by RT-PCR of viral RNA and AflII digestion (B). Lanes 1, 3, 5, 7, and 9, undigested DNA; lanes 2, 4, 6, 8, and 10, AflII-digested DNA. Lanes 1 and 2; wildtype NL/1/00, lane 3 and 4; recombinant NL/1/00, lane 5 and 6; wildtype NL/1/99, lane 7 and 8; recombinant NL/1/99 without AflII site, lane 9 and 10; recombinant NL/1/99 with the AflII site. When the wild-type 603 bp N fragment is digested with AflII, fragments of 469 and 134 are generated. Due to the introduction of the additional AflII site, the recombinant 469 bp fragment is further digested to yield 402 and 67 bp fragments (the latter fragment is indicated with an arrowhead).

In analogy with other paramyxoviruses, we assumed that the leader sequence of hMPV is 3'-UGCGCUUUUUUUGCG-5', and that the truncated versions of this leader sequence that were detected were due to the presence of nuclease activity in the virus preparations. It is interesting to note that Randhawa *et al.* observed truncation of the leader sequence of APV with a single nucleotide in their experiments, but when a hypothetical U residue was added to their minigenome constructs in analogy with other paramyxoviruses, this minigenome was replicated efficiently (187). The leader and trailer of hMPV appear to be less complementary as compared to those of RSV and APV (Fig. 3); only three of the five terminal residues of hMPV are complementary, compared to five of five and four of five for APV and RSV, respectively. With the completion of the full-length genomic sequences described here and those described recently by Biacchesi *et al.*, full-length genomic sequences for each of the two main genetic lineages that represent hMPV serotypes A and B and each of the sublineages (A1, A2, B1, and B2) are now available from public databases (14, 229).

The sequences of the genomic termini were used to generate minigenome constructs, containing CAT or GFP as reporter genes. To increase transcription from the T7 promoter, either two or three G residues were placed between the T7 promoter and the end of the trailer sequence (171). Neither the addition of these two or three G residues at the trailer end nor the absence or presence of the terminal UG residues in the leader significantly affected CAT expression from minigenome vectors, suggesting that the authenticity of the sequence was not critical in minigenome assays. In minigenome assays, the N, P and L proteins were found to be indispensable, whereas the M2.1 protein was not. For RSV, the M2.1 protein was shown to enhance the processivity of the viral polymerase, which is important for the efficient synthesis of full-length mRNA (39) and the read-through of intergenic regions (72, 101). The hMPV M2.1 protein may have the same function, but this cannot be concluded yet from our experiments. Although we were able to rescue virus in the absence of the M2.1 expression vector, it was shown for RSV that the simple expedient of omitting an expression plasmid is invalid for evaluating recovery requirements (35). Thus, the functional analysis of M2.1 requires further study.

When the CAT ORF of the minigenome construct was replaced by a full-length cDNA copy of the NL/1/00 or NL/1/99 genome, we could recover infectious virus from transfected cells. An additional AflII restriction site introduced in the N gene was used to confirm that the rescued viruses were recombinants. The AflII restriction site did not affect the efficiency of virus replication in vitro, since all recombinant viruses produced replicated in Vero cells with similar kinetics as their wild-type counterparts. Although transfections in both 293T cells and BSR-T7 cells resulted in the rescue of recombinant virus, the BSR-T7 cells yielded higher virus titers shortly after transfection, perhaps due to higher expression of T7 RNA polymerase in all cells. When we used a fowlpox virus expressing the T7 RNA polymerase gene as an alternative way for T7 delivery in 293T cells, we also successfully recovered recombinant hMPV NL/1/00. Since the efficiency of virus rescue using fowlpox-T7 was not significantly higher than rescue using plasmid-derived T7 RNA polymerase, the latter system was preferred since only plasmid DNAs are used without the need for infectious viruses. We were able to rescue recombinant NL/1/99 by using both positive- and negative-sense

full-length cDNA plasmids, revealing that the efficiency with negative-sense cDNA resulted in lower titers than for positive-sense cDNA (105.7 and 107.2 TCID50 respectively). This is in agreement with the general belief that for rescue of negative-sense nonsegmented viruses, the simultaneous presence of viral mRNAs and naked negative-sense genomic RNA will result in hybridization, preventing the assembly of the genome into the RNP.

A plethora of live-attenuated viruses and chimeric viruses have been generated using reverse genetics (41, 59, 162, 195, 206, 219), and the same can now be envisaged for hMPV. For instance, recombinant hMPV strains harboring the surface glycoproteins of both serotype A and B isolates of hMPV can be generated that may induce a broad antibody response in infected hosts. Chimeric live-attenuated vaccines based on the hMPV genome, in which genes of RSV and/or parainfluenza viruses are inserted, may be useful as multivalent vaccine candidates. Recently, a chimeric bovine/ human parainfluenza virus type 3 vector expressing the hMPV F protein was described and was found to induce protective hMPV antibody titers in a hamster model (219). In addition to its use for the generation of vaccine candidates, the reverse genetics system described here will be useful for fundamental and applied metapneumovirus research.

ACKNOWLEDGEMENTS
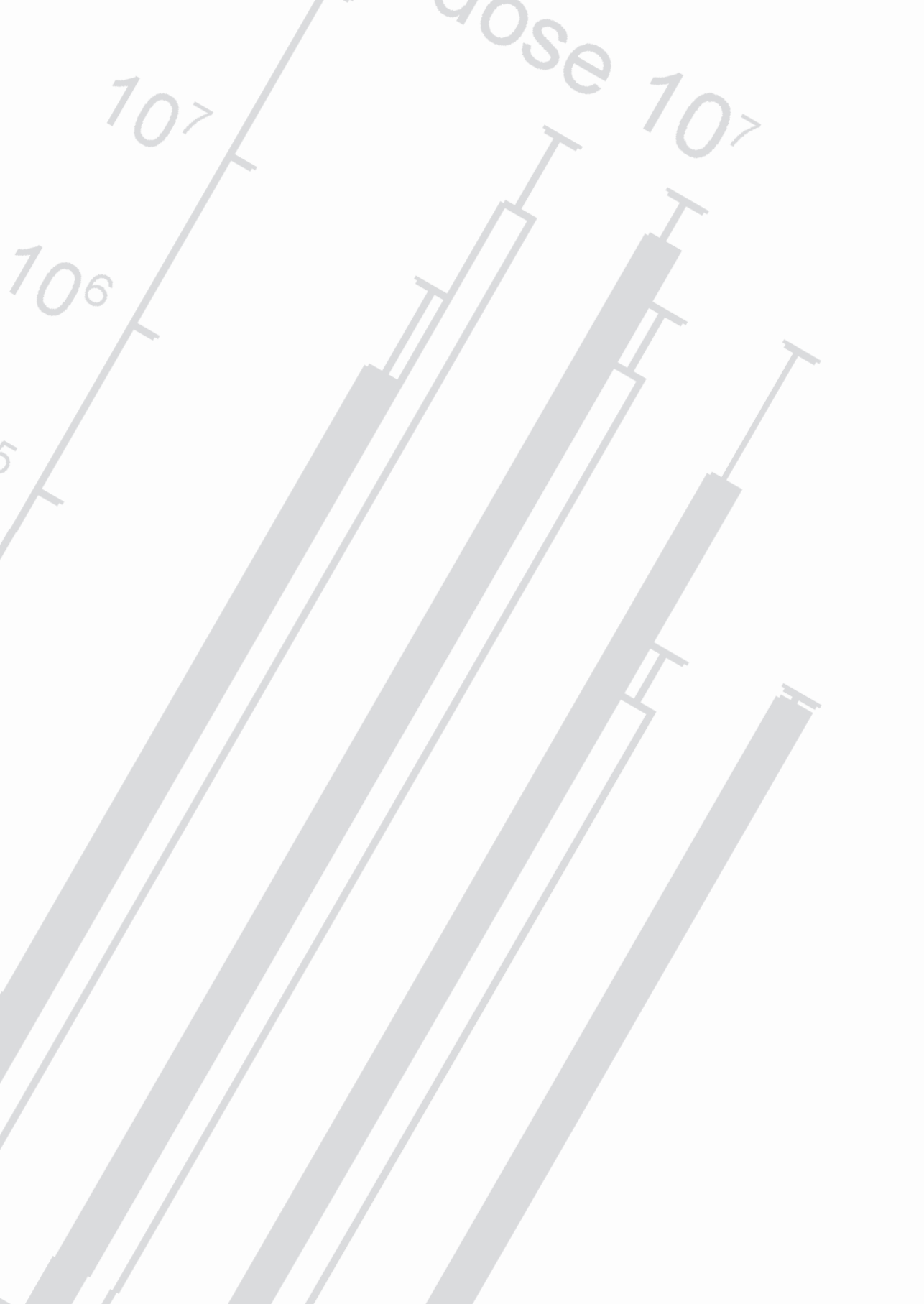
Experimental infection of macaques with human metapneumovirus induces transient protective immunity

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ABSTRACT

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 for 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 (RTI) in humans (227). Clinical studies have identified young infants, immunocompromised patients and the elderly as risk groups for hMPV-associated severe disease (231, 245, 246). HMPV causes clinical symptoms similar to those associated with the closely related human respiratory syncytial virus (RSV) (157, 231, 242, 245, 246). Phylogenetic studies have identified two major genetic lineages of hMPV that circulate worldwide (A and B), with each lineage divided into at least two sub-lineages (1 and 2). A cross-neutralization study with ferret sera collected early after infection indicated that the two main lineages represent two serotypes (229). However, the existence of serotypes has not been confirmed with sera from other animal species and the relevance for humans is still under debate (12, 205).

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 (12, 149, 205). In the human population, reinfections with viruses from homologous and heterologous lineages have been reported to occur throughout life (62, 106, 142, 227), 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 (89), whereas most viruses that are restricted to replication in the respiratory tract, such as parainfluenza virus and RSV, induce transient immunity (30, 36, 60). 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 (96, 106). To date, little is known about the clinical impact of reinfections with hMPV; one heterologous reinfection within one month after the primary infection has been reported (61). 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 parainfluenzaviruses types 1 and 3 and hMPV (15, 108, 149, 218). In animal models, these candidate vaccines induced protective immunity against subsequent infection with either homologous or heterologous hMPV strains 4-6 weeks after immunization (16). 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 longer than 6 weeks has not been reported (23, 149, 218).

Previously, we have demonstrated that cynomologus macaques are a suitable non-human primate model for hMPV infections (135), 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 availabe, 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.

MATERIALS AND 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 (108). After virus rescue from 293-T cells, the virus was passaged twice for 5 days on Vero-118 cells in infection medium [Iscove'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 (135). After freeze-thawing, the supernatants were stored in 25 % sucrose (w/v) at –70 °C. The titre of these stocks, as determined by end-point-titration using spinoculation (166) was 10^8 tissue culture infectious dose 50 ($TCID_{50}$) ml^{-1}.

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 anesthetized 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 (10^4 and 10^6 $TCID_{50}$). In all subsequent experiments, a dose of 10^7 $TCID_{50}$ was used. An overview of the experimental design is shown in Table 1.

Sample collection
To monitor virus replication, animals were lightly anaesthetized 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 of penicillin ml^{-1}, 200 µg of streptomycin ml^{-1}, 100 U of polymyxin B sulfate ml^{-1}, 250 µg of gentamicin ml^{-1}, and 50 U of nystatin ml^{-1} (all from ICN, Zoetermeer, The Netherlands). Samples were vigorously mixed and aliquots of 250 µl were 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 5×10^5 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.

Real time RT-PCR

RNA was isolated from 200 µl throat- or nose-swab material or from 5×10^5 BAL cells in 400 µl lysis buffer by using the High Pure viral RNA kit (Roche Diagnostic). The presence of hMPV genomes was detected by real-time RT-PCR (150). This RT-PCR assay is able to detect viruses from all four hMPV lineages, but the probe used in these assays tends to be less sensitive for detecting NL/1/99 virus (but is sensitive for all other type B viruses) and we therefore used an alternative probe, CAATGCAGTGACACCTTCATCATTACA, for 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 (150). Data are shown as the geometric means of the genome copy numbers in samples from two animals with the same infection history.

Table 1. Study design

Animal	Inoculation 1 (week 0)	Inoculation 2 (week 6)	Inoculation 3 (week 10)	Inoculation 4 (week 58)	Inoculation 5 (week 74)
1 and 2	NL/1/00 (10^4)	NL/1/00 (10^7)	NL/1/00 (10^7)	NL/1/00 (10^7)	-
3 and 4	NL/1/00 (10^6)	NL/1/00 (10^7)	NL/1/00 (10^7)	NL/1/00 (10^7)	-
5 and 6	NL/1/99 (10^4)	NL/1/99 (10^7)	NL/1/99 (10^7)	NL/1/00 (10^7)	-
7 and 8	NL/1/99 (10^6)	NL/1/99 (10^7)	NL/1/99 (10^7)	NL/1/00 (10^7)	-
9 and 10	-	-	-	NL/1/00 (10^7)	NL/1/00 (10^7)

Virus isolation

Virus isolation was performed on Vero-118 cells, as described previously (135). 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 were applied to the cells by spinoculation (166). After 1 h incubation at 37 °C, the sample was removed, the cells were washed and subsequently incubated with infection medium. After 7 days of culture, an immunofluorescence assay was used to identify infected cultures as described previously (229).

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 2^{-3}, 2^{-5}, 2^{-7} and 2^{-9}, were incubated for

1 h at 37 °C with 50 p.f.u. of 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 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 and Muench (1938) (188). 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 (10^4 and 10^6 $TCID_{50}$). Viral genomes were only detected in swabs collected from animals inoculated with 10^6 $TCID_{50}$ of virus (Fig. 1b, d). For NL/1/00-infected animals, viral genomes were detected between days 1 and 10 in the nose and throat. For the NL/1/99-infected 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 number 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 10^6 $TCID_{50}$, 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 x 10^5 genome copies per 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 x 10^5 genomes, and no infectious virus could be detected. The virological data were confirmed by serology: in contrast to the 10^4 $TCID_{50}$-inoculated animals (Fig. 2a, c), the 10^6 $TCID_{50}$-inoculated animals developed a virus-specific VN antibody response (Fig. 2b, d).

Figure 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, f, i, j, m, n, o, p) or NL/1/99 (c, d, g, h, k, l). (a, c) Primary inoculation with 10^4 $TCID_{50}$; (b, d): primary inoculation with 10^6 $TCID_{50}$; (e-p): inoculation with 10^7 $TCID_{50}$; (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.
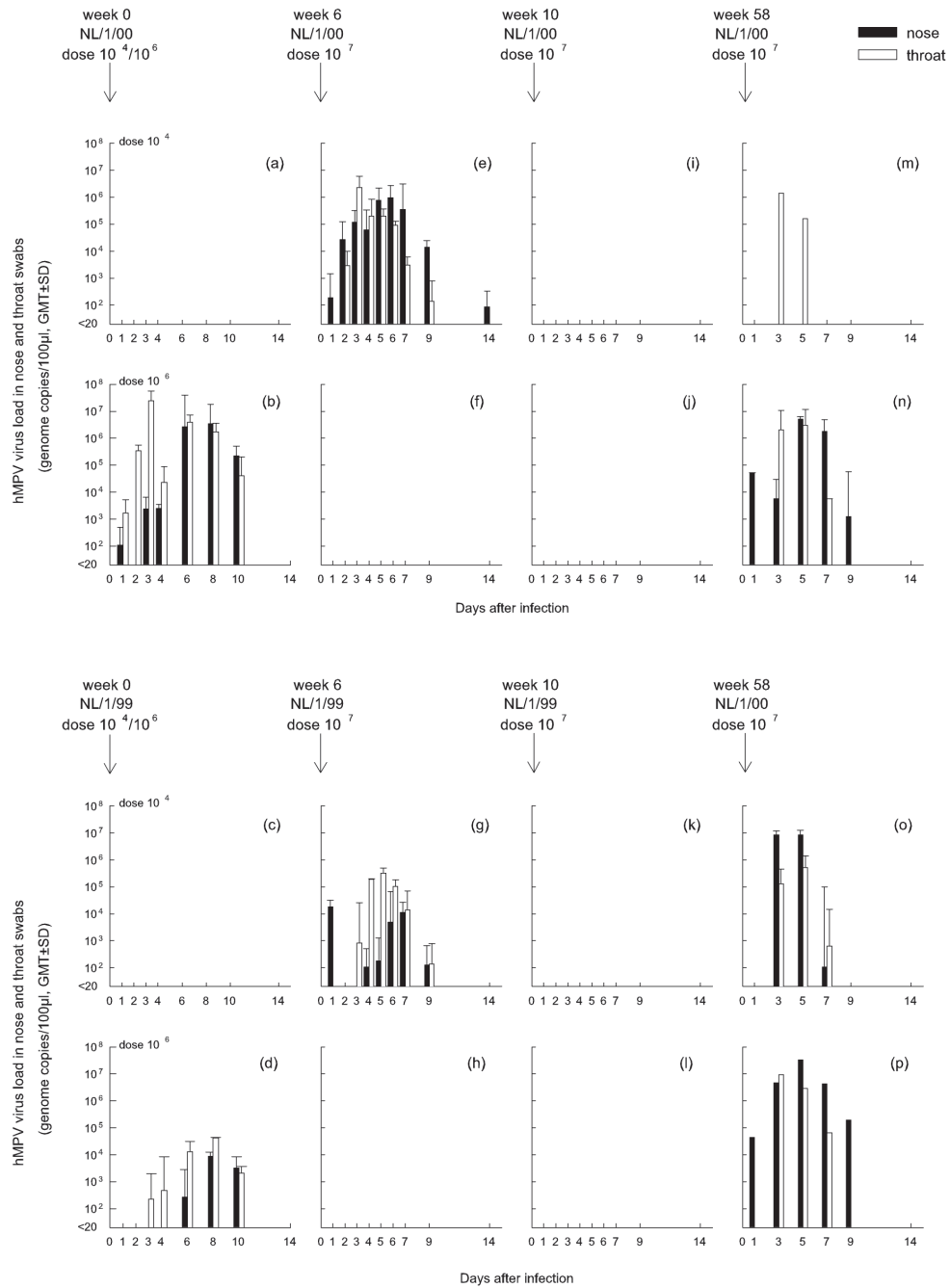
Figure 1.

HMPV infection protects from early reinfection

The animals receiving 10^4 TCID$_{50}$ were subsequently regarded as naive animals, because no virus could be detected upon inoculation and serum antibodies were absent, as measured in VN (Fig. 2a, c), ELISA and immunofluorescence assays (data not shown). We also concluded that 10^6 TCID$_{50}$ 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 (10^7 TCID$_{50}$) for all subsequent inoculations.

Six weeks after the first inoculation, a second inoculation was performed with 10^7 TCID$_{50}$ of the homologous viruses. In the four animals that had previously received 10^4 TCID$_{50}$ but had remained seronegative, virus shedding and specific VN antibody responses were detected and reached similar levels as in the four animals receiving 10^6 TCID$_{50}$ in the first part of the experiment (Figs 1e, g, 2a, c). For the 10^7 TCID$_{50}$ NL/1/00-inoculated animals, virus shedding from the throat was similar to that observed for the 10^6 TCID$_{50}$-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 10^7 TCID$_{50}$ of both viruses resulted in the induction of virus specific antibody responses (Fig. 2a, c).

The animals that had previously been inoculated with 10^6 TCID$_{50}$ were largely protected from subsequent challenge 6 weeks later with 10^7 TCID$_{50}$ (Fig. 1f, h). No viral genomes were detected in nose or throat swabs, although the 5×10^5 BAL cells collected on day 3 post-inoculation contained 0.06-8×10^4 (GMT, 5.6×10^3) genome copies, which was similar to the 0.02-5×10^5 (GMT, 3.3×10^4) 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 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).

Figure 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$_{50}$ of 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.
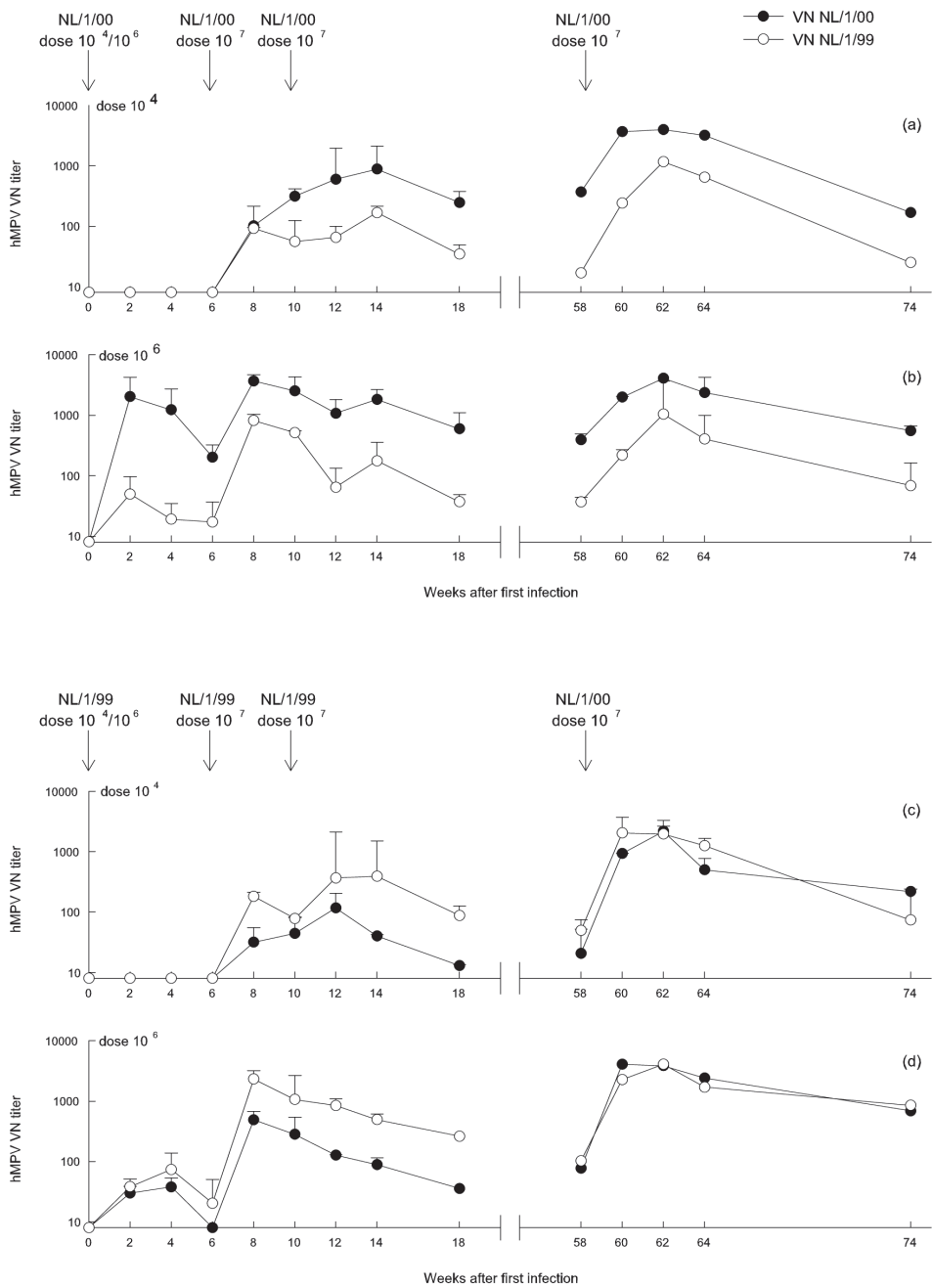
Figure 2.

At the end of inoculation 3, all animals had high levels of virus-specific VN antibody titres. Within 8 weeks after 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 comparable levels as in the control animals (Fig. 1o, p, 3a). Both control animals and the three heterologous-challenged animals demonstrated peak genome copy numbers between day 3 and 5 post-inoculation of around 10^7 genomes per 100 µl. At day 3 and 5 post-inoculation, infectious virus was recovered from the throat samples of all animals. Of the three homologous-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 day 3 and 5 post-inoculation, similar to the control animals and peak genome copy numbers were equally high to those detected in the control animals (Figs 1n, 3a). From all three homologous-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 titres 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$_{50}$ of the 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 seen in inoculation 4 (Fig. 3b). Whilst after inoculation 4 (primary inoculation for these animals), virus shedding was detected between day 1 and 9 post-inoculation (Fig. 3a), after inoculation 5, virus shedding occurred between day 3 and 8 for animal 9 and only at day 3 (only throat) for animal 10. In addition, peak viral genomes 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 (animal 5, 6 and 7 at inoculation 4), antibody levels to the NL/1/00 and NL/1/99 reached similar levels (Fig. 2).
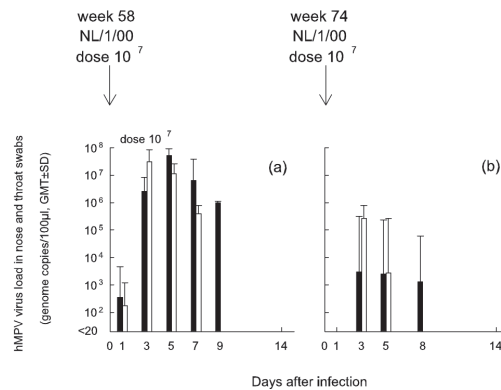
Figure 3. Detection of hMPV by real-time RT-PCR in nose-swab (filled bars) and throat-swab (empty bars) samples of (a) two control animals for inoculation 4, inoculated with 10^7 TCID$_{50}$ NL/1/00, and (b) the same animals homologous-challenged 12 weeks later. Bar represent the GMT ± SD. Time points, virus and dose used for the different inoculations are indicated above the plots. Sampling days are indicated below the *x*-axis.

DISCUSSION

Previously, we established a cynomolgus macaque model for hMPV infection, using 105 TCID50 NL/1/00 (a prototype, uncloned virus isolate for lineage A). In the present study, inoculation with 106 TCID50 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 107 TCID50 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 107 TCID50 of 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 (149, 205). 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 (12, 205), 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 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 (12, 205). 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 Skiadopolous et al. (2004) where captive-bred chimpanzees were positive for hMPV serotype A and negative for serotype B VN antibodies (205). 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 (61), 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, antibody titres are 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 (51). 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 (129, 149, 217, 218), 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.

ACKNOWLEDGEMENTS

Immunization of Syrian golden hamsters with F subunit vaccine of human metapneumovirus induces protection against challenge with homologous or heterologous strains

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ABSTRACT

Human metapneumovirus (hMPV), a newly discovered paramyxovirus, is associated with acute respiratory-tract illness, primarily in young children, individuals with underlying disease and the elderly. Two genetic lineages of hMPV circulate around the world, and viruses from these two lineages demonstrate antigenic differences. The clinical impact of hMPV warrants the development of vaccines. Recombinant soluble fusion (F) proteins of prototype viruses of the two main lineages of hMPV that can be produced in high yields have been constructed. In this study, the antigenicity, immunogenicity and protective efficacy of these soluble F subunit vaccines were evaluated in Syrian golden hamsters (*Mesocricetus auratus*). Immunization of hamsters with the soluble F proteins, adjuvanted with Specol or iscom matrix, induced high virus-neutralization titres, with higher titres against the homologous than the heterologous virus. The neutralizing antibodies protected from subsequent infection of the lungs with both homologous and heterologous virus. Upon challenge, viral titres in the nasal turbinates of immunized animals were reduced significantly compared with those of PBS immunized animals. In conclusion, a soluble F subunit vaccine for hMPV that induces cross-protective immunity for infection of the lower respiratory tract in Syrian golden hamsters has been generated.

INTRODUCTION

The human metapneumovirus (hMPV), a recently described paramyxovirus, was first isolated from respiratory specimens obtained from children in The Netherlands who were hospitalized for acute respiratory-tract illness (RTI) (227). Clinical manifestations of hMPV infections are similar to those caused by respiratory syncytial virus (RSV), ranging from mild respiratory illness to bronchiolitis and pneumonia (231, 246). RSV and hMPV are both members of the subfamily Pneumovirinae within the family Paramyxoviridae. However, within this subfamily, RSV is a member of the pneumovirus genus and hMPV is the first mammalian member of the metapneumovirus genus, based on differences in genome organisation (226). Phylogenetic analysis of fusion (F) and attachment (G) genes of a large number of hMPV isolates revealed the existence of two main genetic virus lineages (A and B), each divided into at least two sublineages (1 and 2). The two main lineages, with prototype viruses NL/1/00 and NL/1/99 for the lineage A and B, respectively, were found to be antigenically distinct in virus-neutralization assays with ferret sera (229).

The genome of HMPV encodes three surface glycoproteins F, G and the small hydrophobic protein (SH). Whereas the G and SH proteins are highly variable, the F protein is highly conserved between the major lineages A and B. Antibodies induced against the F protein are correlates of protection in animal models (204, 218). The fusion protein of hMPV demonstrates similarities with those of other paramyxoviruses (226, 229). The F proteins of paramyxoviruses are class I viral fusion proteins, which are synthesized as an inactive precursor F0 that must be cleaved by host endoproteases, resulting in two disulfide-linked polypeptides, F1 and F2 (139, 190).

The clinical impact of hMPV warrants the development of vaccines. For RSV, subunit vaccines based on the F or G proteins have been developed and tested in a variety of rodent and primate models, where they demonstrated protective efficacy (100, 163, 236). In addition, RSV F subunit vaccines have been evaluated in clinical trials in healthy adults, children older than 1 year with or without underlying pulmonary disease, the elderly, and pregnant women, and have been found to be safe and effective (69, 161, 179, 222). Based on the genetic relationship of RSV and hMPV, we hypothesized that a similar subunit vaccine for hMPV would induce protective immunity against hMPV infection. Soluble fusion proteins of prototype viruses representing the two main lineages of hMPV were produced to high yields in stable NSO murine myeloma cell lines (224). We evaluated the antigenicity, immunogenicity and cross-protective efficacy of these soluble hMPV F proteins in Syrian golden hamsters (Mesocricetus auratus). As adjuvants are known to enhance the immune response, the immunogenicity of the soluble F proteins was tested in combination with different adjuvants: Alum, Specol and iscom matrix (211). Alum induces low antibody titres when used with subunit vaccines, but it was chosen because of its long history of use in humans. Iscom matrix is known to induce high antibody titres and is currently being evaluated in humans. Specol was chosen to function as positive control, as it induces high antibody titres in rodents.

This study demonstrates that the soluble hMPV F proteins adjuvanted with Specol or iscom matrix are promising candidate subunit vaccines for the induction of protective antibody levels against

homologous and heterologous hMPV infections.

MATERIALS AND METHODS

Cells and viruses

Subclone 118 of Vero-WHO cells (Vero-118 cells) (135) were grown in Iscove's Modified Dulbecco's Medium (IMDM; BioWhittaker) supplemented with 10 % fetal calf serum (Greiner Bio-One), 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1} and 2 mM glutamine. The construction of recombinant hMPV NL/1/00 (prototype virus for lineage A) and NL/1/99 (prototype virus for lineage B) has been described previously (108). High-titre virus stocks were produced in Vero-118 cells in infection medium: IMDM supplemented with 4 % bovine serum albumin fraction V (Invitrogen), 100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1}, 2 mM glutamine and 3.75 µg trypsin ml^{-1}. After 7 days the cultures were harvested and stored in 25 % sucrose at –70 °C.

Construction of soluble F proteins

Constructs expressing a truncated version of the hMPV F protein, lacking the transmembrane domain, were generated as described (224). In brief, full-length hMPV cDNA plasmids were used as templates to generate F gene PCR products by using the following oligonucleotides for both the NL/1/00 and NL/1/99 sequences; 5'- AATCAACGGTCCGCCACCATGTCTTGGAAAGTG-3' and 5'-TTAATTGAATTCTTAGTGATGGTGATGGTGATGGCCAGTGTTTCCTTTCT CTGC-3'. The PCR products were digested with restriction endonucleases *Rsr*II and *Eco*RI and ligated to the pEE15.1 vector (Lonza) digested with the same restriction endonucleases. Stable NSO murine myeloma cell lines expressing NL/1/00 or NL/1/99 F proteins were generated as described by Bebbington *et al*. (1992) (9). Soluble F protein (Fsol) was purified by affinity chromatography using a F-specific mAb, 1017, which binds to both NL/1/00 and NL/1/99 F proteins (224), coupled to cyanogen bromide-activated Sepharose and eluted with 0.1 M glycine, pH 2.8. The eluate was neutralized with 0.1 vol. 1 M Tris/HCl, pH 8.0, and was dialysed against PBS.

Vaccine preparations

For the iscom matrix-adjuvanted Fsol preparation, *N*-decanoyl-*N*-methylglucamide (MEGA-10; Sigma-Aldrich) at a final concentration of 2 % was supplemented with cholesterol [1 mg (mg protein)$^{-1}$, Sigma], phosphatidylethanolamine [1 mg (mg protein)$^{-1}$; Sigma] and Quil-laja glucosides [5 mg ISCOPREP 703 (mg protein)$^{-1}$; Isotec] and dialysed against PBS. The iscom matrix preparation was analyzed by negative-contrast electron microscopy, revealing the typical iscom matrix particles with a diameter of approximately 40 nm. The iscom matrix preparation was added to the F protein immediately before immunization, resulting in preparations containing 10 µg Fsol in 100µl.

Concerning the correct choice of an aluminum-containing adjuvant, the charge of adjuvants and antigens is characterized by the point of zero charge (PZC) and isoelectric point (pI) respectively (1). Maximum adsorption of protein by aluminum adjuvants is reached at pH conditions under which the antigen and adjuvant have opposite charges. Based on the approximate pI of 5.9 of the

Fsol proteins (http://au.expasy.org/cgi-bin/pi-tool), Alhydrogel with PZC value 11,1 was chosen as aluminum-containing adjuvant (a gift from Dr. Erik B. Lindblad, Brenntag Biosector, Frederikssund, Denmark). A vaccine dose was prepared by mixing 50 µl of Fsol (200 µg protein ml^{-1} in PBS) with 50 µl 2 % Alhydrogel (10,3 mg Al^{3+} ml^{-1}), resulting in preparations containing 10 µg Fsol and 0,52 mg Al^{3+}.

Doses of Specol vaccine (Stimune; Cedi Diagnostics) were prepared by mixing 4 vols water phase containing 10 µg Fsol with 5 vols Specol while mixing vigorously.

To obtain inactivated whole-virus vaccines, hMPV strains NL/1/00 and NL/1/99 were grown to 70–90 % cytopathic effect on Vero-118 cells. After one freeze-thaw cycle, cell-free supernatants were purified and concentrated using a 30–60 % (w/w) sucrose gradient. Virus was inactivated with β-propiolactone (Sigma-Aldrich) and complete inactivation was confirmed by titration on Vero-118 cells. SDS-PAGE analysis and western blotting with an F monoclonal antibody (mAb) indicated that 100 µg inactivated virus contained approximately 10 µg F protein (data not shown); therefore a vaccine dose of 100 µg inactivated virus (in 100 µl PBS) was used.

Animal experiments

All intramuscular immunizations, intranasal inoculations, orbital punctures and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by an independent, national animal ethics committee (DEC consult) and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines.

Immunization and challenge

Five- to seven-week-old female Syrian golden hamsters (Harlan Sprague-Dawley) were immunized twice intramuscularly at a 3 week interval, with a dose of 100 µl vaccine containing 10 µg Fsol or 100 µg inactivated virus in PBS. Three weeks after the second immunization in the immunization-optimization experiment, or 4 days post-infection in the challenge experiment, blood samples were collected by orbital puncture. Blood samples were stored overnight at room temperature and centrifuged for 15 min at 1200 g; serum was collected and stored at –20 °C.

Animals were challenged intranasally with 100 µl of NL/1/00 virus, diluted in PBS in order to obtain the required dose [10^4, 10^5 or 10^6 TCID$_{50}$ (50 % tissue culture infectious dose) in 100 µl]. Four days after inoculation, lungs and nasal turbinates were collected, snap-frozen immediately and stored at –80 °C until further processing.

F protein-specific ELISA

Ninety-six-well plates were coated overnight at 4 °C with 100 ng Fsol protein (NL/1/00 and NL/1/99, ratio 1:1) per well in PBS. Serum samples, diluted in Meddens reagent (Meddens), were added to the plates and incubated for 1 hour at 37 °C. After washing, plates were incubated for 1 h with goat-anti-hamster IgG-horseradish peroxidase (1:5000; DakoCytomation). 3,3',5,5'-Tetramethylbenzidine (TMB) diluted 1:10 in TMB diluent (Meddens) was used as a substrate. The reaction was stopped

by adding an equal volume of 2 M H_2SO_4, after which the A_{450} was determined. Results are depicted after substraction of background values.

Plaque-reduction virus neutralization (PRVN) assay

Virus neutralizing (VN) antibody titres were determined by a PRVN assay. Heat-inactivated (30 min., 56 °C) serum samples, diluted by 2^{-3}, 2^{-5}, 2^{-7} and 2^{-9}, 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 two h, the supernatants were removed, and 1 ml IMDM containing 2 % BSA fraction V, 1 % methyl cellulose (MSD) and 3.75 µg trypsin ml^{-1} was added to the wells. Six days later, fluorescent plaques were counted using a Typhoon 9410 variable-mode imager (GE Healthcare). VN antibody titres are expressed as the dilution resulting in 50 % reduction of the number of plaques, calculated according to the method of Reed & Muench (1938) (188). Per assay, each serum was tested in duplicate against hMPV NL/1/00 and NL/1/99.
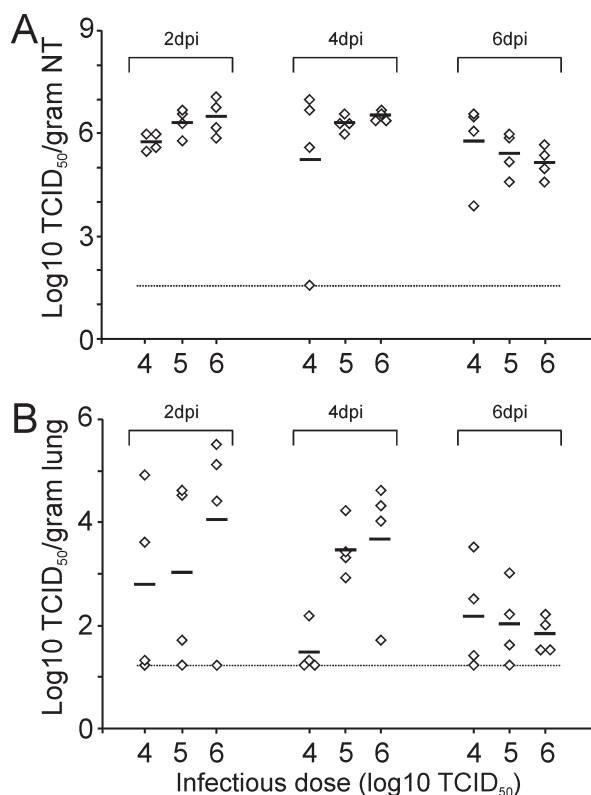
Figure 1. Infectious virus titres in (a) nasal turbinates (NT) and (b) lungs of Syrian golden hamsters challenged with 10^4, 10^5 or 10^6 TCID$_{50}$ hMPV NL/1/00. NT and lungs were collected at day 2, 4 or 6 p.i. Virus present in tissues was quantified by serial dilution in Vero-118 cell monolayers. The lower limit of detection is indicated by the dotted line.

Virus titrations

Tissues from the inoculated hamsters were homogenized using a Polytron homogenizer (Kinematica AG) in infection medium. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titrations were performed with tenfold serial dilutions in 96-well plates (Greiner Bio-One). Confluent monolayers of Vero-118 cells were spin-inoculated (15 min, 2000 g) with 100 µl tenfold serial dilutions of each sample and incubated at 37° C. Two hours after the spin inoculation, the inoculum was replaced with fresh infection medium. After 3-4 days, 100 µl fresh infection medium was added to each well. Seven days after inoculation, infected wells were identified by immunofluorescence assays with hMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (227). Titres expressed as $TCID_{50}$, were calculated as described by Reed & Muench (1938) (188).Titres were calculated (g tissue)$^{-1}$, with a detection limit of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ (g tissue)$^{-1}$ for nasal turbinates and lungs, respectively.

RESULTS

Challenge model

We first set up a challenge model by inoculating hamsters with different virus doses and euthanasia of the animals at different days post-inoculation (2, 4 and 6 days p.i.). Three groups of 12 hamsters were inoculated intranasally with 10^4, 10^5 or 10^6 $TCID_{50}$ NL/1/00. From each group, four animals were euthanized at 2, 4 or 6 days p.i. In general, both in the nasal turbinate and in the lungs, virus shedding was higher at 2 and 4 days p.i. than at 6 days p.i. (Fig. 1), except for viral shedding from the nose at 4 days p.i. at the lowest inoculation dose (Fig. 1a). At 2 and 4 days p.i., animals inoculated with 10^6 $TCID_{50}$ harboured more virus in their nose and lungs than did those inoculated with lower virus doses. Only at 4 days p.i., and only with the two highest doses (10^5 and 10^6), virus was detected in the lungs of all animals (Fig. 1b). Based on these results, challenge experiments were conducted with 10^6 $TCID_{50}$ with subsequent euthanasia at 4 days p.i.

Immunogenicity of Fsol

Soluble F proteins of the two prototypes of hMPV (NL/1/00 and NL/1/99) were produced: Fsol/1/00 and Fsol/1/99. NSO murine myeloma cells were choosen for their capacity to produce high yields of protein, as shown before (224). By using these stable NSO murine myeloma cell lines, we were able to affinity-purify up to 20 mg soluble hMPV F protein ml^{-1}. Analyses of these proteins by SDS-PAGE and Coomassie blue staining demonstrated that the affinity-purified proteins were >90 % pure, with a predicted molecular weight of around 55 kDa (Fig. 2).

The immunogenicity of these two proteins was tested in combination with three different adjuvants and compared with that of inactivated viruses containing the native form of the F protein.

Six Syrian golden hamsters per group were immunized twice intramuscularly with 10 µg Fsol alone, Fsol with adjuvant (iscom matrix, alum or Specol), 100 µg inactivated virus or PBS. Three weeks later, sera were subjected to F-specific ELISA at a 1:1600 dilution. All animals, except the PBS-immunized animals, demonstrated the presence of F-specific antibodies (Fig. 3). Animals
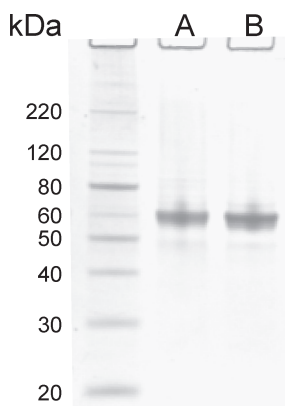
Figure 2. Analysis of affinity-purified soluble F proteins by SDS-PAGE. Fsol/1/00 (A) and Fsol/1/99 (B) were separated on a 10 % NuPage gel in a MES buffer system (Invitrogen), stained with Coomassie blue and photographed using a digital CCD camera. A molecular mass marker is shown in the left-hand lane.

immunized with an NL/1/00 F protein preparation displayed higher ELISA antibody titres than those immunized with NL/1/99 preparations, although a mixture of antigens from both prototype viruses was used to coat the ELISA plates. The non-adjuvanted Fsol proteins induced slightly lower antibody titres compared with to the whole virus preparation. Addition of adjuvants increased the immunogenicity of the Fsol proteins, with the highest titres induced by iscom matrix- and Specol-adjuvanted vaccines (Fig. 3). High ELISA antibody titres correlated with high VN antibody titres (data not shown). Based on the high antibody titres induced by immunization, the protective
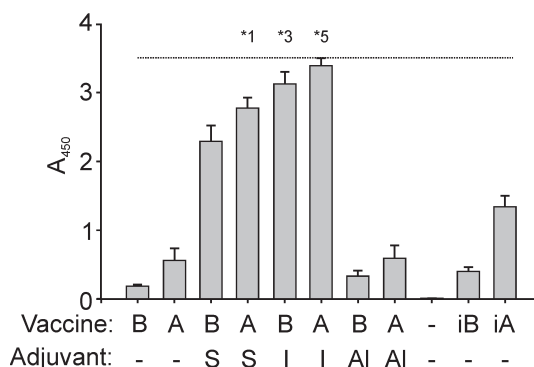
Figure 3. Induction of F-specific antibody titres in Syrian golden hamsters immunized with hMPV F protein preparations or controls as measured by ELISA. Serum samples were diluted 1:1600 in PBS. The upper limit of detection is indicated by the dotted line. *Number of sera with values above detection limit (A_{450}>3.5). Each bar represents the geometrical mean + SEM of all animals of the same group. A_{450} values are given after substraction of background. Vaccine: A, Fsol/1/00; B, Fsol/1/99; iA, inactivated NL/1/00; iB, inactivated NL/1/99; -, no vaccine. Adjuvant: Al, Alum; I, iscom matrix; S, Specol; -, no adjuvant.
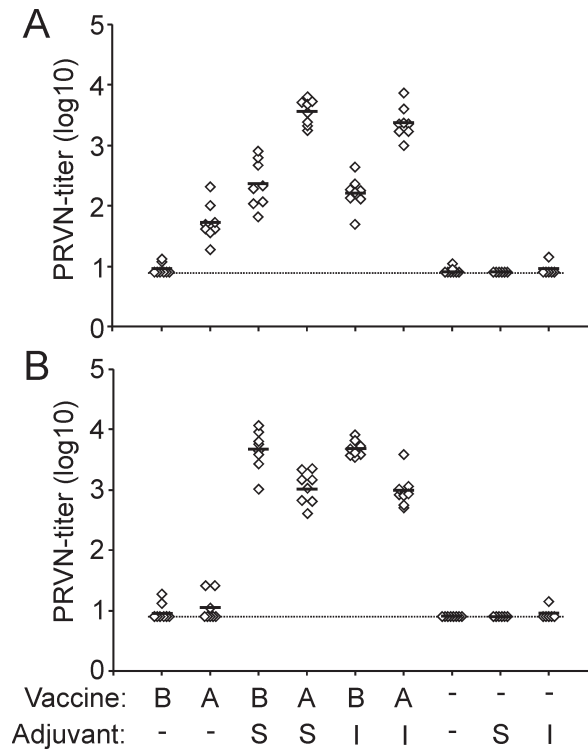
Figure 4. Fifty per cent PRVN titres against NL/1/00 (a) and NL/1/99 (b) after immunization with Fsol vaccines. Blood samples were collected by orbital puncture 4 days after challenge. Titres were calculated according to the method of Reed & Muench (1938). The lower limit of detection is indicated by the dotted line. Vaccine and adjuvant designations are as described in the legend to Fig. 3.

efficacy of the iscom matrix and Specol adjuvanted F subunit vaccines was tested in immunization/ challenge experiments.

Immunization/challenge experiment

Nine groups of eight hamsters (six hamsters for the adjuvant-control groups) were immunized twice with 10 μg Fsol alone, Fsol with adjuvant (iscom matrix or Specol), adjuvants alone or PBS. Three weeks after the second immunization, all hamsters were inoculated intranasally with 10^6 $TCID_{50}$ NL/1/00.

Immunization of control animals with PBS, iscom matrix or Specol (without antigen) did not induce detectable virus-specific antibodies (Fig. 4), which upon challenge resulted in virus shedding in the nose of all animals (Fig. 5a). Unfortunately, in contrast to the animals in the challenge optimization experiment (Fig. 1), some of these control animals (e.g. three of eight, two of six and one of six for the PBS, iscom matrix and Specol groups, respectively) did not secrete virus from the lower respiratory tract (LRT) (Fig. 5b). Similarly, immunization of hamsters with Fsol alone induced low

to undetectable levels of antibodies (Fig. 4). Upon challenge, virus was detected in the upper respiratory tracts (URTs) of all these animals and in the LRTs of five and six out of eight animals (Fsol/1/99 and Fsol/1/00, respectively) (Fig. 5).

Despite these uninfected-control animals, significant protective efficacies were observed for animals immunized with Fsol adjuvanted with iscom matrix or Specol. Immunization with these vaccines induced high virus-specific antibody titres (Fig. 4). Upon challenge, these antibody titres partly protected from infection of the URT, with viral titres in the nose being statistically significant lower than those observed in the nasal turbinates of the PBS-immunized animals (Mann-Whitney test, $P<0.05$). Complete (cross-) protection of the LRT was induced by immunization with these adjuvanted Fsol proteins. None of the animals demonstrated the presence of infectious virus (Fig. 5b) or viral genomes in their lung samples (data not shown). Immunization with adjuvanted Fsol vaccines induced significantly higher protective efficacy for LRT than did immunization with PBS
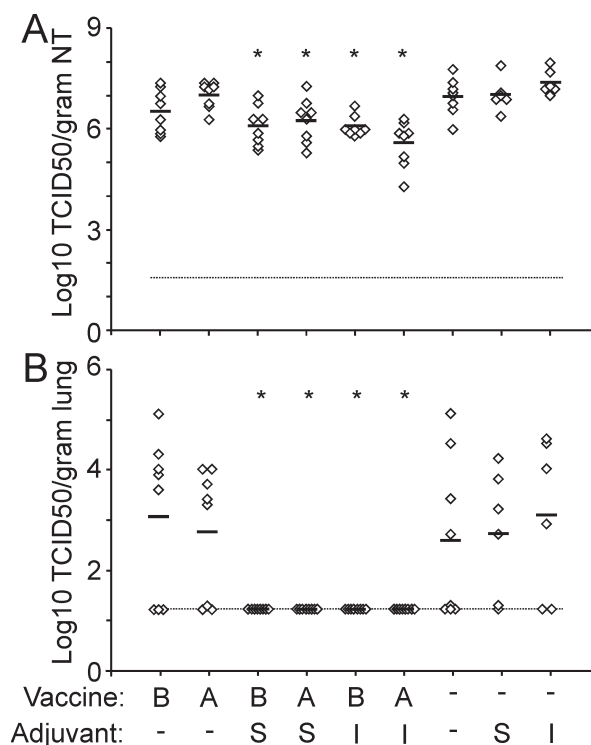
Figure 5. Infectious virus titres in (a) nasal turbinates (NT) and (b) lungs of Syrian golden hamsters. Animals were immunized twice at a 3 week interval. Three weeks after the second immunization, animals were challenged with 10^6 $TCID_{50}$ hMPV NL/1/00. Animals were euthanized 4 days after infection. Virus present in tissues was quantified by serial dilution on Vero-118 cell monolayers. Asterisks indicate a significant difference between the indicated group and the PBS control group (Mann-Whitney, $P<0,05$). The lower limit of detection is indicated by the dotted line. Vaccine and adjuvant designations are as described in the legend to Fig. 3.

(Mann-Whitney test, P=0,038). Immunization with adjuvanted Fsol/1/00 as well as Fsol/1/99 (cross-) protected the LRT against challenge infection with hMPV/NL/1/00.

All sera were tested in (PRVN) assays against NL/1/00 (Fig. 4a) and NL/1/99 (Fig. 4b). Sera from all animals from the control groups (PBS, iscom matrix, Specol) were negative for neutralizing antibodies. Animals immunized with adjuvanted vaccines had higher antibody titres than those immunized with Fsol alone. In general, homologous VN antibody titres were higher than heterologous VN antibody titres, with the highest homologous titres observed in the NL/1/00-immunized animals. Mean homologous titres induced by the adjuvanted vaccines were 20–30-fold and 2.5–3.4-fold higher than the heterologous titres for NL/1/99 and NL/1/00, respectively (Table 1). The difference in homologous and heterologous titres was not reflected in differences in protective efficacy, as both homologously and heterologously challenged animals were protected from LRT infection and displayed an equal reduction of viral titres in the nasal turbinates.

Table 1. HMPV NL/1/00- and NL/1/99-specific PRVN antibody titres in sera collected from Syrian golden hamsters immunized twice with Fsol NL/1/99 (B) or NL/1/00 (A) adjuvanted with Specol or iscom matrix. Mean PRVN antibody titres are depicted for each group. Homologous PRVN antibody titres are shown in bold; ratios are given as the homologous PRVN titres divided by the heterologous PRVN antibody titres.

	F1/99 (B) Specol	F1/00 (A) Specol	F1/99 (B) iscom	F1/00 (A) iscom
NL/1/00 (A)	230	**3563**	163	**2363**
NL/1/99 (B)	**4546**	1044	**4816**	963
Ratio A-B		3.4		2.5
Ratio B-A	20		30	

DISCUSSION

The clinical impact of hMPV warrants the development of vaccines, and subunit vaccines are promising candidate vaccines for boosting pre-existing antibody titres as described for RSV subunit vaccines (69, 161, 179, 222). We have produced recombinant soluble F proteins (Fsol proteins) for the prototypes of the two main lineages of hMPV (NL/1/00, lineage A; NL/1/99, lineage B) in a system that allows production of high yields of recombinant proteins. The use of these proteins as subunit vaccines was evaluated in Syrian golden hamsters.

The F proteins of paramyxoviruses are class I viral fusion proteins, which are synthesized as an inactive precursor, F0. This immature F protein is subsequently modified by the addition of N-linked carbohydrate and then assembled into homo-oligomers in the rough endoplasmic reticulum. F0 is subsequently cleaved endoproteolytically, resulting in two polypeptides, F1 and F2, that are covalently linked to each other by disulfide bonds (139, 190). Thus, the F protein is present during infection in immature and mature forms (192).

The F glycoprotein is one of the major targets of RSV- or hMPV-neutralizing antibodies (204, 218)

and is therefore an ideal candidate to use as subunit vaccine. Upon vaccination and/or natural infection with RSV, both neutralizing and non-neutralizing antibodies can be detected (223). Several studies have suggested that non-neutralizing antibodies are directed against the uncleaved form of the F protein, whilst neutralizing antibodies are directed against the cleaved form of the protein (192, 223).

Both Fsol proteins used in this study were produced from viruses containing a serine at position 101 in the RQSR motif at the putative cleavage site of the F protein. It has previously been demonstrated that hMPV expressing a fusion protein with 101S is incapable of initiating multicycle growth without the addition of trypsin, as the F protein was not efficiently cleaved proteolytically (194). In the present study, the production of the Fsol proteins of hMPV occurred in the presence of serum and in the absence of trypsin, so most of the Fsol proteins were not cleaved proteolytically. This was also confirmed by performing Western blot analysis on the Fsol proteins after incubation with or without trypsin. After the addition of trypsin, a smaller band was revealed, corresponding to the predicted size of cleavage fragment hMPV F1, by using F-specific mAb 1017 (data not shown).

Although most of the proteins used for the vaccine preparation were uncleaved, we have demonstrated that these truncated Fsol proteins were as immunogenic as the proteins in the native form. Immunization with the soluble proteins induced neutralizing-antibody titres similar to those induced by immunization with whole inactivated virus. However, antibody titres in animals immunized with Fsol were low. Therefore, several adjuvants were tested to induce higher neutralizing antibody titres.

Alum was chosen because of its long history of use in humans, although it is known to induce low antibody titres when used with subunit vaccines (93). Specol and iscom matrix were chosen because they both generate long-lasting, functional antibody responses, but neither adjuvant is licensed for human use (10, 141, 193). However, several studies in human volunteers have shown that iscom-based vaccines are highly immunogenic as well as safe and well-tolerated (49, 77). The addition of Alum to the Fsol proteins resulted in antibody levels similar to those induced by the non-adjuvanted Fsol. This weak response might be the result of a low degree of adsorption of Fsol to Alhydrogel, which is considered to be an important parameter for the function of aluminum adjuvants (145).

Addition of both Specol and iscom matrix to the Fsol protein enhanced the hMPV-specific antibody response. This enhanced effect was also seen in cotton rats immunized with 25 µg soluble hMPV F protein adjuvanted with Titermax Gold (47). Although, Titermax Gold is known to induce high antibody titres, the adjuvant induced sterile abscesses in non-human primates and is not licensed for human use (55).

The iscom matrix- and Specol-adjuvanted F-subunit vaccines were tested for protective efficacy against homologous and heterologous infection in Syrian golden hamsters. For this purpose, we developed a challenge model for NL/1/00 infections in Syrian golden hamsters. An optimum inoculation dose of 10^6 TCID50, followed by collection of lungs and nasal turbinates at 4 days p.i.,

resulted in virus detection in samples collected from all animals.

Using this challenge model in an immunization/challenge experiment, none of the vaccines induced complete protection against URT infection, although viral titres in the nose of adjuvanted Fsol-immunized animals were significantly lower than those in the PBS-immunized animals. However, the primary goal of immunization against respiratory viruses is prevention of serious LRT illnesses. The control vaccines (iscom matrix or Specol without antigen, PBS or non-adjuvanted Fsol) did not prevent LRT infections, whilst immunization with adjuvanted Fsol induced complete protection against LRT infection. Even after optimization of the challenge model, subsequent challenges of the control animals in the immunization/challenge experiment resulted in <100% infection. Although intranasal inoculation of Syrian golden hamsters has been used successfully as the infection procedure in other studies, this route of infection might be less efficient for the virus strains used in the present study. Perhaps a higher inoculum dose or even intratracheal infection would have resulted in 100% infection. However intranasal infection, with a risk for a less robust infection, resembles a natural infection more closely. Despite the fact that not all control animals harboured virus in their lungs upon challenge, significant protective efficacy was demonstrated for the adjuvanted Fsol proteins. All animals immunized with Fsol adjuvanted with iscom matrix or Specol were protected from infection of their lungs, compared with three of eight animals in the PBS-immunized group (Mann-Whitney test, P=0,038).

In passive-transfer experiments in which RSV-specific mAbs were used, it was demonstrated that URT protection requires significant higher antibody doses than doed protection of the LRT (200). This suggests that antibody titres induced by the studied vaccines might be too low to protect against URT infection. A third vaccination could be considered to increase the antibody levels or the use of alternative adjuvants in order to achieve protection of the URT. Alternatively, although the uncleaved Fsol proteins induced neutralizing antibodies, immunization with a cleaved form of Fsol might induce higher neutralizing antibody titres that protect against URT infection. Both the adjuvanted Fsol/1/00 and Fsol/1/99 proteins induced higher homologous than heterologous VN antibody titres, indicating serological differences between the two main lineages of hMPV, as described previously (229). However, antibodies raised against the NL/1/99 F protein provided cross-protection against heterologous NL/1/00 virus infection of the LRT. Although we demonstrate cross-protection in only one direction, it seems likely that cross protection will be obtained in both directions. This has been observed previously with the F protein in its native form (205, 218).

Vaccine development aimed at protecting naïve individuals from infection with RSV or hMPV is hampered by the experience with a formalin-inactivated RSV vaccine. Immunization of naïve children with this vaccine induced enhanced disease upon subsequent infection (134). Enhanced disease has so far not been observed in employment of F subunit vaccines for RSV in patients with pre-existing immunity and is not to be expected for hMPV subunit vaccines. Therefore, the focus of this study was the protective efficacy of F subunit vaccines. Syrian golden hamsters are not the ideal animal model to study vaccine-induced immune pathology, as immunological tools are scarce. The presented results justify subsequent studies in non-human primates, in which the safety issue

can be addressed properly.

Our results demonstrate that immunization of Syrian golden hamsters with adjuvanted hMPV Fsol subunit vaccines induced a strong antibody response that provided complete protection against LRT infection with homologous and heterologous viruses. The availability of this protein in high quantities, in combination with the demonstrated beneficial use of the safe adjuvant iscom matrix, makes this F subunit vaccine an excellent candidate for further exploration to boost antibody titres in humans with pre-existing antibody titres.

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Generation of temperature-sensitive human metapneumovirus strains that provide protective immunity in hamsters

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ABSTRACT

Human metapneumovirus (HMPV) causes acute respiratory tract illness primarily in young children, immunocompromised individuals and the elderly. Vaccines would be desirable to prevent severe illnesses in these risk groups. Here, we describe the generation and evaluation of cold-passage (cp) temperature-sensitive (ts) HMPV strains as vaccine candidates. Repeated passage of HMPV at low temperatures in Vero cells resulted in the accumulation of mutations in the viral genome. Introduction of these mutations in a recombinant HMPV by reverse genetics resulted in a ts-phenotype, judged on the decreased shut-off temperature for virus replication *in vitro*. As an alternative approach, three previously described cp-respiratory syncytial virus (cp-HRSV) mutations were introduced in a recombinant HMPV, which also resulted in a low shut-off temperature *in vitro*. Replication of these ts-viruses containing either the cp-HMPV or cp-HRSV mutations was reduced in the upper respiratory tract (URT) and undetectable in the lower respiratory tract (LRT) of hamsters. Nevertheless, high titres of HMPV-specific antibodies were induced by both ts-viruses. Upon immunization with the ts-viruses, the LRT of hamsters were completely protected against challenge infection with a heterologous HMPV strain, and URT viral titres were reduced by 10 000-fold. In conclusion, we provide proof-of-principle for two candidate live-attenuated HMPV vaccines that induce cross-protective immunity to prevent infection of the LRT in Syrian golden hamsters. Further mapping of the molecular determinants of attenuation of HMPV should be the subject of future studies.

INTRODUCTION

The human metapneumovirus (HMPV) was first isolated from respiratory specimens obtained from children hospitalized for acute respiratory tract illness (RTI) in The Netherlands (227). Based on sequence information and genome organization, HMPV was classified as the first mammalian member of the Paramyxoviridae family, subfamily Pneumovirinae, genus Metapneumovirus. Clinical manifestations of HMPV infections are similar to those caused by the closely related respiratory syncytial virus (HRSV), ranging from mild respiratory illness to bronchiolitis and pneumonia (231, 246). Phylogenetic analysis of a large number of HMPV isolates revealed the existence of two main genetic virus lineages, which were found to be antigenically distinct in virus-neutralization assays with ferret sera (229). Whereas two surface glycoproteins, the attachment protein (G) and the short-hydrophobic protein (SH) are highly variable among virus isolates, the fusion protein (F) is highly conserved, and antibodies induced against F correlate with protection in animal models (204, 218).

A variety of vaccination strategies may be required to prevent HMPV respiratory tract infections in different risk groups, such as young children, individuals with underlying disease, and the elderly. Several vaccination strategies have been explored since the discovery of HMPV (111), including subunit vaccines (47, 109), a T-cell epitope vaccine (107), live attenuated vaccines (LAVs) (12, 178, 218) and formalin-inactivated (FI-) HMPV (52, 98, 252). Immunization with FI-HMPV primed for hypersensitivity responses upon challenge infection (52, 98, 252), suggesting that classical inactivated vaccines for HMPV may predispose for enhanced disease when used in immunologically naïve recipients, similar to what was previously described for HRSV and measles (79, 134, 180). For LAVs, no enhanced disease has been observed in studies performed in naïve animals with HRSV and HMPV. In addition, live attenuated measles virus vaccines have also not been associated with vaccine-mediated enhanced disease, either in humans or in animal models. LAVs may be useful to prime or boost HMPV-specific immune responses since such viruses have the advantage of mimicking a natural infection, and thus could provide protection against subsequent infections without inducing enhanced disease. Recently developed reverse genetics systems for HMPV (15, 108) facilitate the modification of viral genomes and thus provide a powerful tool to design LAVs. Several LAVs for HMPV have recently been described, including HMPV deletion mutants, chimeric viruses based on HMPV and avian metapneumovirus (AMPV), and a human/bovine parainfluenza virus type 3 (B/HPIV3) expressing the F protein of HMPV (12, 178, 218). Here, classical methods of virus adaptation to replication at low temperatures (cold-passage, cp) were used to attenuate HMPV, and the associated sequence changes in the viral genome were identified. Recombinant viruses containing HMPV or HRSV cp-mutations were generated by reverse genetics. These recombinant viruses were found to be temperature-sensitive (ts) in vitro, attenuated for replication in hamsters, yet highly immunogenic in this animal model. Hamsters immunized with ts-HMPV strains were protected against heterologous virus infection in the lower respiratory tract (LRT), and had significantly reduced virus titers in the URT. Thus, cp/ts-HMPV strains represent promising LAV candidates to protect against HMPV infections.

MATERIALS AND METHODS

Cells

Vero cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10 % fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 2 mM glutamine. Subclone 83 of WHO Vero cells was selected for virus passaging at low temperatures, and subclone 118 (135) which was selected for being permissive for the four genetic HMPV lineages, was used for all other experiments. To produce purified and concentrated virus stocks, virus strains were grown in infection medium consisting of IMDM supplemented with 4 % bovine serum albumin fraction V (Invitrogen, Breda, The Netherlands), 100 IU ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM glutamine and 3.75 µg ml^{-1} trypsin until 70–90 % of the cells displayed cytopathic effects. After one freeze-thaw cycle, cell-free supernatants were purified and concentrated using a 30–60 % (w/w) sucrose gradient.

Cold-passaging of virus

HMPV isolate NL/1/99 (229) (lineage B, passage 3 at 37 °C) was serially passaged in Vero-83 cells at decreasing temperatures. Virus was cultured at 34 °C, 31 °C, 28 °C and 25 °C for 3, 3, 2 and 2 passages respectively. When the temperature was decreased further to 22 °C or 20 °C, virus replication was impaired too much, and passaging was thus continued at 25 °C until passage 35 was reached. Cultures were harvested from every passage approximately 7 days after inoculation and stored in 25 % sucrose at –80 °C.

Sequence analysis

Viral RNA was isolated from virus stocks of cp-NL/1/99 passage 35, and intermediate passages 14, 23 and 29, using the High Pure RNA Isolation Kit (Roche Diagnostics, Almere, The Netherlands) according to instructions from the manufacturer. RNA was subsequently used in reverse transcriptase polymerase chain reaction (RT-PCR) assays using primer sets designed on the basis of the full-length genome sequence of NL/1/99 (GenBank accession no. AY525843). Both strands of the overlapping PCR-fragments were sequenced without prior cloning, to minimize amplification and sequencing errors. The nucleotide sequence of the cp-NL/1/99 genome was compared with the genome of the wild-type virus to identify nucleotide substitutions. All primer sequences are available upon request.

Sequence comparison of cold-passaged HRSV and HMPV

Genome sequences of HRSV strains containing mutations responsible for temperature sensitivity *in vitro* and attenuation *in vivo* (74, 127, 128, 240) were aligned with the full-length sequence of HMPV NL/1/99 using BioEdit software (97). Regions containing known ts-mutations in the HRSV genome were compared with their counterparts of HMPV, to determine whether HRSV ts-mutations could be introduced in homologous sites, conserved in the HMPV genome.

Recombinant viruses

The construction of wild-type recombinant HMPV NL/1/00 and NL/1/99 (genetic lineage A and B respectively) has been described previously (108). Mutations that were found in cp-NL/1/99, or identified upon sequence comparison of ts-HRSV and HMPV, were generated in the context of NL/1/99 using the QuickChange multi site-directed mutagenesis kit (Stratagene, Leusden, The Netherlands) according to instructions of the manufacturer. All primer sequences used for mutagenesis are available upon request.

Virus growth at different temperatures

To generate virus growth curves, 25 cm^2 flasks containing confluent Vero-118 cells were inoculated at 37 °C for 2 hours with wild-type or mutant HMPV at a multiplicity of infection (m.o.i.) of 0.1. After adsorption of the virus to the cells, the inoculum was removed and cells were washed 2 times with media before addition of 7 ml of fresh media, and incubation at 32 °C, 37 °C, 38 °C, 39 °C or 40 °C. Every day, 0.5ml of the supernatant was collected and replaced by fresh media. To determine viral titres, supernatants were subjected to plaque assays as described previously (108), with the exception that cells were incubated at 32 °C. Wild-type NL/1/99 virus and the viruses containing cp-HMPV mutations were incubated for 6 days, whereas the virus harboring the cp-HRSV mutations was incubated for 8 days, since only very small plaques were observed after 6 days.

Hamster experiments

The replication kinetics and immunogenicity of the recombinant candidate LAVs were studied in Syrian golden hamsters (*Mesocricetus auratus*; Charles River, Sulzfeld, Germany). Groups of 12 female hamsters, 5-7 week old, were inoculated intranasally with 10^6 50% tissue-culture infectious dosis (TCID$_{50}$) of NL/1/99 or LAV in a 100 µl volume. Four days post infection (dpi), lungs and nasal turbinates (NT) were collected from six animals per group, snap-frozen immediately and stored at –80 °C until further processing. From the other animals, blood samples were collected by orbital puncture at 21 dpi. Blood samples were stored overnight at room temperature and centrifuged 15 min at 1200 g; serum was collected and stored at –20 °C.

For the immunization and challenge experiment, animals were immunized by virus inoculation as described above, with 10^6 TCID$_{50}$ of LAV or NL/1/99, or PBS as control. At 21 dpi, animals were challenged intranasally with 10^7 TCID$_{50}$ of NL/1/00 virus. Four days after heterologous challenge infection, lungs, NT and blood samples were collected for further processing.

All intranasal inoculations, orbital punctures and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and were carried out in accordance with animal experimentation guidelines.

Plaque reduction virus neutralization assay

Virus neutralizing (VN) antibody titres were determined in serum samples by a plaque reduction virus neutralization (PRVN) assay as described previously (50). In brief, serum samples were diluted

and incubated for 60 minutes at 37 °C with approximately 50 plaque forming units (pfu) of NL/1/00 or NL/1/99, expressing the enhanced green fluorescent protein (eGFP). Subsequently, the virus-serum mixtures were added to Vero-118 cells in 24 well plates, and incubated at 37 °C. After two hours, the supernatants were replaced by a mixture of equal amounts of infection medium and 2 % methyl cellulose. Six days later, fluorescent plaques were counted using a Typhoon 9410 Variable Mode Imager (GE Healthcare, Diegem, Belgium). VN antibody titres were expressed as the dilution resulting in 50 % reduction of the number of plaques, calculated according to the method of Reed and Muench (188). Per assay, each serum was tested in duplicate against HMPV NL/1/00 and NL/1/99.

Virus titrations

Tissues from the inoculated hamsters were homogenized using a polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) in infection media. After removal of tissue debris by centrifugation, supernatants were used for virus titration in Vero-118 cells. Titrations were performed with 10-fold serial dilutions in 96-well plates (Greiner Bio-One). Confluent monolayers of Vero-118 cells were spin-inoculated (15 min., 2.000 *g*) with 100 µl of 10-fold serial dilutions of each sample and incubated at 37 °C. Two hours after the spin-inoculation, the inoculum was replaced with fresh infection media. After 3 - 4 days, 100 µl of fresh infection media was added to each well. Seven days after inoculation, infected wells were identified by immunofluorescence assays with HMPV-specific polyclonal antiserum raised in guinea pigs, as described previously (227). Titres expressed as $TCID_{50}$ were calculated as described by Reed and Muench (188). Titres were calculated per gram tissue, with a detection limit of $10^{1.6}$ and $10^{1.2}$ $TCID_{50}$ per gram of tissue for the NT and lungs, respectively.

RESULTS

Sequence analysis of cp-NL/1/99

HMPV isolate NL/1/99 was serially passaged in Vero-83 cells at slowly decreasing temperatures until a temperature of 25 °C was reached. When the temperature was further decreased to 22 °C or 20 °C, virus replication was severely impaired and virus yield was negligible. Therefore, passaging was continued at 25 °C until passage 35 was reached, when no changes in replication kinetics had been observed for 5 passages. Viral RNA of cp-NL/1/99 obtained after 35 passages was subjected to RT-PCR, followed by direct sequencing. Analysis of the full viral genome sequence and comparison with the original NL/1/99 genome revealed the presence of 19 nucleotide changes, resulting in 17 amino acid substitutions (Table 1). Analysis of virus genome sequences after fewer passages (passage 14, 23, and 29) indicated the gradual accumulation of these mutations (data not shown). One mutation that was found in the L gene after 29 passages had disappeared in the passage 35 virus, but this mutation was also included in further studies. Mutations were found throughout the viral genome in all genes, except the genes encoding the nucleoprotein (N) and the small hydrophobic protein (SH) (Table 1).

Table 1. Nucleotide and amino acid substitutions found after 35 rounds of cold passaging of HMPV NL/1/99 in Vero cells.

Position[*]	Gene	nt (wt)	nt (cp)	aa[‡]	aa (wt)	aa (cp)	HMPV$_{M19}$[§]	HMPV$_{M8}$	HMPV$_{M11}$	HMPV$_{M2}$
1458	P	GAA	GTA	66	Glu	Val	X	-	X	-
2203	M	TAT	CAT	9	Tyr	His	X	-	-	-
2291	M	TTA	TCA	38	Leu	Ser	X	-	-	-
2333	M	CTA	CCA	52	Leu	Pro	X	-	-	-
2514	M	GTT	GTG	112	Val	Val	X	-	-	-
2572	M	TCA	CCA	132	Ser	Pro	X	X	X	-
2614	M	CTA	TTA	146	Leu	Leu	X	X	X	-
3341	F	GAG	AAG	93	Glu	Lys	X	-	-	-
3365	F	TCA	CCA	101	Ser	Pro	X	X	X	-
3903	F	GAT	GGT	280	Asp	Gly	X	X	X	-
4476	F	CAG	CGG	471	Gln	Arg	X	X	X	-
4658	F	AAC	TAC	532	Asn	Tyr	X	-	-	-
4676	F	CAT	TAT	538	His	Tyr	X	-	-	-
5255	M2	AGC	ATC	187	Ser	Ile	X	X	X	-
6609	G	ACA	CCA	139	Thr	Pro	X	X	X	-
6685	G	CAA	CCA	164	Gln	Pro	X	-	-	-
7826[†]	L	GGA	AGA	235	Gly	Arg	X	-	X	X
8090	L	AAC	GAC	323	Asn	Asp	X	-	X	X
11480	L	TTC	CTC	1453	Phe	Leu	X	X	X	-

[*]Position is specified as the nucleotide position numbered from the 3'-end of negative-sense RNA (GenBank accession no. AY525843). [‡]Position is specified as the amino acid number in the indicated HMPV open reading frame. P, phosphoprotein; M, matrix protein; F, fusion protein; M2, putative 22K protein; G, attachment protein; L, large polymerase protein. [§]HMPV$_{M19}$ indicates that this virus contains 19 mutations found after cold passaging. X indicates the presence of this mutation in the recombinant virus. Nucleotide changes in each codon are underlined. [†]Transient mutation in passage 29 at 25°C.

Sequence comparison of cp-HRSV and HMPV

For HRSV, numerous mutations that accumulated in the viral genome after cold-passaging have been identified. After extensive studies, the ts-phenotype of cp-HRSV could be assigned to single mutations, or combinations of mutations. To explore the possibility of introducing these known cp/ts-mutations of HRSV into the HMPV genome, sequences of HRSV genes containing known cp/ts-mutations were aligned with their counterparts of HMPV NL/1/99. Most mutations could not be introduced in HMPV, because of a lack of similarity between the genes of HRSV and HMPV. However, four mutations at position 521 (128), 1169 (127) and 1321 (240) of the L gene and in the gene start (GS) of M2 (74) were identified, for which the HMPV genome was identical to the wild-type HRSV sequence (Table 2). Thus, these cp/ts-mutations of HRSV could be introduced easily in the genome of HMPV NL/1/99.

Construction of recombinant HMPV cp-NL/1/99

Wild-type recombinant HMPV NL/1/99 was used as a backbone for the introduction of mutations as listed in tables 1 and 2. Three different viruses containing all mutations or subsets of cp-HMPV

Table 2. Nucleotide substitutions in cp-HRSV that were introduced in recombinant HMPV/NL/1/99.

Position[*]	Position[†]	Gene	Virus origin	nt (wt)	nt (cp)	aa (wt)	aa (cp)	Ref.
521	456	L	cpts530	TTC (HRSV) TTT (HMPV)	TT<u>A</u>	Phe	Leu	128
1169	1094	L	cpts530/1009	ATG	<u>G</u>TG	Met	Val	127
1321	1246	L	cpts530/1030	TAT	<u>A</u>AT	Tyr	Asn	240
-	-	GS-M2	cpts248/404	AATA	AA<u>C</u>A	-	-	74

[*]Position is specified as the amino acid number of the L gene of HRSV (GenBank accession no. U63644). [†]Position is specified as the amino acid number of the L gene of HMPV. L, large polymerase protein; GS-M2, gene-start sequence of the M2 gene. Nucleotides changes in each codon or nucleotide sequence are underlined.

mutations were constructed. These viruses containing 19, 8 or 11 nucleotide substitutions were named $HMPV_{M19}$, $HMPV_{M8}$ and $HMPV_{M11}$ respectively, based on the number of mutations that were introduced (Table 1). Mutant virus $HMPV_{M19}$ could not be rescued by reverse genetics after three attempts. The parental virus obtained after 35 passages at 25 °C also replicated very poorly, to low virus titres. Therefore, we next attempted to rescue recombinant viruses that contained only a selection of the cp-mutations, 8 and 11 respectively, which were generated as cloning intermediates during the cloning of $HMPV_{M19}$.

Upon introduction of the four cp-HRSV mutations in the NL/1/99 backbone, no virus could be recovered after three attempts. Therefore, four viruses containing each possible combination of three mutations were generated, thus omitting one of the mutations (Table 2). Only the virus in which the L1321 mutation was omitted (named $HMPV_{HRSV3}$ hereafter) could be rescued.

Temperature-sensitivity

To study the possible ts-phenotype of recombinant viruses, virus growth curves were generated in Vero cells at 32 °C, 37 °C, 38 °C, 39 °C or 40 °C. Plaque assays were performed to determine the viral titres in the supernatants of samples that were collected daily. Wild-type HMPV was able to replicate at all temperatures, with the highest virus titre obtained at 37 °C. At 40 °C, the virus titre was more than 100-fold reduced compared to the optimal temperature of 37 °C (Fig. 1a). $HMPV_{M8}$ which was an intermediate virus in the cloning procedure of $HMPV_{M19}$, also replicated at all temperatures, but with higher titres as compared to wild-type HMPV, and an optimal replication temperature of 32 °C (Fig. 1b). Even at 40 °C, $HMPV_{M8}$ displayed faster replication kinetics in Vero cells and at all temperatures higher peak virus titres were reached compared to wild-type HMPV. Mutant $HMPV_{M11}$ also displayed optimal virus growth at a temperature of 32 °C. Peak titres were reached later for $HMPV_{M11}$, but virus titres at 6 dpi at 32 °C were higher as compared to $HMPV_{M8}$ (Fig. 1c). This virus did not replicate at 39 °C and 40 °C, indicating that this virus was temperature-sensitive. The only differences between $HMPV_{M11}$ and $HMPV_{M8}$ were two mutations in the L gene and one mutation in the P gene (Table 1). $HMPV_{M9}$ that was also generated as a cloning intermediate, containing all mutations of $HMPV_{M8}$ and the mutation in the P gene turned out to be insensitive to

higher temperatures (data not shown). Therefore, $HMPV_{M2}$ was constructed containing only two L mutations (nt 7826 and 8090, Table 1) as compared to wild-type NL/1/99. The replication kinetics of $HMPV_{M2}$ was most similar to that of the wild-type NL/1/99 virus (compare Fig. 1a and 1d), suggesting that these two L mutations alone are not ts- or Vero-cell adaptive mutations.
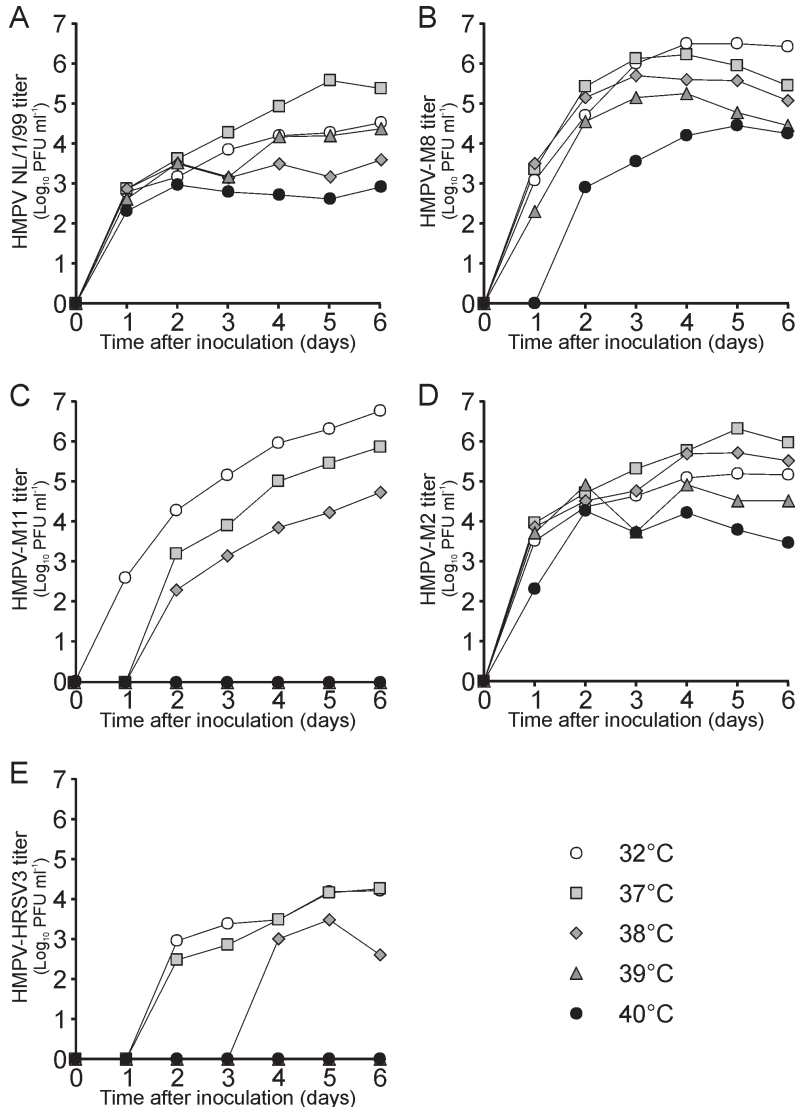

Figure 1. Replication kinetics in Vero cell cultures of wt HMPV and recombinant viruses in which cp-mutations were introduced. Vero cells, infected at an m.o.i. of 0.1 with HMPV NL/1/99 (A), $HMPV_{M8}$ (B), $HMPV_{M11}$ (C), $HMPV_{M2}$ (D) or $HMPV_{HRSV3}$ (E) were washed and incubated for 6-8 days at 32 (open circle), 37 (square), 38 (diamant), 39 (triangle) or 40 °C (closed circle). Shading of symbols varies from white to black with increasing temperature. Samples were collected daily, and virus titres were determined by plaque assay.

The only viable NL/1/99 with cp-HRSV mutations, $HMPV_{HRSV3}$, replicated slowly and to a 10-fold lower peak titre at 37 °C, but at 32°C the peak titre was comparable to wild-type HMPV. At 38 °C, no virus was detected until 4 dpi, and at 39 °C and 40 °C the virus did not replicate at all. Thus, $HMPV_{HRSV3}$ appeared to be temperature-sensitive in vitro (Fig. 1e).

Replication kinetics and immunogenicity in hamsters

For the two viruses with a ts-phenotype in vitro, $HMPV_{M11}$ and $HMPV_{HRSV3}$, we tested the level of attenuation in hamsters. Syrian golden hamsters were inoculated with 10^6 $TCID_{50}$ $HMPV_{M11}$, $HMPV_{HRSV3}$, or wild-type NL/1/99 (12 animals per group), after which virus titres in the lungs and NT were compared at four dpi (6 animals per group), and virus neutralizing antibody titres were determined at 21 dpi (6 animals per group). In the NT of animals inoculated with wild-type HMPV, virus titres up to 10^7 $TCID_{50}$ $gram^{-1}$ NT were detected (Fig. 2a). In the animals inoculated with each of the candidate LAVs however, mean virus titres ranged from 10^2 to 10^4 $TCID_{50}$ $gram^{-1}$ NT, indicating that virus replication was ~10 000-fold reduced in the URT. In the lungs of animals inoculated with wild-type HMPV, the mean virus titre was $10^{2.2}$ $TCID_{50}$ $gram^{-1}$ lung, while in the animals inoculated with $HMPV_{M11}$ or $HMPV_{HRSV3}$ virus titres were below the detection limit of $10^{1.2}$ $TCID_{50}$, with the exception of a single animal in the $HMPV_{M11}$ inoculated group ($10^{1.3}$ $TCID_{50}$). Thus, both ts viruses appeared to be highly attenuated *in vivo* and virus replication was restricted to the URT, where virus titres were ~10 000-fold reduced compared to wild-type HMPV.
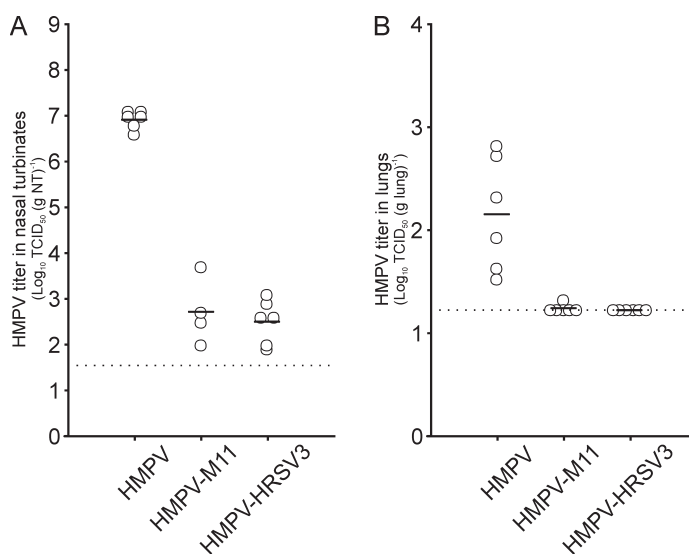
Figure 2. Infectious virus titres in NT (A) and lungs (B) of Syrian golden hamsters inoculated with 10^6 $TCID_{50}$ of NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$. NT and lungs were collected at 4 dpi. Virus in tissues was quantified by serial dilution in Vero-118 monolayers. The solid lines represent the geometric mean titre (GMT), the lower limit of detection is indicated with a dotted line.

From the remaining six animals of each group, serum samples were collected and subjected to a PRVN assay to determine virus neutralizing antibody titres against HMPV NL/1/99, induced by the candidate LAVs (Fig. 3). The PRVN titres in the wild-type HMPV inoculated animals were slightly higher than those observed in the HMPV$_{M11}$ or HMPV$_{HRSV3}$ inoculated animals (mean VN antibody titres of 90, 25, and 28 respectively, not significantly different, Mann-Whitney test).
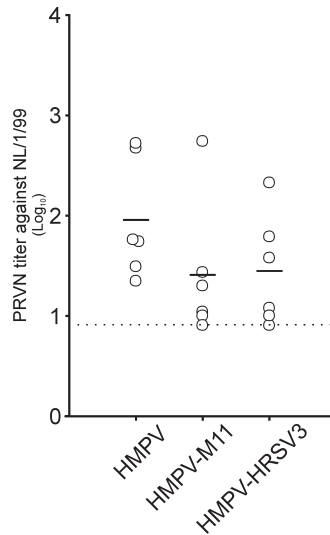
Figure 3. PRVN titres (50%) measured against NL/1/99, after immunization with HMPV NL/1/99, HMPV$_{M11}$ or HMPV$_{HRSV3}$. Blood samples were collected by orbital puncture at 21 dpi. Titres were calculated according to the method of Reed & Muench (1938). The solid lines represent the GMT, the lower limit of detection is indicated with a dotted line.

Immunization-challenge experiment

Since both HMPV$_{M11}$ and HMPV$_{HRSV3}$ induced a detectable but low virus neutralizing antibody response, we investigated whether these viruses had induced sufficient protective immunity to prevent subsequent HMPV infection. Groups of six animals were immunized with 10^6 TCID$_{50}$ of HMPV$_{M11}$, HMPV$_{HRSV3}$, wild-type HMPV NL/1/99 or PBS. Three weeks after immunization, animals were challenged with 10^7 TCID$_{50}$ of the heterologous HMPV strain NL/1/00, to evaluate whether the induced immune response was robust enough to provide cross-protection against heterologous infection. Four days after challenge infection, lungs, NT and blood samples were collected. In PBS-immunized control hamsters, virus titres upon challenge reached >10^8 TCID$_{50}$ gram^{-1} tissue in the NT samples. These virus titres were more than 1000-fold reduced in animals immunized with HMPV$_{HRSV3}$, and >10 000-fold reduced in the animals immunized with HMPV$_{M11}$ or wild-type HMPV (Fig. 4a). In the lungs of PBS-immunized animals, the mean virus titre after challenge infection was $10^{4.3}$ TCID$_{50}$ gram^{-1} lung tissue. Virus was undetectable in lungs of all animals immunized with HMPV$_{M11}$, HMPV$_{HRSV3}$, and wild-type HMPV NL/1/99 (Mann-Whitney test, P<0,05) (Fig. 4b).
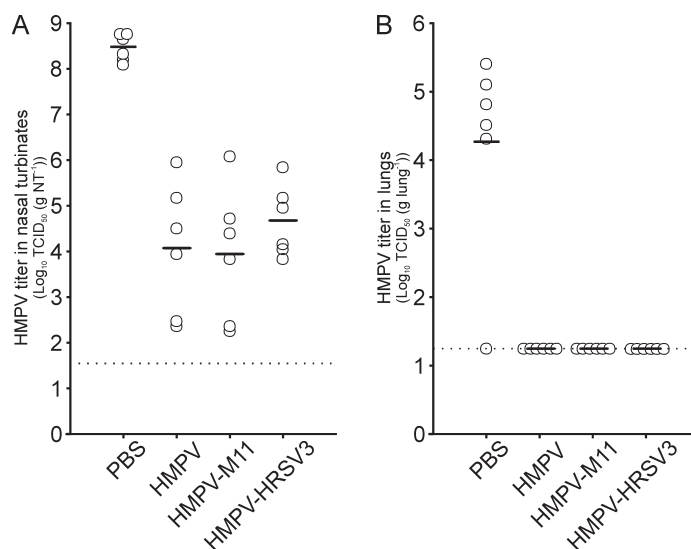
Figure 4. Infectious virus titres in NT (A) and lungs (B) of Syrian golden hamsters. Animals were immunized with PBS, HMPV NL/1/99, HMPV$_{M11}$ or HMPV$_{HRSV3}$. Three weeks after immunization, animals were challenged with 10^7 TCID$_{50}$ of the heterologous virus HMPV NL/1/00. Animals were euthanized at 4 dpi. Virus present in tissues was quantified by serial dilution in Vero-118 monolayers. The solid lines represent the GMT, the lower limit of detection is indicated with a dotted line.

Serum samples of all animals inoculated with wild-type NL/1/99 or one of the ts-HMPV candidates based on NL/1/99, were tested in a PRVN assay against both NL/1/99 (lineage B) and NL/1/00 (lineage A) virus. Although HMPV$_{M11}$ and HMPV$_{HRSV3}$ are highly attenuated, PRVN titres in animals inoculated with these viruses were comparable to wild-type HMPV inoculated animals (Fig. 5). As expected, PRVN titres against homologous virus were higher than against heterologous virus. Thus, we conclude that HMPV$_{M11}$ and HMPV$_{HRSV3}$ are attenuated in hamsters, yet induce an HMPV-specific immune response that is sufficient to provide protective immunity to prevent HMPV lower respiratory tract infections.

DISCUSSION

The clinical impact of HMPV warrants the development of vaccines to prevent serious respiratory tract disease in young children, immunocompromised individuals and the elderly. Here, a classical method for obtaining live attenuated viruses (LAVs), by passaging virus at low temperatures, was explored for HMPV. HMPV isolate NL/1/99 was passaged at gradually decreasing temperatures, until passage 35 at a temperature of 25 °C was reached. After sequencing of this passage 35 virus, 19 nucleotide mutations were found resulting in 17 amino acid substitutions (Table 1). The mutations at nt position 3341 (E93K) and 3365 (S101P) have been described previously (13, 50, 194). The S101P mutation is located in the putative cleavage site motif of the F protein and viruses

containing this mutation did not require trypsin for growth in tissue culture. Moreover, this trypsin-independent cleavage of the HMPV F protein containing the 101P was enhanced by the amino acid substitution E93K. Thus, repeated passaging of HMPV NL/1/99 in Vero cells resulted in the introduction of mutations that render the virus to be relatively independent of trypsin.
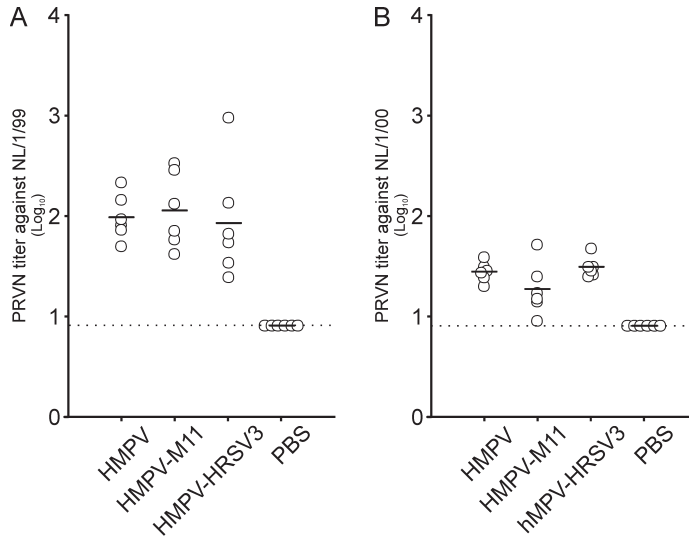
Figure 5. PRVN titres (50%) measured against HMPV NL/1/99 (A) or HMPV NL/1/00 (B), after immunization with HMPV NL/1/99, $HMPV_{M11}$ or $HMPV_{HRSV3}$ or PBS and subsequent challenge with HMPV NL/1/00. Blood samples were collected by orbital puncture 4 days post challenge. Titres were calculated according to the method of Reed & Muench (1938). The solid lines represent the GMT, the lower limit of detection is indicated with a dotted line.

Initial attempts to rescue a recombinant virus with all 19 passage-35 mutations failed repeatedly. The parental passage-35 virus replicated very slowly, and to low virus titres. It is possible that the consensus sequence generated on the basis of the passage-35 virus was derived from a variety of quasi-species in the culture supernatant, which were replication-deficient upon clonal passage. Since HMPV generally replicates poorly in in-vitro cell cultures, which was even more severe for the passage-35 virus, plaque purification of this virus was not attempted. Rather, we next tested whether viruses with a subset of the mutations of the passage-35 virus could be rescued. A virus with 11 of the 19 mutations, $HMPV_{M11}$ turned out to have a ts-phenotype in vitro (Fig. 1c). Ten of these 11 mutations were non-silent, and were located in the P, M, F, M2, G, and L genes. To our knowledge, none of these mutations has been observed in cp/ts-HRSV. A recombinant virus with 8 of the 11 cp-mutations, $HMPV_{M8}$, did not display a ts-phenotype in vitro. The only differences between $HMPV_{M11}$ and $HMPV_{M8}$ were two mutations in the L gene (nt 7826 and 8090), and one mutation in the P gene (nt 1458, Table 1). $HMPV_{M9}$, containing all mutations of $HMPV_{M8}$ and the mutation in the P gene was not temperature sensitive. A recombinant virus containing only the two

mutations in L also did not display a ts-phenotype. Therefore it seems likely that one or both of the L mutations in combination with one or more other mutations is responsible for the ts-phenotype of HMPV$_{M11}$. Further studies are needed to map the phenotype of all cp-mutations, and especially the phenotype of HMPV harboring all 3 mutations that are different between HMPV$_{M8}$ and HMPV$_{M11}$ should be evaluated.

For HRSV, this classical approach of generating attenuated cp-viruses has resulted in several candidate LAVs that have been tested extensively in animal models and even in human volunteers. Sequence comparison of HMPV with different cp-HRSV strains resulted in the identification of four cp-HRSV mutations that could be introduced in the HMPV genome. When three of these mutations were introduced in the HMPV genome, omitting the L1321 mutation (Table 2), virus could be rescued (HMPV$_{HRSV3}$). Upon introduction of the HRSV L1321 mutation in the HMPV genome, recombinant HMPV could not be rescued. It is possible that introduction of L1321 in HMPV yields a stronger ts-phenotype if other, potentially compensatory, mutations are present. Virus replication curves generated at different temperatures revealed that HMPV$_{HRSV3}$ was restricted for replication at 39 °C and 40 °C, indicating that this virus had a ts-phenotype (Fig. 1e). The L521 phenylalanine (Phe) to leucine mutation, present in HMPV$_{HRSV3}$, has previously been mutagenized at the analogous Phe at amino acid position 456 of recombinant HPIV3 (207). This amino acid substitution resulted in a virus bearing a ts phenotype with virus replication 10-fold reduced in the upper, but not the lower respiratory tract of hamsters. Substitution of this amino acid in two cp-HPIV3 candidates (r*cp*45 and r*cp*45$_L$) induced a 100- to 1000-fold more restricted replication in hamsters than their cp parents (207).

Both HMPV$_{M11}$ and HMPV$_{HRSV3}$ were found to be attenuated in hamsters, with ~10 000-fold reduction of virus replication in the URT, and no detectable virus in the LRT as compared to wild-type virus (Fig. 2). In immunized animals, the HMPV-specific antibody titres were slightly lower as compared to animals inoculated with wild-type virus (Fig. 3 and Fig. 5). Nevertheless, immunized animals were completely protected from HMPV LRT infection, and virus titres in the URT were reduced to the same extent as seen in hamsters exposed to wild-type HMPV (Fig. 4). Viral titres in both the lungs and NT of PBS-immunized animals that were challenged with NL/1/00 were approximately 100-fold higher than the titres obtained in NL/1/99-infected animals (Fig. 2). Similar differences in replication between these two viruses have been observed before in cynomolgus macaques (228), but may here also be caused by differences in inoculum size, 10^6 versus 10^7 for NL/1/99 and NL/1/00, respectively.

Altogether, it seems that the balance between the level of attenuation and the level of induction of specific immune responses for both HMPV$_{M11}$ and HMPV$_{HRSV3}$ in hamsters is appropriate for these viruses to serve as vaccines. The results of this study justify subsequent studies in non-human primates, in which the attenuation, stability, immunogenicity and safety issues can be further addressed.

Future studies should determine the contribution to the ts-phenotype of each individual mutation

that was introduced in $HMPV_{M11}$ and $HMPV_{HRSV3}$. During passaging at lower temperatures, both ts-mutations, mutations associated with adaptation to Vero cells, and mutations without apparent phenotype changes may be observed, and it would be good to discriminate amongst these. The S101P and E93K mutations in the F protein may be good examples of cell culture adaptation mutations. When the mutations responsible for the attenuated phenotype have been defined, amino acid point mutations should be generated using codons that differ from the wild-type codon by preferably two or three nucleotides, in order to reduce the frequency of reversion.

In addition to the use of LAVs for the induction of protective immunity against the attenuated virus itself, attenuated candidate HMPV may be used as a vector to induce immunity against a second or even a third viral respiratory pathogen, such as HRSV or HPIV3. A similar approach has already been conducted with a chimeric human/bovine parainfluenza virus type 3 (B/HPIV3) expressing the F protein of HMPV, that induced protective immunity against both HMPV and HPIV3 infection in African green monkeys (218).

Our results demonstrate that immunization of Syrian golden hamsters with attenuated recombinant viruses containing cp-HMPV or cp-HRSV mutations induced a good antibody response, and provided complete protection against LRT infection with a heterologous strain of HMPV. The high degree of attenuation and the high level of immunogenicity suggest that $HMPV_{M11}$ and $HMPV_{HRSV3}$ represent excellent candidate LAVs for further exploration to prime the HMPV-specific immune response in non-human primates, and perhaps humans.

ACKNOWLEDGEMENTS

Immunogenicity and efficacy of two candidate human metapneumovirus vaccines in cynomolgus macaques

Vaccine, accepted with minor modifications

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ABSTRACT

Human metapneumovirus (HMPV) is an important cause of acute respiratory tract disease for which the development of vaccine candidates is warranted. We have previously described the generation of an iscom matrix-adjuvanted HMPV fusion protein subunit vaccine (Fsol) and a live-attenuated vaccine ($HMPV_{M11}$). Here, we evaluate the immunogenicity and efficacy of these vaccines in cynomolgus macaques. Immunization with Fsol induced HMPV F-specific antibody responses, virus neutralizing antibody titers, and cellular immune responses, but the induced humoral immune response waned rapidly over time. $HMPV_{M11}$ was strongly attenuated and displayed limited immunogenicity, although immunization with this virus primed for a good secondary HMPV-specific lymphoproliferative response after challenge infection. The duration of virus shedding in $HMPV_{M11}$-immunized animals was reduced compared to sham-immunized animals. Both vaccines induced HMPV-specific immune responses, but the rapid waning of immunity is a challenging obstacle for vaccine development.

INTRODUCTION

The human metapneumovirus (HMPV) was first isolated from respiratory specimens obtained from children hospitalized for acute respiratory tract illness (RTI) in The Netherlands (227), and was characterized as the first mammalian member of the Metapneumovirus genus of the subfamily Pneumovirinae, within the family Paramyxoviridae. Phylogenetic analysis revealed the existence of two main genetic lineages (A and B), each of which could be further divided into two sublineages (A1, A2, B1 and B2) (229).

Clinical manifestations of HMPV infections are comparable to those caused by the closely related human respiratory syncytial virus (HRSV), ranging from mild respiratory illness to bronchiolitis and pneumonia (231, 246). The clinical impact warrants the development of vaccines, but different vaccination strategies may be required to prevent HMPV-associated respiratory disease in the different risk groups, especially young children, immunocompromised individuals and the elderly. Since the discovery of HMPV, several vaccination strategies have been explored in animal models (111), including subunit vaccines (47, 109), live attenuated vaccines (12, 110, 178, 218), a T-cell epitope vaccine (107), heat-inactivated- (98) and formalin-inactivated- (FI-) HMPV vaccines (52, 252). The studies on FI-inactivated vaccines indicated that immunization with FI-HMPV primed for hypersensitivity responses upon challenge infection (52, 252), similar to what has been described for FI-HRSV and FI-measles virus (51, 79, 134, 180). This suggests that classical inactivated vaccines for HMPV may predispose for enhanced disease when used in immunologically naïve individuals.

For HRSV, a variety of subunit vaccines based on the fusion (F) protein or attachment protein (G) have been developed and tested in various animal models (100, 163). Additionally, HRSV F subunit vaccines have been evaluated in clinical trials in healthy adults, the elderly, pregnant women, and children below the age of one year (69, 161, 179, 222). In all studies, these F subunit vaccines turned out to be protective, safe and well-tolerated. Since HMPV and HRSV are closely related viruses, we have evaluated soluble HMPV F proteins (Fsol) as a candidate HMPV vaccine (109). The immunogenicity and protective efficacy of Fsol proteins of HMPV NL/1/00 and NL/1/99, representing the main genetic lineages A and B respectively, were evaluated in Syrian golden hamsters (mesocricetus auratus) (109). Addition of adjuvants such as iscom matrix or Specol to Fsol enhanced the immune response in immunized animals, as demonstrated by increased virus neutralizing (VN) antibody titers compared to the non-adjuvanted Fsol. Immunization with these adjuvanted Fsol preparations induced complete protection of the lower respiratory tracts of the animals against either homologous or heterologous challenge infection (109).

We have recently also reported the generation of live-attenuated vaccine candidates for HMPV (110). The classical method of virus adaptation to replication at low temperatures (cold-passaging, cp) was used to attenuate HMPV, and the associated sequence changes in the viral genome were identified. Reverse genetics techniques were used to introduce subsets of these cp-mutations into the viral genome, resulting in recombinant viruses that were temperature-sensitive (ts) in vitro, and attenuated yet highly immunogenic in hamsters. Animals immunized with ts-HMPV strains were

protected against heterologous virus infection in the LRT, and had significantly reduced virus titers (10.000 fold) in the upper respiratory tract (URT) (110).

These two different immunization strategies, HMPV F subunit vaccines and live attenuated HMPV vaccines, may be used to prevent severe disease associated with HMPV in different risk groups for HMPV. For instance, the F subunit vaccine may be a good candidate to boost existing HMPV-specific immune responses in immunocompromised individuals and the elderly, similar to subunit influenza virus vaccines. Live-attenuated vaccines may be more useful to prime or boost HMPV-specific immune responses in young children. Live-attenuated viruses have the advantage of mimicking a natural infection, and thus could provide better protection against subsequent infections in immunologically naïve individuals. We have previously shown that both vaccines provided protection against infection of the lower respiratory tract in hamsters, and these results justify the further exploration of these vaccines in an animal model that is more related to humans. In this study, we evaluate the immunogenicity and efficacy of both vaccines in cynomolgus macaques.

MATERIALS AND METHODS

Cells and viruses

Subclone 118 of WHO Vero cells (135) was grown in Iscove's Modified Dulbecco's Medium (IMDM, BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS, Greiner Bio-One, Alphen aan den Rijn, The Netherlands), penicillin (100 IU/ml), streptomycin (100 μg/ml) and glutamine (2 mM). To produce purified and concentrated virus stocks, virus strains were grown in infection medium consisting of IMDM supplemented with 4 % bovine serum albumin fraction V (Invitrogen, Breda, The Netherlands), penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2mM) and trypsin (3.75 μg/ml) until 70 – 90 % of the cells displayed cytopathic effects. After one freeze-thaw cycle, cell-free supernatants were purified and concentrated using a 30 – 60 % (w/w) sucrose gradient.

The construction of wild-type recombinant HMPV NL/1/00 (genetic lineage A) and HMPV$_{M11}$ (in the NL/1/99 backbone, lineage B) have been described previously (108, 110).

Vaccine preparations

Adjuvanted soluble F proteins (Fsol) were generated as described (109). In brief, plasmids expressing a truncated version of the NL/1/00 or NL/1/99 F protein, lacking the transmembrane domain, were created (224). Stable NSO murine myeloma cell lines expressing NL/1/00 or NL/1/99 F proteins were generated as described (9). F proteins were subsequently purified by affinity chromatography. For the iscom matrix preparation, decanoyl-N-methylglucamide (MEGA-10, Sigma-Aldrich, Zwijndrecht, The Netherlands) at a final concentration of 2 % was supplemented with cholesterol (1 mg/mg protein, Sigma), phosphatidylethanolamine (1 mg/mg protein, Sigma) and Quil-laja glucosides (5 mg/mg protein ISCOPREP 703, Iscotec, Lulea, Sweden) and dialyzed against PBS. The iscom matrix preparation was analyzed by negative contrast electron microscopy, revealing the typical iscom matrix particles with a diameter of approximately 40 nm. Iscom matrix preparation was added to the purified Fsol immediately before immunization, resulting in preparations containing 10 μg

Fsol/500 µl.

HMPV$_{M11}$, a temperature-sensitive recombinant HMPV was generated after introduction of eleven mutations that were identified in NL/1/99 after serial passaging at slowly decreasing temperatures (110). This virus was grown, purified and concentrated as described above, resulting in a virus stock with $10^{9.7}$ TCID$_{50}$/ml.

Animal experiments

Eighteen young cynomolgus macaques (*Macaca fascicularis*, age range 8–18 months at first vaccination) were immunized with Fsol (n=6, group 2), PBS as a control (n=6, group 1), or HMPV$_{M11}$ (n=6, group 3). All animals were seronegative for both HMPV lineages, as tested by HMPV F-specific ELISA (109), immunofluorescence and plaque reduction virus-neutralization assays against both HMPV serotypes (50). Animals were immunized twice, with a four week interval. Animals of groups 1 and 2 were vaccinated by intramuscular (i.m.) injection. The third group was inoculated with 10^6 and $10^{5.5}$ TCID$_{50}$ HMPV$_{M11}$ for the first and second immunization respectively, in a final volume of 6 ml, which was administered intratracheally (i.t.; 5 ml, inoculated just below the larynx) and intranasally (i.n.;1ml, divided over both nostrils). Eight weeks after the second immunization, all animals were challenged with $10^{6.5}$ TCID$_{50}$ of HMPV/NL/1/00 in 6ml (5 ml i.t., 1 ml i.n.). Before inoculations and bronchoalveolar lavages (BAL, see below), animals were anaesthetized by using a cocktail of Ketamine® and Domitor® (50 / 50 v/v) at an i.m. dose of 0.2 ml/kg, followed by Antisedan® (antagonist of Domitor®) at an i.m. dose of 0.05 ml/kg after inoculation or collection of BAL samples. This animal study was approved by an independent Animal Ethics Committee and the Dutch authority for working with genetically modified organisms, and was carried out in accordance with animal experimentation guidelines.

Sample collection

To monitor virus replication, pharyngeal and nasopharyngeal swabs were collected at day 4 and 14 after both inoculations with HMPV$_{M11}$, and at day 1, 3, 5 and 9 after challenge infection with NL/1/00. Swabs were collected in 2 ml transport medium consisting of Hanks' balanced salt solution supplemented with glycerol (10%), penicillin (200 U/ml), streptomycin (200 mg/ml), polymyxin B sulfate (100 U/ml), gentamicin (250 mg/ml) and nystatin (50 U/ml). Samples were mixed vigorously, aliquotted, and stored at -70°C until further processing. On day 1, 3, 5 and 9 after challenge infection, BAL samples were collected by i.t. infusion and subsequent recovery of 10ml PBS with a flexible catheter. BAL cells were centrifuged (5 min, 400 g), resuspended in PBS, counted, and 5 x 10^5 cells were added to 200 µl transport medium, and stored at -70°C until processing. To test for serum antibody responses, blood samples were collected at week 0 (prior to first immunization), 2, 4 (prior to second immunization), 6, 8, 12 (prior to challenge infection) and day 5 and 14 after challenge infection. Sera were heat-inactivated (HI; 30 min, 56°C) and stored at -20°C. On the day of challenge infection, and two weeks later, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using density gradient centrifugation, and stored in aliquots at −135°C in medium supplemented with 20% HI-FCS and 10% DMSO.

Real-time RT-PCR

Viral RNA was isolated from 200 μl pharyngeal or naspharyngeal swab-material, or 5×10^5 BAL cells in 200 μl, using the MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, The Netherlands), with an elution volume of 50 μl. HMPV genome copies were detected by Taqman real-time PCR as described (150, 228).

F-ELISA

F-specific enzyme-linked immunosorbent assays were carried out as described previously (109). Briefly, 96-well plates (Greiner Bio-One) were coated overnight at 4 °C with 100 ng per well of Fsol protein (NL/1/00 and NL/1/99, ratio 1:1) in PBS. Serum samples were diluted 1:150, added to the plates, and incubated for 1 hour at 37 °C. After washing, plates were incubated for 1 hour with goat anti-human Ig-horseradish peroxidase (1:5000; Southern Biotech), followed by incubation with substrate (3,3',5,5'-Tetramethylbenzidine [TMB]). The reaction was stopped by adding an equal volume of 2 M H2SO4, after which the OD_{450} was determined. Results are depicted after subtraction of background values.

Plaque reduction virus neutralization assay

Virus neutralizing (VN) antibody titers were determined in serum samples by a plaque reduction virus neutralization (PRVN) assay as described previously (50). In brief, serum samples were diluted and incubated for 60 minutes at 37°C with approximately 50-60 plaque forming units (pfu)
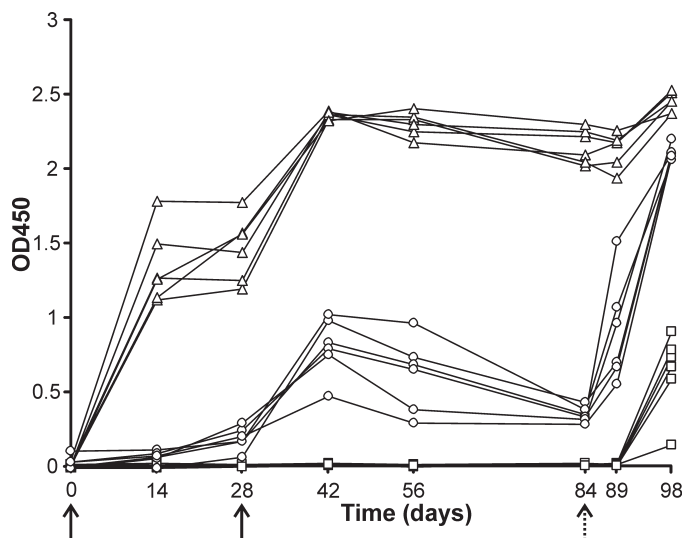
Figure 1. Induction of F-specific antibody titers in cynomolgus macaques immunized with PBS (squares), HMPV$_{M11}$ (circles), or iscom matrix-adjuvanted HMPV Fsol preparations (triangles), as measured by ELISA. Serum samples were diluted 1:150 in PBS. OD_{450} values of serum samples are given after subtraction of background. Time-points of immunizations and challenge infection are indicated by the solid and dotted arrows respectively.

of NL/1/00 or NL/1/99, expressing the enhanced green fluorescent protein (eGFP). Subsequently, the virus-serum mixtures were added to Vero-118 cells in 24 well plates (Greiner Bio-One), and incubated at 37°C. After two hours, the supernatants were replaced by a mixture of equal amounts of infection medium and 2% methyl cellulose. Six days later, fluorescent plaques were counted using a Typhoon 9410 Variable Mode Imager (GE Healthcare, Diegem, Belgium). VN antibody titers were expressed as the dilution resulting in 50% reduction of the number of plaques, calculated according to the method of Reed and Muench (188). Per assay, each serum was tested in duplicate against HMPV NL/1/00 and NL/1/99.

Lymphoproliferation assays

Lymphoproliferation assays were carried out as described previously (52). In short, PBMCs were thawed and cultured overnight in RPMI-1640 medium (BioWhittaker) containing penicillin (100 U/ml), streptomycin (100 g/ml), glutamine (2 mM) and 2-mercapto-ethanol (10^{-5} M) supplemented with 10% HI-FCS and 1% HI pooled macaque serum. The next day, 1.5×10^5 PBMCs were plated in 150µl per well in 96-well round-bottom plates (Greiner Bio-One), followed by stimulation in triplicate with beta-propiolactone (BPL)-inactivated Vero-118 cell antigen (negative control) or BPL-inactivated (non-purified) HMPV/NL/1/00. After 4 days of culture, 100µl medium was replaced by fresh medium containing 10µM BrdU. The next day cells were harvested, pooled per stimulation, and processed for FACS staining with FITC-labeled mouse-anti-BrdU (BD Biosciences), and PerCP-labeled mouse anti-human CD3 (BD Pharmingen). Results are shown as the percentage HMPV-stimulated BrdU-positive cells in the CD3-positive lymphocyte population, after subtraction of values for non-specific stimulation (after gating on basis of FSC/SSC profile).

RESULTS

Immunogenicity of HMPV Fsol

Cynomolgus macaques immunized with iscom matrix-adjuvanted Fsol NL/1/99 showed high serum levels of F-specific antibodies 14 and 28 days after the first immunization (t0), and this antibody response was boosted after the second immunization (t28) (Fig. 1). The antibodies were able to neutralize both homologous (NL/1/99) and heterologous (NL/1/00) strains of HMPV, with homologous titers approximately 10-fold higher than heterologous titers (Fig. 2c, d). Peak VN antibody levels were detected two weeks after the second immunization, after which antibody levels slowly waned over time until the day of challenge (t84).

In PBMCs collected after vaccination but prior to challenge HMPV-specific lymphoproliferative responses detected in animals immunized with Fsol were significantly higher than those in the PBS-immunized group (Mann-Whitney, $P<0.05$) (Fig. 3).

Immunogenicity of HMPV$_{M11}$

In macaques immunized with HMPV$_{M11}$ (first dose 10^6 TCID$_{50}$, second dose $10^{5.5}$ TCID$_{50}$), no virus could be detected in throat- or nose-swabs collected 4 and 14 days after immunizations, either by
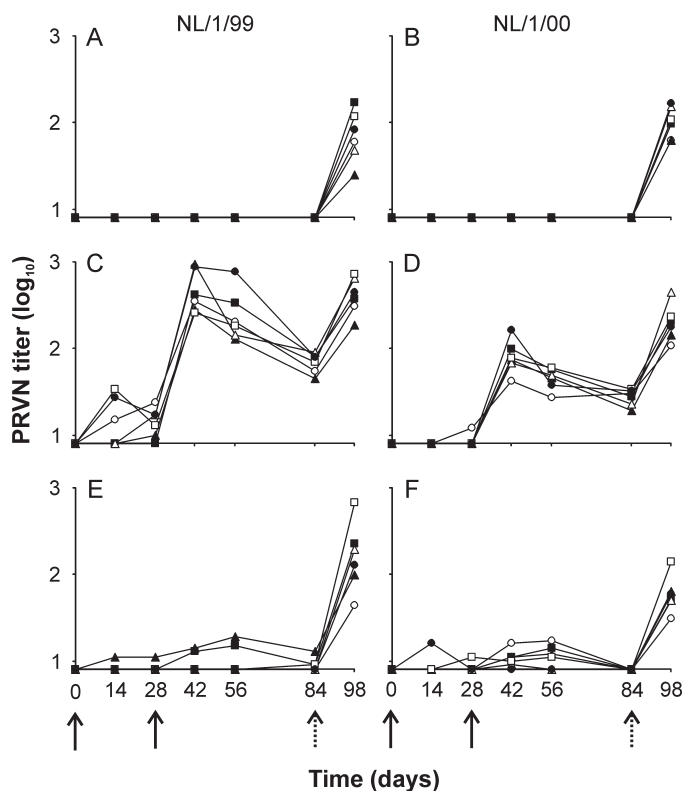
Figure 2. Plaque reduction virus neutralization (PRVN) titers against NL/1/99 (left panels) and NL/1/00 (right panels), after immunization with PBS (A and B), iscom matrix adjuvanted Fsol (C and D) or HMPV$_{M11}$ (E and F). Titers were calculated according to the method of Reed and Muench, and VN titers of individual animals are indicated by different symbols. Time-points of immunizations and challenge infection are indicated by the solid and dotted arrows respectively.

virus isolation in Vero cells or by real-time RT-PCR (data not shown). This demonstrates that the virus was attenuated in cynomolgus macaques. HMPV F-specific antibody levels could be demonstrated in the sera of HMPV$_{M11}$-immunized group after one immunization, but were substantially lower than those detected in Fsol-immunized animals. The HMPV$_{M11}$-induced antibody responses showed a clear booster response after the second immunization. Homologous and heterologous VN antibody levels were around the detection limit of the PRVN-assay after the two immunizations (Fig. 2 e, f). A very low lymphoproliferative response was observed in HMPV$_{M11}$-immunized animals, only marginally higher than those in PBS-immunized animals on the day of challenge infection. HMPV$_{M11}$ thus seemed to be over-attenuated in this macaque model, and displayed limited immunogenicity.

Virus detection after challenge with NL/1/00

Eight weeks after the second immunization, all animals were challenged with a heterologous virus, NL/1/00. No virus could be detected by RT-PCR in throat samples collected at day 1 post-challenge (Fig. 4a). However, at day 3 and 5 virus was detected in all animals at similar levels. The Ct- (cycle

threshold) value indicates the first positive cycle at which viral RNA is detected by RT-PCR. A difference of 3 Ct correlates with approximately 1 Log difference in virus titer (150). At day 9, only one animal was positive in the HMPV$_{M11}$-immunized group, whereas virus could be detected in 5 and 6 out of 6 animals in the Fsol- and PBS-immunized groups, respectively. Virus was also detected in nose-samples, with a similar trend as observed in the throat samples (data not shown). In BAL samples from the groups that received Fsol vaccine or the live-attenuated HMPV$_{M11}$, virus was detected 1 day after challenge, with higher mean Ct-values than in the PBS control group (Fig. 4b). At day 3, the differences in virus loads between the groups were smaller. At the last day of sampling, 9 days post challenge, virus could not be detected in animals immunized with HMPV$_{M11}$, while virus was still present in all animals of the PBS control group, and also in 4 animals of the Fsol-immunized group. Thus, both the Fsol and HMPV$_{M11}$ vaccine reduced the virus load in the lower respiratory tract upon challenge infection. Moreover, the duration of virus shedding was reduced in the lower and upper respiratory tracts of the HMPV$_{M11}$-immunized animals and in some of the Fsol-immunized animals.

Two weeks after challenge, lymphoproliferative responses in PBMC collected from Fsol-immunized animals were higher than observed prior to challenge. Virus neutralizing antibodies were also boosted after challenge, indicating that immunization with Fsol primed for both HMPV-specific
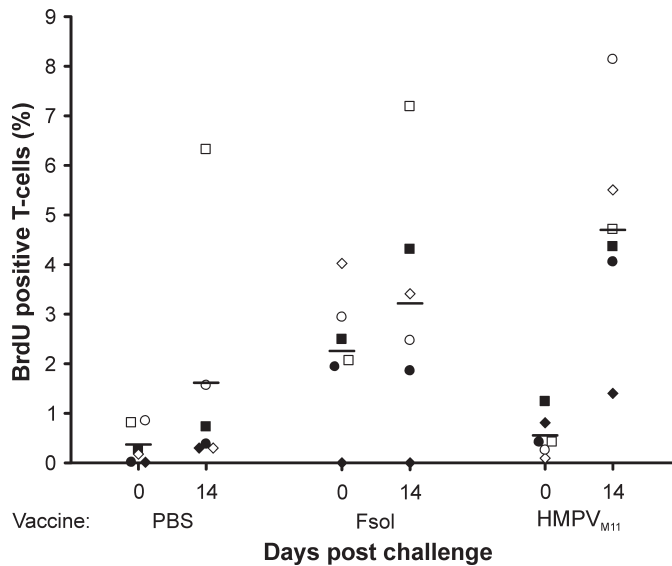
Figure 3. Lymphoproliferative responses in immunized macaques. PBMCs were collected at the moment of challenge, and 2 weeks post challenge infection, and were stimulated in vitro with BPL-inactivated Vero-118 cell antigen or BPL-inactivated HMPV antigen. Culture supernatants were harvested after 4 days, and fresh medium supplemented with BrdU was added. The next day the cells were harvested, after which T-cells that had incorporated BrdU were detected by FACS analysis. Mean percentages of positive cells per group are indicated by the horizontal line, while responses of individual animals are indicated by different symbols.
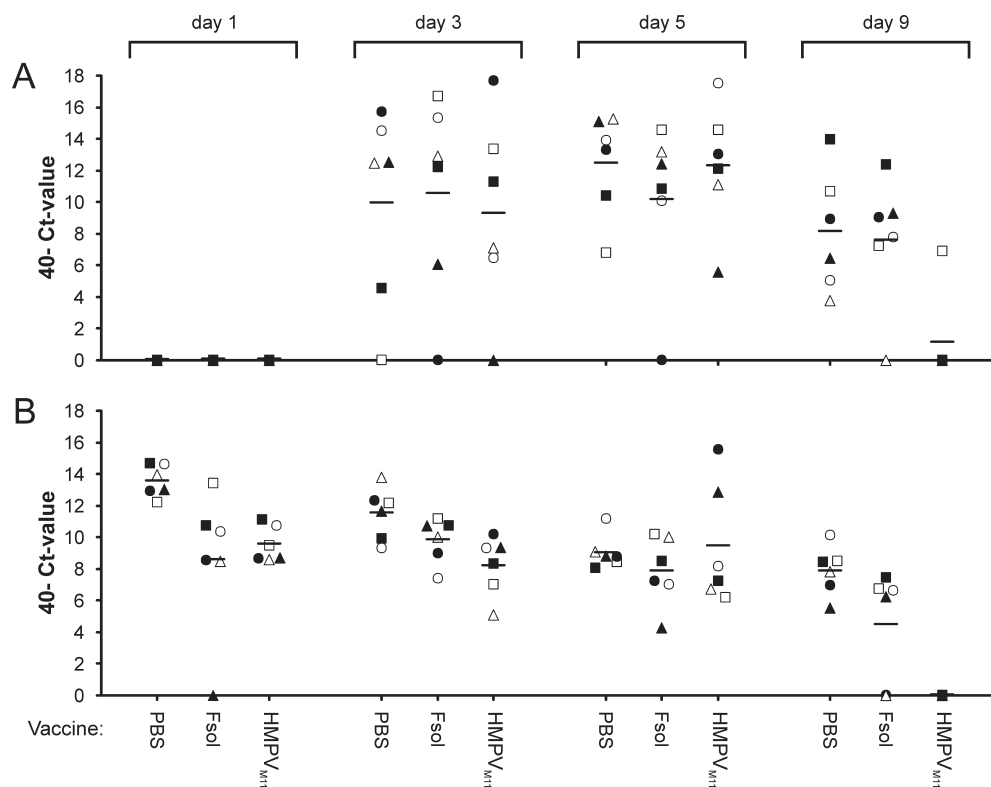
Figure 4. Detection of HMPV by real-time RT-PCR in throat-swabs (A) and BAL samples (B) obtained from immunized cynomolgus macaques at day 1, 3, 5, and 9, after challenge infection with NL/1/00. Ct-values >40 were considered to be negative. Values are given after subtraction of the Ct-value from 40. Mean Ct-values per group are indicated by the horizontal line, different symbols indicate Ct-values of each individual animal.

T-cell and antibody responses. Low antibody levels were present prior to challenge in HMPV$_{M11}$-immunized animals, but an increase in VN titer was observed in all animals 2 weeks after challenge infection, comparable to VN titers induced in the PBS-immunized animals (Fig. 2a, b). As expected, no antibodies were present in the PBS-immunized group, five days after challenge (Fig. 1, t 84), but all animals seroconverted at day 98 (two weeks post challenge) (Fig. 1, 2a, 2b). Prior to challenge, lymphoproliferative responses in animals that had received HMPV$_{M11}$ were only marginally higher than those observed in PBS-immunized animals. However, a significant increase in response was observed two weeks later (Mann-Whitney, *P*<0.05), which was much higher than observed in the PBS-immunized animals.

We thus conclude that immunization with HMPV$_{M11,}$ although not very immunogenic, primed for strong cellular and humoral secondary immune responses, resulting in accelerated clearance of the challenge virus.

DISCUSSION

Development of vaccines to prevent serious HMPV respiratory tract disease in young children, immunocompromised individuals and the elderly is warranted. The immunogenicity and protective efficacy of two candidate vaccines have previously been evaluated in Syrian golden hamsters; an iscom matrix adjuvanted HMPV Fsol subunit vaccine and a cold-passaged, live-attenuated HMPV vaccine, $HMPV_{M11}$. Two immunizations with these vaccines were found to protect hamsters against heterologous virus infection of the lower respiratory tract (109, 110).

Here, the immunogenicity and efficacy of both vaccines was evaluated in a cynomolgus macaque model. Both vaccines induced HMPV-specific immune responses after two immunizations, as determined by the presence of HMPV-specific VN antibodies in serum and/or HMPV-specific T-cell responses in PBMC. We have previously demonstrated in macaques, that immunity against HMPV induces only transient protection (228). In the present study animals were challenged 8 weeks after the last immunization, using a heterologous challenge virus strain. We reasoned that if the vaccine would provide protection from heterologous challenge, it would definitely protect against homologous challenge. Although the animals proved not to be protected, virus shedding was > 1 Log reduced in Fsol- or $HMPV_{M11}$-immunized animals at the peak of virus replication. In addition viral clearance was accelerated, since no virus was present in 2 and 6 out of 6 animals in the Fsol- and $HMPV_{M11}$-immunized groups, respectively, while virus could still be detected in the lower respiratory tract of all PBS-immunized animals at day 9 after challenge infection. At this time point, virus could also not be detected in the lower respiratory tract in 5 out of 6 $HMPV_{M11}$-immunized animals, indicating that the overall duration of virus shedding was reduced. When we calculated the area under the curves in figure 4, as a measurement of the total virus loads, values in the PBS-immunized animals were significantly higher compared to the Fsol- and $HMPV_{M11}$-immunized animals (Mann-Whitney, P<0.05), indicating that immunization with both vaccines significantly reduced virus shedding.

The Fsol-vaccine induced detectable levels of HMPV-neutralizing antibodies in only 3 animals after the first immunization, but all animals seroconverted after the second immunization. However, as previously described for VN-antibodies that were induced after wild type HMPV infection (228), antibody levels declined rapidly, resulting in VN titers that were approximately 50% reduced at the time of challenge infection, compared to peak VN antibody levels, 6 weeks earlier. Lymphoproliferative responses could be demonstrated in Fsol-immunized animals at the day of challenge, in 5 out of 6 animals, which were enhanced 2 weeks later after challenge infection. Iscom matrix is known to have the potential to induce both CD4+ and CD8+ T cells against the antigens with which it is administered, as was first demonstrated by Takahashi et al., using influenza or HIV iscom-based vaccines containing viral envelope proteins (215).

In animals immunized with $HMPV_{M11}$, no vaccine virus could be recovered from the upper respiratory tract, 4 or 14 days after the first and second immunization. HMPV F-specific antibody levels, as determined by ELISA, were demonstrated after the second immunization, but VN antibody levels were only just above the detection limit. No substantial responses were measured in PBMCs

collected on the day of challenge, but a significant increase in proliferating T cells was induced after challenge infection, with levels higher than found in the Fsol-immunized animals and PBS controls. Although no robust immune response seemed to be induced before the challenge infection, it appeared that immunization with $HMPV_{M11}$ resulted in efficient priming of the immune response, as indicated by an obvious secondary immune response after challenge as compared to the PBS-immunized control animals. We speculate that although $HMPV_{M11}$ seemed over-attenuated, the replication of the attenuated virus for at least one round at a multiplicity of infection of 107 was sufficient to prime T and B cell responses and induce mucosal immune responses, resulting in a shortened period of virus shedding in immunized animals.

HMPV-specific immune responses were induced by both vaccines, but these were not sufficient to protect against HMPV lower respiratory tract infection eight weeks later, the main goal of immunization. With the Fsol vaccine, we aimed at inducing protection by inducing high levels of virus neutralizing antibodies. HMPV-specific antibody levels were achieved, but as postulated before, a certain threshold of VN antibody titers may be needed to protect against subsequent infection (228). The VN antibody titers that were induced in this experiment were 10-fold lower than those in Syrian golden hamsters, 4 weeks after immunization (109). At the time of challenge infection in macaques, 8 weeks after the last immunization, VN antibody titers had declined even further. In previous immunization experiments in cotton rats with alum-adjuvanted HRSV F protein, a dose-range study of 0.05, 0.5 and 5 µg F protein, resulted in higher VN antibody titers for the higher protein doses. However, even immunization with the lowest dose resulted in reduced virus replication in the LRT, when challenged 3 or 6 months after immunization. Here, 10 µg F protein was used, in the same dose range as used for HRSV F vaccines in animals and humans (179). Therefore, similarly robust immune responses were expected for HMPV. Dose-range studies in cynomolgus macaques are difficult because of ethical reasons, but eventually a dose-finding study could be considered to increase vaccine efficacy.

The declining levels of HMPV-specific antibodies over time, is an important issue that has to be addressed in future studies. The HMPV-specific immune response induced by immunization should preferably be more efficient than the response induced by wild type virus infection, since it was previously shown that macaques can be reinfected with homologous virus only 12 weeks after the previous infection (228). Moreover, Ebihara et al. showed that reinfections in humans with either homologous or heterologous strains can occur, even during the same winter season (61). In previous vaccination studies in African green monkeys, animals were already challenged 4 or 5 weeks after immunization (12, 178, 205, 218). Longer intervals and heterologous challenge infection should be considered between immunization and challenge, to evaluate the protective efficacy of the administered vaccine, and to study the effect of waning immunity on the induced immune responses.

$HMPV_{M11}$ could not be recovered from throat- and nose-swabs from animals that were immunized with this candidate virus, and the induced immune response was insufficient to protect against infection. This suggests that $HMPV_{M11}$ may be too attenuated to induce protective immunity.

Therefore, the contribution of each individual mutation to the attenuated phenotype should be characterized. Introduction of multiple attenuating mutations may eventually result in enhancement of the attenuated phenotype. Ultimately, viruses harbouring different combinations of mutations may be generated and evaluated, to yield a candidate virus that is highly immunogenic, yet sufficiently attenuated.

In conclusion, the present study showed that immunization with an iscom matrix-adjuvanted Fsol subunit vaccine for HMPV does not provide protection against subsequent heterologous challenge infection, even though HMPV-specific VN antibodies were induced. Our results confirm that the waning immunity against HMPV is a challenging obstacle for vaccine development. Furthermore, the live-attenuated HMPV candidate that was evaluated successfully in Syrian golden hamsters turned out to be over-attenuated in this macaque model. However, this macaque model may not be the most permissive model for HMPV and the attenuated phenotype of $HMPV_{M11}$ may be further evaluated in the more permissive African green monkey model, and eventually humans.

ACKNOWLEDGEMENTS

Low pH induced membrane fusion mediated by human metapneumoviruses F protein is a rare, strain dependent phenomenon

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ABSTRACT

Membrane fusion promoted by human metapneumovirus (HMPV) fusion (F) protein was suggested to require low pH (Schowalter et al., J. Virol. 80:10931-41). Using prototype F proteins representing the four HMPV genetic lineages, we detected low pH dependent fusion only for some lineage A strains. A glycine at position 294, present in only 4% of publicly available HMPV F sequences, was found to be responsible. Other substitutions in F, introduced after repeated virus passage, also influenced membrane fusion, but independently of pH. Thus, adaptation of HMPV to cell cultures may select for more fusogenic F proteins, either pH-dependent or independent.

The human metapneumovirus (HMPV) was first described in 2001 when it was classified as the first mammalian member of the Paramyxoviridae family, subfamily Pneumovirinae, genus Metapneumovirus (16). Sequence analyses of the surface glycoprotein genes of several HMPV isolates revealed the existence of two main genetic virus lineages (A, B), each divided into at least two sublineages (A1, A2, B1, B2) (17).

One of the major HMPV glycoproteins is the fusion (F) protein, which is relatively conserved among HMPV strains (17) and shares structural features with other paramyxovirus F proteins. These proteins are class I viral fusion proteins (18), which are synthesized as inactive precursors, F0, that must be cleaved to yield fusion-competent disulfide-linked F2-F1 chains (reviewed in 7, 8). They mediate fusion of the viral and cell membranes to facilitate virus entry into the cell and commonly promote cell-cell fusion leading to syncytia formation.

The fusion proteins of virus families that enter the cell via acidic endosomes require low pH for triggering membrane fusion (13, 18). In contrast, membrane fusion promoted by paramyxovirus F proteins generally occurs at the cell surface and at neutral pH (6, 11). It is believed that in viruses of the Paramyxovirinae subfamily the F protein is maintained in the virion in a metastable pre-fusion conformation by specific interactions with the attachment protein. Following attachment to the target cell receptor, conformational changes in the attachment protein are transduced to the F protein to activate it for fusion "at the right time and in the right place" (6, 10). However, it is unlikely that this applies to the Pneumovirinae, including human respiratory syncytial virus (HRSV) and HMPV, since spontaneous mutants of HRSV (5) or genetically engineered recombinants of HRSV (14, 15) and HMPV (1) expressing F as the only surface glycoprotein are infectious. In addition, whereas Paramyxovirinae F proteins require cooperation of the attachment protein for syncytia formation, expression of HRSV (3, 20) or HMPV F (12) proteins alone in transfected cells is sufficient to induce syncytia formation. Thus, the precise mechanism of regulating the activation of the F protein of viruses belonging to the Pneumovirinae subfamily remains unclear.

Schowalter et al. (12) recently showed that syncytia formation promoted by the F protein of HMPV, strain CAN97-83, was only detected when cells transfected with an F-expressing plasmid were treated with trypsin (required to cleave the F0 precursor) and exposed to low pH. This low pH dependency suggested a unique way of triggering fusion among paramyxovirus F proteins. To test the generality of this statement, HMPV F mediated membrane fusion was assayed with proteins derived from strains NL/1/00 (lineage A1), NL/17/00 (A2), NL/1/99 (B1), and NL/1/94 (B2) (17). These genes were cloned in plasmid pCAGGS (9) and used in a syncytium formation (SF) assay, essentially as described by Schowalter et al. (12). Briefly, Vero cells growing in 8-well plates were transfected with 1μg of pCAGGS-F plasmids, using Fugene. The next day, cells were incubated for two hours in media with 1 μg/ml trypsin and subsequently exposed to PBS at pH 5 or pH 7.4 for four minutes. This pH-pulse was repeated three times, two hours apart. Twenty-four hours after the last pH-pulse, cells were fixed and syncytia visualized with HMPV F-specific antibodies.

Fusion was also tested in a cell content mixing (CM) assay in which two wells of a 6-well plate
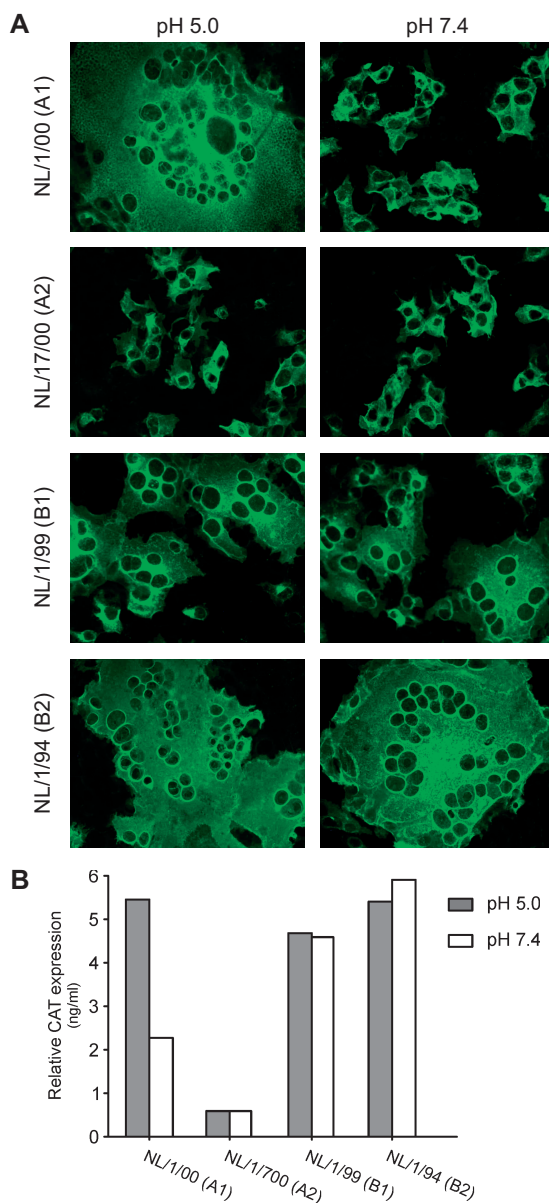
Figure 1. Evaluation of cell-cell fusion mediated by F proteins representing the genetic lineages A1, A2, B1 and B2 of HMPV. Syncytium formation (A) in Vero cells transfected with pCAGGS expression vectors carrying the F genes of the HMPV viruses shown at the left margin (genetic lineage indicated between parentheses). The cells were exposed to low pH (pH 5.0, left panels) or neutral pH (pH 7.4, right panels), before being stained with a rabbit polyclonal anti-F antibody. Content mixing assays (B) were performed in Vero cells transfected with plasmids carrying F, ß-gal and LTR-CAT genes or F, ß-gal and tat genes. The next day, the two cell populations were mixed, followed by low pH (pH 5.0, black bars) or neutral pH (pH 7.4, white bars) treatments. Twenty four hours later, the CAT-values were measured and normalized based on ß-gal expression to correct for differences in transfection efficiency and sample processing.
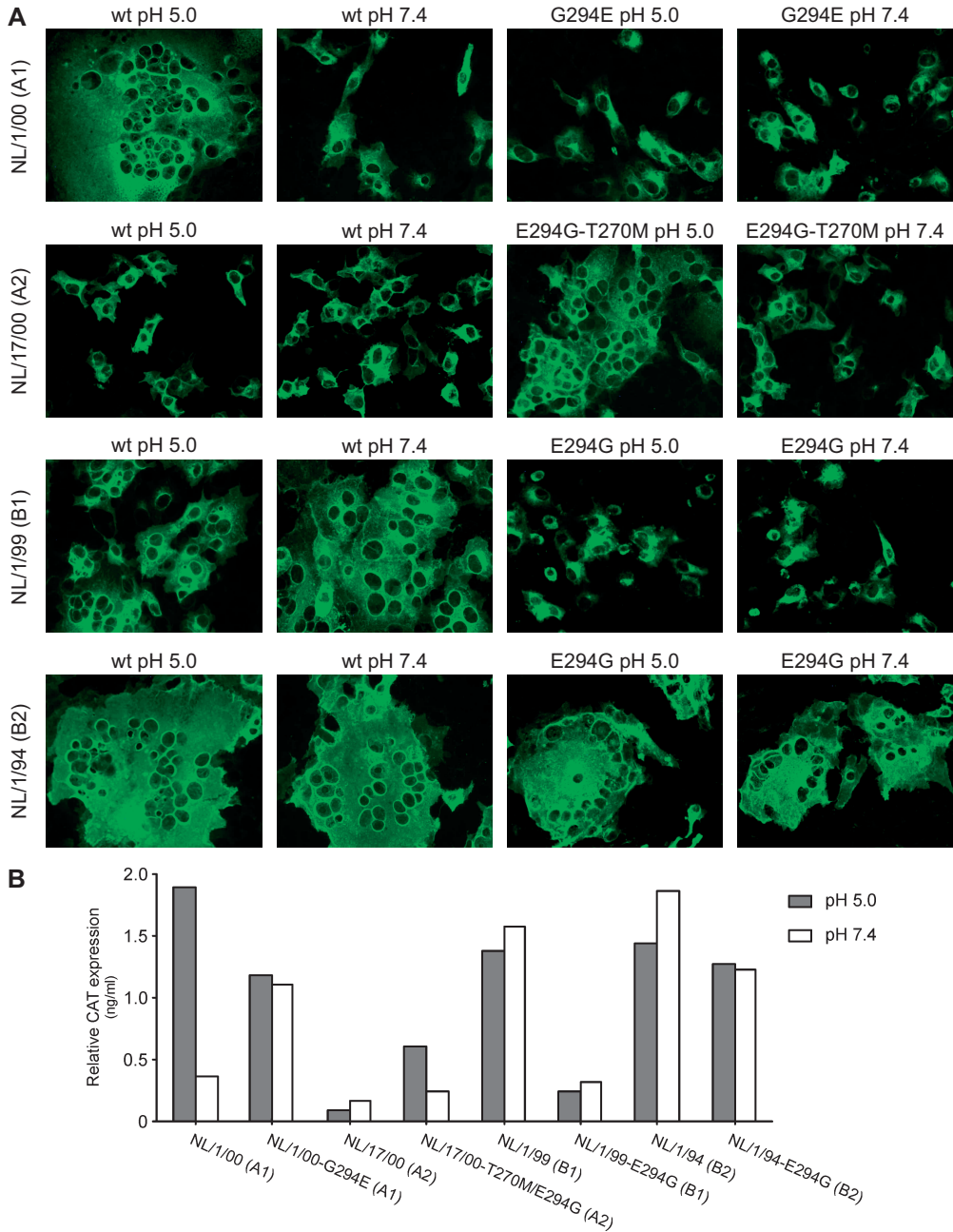
Figure 2. Evaluation of cell-cell fusion with F protein mutants. Syncytium formation assays (A) and content mixing assays (B) were done with the wild type and mutant F proteins shown in each panel, as described in the legend to Figure 1.

containing Vero cells were each transfected with 2µg of pCAGGS-F and 0.4µg of pTS27 plasmid, a constitutive ß-galactosidase (ß-gal) expression vector, using Lipofectamine 2000. One well was co-transfected with 2µg of pLTR-CAT (containing the chloramphenicol acetyl transferase (CAT) gene under control of the HIV-1 LTR) and the other well with 2µg of pTat (expressing the HIV-1 trans-activator of transcription) (4). One day after transfection, both cell populations were harvested using trypsin-EDTA, mixed, and plated in three wells of a 6-well plate. The next morning, cells were exposed to three pH-pulses as described above. Cell lysates were harvested twenty-four hours after the last pH-pulse, and CAT and ß-gal expression was quantified by ELISA. In this assay, cell contents mixing, as a result of F-mediated fusion, resulted in tat-mediated transactivation of the HIV-1 LTR, and hence induction of CAT expression. CAT expression was normalized based on ß-gal expression.

Fig. 1 shows SF and CM assays performed with the four prototype HMPV proteins. Transfection of empty pCAGGS vector or exclusion of trypsin resulted in no measurable membrane fusion in both assays (not shown). A clear low pH dependency could be observed for $F_{NL/1/00(A1)}$, since large syncytia were observed upon exposure to pH 5, whereas only small syncytia were visible after exposure to pH 7.4 (Fig. 1A). In the CM assay, low pH treatment also resulted in significant enhanced CAT-expression (Fig. 1B). Small syncytia and low levels of CAT expression were observed for the $F_{NL/17/00(A2)}$ protein at low and neutral pH. Transfection of $F_{NL/1/99(B1)}$ and particularly $F_{NL/1/94(B2)}$ resulted in large syncytia at low and neutral pH, indicating that fusion promoted by lineage B F proteins was not enhanced at low pH, a finding that was confirmed in the CM assay (Fig. 1). These first results indicated that low pH dependency is not a general phenomenon for the F proteins of HMPV. Similar results were obtained with LLC-MK2 cells in the syncytia formation assay, ruling out that they were cell type specific (not shown).

The results obtained with $F_{NL/17/00(A2)}$ were unexpected since Schowalter et al.(12) had made use of an almost identical F protein, $F_{CAN97-83\ (A2)}$, which differs only in two amino acids, T270M and E294G, from $F_{NL/17/00(A2)}$. Site directed mutagenesis was used to change T270M in $F_{NL/17/00(A2)}$ because all HMPV F sequences in public databases, except this strain, have Met at that position. This change had no effect on F protein activity. The single mutation E294G increased moderately the fusogenic activity of $F_{NL/17/00(A2)}$ in a low pH-dependent manner (not shown). However, it was the double mutation T270M/E294G that increased significantly membrane fusion induced by $F_{NL/17/00(A2)}$, in both SF and CM assays, but only at low pH (Fig. 2), mimicking the results reported for $F_{CAN97-83(A2)}$. It was interesting to note that the other F protein that displayed enhanced fusion upon low pH treatment, $F_{NL/1/00(A1)}$, also had 294G. Therefore, 294G might represent a determinant of low pH enhanced fusion for lineage A F proteins, since the change G294E in $F_{NL/1/00(A1)}$ also resulted in reduced fusion, independent of pH (Fig. 2).

Residue 294 is located at the base of the globular head in a model of the pre-active conformation of the HMPV F trimer (Fig. 3). Since aa294 is near 368H in the 3-D model, it is envisaged that the presence of either 294E or 294G could influence protonation of 368H. Protonation of histidines is important for activation of certain fusion proteins that require low pH for fusion (2). It is then

possible that 294E facilitates protonation of 368H in HMPV F protein even at neutral pH, whereas protonation of 368H may require low pH when it is 294G. This would explain the low pH requirement for fusion mediated by lineage A F proteins that have 294G. However, this does not seem to be the case for lineage B F proteins. Indeed, the change E294G had contrasting effects in $F_{NL/1/99(B1)}$ and $F_{NL/1/94(B2)}$ and different than in lineage A proteins (Fig. 2). In the B1 strain, it inhibited F protein induced fusion at low and neutral pH and in the B2 strain reduced marginally F mediated fusion at either pH. The different responses of lineage A and B proteins to the E294G change are likely determined by other amino acid differences between the two lineages that may alter the local structure of HMPV F near 368H.

The absence of 294G in lineage B F protein sequences in public databases supports the hypothesis that low pH enhanced fusion is restricted to lineage A viruses. Furthermore, because HMPV strains with 294G represent only a small proportion of all lineage A strains (27 of 433 lineage A sequences), low pH dependent fusion is probably a rare characteristic of HMPV.

The $F_{NL/1/94(B2)}$ construct used in Fig. 1 and 2 was derived from a virus stock that had been passaged repeatedly in Vero cell cultures. It appeared that two amino acid substitutions, V231I and Q307R, were introduced upon passaging, compared to the original F sequence (17). Since all HMPV F sequences in public databases contain Val and Gln at these positions, they were reverted in $F_{NL/1/94\ hp}$ (renamed hp, for high passage), to generate $F_{NL/1/94\ lp}$ (low passage). In the SF and CM assays, $F_{NL/1/94\ lp}$ was much less fusogenic than $F_{NL/1/94\ hp}$ at low and neutral pH (Fig. 4). These data suggest that propagation of HMPV in cell cultures may select for mutations that alter the fusogenic properties of the F protein. HMPV isolates are generally poor inducers of syncytia in infected cultures. It is conceivable that in vitro passage may select mutations in the F protein that improve virus replication and perhaps syncytia formation.

Both $F_{CAN97-83(A2)}$ and $F_{NL/1/00(A1)}$ were derived from HMPV strains obtained by virus isolation in cell culture, while the majority of F sequences in the database were derived by direct sequencing of PCR fragments obtained from clinical specimens without virus isolation. The 294G mutation present in both F proteins might thus have been selected for in cell culture systems, just like the mutations in $F_{NL/1/94\ hp}$. The data reported by Schowalter et al. (12) and the data presented here indicate that some HMPV F proteins require low pH for efficient fusion, but that this is an exception rather than a general phenomenon. The trigger for HMPV F protein to initiate the process of membrane fusion thus still remains unknown.

A

B

368H

294E
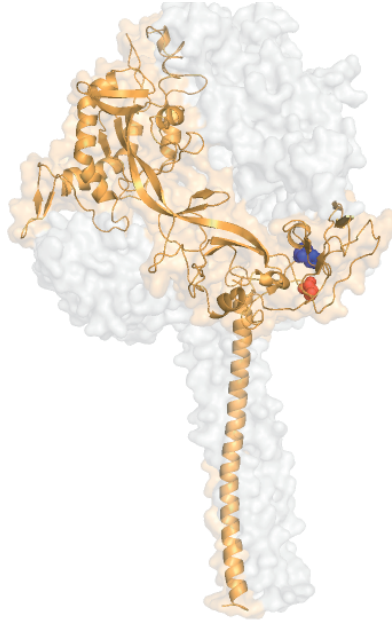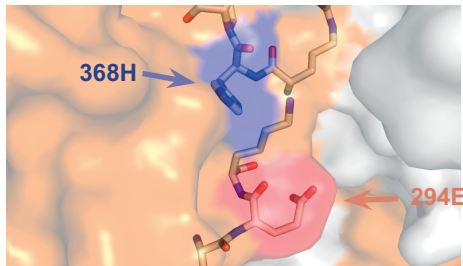
NL/17/00 (A2)
NL/1/99 (B1)
NL/1/94 (B2)

C

368H

294E
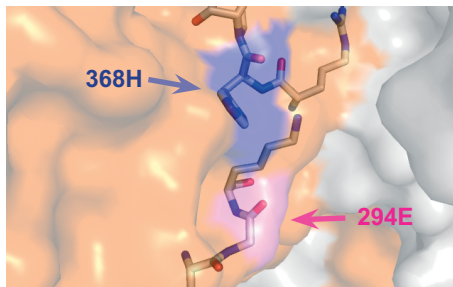
NL/1/00 (A1)
CAN/97/83 (A2)

Figure 3. Location of critical residues in the model of HMPV F protein. A) The model represents the prefusion conformation of the HMPV F trimer, built with the atomic coordinates of the pre-active structure of the parainfluenza virus 5 F protein, reported by Yin et al. (19). One of the monomers is shown as golden ribbon in which residues 368 (blue) and 294 (red) are shown as balls. B) and C) are partial blown ups of the structure showing the lateral side chains of 368H and 294E or 294G. The strains mentioned in the text, and that have either 294E or 294G, are indicated below panels B and C, respectively.
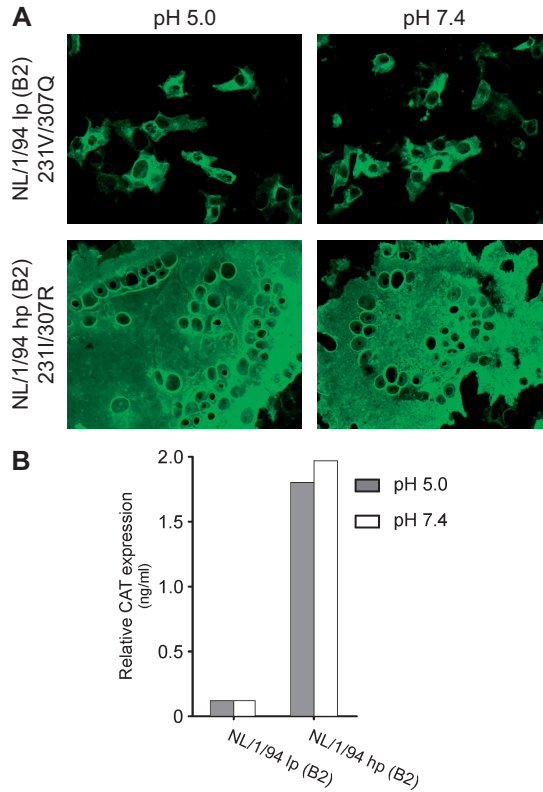
Figure 4. Cell-cell fusion mediated by $F_{NL/1/94(B2)}$. Syncytium formation assays (A) and content mixing assays (B) were performed as described in the legend to Figure 1. The assays were done with the plasmids used in Figure 1 and 2, expressing $F_{NL/1/94(B2)}$, obtained from a virus passed repeatedly in vitro (hp, for high passage), or after reintroduction of the changes I231V and R307Q in the same protein to revert it to the originally reported sequence (17), obtained from low passage (lp) virus.
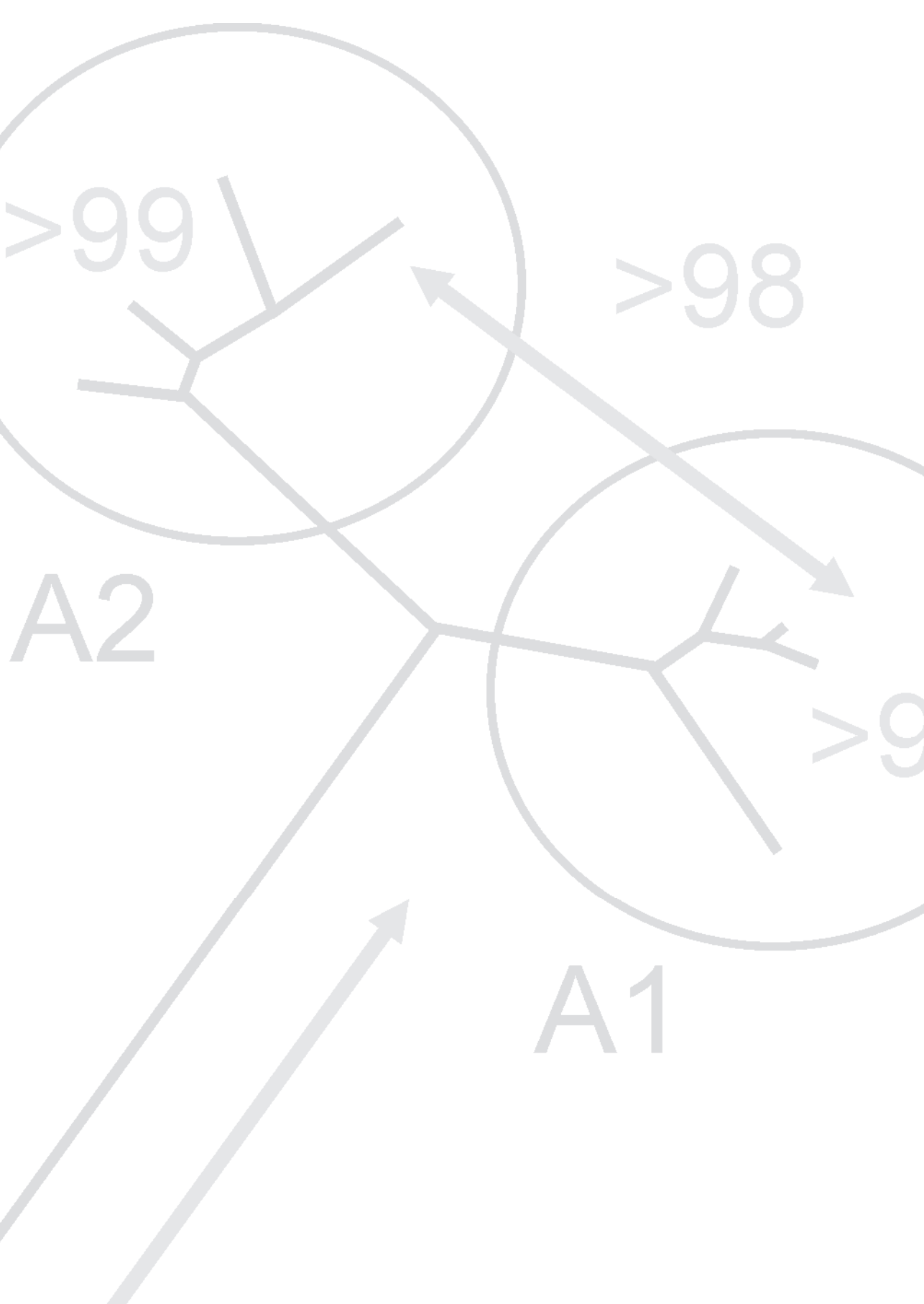
Summarizing discussion

Since the discovery of HMPV, numerous studies have demonstrated that HMPV is responsible for a substantial proportion of lower respiratory tract (LRT) infections in infants and young children and is second only to HRSV as a cause of bronchiolitis in early childhood (18, 78, 82, 104, 160, 231, 233, 244). A wide spectrum of clinical symptoms associated with HMPV infection, ranging from mild upper respiratory tract (URT) infection to severe respiratory disease requiring hospitalization, has been reported in patients of all ages. Whereas HMPV-infected adults usually suffer from relatively mild common cold-like respiratory symptoms (67, 123, 212, 225), HMPV-induced disease in children, patients with underlying disease, immune-compromised individuals and fragile elderly, tends to be more severe (17, 65, 174, 231).

GENETIC AND ANTIGENIC VARIABILITY OF HMPV

The relatively high clinical impact of HMPV warrants the development of a vaccine. However, the extent of genetic and antigenic variability of the major HMPV transmembrane glycoproteins, the most logical targets for vaccine development, was unknown. Therefore, we addressed the genetic and antigenic diversity of HMPV by analysis of 84 partial fusion (F) protein sequences, 4 complete F protein sequences and 35 complete attachment (G) protein sequences (chapter 2) (229). Phylogenetic analyses of these sequences revealed the existence of two main genetic lineages (A and B) which each can be divided into two sublineages (A1, A2, B1 and B2). As previously described for HRSV (120, 213) and AMPV (164, 199), the F proteins of HMPV were highly conserved. In contrast, the nucleotide and predicted amino acid (aa) sequences of HMPV G proteins showed low sequence conservation, as low as 30% - 37% aa identity, similar to data for HRSV and AMPV (Fig. 1) (122, 126). Besides the high sequence variation, we observed variation in the lengths of the different predicted G ORFs. The genetic variability of HMPV genes has been studied by many other research groups, yielding results that were in agreement with the data described here (6, 14, 20, 118, 233). Next, we tested ferret serum samples raised against viruses from the four sublineages in virus neutralization (VN) assays, to address the antigenic relationship between members of the different HMPV lineages. Studies with serum samples collected 21 days post infection showed that viruses within the main lineages (A or B) were antigenically closely related. The homologous VN titers for members of the two lineages were 12- to 128-fold higher than heterologous titers, indicating a difference in antigenicity between lineage A and B. Classic virology studies have used a definition of a homologous-to-heterologous virus neutralization titer ratio of >16 for defining serotypes. This same definition noted that if neutralization shows a certain degree of cross-reaction between two viruses in either or both directions (homologous-to-heterologous titer ratio of 8 or 16), distinctiveness of serotype is assumed if substantial differences in sequences are observed. On the basis of our results, and the definition mentioned, we proposed to define the two main lineages of HMPV as serotypes A and B. However, the existence of serotypes has not been confirmed with serum samples from some other animal species (12, 205), and is thus still subject of debate.
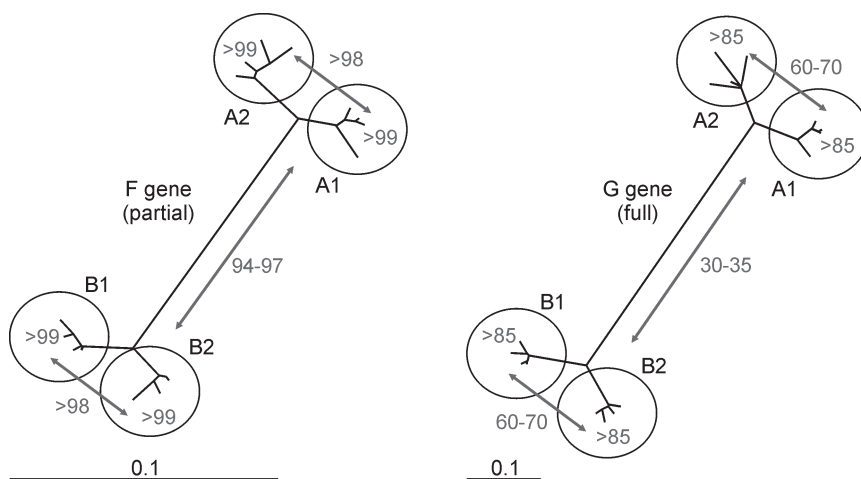
Figure 1. Phylogenetic trees for the fusion (F) and attachment (G) genes of selected HMPV isolates. From each of the four genetic lineages, four representative isolates were selected, and maximum likelihood trees were generated for the G gene (right) and 451 nucleotides in the F gene (left). Numbers in trees represent percentages amino acid identity between virus isolates.

REVERSE GENETICS

We selected two HMPV isolates, NL/1/00 and NL/1/99, representing the two main genetic lineages A1 and B1 respectively (227), for the establishment of reverse genetics systems (chapter 3) (108). These viruses were selected because they were found to replicate efficiently in tertiary monkey kidney cells and Vero cells. Two different approaches were followed to determine the extreme termini of the viral genomes of NL/1/00 and NL/1/99. The first approach, in which viral RNA was circularized by T4 RNA ligase, followed by RT-PCR amplification and sequencing, resulted in leader sequences that were 2 or more nucleotides shorter than expected from the leader sequence of HRSV (155). For this reason, a 3'RACE method was used as an alternative strategy to determine the cDNA leader sequence of HMPV, revealing the presence of an additional AC dinucleotide in a proportion of the sequenced clones. The leader and trailer of HMPV appeared to display less complementarity as compared to those of HRSV and AMPV. The newly derived sequences of the genomic termini were used to generate minigenome constructs, containing CAT or GFP as reporter genes, resulting in pSP72-P_{T7}-Tr-CAT-Le-δ-T_{T7} and pSP72-P_{T7}-Tr-GFP-Le-δ-T_{T7} (P_{T7}: T7 promotor, Tr: trailer, Le: leader, δ: hepatitis delta virus ribozyme, T_{T7}: T7 terminator). Two or three G residues were placed between the T7 promotor and the end of the trailer sequence to increase transcription from the T7 promoter (171). When plasmids encoding the viral N protein, the polymerase proteins L and P, and the transcription antitermination protein (also called transcription elongation factor) M2.1, also under the control of T7-promotors, are co-transfected with the minigenome, expression of the reporter gene can be measured. By using these minigenome assays, we could test whether the sequences of the genomic termini were correct and the plasmids encoding the N, P, L and M2.1

proteins were functional. The N, P and L proteins were found to be indispensable in minigenome assays, whereas the M2.1 protein was not.

When the CAT ORF of the minigenome construct was replaced by a full-length cDNA copy of the NL/1/00 or NL/1/99 genome, we recovered infectious virus from transfected cells. An additional *AflII* restriction site that was introduced in the N gene did not affect the efficiency of virus replication *in-vitro*. This additional AflII restriction site was used to confirm that the rescued viruses were recombinants. Different ways of delivering T7 polymerase to the reverse genetics system were tested. The use of BSR-T7 cells constitutively expressing T7 polymerase, 293T-cells which were cotransfected with a T7 polymerase expressing vector, or 293T cells that were infected with fowlpox virus expressing the T7 RNA polymerase, all resulted in the rescue of recombinant virus. However, the BSR-T7 cells yielded higher virus titers shortly after transfection, perhaps due to higher expression of T7 RNA polymerase in all cells. Furthermore, we were able to rescue recombinant NL/1/99 using both positive and negative sense full-length cDNA plasmids, revealing that the efficiency with negative sense cDNA resulted in lower titers compared to positive sense cDNA ($10^{5.7}$ and $10^{7.2}$ TCID50 respectively). This was in agreement with the general belief that for rescue of negative sense nonsegmented viruses, the simultaneous presence of viral mRNAs and naked negative-sense genomic RNA will result in hybridization, thereby preventing the assembly of the genome into the RNP.

We were able to rescue virus in the absence of the M2.1 expression vector, however it was shown for HRSV that the simple expedient of omitting an expression plasmid is invalid for evaluating recovery requirements (35). Later research by others revealed that HMPV M2.1 deletion mutants, in contrast to HRSV, could be recovered, but were found to be replication deficient in hamsters (23).

The various reverse genetics systems that are available for paramyxoviruses in general (5, 39, 81, 103, 114, 185, 253) and recently also for AMPV (88) and HMPV (15, 108), facilitate specific manipulation of the viral genome, and are thus important tools to design live attenuated vaccine strains, and for fundamental and applied paramyxovirus research. For example, the introduction of GFP into the third position of the viral genome of both HMPV NL/1/00 and NL/1/99 resulted in viruses that could be used in a plaque reduction virus neutralization (PRVN) assay for HMPV (50). This improved PRVN assay was faster, more suitable for high throughput testing, and 10-fold more sensitive than conventional virus neutralization assays (50).

PROTECTIVE IMMUNITY AGAINST HMPV

In previous experiments, we established a cynomolgus macaque infection model (*Macaca fascicularis*) for HMPV, using 10^5 TCID$_{50}$ of the serially passaged wild-type virus isolate NL/1/00 (the A1 lineage prototype) (135). Because molecularly cloned viruses (NL/1/00 and NL/1/99), representing both genetic lineages of HMPV had become available (108), we wished to re-evaluate this model. Furthermore, we wished to evaluate the duration and specificity of antibody responses upon repeated infections with molecularly cloned prototype viruses (chapter 4) (228).

Inoculation with 10^6 $TCID_{50}$ of molecularly cloned NL/1/00 resulted in infection of the respiratory tract and seroconversion in macaques. Inoculation with cloned NL/1/99 virus did not result in robust infection, since lower virus genome copy numbers were obtained, and we were not able to re-isolate virus from nose or throat samples. For this reason we used 10^7 $TCID_{50}$ as a standard dose for all subsequent inoculations with cloned low-passage stocks of NL/1/00 and NL/1/99. However, even after inoculations with 10^7 $TCID_{50}$ NL/1/99, infectious virus could not be recovered. In studies with African green monkeys (AGMs) 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 (149, 205). Thus, it is possible that lineage B viruses in general replicate less efficiently in non-human primate models. The rather high dose that was required for robust HMPV infection was previously also seen for HRSV infections in these animals (51).

Next, we evaluated the protective immunity in cynomolgus macaques against homologous and heterologous re-infection with HMPV at different time points after primary infection. Macaques were protected from homologous challenge infection at 4 – 6 weeks after primary infection, which was in agreement with data obtained from AGMs and chimpanzees (12, 205). However, challenge at 11 months after the last infection resulted in a robust infection, despite two or three previous infections. Additionally, a homologous challenge 12 weeks after the primary infection resulted in detection of viral genomes in the respiratory tract, although with lower titers than in 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, largely dependent on infection history. Furthermore, these data clearly demonstrated that immunity against HMPV wanes over time and that vaccination studies should thus not only test protection at 4–6 weeks after vaccination, but should also aim at inducing protection for a longer period of time. Although VN antibody titers were still present 8 months after the previous inoculation, animals were not protected against subsequent inoculation with HMPV. This indicated that the animals need a certain threshold of VN antibody titer or that other correlates of protection, such as mucosal or cellular immunity, must exist.

We previously proposed to define the two main lineages of HMPV as serotypes A and B (chapter 2), but the relevance of this conclusion was subsequently challenged by others (12, 205). The macaque re-infection study showed that also in non-human primates homologous titers were, in general, higher than heterologous titers. Although the full impact of homologous and heterologous protection is still not resolved, it seemed that the heterologous challenge at 8 months was more successful than the homologous challenge. Our data demonstrated that only after infection with both virus serotypes, VN antibody titers were equally high to both lineages of HMPV, which indicates that for optimal protection both types of viruses should be included in future vaccines, especially when vaccines target the variable surface glycoproteins G and SH.

VACCINATION APPROACHES TO COMBAT HMPV

Since the discovery of HMPV, several research groups have developed vaccine candidates that may

be used to protect different risk groups against HMPV-induced respiratory disease. As expected from our knowledge of HRSV and AMPV, it was found that antibodies induced against the relatively conserved HMPV F protein correlated with protection in animal models (204, 218). The studies in both rodent and non-human primate models were promising, but none of the vaccine candidates has been tested in human volunteers yet (111). Here, we discuss the immunogenicity and protective efficacy of a variety of subunit, live attenuated virus, inactivated virus and cytotoxic T-lymphocyte epitope vaccines that have been evaluated in animal models.

Subunit vaccines

In chapter 5, we evaluated the antigenicity, immunogenicity and cross-protective efficacy of a soluble HMPV F protein (Fsol) subunit vaccine in Syrian golden hamsters (109) (Table 1). Because the F glycoprotein is one of the major targets of HRSV- and HMPV-neutralizing antibodies (204, 218), we produced recombinant Fsol proteins for the prototypes of the two main genetic lineages of HMPV, NL/1/00 (A1) and NL/1/99 (B1). Immunization of animals with Fsol alone resulted in low antibody titers. Therefore, three adjuvants (Alum, Specol and iscom matrix) were tested to induce higher neutralizing antibody titers. Addition of Alum to the Fsol proteins resulted in similar antibody levels as induced by the non-adjuvanted Fsol, but addition of Specol or iscom matrix to the Fsol protein enhanced the HMPV-specific antibody response.

Next we tested the iscom matrix and Specol adjuvanted Fsol vaccines for protective efficacy against homologous and heterologous infection. First, we developed a challenge model for NL/1/00 infections in Syrian golden hamsters. Using this infection model in an immunization/challenge experiment, none of the vaccines induced complete protection against URT infection, although viral titers in the nose of adjuvanted Fsol immunized animals were significantly lower than those in the PBS immunized animals. However, the primary goal of immunization against respiratory viruses is prevention of serious LRT illnesses. All animals immunized with Fsol adjuvanted with iscom matrix or Specol were protected from infection of the lungs, compared to three animals in the PBS immunized group (Mann-Whitney test, P=0,038). For HRSV it was demonstrated that URT protection requires significantly higher antibody levels than protection of the LRT (200). Therefore, a third vaccination, or alternative adjuvants, could be considered to increase the antibody levels in order to achieve protection of the URT.

Both the adjuvanted Fsol/1/00 and Fsol/1/99 proteins induced higher homologous than heterologous VN antibody titers, again pointing at serological differences between the two main lineages of hMPV, as described before (229). However, immunity raised against the NL/1/99 F protein provided cross-protection against heterologous NL/1/00 virus infection of the LRT. Although we demonstrated cross-protection in only one direction, it seems likely that cross protection will be obtained in both directions. This has indeed been observed with the F protein in its native form (205, 218). The availability of the F protein in high quantities in combination with the demonstrated beneficial use of the safe adjuvant iscom matrix in hamsters, made this F subunit vaccine an excellent candidate for further exploration in a non-human primate model.

The immunogenicity of this iscom matrix-adjuvanted was therefore evaluated in a cynomolgus macaque model. Groups of 6 animals were immunized twice, with a 4-week interval, with Fsol or PBS. Animals were challenged 8 weeks after the last immunization, because we have previously demonstrated in macaques, that immunity against HMPV induces only transient protection (228). None of the animals was protected against subsequent heterologous infection with HMPV NL/1/00. However, the period of virus shedding in the lower respiratory tract was reduced in 2 out of 6 animals in the Fsol-immunized group. Detectable levels of HMPV-neutralizing antibodies were induced in only 3 animals after the first immunization, but all animals seroconverted after the second immunization. However, as previously described for VN-antibodies that were induced after wild type HMPV infection (228), antibody levels declined rapidly, resulting in VN titers that were reduced with approximately 50% at the time of challenge infection, compared to peak VN antibody levels, 6 weeks earlier. Lympho-proliferative responses could be demonstrated in Fsol-immunized animals at the day of challenge, in 5 out of 6 animals, which were enhanced 2 weeks later after challenge infection. Iscom matrix is known to have the potential to induce both CD4+ and CD8+ T cells against the antigens with which it is administered.

With the Fsol vaccine, we aimed at inducing protection by the induction of high levels of virus neutralizing antibodies. HMPV-specific antibody levels were achieved, but as postulated before, a certain threshold of VN antibody titers may be needed to protect against subsequent infection (228). This indicated that the declining levels of HMPV-specific antibodies over time, is an important issue that has to be addressed in the future.

The protective efficacy of a soluble F protein of HMPV lacking the transmembrane domain (FΔTM) has also been tested in cotton rats by Cseke *et al.* (47) (Table 1). In this study, three different prime/boost protocols were compared for their ability to induce protection: 2 immunizations with 100 μg of plasmid DNA encoding HMPV F, 2 immunizations with 25μg adjuvanted FΔTM protein, and a primary immunization with DNA plasmid followed by a boost-immunization with adjuvanted FΔTM. The highest serum neutralizing antibody titers were induced by the FΔTM protein. In these animals, virus replication was significantly reduced in the URT and more than 1.500 fold reduced in the LRT after homologous virus challenge infection.

Live attenuated vaccines
Live attenuated vaccines (LAV) have the advantage of mimicking a natural infection and may provide protection against subsequent infections without inducing enhanced disease. The development of reverse genetics systems for HMPV provided a powerful tool to design LAV (15, 108). A number of strategies for attenuating HRSV have been explored in the past, which may be helpful for the development of LAV against HMPV.

Cold-passaged temperature-sensitive HMPV
In chapter 6, we describe a classical method for obtaining live attenuated viruses (LAVs), by passaging HMPV at low temperatures (110) (Table 1). HMPV NL/1/99 was passaged at gradually decreasing temperatures, until passage 35 at a temperature of 25°C was reached. After sequencing of

Candidate vaccine	Animal model	Outcome	Reference
Soluble F protein	Hamsters, cynomolgus macaques	Immunization with adjuvanted soluble F protein induced complete protection of the LRT against heterologous challenge infection in hamsters. In macaques, both humoral and cellular immune responses were induced, but animals were not protected against challenge infection.	Herfst et al. (109, 113)
Soluble F protein and F DNA vaccine	Cotton rats	Viral replication after challenge infection was 10 fold reduced in the LRT. No significant reduction was observed in the URT compared to non-immunized animals.	Cseke et al. (47)
Cpts-virus	Hamsters, cynomolgus macaques	Viruses with cp-HMPV or cp-HRSV mutations protected against heterologous LRT infection in hamsters. In macaques, cp-HMPV-immunization primed for good cellular response. Animals were not protected, but duration of virus shedding was reduced.	Herfst et al. (110, 113)
B/HPIV3 expressing HMPV F	Hamsters, AGM	B/HPIV3 hMPV F2 was sufficiently attenuated in rhesus monkeys. Immunization of AGMs resulted in complete protection of the LRT and virus titers in the URT were > 100 fold reduced.	Tang et al. (218, 219)
HMPV deletion mutants	Hamsters, AGM	ΔG and ΔM2-2 viruses were attenuated in AGMs. After challenge, virus shedding in the LRT was virtually undetectable.	Biacchesi et al. (12, 16); Buchholz et al. (23)
Chimeric HMPV/ AMPV-C	Hamsters, AGM	The P-chimera was attenuated 100 and 1.000 fold in the URT and LRT of AGMs. Protective efficacy is comparable with wt HMPV.	Pham et al. (178)
FI-HMPV	Macaques, cotton rats	Eosinophilic bronchitis and bronchiolitis, indicative of a hypersensitivity response in macaques. Almost complete protection in lungs, but dramatic increase in lung pathology in mice.	De Swart et al. (52); Yim et al. (252)
HI-HMPV	Mice	More significant lung inflammation associated with eosinophilic infiltration, 26 % of mice died one day post immunization with HI-HMPV in incomplete Freund's adjuvant.	Hamelin et al. (98)
T-lymphocyte vaccine	Mice	Reduced viral load in the lungs of HMPV challenged mice. Enhanced expression of Th1-type cytokines in lungs and regional lymph nodes. Reduced Th2-type cytokines.	Herd et al. (107)

Table 1. FI: formalin inactivated, HMPV: human metapneumovirus, B/HPIV3: bovine/human parainfluenzavirus type 3, AGM: African green monkeys, F: fusion protein, LRT: lower respiratory tract, URT: upper respiratory tract, G: attachment protein, M2-2: second ORF of M2, P: phosphoprotein, wt: wild-type.

this passage 35 virus, 19 nucleotide mutations were found, resulting in 17 aa substitutions. Since we were not able to rescue recombinant virus with all 19 mutations, we tested whether viruses with a subset of the mutations of the passage-35 virus could be rescued. A virus with 11 of the 19 mutations (HMPV$_{M11}$, 1 silent, 10 non-silent), turned out to have a ts-phenotype in vitro, whereas HMPV$_{M8}$, with 8 of the 11 cp-mutations, did not. The only differences between HMPV$_{M11}$ and HMPV$_{M8}$ were two mutations in the L gene, and one mutation in the P gene. HMPV$_{M9}$, containing all mutations of HMPV$_{M8}$ and the mutation in the P gene was not temperature sensitive. A recombinant virus containing only the two mutations in L also did not display a ts-phenotype. Therefore, it seems likely that one or both of the L mutations in combination with one or more other mutations was responsible for the ts-phenotype of HMPV$_{M11}$.

Sequence comparison of HMPV with different cp-HRSV strains resulted in the identification of four cp-HRSV mutations that could be introduced in the HMPV genome. Only when three of these mutations were introduced in the HMPV genome, thereby omitting the L1321 mutation, virus could be rescued (HMPV$_{HRSV3}$). Virus replication curves generated at different temperatures revealed that HMPV$_{HRSV3}$ was restricted for replication at 39°C and 40°C, indicating that this virus had a ts-phenotype.

Both HMPV$_{M11}$ and HMPV$_{HRSV3}$ were found to be attenuated in hamsters, with ~10.000-fold reduction of virus replication in the URT, and no detectable virus in the LRT as compared to wild-type virus. Although HMPV-specific antibody titers were slightly lower in immunized animals as compared to animals inoculated with wild-type virus, immunized animals were completely protected from HMPV LRT infection, and virus titers in the URT were reduced to the same extent as seen in hamsters exposed to wild-type HMPV. The high degree of attenuation and the high level of immunogenicity suggested that HMPV$_{M11}$ and HMPV$_{HRSV3}$ represent excellent candidate LAVs for further exploration to prime the HMPV-specific immune response in naïve individuals.

For this reason, we evaluated the immunogenicity of HMPV$_{M11}$ in cynomolgus macaques. Six animals were immunized twice, with a 4-week interval, and were subsequently challenged 8 weeks after the second immunization. Vaccine virus could not be recovered from the upper respiratory tract of immunized animals, 4 or 14 days after the first and second immunization. HMPV F-specific antibody levels, as determined by ELISA, were demonstrated after the second immunization, but VN antibody levels were only just above the detection limit. No substantial responses were measured in PBMCs collected on the day of challenge, but a significant increase in proliferating T cells was induced after challenge infection, with levels higher than found in the Fsol-immunized animals and PBS controls. Although no robust immune response seemed to be induced before the challenge infection, it appeared that immunization with HMPV$_{M11}$ resulted in efficient priming of the immune response, as indicated by an obvious secondary immune response after challenge as compared to the PBS-immunized control animals. None of the animals was protected against heterologous infection with HMPV NL/1/00, but the period of virus shedding was reduced and peak virus titers in the upper respiratory tract were reduced by ~ 1 log. Virus could not be detected at day 9 after challenge infection in the upper respiratory tract of HMPV$_{M11}$-immunized animals, and virus

was only present in the lower respiratory tract of 1 animal at this timepoint. In contrast, virus could still be detected in the upper and lower respiratory tract of all PBS-immunized animals 9 days after challenge.

Immunization with HMPV$_{M11}$ thus did not result in robust protection against infection, which suggests that HMPV$_{M11}$ may be too attenuated to induce protective immunity. Therefore, the contribution of each individual mutation to the attenuated phenotype should be characterized. Different combinations of these attenuating mutations may be generated and evaluated, to yield a candidate virus that is highly immunogenic, yet sufficiently attenuated.

Vectored vaccines
Tang et al. (2003) described the first LAV against HMPV, a chimeric bovine PIV3 harboring the F and HN genes of human PIV3 (Table 1). This B/HPIV3 virus was used as a vector to express the F protein of HMPV from position 2 of the genome (B/HPIV3/HMPV F2) (219). Immunization of hamsters and AGMs with B/HPIV3/HMPV F2 induced both HPIV3 and HMPV-specific neutralizing antibodies that protected against HPIV3 and HMPV challenge infection, 4 weeks after immunization (218, 219). In AGMs, no HMPV replication was observed in the LRT after challenge infection and the virus titer in the URT was more than 100 fold reduced. Additional studies in rhesus monkeys demonstrated that the B/HPIV3/HMPV F2 virus replicated to the same extend as a recombinant bovine PIV3 (rBPIV3), that was previously found to be attenuated and safe in human infants (133, 218).

Deletion mutants
Recombinant HMPVs lacking the small hydrophobic (ΔSH), attachment (ΔG) or second ORF of the M2 (ΔM2-2) proteins were constructed by Biacchesi et al. and Buchholz et al. These viruses replicated efficiently in vitro, while being attenuated in vivo (16, 23) (Table 1). After initial experiments in hamsters, replication kinetics, immunogenicity and protective efficacy of these viruses were studied in AGMs (12). Replication of ΔG and ΔM2-2 viruses was reduced 6 and 160 fold in the URT and 3.200 and 4.000 fold in the LRT respectively, whereas viral titers for the ΔSH virus were only slightly lower as compared to wild-type HMPV. Upon challenge infection, 5 weeks after immunization with the virus deletion mutants, only trace amounts of virus were detected in the URT, and virus shedding in the LRT was virtually undetectable.

Chimeric viruses
Pham et al. (2005) generated chimeric viruses by replacing the N or P proteins of HMPV with their counterparts of AMPV-C, a close relative of HMPV that causes respiratory illnesses in poultry (178) (Table 1). In hamsters, high levels of protective neutralizing antibodies were induced after intranasal infection with such chimeric metapneumoviruses, although virus titers in the lungs and nasal turbinates were approximately 100-fold reduced compared to wild-type HMPV at three dpi. At five dpi, there was only a small difference between viral titers of the chimeras and wild-type HMPV. In AGMs, the N-chimera replicated to ~ 10-fold lower titers in the lower respiratory tract (LRT), whereas the P-chimera was reduced 100 - 1.000 fold in the upper and lower respiratory tract. All

animals were challenged 4 weeks after immunization. The immunogenicity and protective efficacy of both chimeras were comparable with wild-type HMPV.

Inactivated virus vaccines

Formalin-inactivated HMPV
As described before, immunization with FI-HRSV or FI-MV in the 1960s, resulted in predisposition for enhanced disease upon natural infection (79, 134). We immunized young macaques with a FI-HMPV preparation formulated in alum, and observed induction of virus-specific humoral and cellular immune responses associated with production of Th2-cytokines (52). Upon challenge with HMPV three months after the third vaccination, none of the animals were protected against infection and all FI-HMPV-primed animals developed an eosinophilic tracheo-bronchitis six days after challenge infection, indicative of a hypersensitivity response.

Comparable results were obtained by Yim et al. in cotton rats that were immunized twice with a FI-HMPV preparation. In contrast to the macaque study, immunized animals showed almost complete protection from viral replication in the lungs. However, there was a dramatic increase in the lung pathology, particularly interstitial pneumonitis and alveolitis with elevated serum neutralizing antibody titer prior to challenge (252).

Heat-inactivated HMPV
Hamelin et al. immunized BALB/c mice intraperitoneally with supernatant from heat-inactivated (HI-) influenza A virus or HI-HMPV in incomplete Freund's adjuvant, or with HI-HMPV without adjuvant (98). Animals were challenged after three immunizations, two weeks apart. On day 1, 26% of mice immunized with inactivated HMPV and adjuvant died, compared to none in the other groups. Infection of mice previously immunized with HI-HMPV, with or without adjuvant, resulted in enhanced pulmonary disease associated with an important eosinophilic infiltration in BAL and a Th2-cytokine response.

Thus, as for MV and HRSV, immunization with inactivated and adjuvanted HMPV vaccines seemed to predispose for enhanced disease upon subsequent infection.

T-lymphocyte vaccine
Herd et al. used predictive bioinformatics, peptide immunization, and functional T-cell assays to identify HMPV cytotoxic T-lymphocyte (CTL) epitopes in mice (107). The CTL epitopes defined in this study were recognized by mouse T cells restricted through several major histocompatibility complex class I alleles, including HLA-A*0201. Peptide immunization reduced viral load in the lungs of HMPV challenged mice and was found to enhance the expression of Th1-type cytokines in lungs and regional lymph nodes. Additionally, it was shown that levels of Th2-type cytokines (IL-10 and IL-4) were significantly lower in HMPV CTL epitope-immunized mice challenged with HMPV. This study established the proof of principle of protection against HMPV infection by immunization with HMPV CTL epitopes.

VACCINATION AGAINST HMPV: CONCLUDING REMARKS

Experiences with inactivated virus or purified protein vaccines for HRSV and MV demonstrated that caution is warranted when exploring these vaccines. Immunization of naïve individuals with such vaccines can prime for enhanced disease upon subsequent exposure to virus infection. Studies in macaques and cotton rats revealed that immunization with FI-HMPV induced enhanced pathology in the lungs of animals after subsequent infection with HMPV (52, 252), suggesting that the problems observed with HRSV and MV vaccines may also apply to HMPV. A variety of alternative vaccination strategies has been explored to protect against HMPV-induced respiratory illness. LAV may be useful in young children to prime the HMPV-specific immune response, while inactivated or subunit vaccines may be useful to boost the immune response in individuals that have previously been exposed to HMPV. Cynomolgus macaques that had been inoculated three times with HMPV within 10 weeks, were not protected against challenge infection 8 months after the last inoculation (228). This indicated that only transient protective immunity was induced after wild-type HMPV infection, and that an effective candidate vaccine for HMPV should ideally be more immunogenic and protective than natural HMPV infection. F subunit vaccines seem to be promising for boosting the HMPV-specific immune response (47, 109, 113). Such vaccines might have potential in particular risk groups, such as immune-compromised individuals and the elderly, as they could be administered together with the annual influenza vaccine. A variety of LAVs, including cpts-viruses, chimeric viruses, vectored vaccines and deletion mutants, look promising for priming of the immune response, for instance in young children (12, 16, 110, 113, 178, 218, 219). However, the balance between a satisfactory degree of attenuation and a satisfactory level of immunogenicity is crucial for such vaccines and need to be evaluated carefully in the ultimate target population.

LOW PH-INDEPENDENT FUSION OF HMPV

The fusion proteins of virus families that enter the cell via acidic endosomes require low pH for triggering membrane fusion (203, 237). In contrast, membrane fusion promoted by paramyxovirus F proteins generally occurs at neutral pH (136, 191). Schowalter et al. (197) recently described that syncytia formation promoted by a HMPV A2 lineage F protein was only detected after treatment with trypsin (to cleave the F0 precursor) and exposure to low pH. Since this low pH dependency suggested a unique mechanism of triggering fusion among paramyxovirus F proteins, we tested the generality of this low pH dependent fusion for the four genetic lineages of HMPV (chapter 8) (112).

The F genes of four HMPV prototypes, NL/1/00 (lineage A1), NL/17/00 (A2), NL/1/99 (B1), and NL/1/94 (B2) (229), were cloned in plasmid pCAGGS (165) and used in syncytium formation assays and cell content mixing assays. In both assays, a clear low pH dependency could be observed for $F_{NL/1/00(A1)}$, whereas small syncytia and low levels of CAT expression were observed for $F_{NL/17/00(A2)}$ at low and neutral pH. Fusion promoted by the lineage B F proteins was not enhanced at low pH, which suggested that low pH dependency was not a general phenomenon for the F proteins of HMPV.

Although there were only 2 amino acid differences (T270M and E294G) between our A2 lineage F protein and the A2 lineage F protein ($F_{CAN97-83}$) used by Schowalter et al. (197), they responded different to exposure to low pH. The T270M mutation was found in all HMPV F sequences in public databases, and this change had no effect on F protein activity. Introduction of the E294G however, resulted in a clear increase of fusogenic activity of the F protein, which was in agreement with the data reported for $F_{CAN97-83}$. Changing E294G in $F_{NL/1/99(B1)}$ inhibited F protein induced fusion at low and neutral pH, whereas this change in $F_{NL/1/94(B2)}$ reduced fusion at either pH only marginally. These results in combination with the absence of 294G in public databases supports the hypothesis that low pH enhanced fusion is restricted to lineage A viruses. Furthermore, because HMPV strains with 294G represent only a small portion of all lineage A strains, low pH dependent fusion is probably a rare characteristic of HMPV F proteins.

The $F_{NL/1/94(B2)}$ that we initially used, appeared to have two mutations that were introduced during passaging in Vero cells. Reversion of these mutations resulted in a much less fusogenic F protein, independent of pH. This suggests that propagation of HMPV in cell cultures may select for mutations that alter the fusogenic properties of the F protein, especially since HMPV isolates are generally poor inducers of syncytia in infected cell cultures.

Both $F_{CAN97-83}$ and $F_{NL/1/00(A1)}$ were derived from HMPV strains obtained by virus isolation in cell culture. The 294G mutation present in both F proteins might thus have been selected for in cell culture systems, just like the mutations in $F_{NL/1/94(B2)}$. These results indicate that some HMPV F proteins require low pH for efficient fusion, but this is an exception rather than a general phenomenon, possibly related to cell culture adaptation.

FUTURE PERSPECTIVES

Since the discovery of HMPV about 7 years ago, many researchers have conducted clinical, applied and fundamental research on HMPV. The development of reverse genetics systems for HMPV has greatly expanded our possibilities to study this virus, since it facilitates the manipulation of the viral genome by deleting complete genes or by introducing mutations. While reverse genetics systems have provided important tools to study adaptive immune responses and vaccine candidates, they can also be used to study innate immunity and interferon antagonist activity of the virus. The use of minigenome systems and reverse genetics can furthermore provide insight into the host tropism of HMPV and AMPV, by exchanging the F or G genes, or proteins of the polymerase complex. In addition, specific functions of individual viral proteins and their role in the replication cycle of HMPV can be studied.

Although some large retrospective studies have been conducted, there is only limited data available to assess the clinical impact of HMPV in the human population and especially in the different risk groups. More long-term multi-center retrospective and prospective studies are required to define the burden of disease due to HMPV, and to study the risk factors associated with severe HMPV disease. Two main genetic lineages of HMPV have been described, and re-infections with both

lineages occur. Therefore, the impact of these genetic lineages on the development of vaccines and therapeutics needs to be elucidated.

Enhanced disease has so far not been observed upon use of HRSV F subunit vaccines in patients with pre-existing immunity, and is therefore not anticipated for HMPV F subunit based vaccines (69, 161, 179, 222). Live-attenuated HRSV vaccine candidates have been evaluated safely in infants as young as 1 – 2 months, with no apparent adverse effects (132, 248). Therefore, our vaccination studies have focused mainly on the immunogenicity and protective efficacy of F subunit and LAV candidates. In the end, the safety of these vaccines should be addressed and vaccine induced immune pathology needs to be studied. For this purpose, Syrian golden hamsters are not the ideal animal model, since immunological tools are scarce. The cynomolgus macaque model that we used to evaluate the immunogenicity of vaccine candidates provided useful immunological data on the induced HMPV-specific response. However, it turned out that macaques were not the most susceptible non-human primate species for HMPV infection especially of the B-type. For future studies, two alternative primate species should be considered that may be more susceptible to HMPV infection. AGMs have been used previously for HRSV and HMPV (129, 149, 217, 218), but the limited availability of reagents to study specific immune responses is a disadvantage. Also their limited susceptibility to HMPV type B makes them less suitable. Chimpanzees are probably the most susceptible species (205), but studies in this species are restricted for ethical and practical reasons and even banned in Europe. Thus a single ideal experimental animal model is not available, and development of better models may be needed. Furthermore, a longer time frame should be considered between immunization and challenge, to evaluate the protective efficacy of the administered vaccine, and to study the effect of waning immunity on the induced immune responses. In most HMPV vaccination studies in African green monkeys, animals were already challenged 4 or 5 weeks after immunization (12, 178, 205, 218).

More efforts should also be dedicated to define the mutations responsible for the attenuated phenotype of the cpts-vaccine candidates. The LAV HMPVM11 induced a HMPV-specific response, but may be too attenuated to induce protective immunity. Therefore, the contribution of each individual mutation to the attenuated phenotype should be characterized. Introduction of multiple attenuating mutations may eventually result in enhancement of the attenuated phenotype. At the end, viruses harbouring different combinations of mutations may be generated and evaluated, to yield a candidate virus that is sufficiently immunogenic and attenuated. In addition to the mutations that were obtained after cold-passaging of NL/1/99 (chapter 6), we also identified sets of cp-mutations of the other HMPV prototypes; NL/1/00, NL/94/1 and NL/17/00 (data not shown). In the future, these mutations can be used to improve the attenuated phenotype of candidate LAVs. The first step in selecting potentially attenuating mutations, which were found in the polymerase proteins of HMPV, can be easily assessed by screening cp-mutations in minigenome systems. But the attenuated phenotype should be evaluated in an animal model that is susceptible for HMPV, and ultimately in humans.

In chapter 8, we demonstrated that the low pH dependency for HMPV F promoted fusion that was

described by others, is rather a rare strain specific phenomenon than a general property (112). The impact of amino acid substitutions in the HMPV F protein on pH dependency and fusogenicity, as shown for lineage A and lineage B2 viruses, respectively, is more likely to be an in vitro artefact, rather than an advantage for the virus in vivo. This assumption is supported by the low prevalence of these mutations in circulating viruses. Obviously, this hypothesis should be tested in an animal model, in which the replication kinetics of viruses containing mutations that were found to be responsible for altered fusogenicity in vitro, should be compared with wild-type viruses. Our collection of F proteins containing different fusogenic properties, offers the opportunity to gain a better understanding of the trigger for paramyxovirus F proteins to initiate the process of membrane fusion, a process that is largely still not understood.

GENERAL CONCLUSION

To date, several HMPV vaccination strategies have been explored in a variety of animal models. Whether there will ever be an HMPV vaccine in the future depends not only on the clinical impact of HMPV and factors like cost-effectiveness among the different risk groups, but also on whether immune responses can be induced that are robust enough to overcome the waning immunity to HMPV.

Most efforts have concentrated on the development of LAVs to prevent serious HMPV-induced respiratory disease in young children. Although some of these LAVs look promising in animal models, the real level of attenuation of these candidates should eventually be studied in the ultimate recipients, which are young infants. Most live-attenuated HMPV candidates that have been tested so far were indeed attenuated, but there was quite some variation in the level of attenuation between these candidates in non-human primates. Our candidate vaccine HMPVM11 seemed to be over-attenuated in macaques, but may still be a good candidate for exploration in clinical trials in human volunteers. It may be better to initiate such trials with a possibly over-attenuated candidate rather than exposing the recipients to a virus that may not be attenuated enough. Although vaccination with such live-attenuated vaccines will not induce life-long protection, it may prevent serious disease symptoms in the period that infants are most susceptible for serious HMPV-induced disease.

For elderly and immune-compromised individuals, our F subunit vaccine may be the best candidate vaccine that is currently available. In rodents, we demonstrated that less antigen is needed per vaccine dose compared to the F subunit vaccine described by Cseke et al. Since high virus neutralizing antibody titers are required to provide protection against infection, the efficacy of alternative adjuvants, like for example MF59 or AS03, may be explored. Besides inducing higher antibody titers, it is desired to improve the longevity of the response.

For both approaches discussed above, the biggest challenge is to create multivalent vaccines to protect not only against HMPV, but also HRSV and HPIV3. The HMPV genome allows the introduction of foreign genes without having much impact on virus replication. Once there is a candidate virus available that is satisfactorily attenuated, yet sufficiently immunogenic, it should

be possible to introduce extra genes to create a live-attenuated multivalent vaccine. For a subunit vaccine, the addition of an extra protein of HRSV and HPIV3 will result in the same multivalent protection. It may be considered to combine vaccination with such a multivalent subunit vaccine with the yearly influenza vaccination.

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Nederlandse samenvatting

Het humaan metapneumovirus (HMPV) behoort tot de familie *Paramyxoviridae*, subfamilie *Pneumovirinae*, genus *Metapneumovirus*, en is voor het eerst beschreven in 2001. HMPV is verwant aan het humaan respiratoir syncytieel virus (HRSV), een belangrijke veroorzaker van luchtweginfecties. Na HRSV is HMPV een van de belangrijkste veroorzakers van bronchiolitis in jonge kinderen. Bij volwassenen resulteert infectie met HMPV normaliter in relatief milde, verkoudheid-achtige symptomen. Jonge kinderen, ouderen en individuen met een verzwakte weerstand vormen de risicogroepen voor infecties met HMPV. In deze belangrijkste risicogroepen kunnen ernstige infecties aan zowel de bovenste als onderste luchtwegen ontstaan en om die reden is het ontwikkelen van een vaccin tegen HMPV gewenst. Dergelijke vaccinatie strategieën moeten vooral gericht zijn op het voorkomen van ernstige infecties in de onderste luchtwegen.

Het voorkomen van verschillende varianten van HMPV zou van invloed kunnen zijn op de werkzaamheid van een vaccin. Een vaccin dat bescherming biedt tegen infectie met de ene variant hoeft namelijk niet vanzelfsprekend bescherming te bieden tegen infectie met een andere variant. Omdat er aanwijzingen waren voor de aanwezigheid van meerdere HMPV varianten, hebben we in hoofdstuk 2 de genetische en antigene eigenschappen van verschillende HMPV-isolaten bestudeerd. Hiervoor zijn de sequenties (genetische blauwdruk) bepaald van 84 fusie eiwitten (F) en 35 aanhechtingseiwitten (G). Dit zijn eiwitten aan de buitenkant van virusdeeltjes waartegen de afweerreactie van de mens voornamelijk gericht is. Aan de hand van deze sequenties konden de virus-isolaten worden ingedeeld in twee genetische hoofdgroepen (A en B), die beiden verder konden worden verdeeld in 2 subgroepen (A1, A2, B1 en B2). Tevens werden tussen deze twee hoofdgroepen grote antigene (serologische) verschillen aangetoond. Antistoffen gericht tegen een groep A virus bleken veel beter in staat te zijn om groep A virussen dan groep B virussen te herkennen. Op basis van deze genetische en antigene verschillen hebben wij twee HMPV-serotypen gedefinieerd.

In hoofdstuk 3 wordt beschreven hoe er voor twee virussen, HMPV/NL/1/00 en HMPV/NL/1/99 (representatief voor subgroepen A1 en B1), een zogenaamd 'reverse genetics' systeem is ontwikkeld. Met een reverse genetics systeem kunnen virussen worden geproduceerd, waarvan de genetische eigenschappen kunnen worden veranderd. Bij deze techniek worden plasmiden (DNA) die het volledige erfelijke materiaal van HMPV bevatten, in zoogdiercellen gebracht door transfectie. Deze plasmiden bevatten alle informatie om virus RNA, dat de genetische blauwdruk bevat, en virus eiwitten te produceren. Met dit RNA en deze eiwitten worden vervolgens nieuwe virusdeeltjes gemaakt.

Herinfecties met HMPV komen vaak voor. Tot voor kort was er nog maar weinig bekend over de afweerreactie die wordt opgewekt tegen HMPV na infectie. Ook was niet bekend in welke mate deze afweerreactie bescherming kan bieden tegen een nieuwe infectie met HMPV en of er verschil is in bescherming tegen groep A en B virussen. Om deze redenen hebben we de afweerreactie en het beschermend vermogen hiervan, bestudeerd in een infectiemodel in Java-apen (*Macaca fascicularis*), zoals beschreven in hoofdstuk 4. Dieren werden via de neus en de luchtpijp geïnfecteerd met een groep A of B virus. Vervolgens werden de dieren op verschillende

tijdstippen nogmaals met hetzelfde virus geïnfecteerd. Een eerste infectie met HMPV resulteerde in het opwekken van virus-neutraliserende antistoffen. Dit zijn antistoffen die aan virusdeeltjes binden, waardoor deze virusdeeltjes niet meer in staat om cellen te infecteren. Op deze manier word het virus uitgeschakeld, oftewel geneutraliseerd. Antistoffen die werden opgewekt na infectie met een A virus, bleken *in vitro* beter in staat om een groep A virus te neutraliseren dan een groep B virus virus. Deze resultaten bevestigden het bestaan van verschillende HMPV serotypen zoals beschreven in hoofdstuk 2. Dieren die 4 of 6 weken na een eerste infectie opnieuw werden geinfecteerd met HMPV waren volledig beschermd tegen herinfectie. Na 12 weken waren de dieren nog maar gedeeltelijk beschermd tegen herinfectie. Acht maanden later was de afweerreactie die door de eerdere infecties was opgewekt bijna ondetecteerbaar. Uit deze studie bleek dat experimentele infecties met HMPV slechts een kortdurende bescherming opwekken. Daarom moeten toekomstige HMPV-vaccines uiteindelijk een sterkere afweerreactie opwekken dan een natuurlijke infectie.

Ouderen en individuen met een verzwakte weerstand kunnen met virus eiwitten worden gevaccineerd om de bestaande afweerreactie tegen een virus te versterken ('boosten'). Om die reden hebben we HMPV F eiwitten geproduceerd voor de twee genetische hoofdgroepen van HMPV, A en B. In hoofdstuk 5 hebben we bestudeerd of vaccinatie met deze F eiwitten (Fsol-vaccin) een afweerreactie konden opwekken in Syrische goudhamsters (*Mesocricetus auratus*), en of deze afweerreactie bescherming kon geven tegen infectie. Adjuvantia kunnen het afweersysteem stimuleren en worden om die reden vaak toegevoegd aan vaccins. Twee vaccinaties met Fsol, waaraan de adjuvantia Specol of iscom-matrix werden toegevoegd, resulteerden in het opwekken van grote hoeveelheden virus-neutraliserende antistoffen in hamsters. Vier weken na de tweede vaccinatie met een groep B Fsol-vaccin, werden de dieren via de neus geïnfecteerd met een groep A virus. De hoeveelheid virus die na infectie kon worden terug gevonden in de bovenste luchtwegen van de gevaccineerde dieren, was significant minder dan in de dieren die niet waren gevaccineerd. In de onderste luchtwegen van gevaccineerde dieren kon geen virus worden aangetoond, terwijl in de onderste luchtwegen van de niet-gevaccineerde dieren wel virus kon worden aangetoond. Hieruit konden we concluderen dat de geadjuveerde Fsol-vaccines, een kruis-beschermende afweerreactie opwekken in de onderste luchtwegen van Syrische goudhamsters.

In hoofdstuk 6 beschrijven we de ontwikkeling van levende, verzwakte virussen die als vaccin gebruikt kunnen worden. Zulke virussen zijn te verzwakt om ziekte te veroorzaken, maar zijn nog wel in staat om na infectie een afweerreactie op te wekken. Door virussen herhaaldelijk bij een lage temperatuur te groeien in cellijnen, ontstond er een opeenstapeling van mutaties in het erfelijke materiaal van het virus. Deze mutaties ontstonden omdat het virus zich ging aanpassen aan het groeien bij deze lage temperatuur. Uiteindelijk werden er 19 mutaties gevonden. Een virus waarin 11 van deze 19 mutaties waren geïntroduceerd, bleek temperatuur-gevoelig te zijn *in vitro*. Dit betekent dat dit virus, HMPV$_{M11}$, minder goed of niet groeit bij hogere temperaturen in vergelijking met wild-type virussen (virussen zonder mutaties). Voor HRSV zijn in het verleden al een aantal van dit soort mutaties beschreven. Als alternatief voor onze temperatuur-gevoelige HMPV$_{M11}$, hebben we bekende HRSV mutaties geintroduceerd in het erfelijke materiaal van HMPV. Een virus waarin

3 HRSV mutaties waren aangebracht, $HMPV_{HRSV3}$, bleek ook temperatuur-gevoelig te zijn. Beide temperatuur-gevoelige virussen bleken zich veel minder goed te kunnen vermenigvuldigen in de bovenste luchtwegen van hamsters dan wild-type virussen. In de onderste luchtwegen konden beide virussen zelfs helemaal niet worden aangetoond. Ondanks dit verzwakte fenotype, wekten beide virussen de productie van HMPV-specifieke antistoffen op. Na 2 vaccinaties, via de neus, met de levend verzwakte vaccin kandidaten $HMPV_{M11}$ of $HMPV_{HRSV3}$ (beiden gebaseerd op een B virus), waren de onderste luchtwegen van de dieren volledig beschermd tegen infectie met een groep A virus. De hoeveelheid virus dat kon worden terug gevonden in de bovenste luchtwegen van gevaccineerde dieren, was met een factor 10.000 verminderd, in verhouding tot de dieren die niet waren gevaccineerd. Beide kandidaat-vaccins wekten dus een afweerreactie op die bescherming bood tegen infectie met HMPV in Syrische goudhamsters.

Aangezien de Fsol- en $HMPV_{M11}$-vaccins beiden een afweerreactie opwekten die bescherming bood tegen infectie met HMPV in hamsters, besloten we om de werkzaamheid van deze vaccins te bestuderen in een meer aan de mens gerelateerd diermodel, de Java-aap (hoofdstuk 7). Dieren werden 2 keer gevaccineerd met het groep B Fsol-vaccin, of het levend, verzwakte $HMPV_{M11}$, gevolgd door infectie met groep A virus, 8 weken later. HMPV-specifieke antistoffen en cellulaire afweerreacties werden opgewekt na vaccinatie met Fsol. Helaas nam de opgewekte bescherming met de tijd weer af, en de dieren bleken uiteindelijk niet goed beschermd tegen infectie met wild-type HMPV. Vaccin kandidaat $HMPV_{M11}$ bleek te verzwakt, aangezien dit virus na vaccinatie niet kon worden aangetoond in de bovenste en onderste luchtwegen. Desondanks resulteerden de vaccinaties in het opwekken van een cellulaire afweerreactie, aangezien er na infectie met HMPV een significante stijging was in het percentage HMPV-specifieke T-cellen. Deze T-cellen zijn onderdeel van het cellulaire afweersysteem en spelen een belangrijke rol in de afweer tegen het virus. Deze dieren waren niet goed beschermd tegen infectie met HMPV, maar de virus vermeerdering en de duur van virus uitscheiding was in $HMPV_{M11}$-gevaccineerde dieren verminderd ten opzichte van de dieren die niet waren gevaccineerd. Ondanks dat er HMPV-specifieke afweerreacties werden opgewekt na vaccinatie, boden beide vaccins helaas geen bescherming tegen infectie. De snel afnemende bescherming na vaccinatie vormt een groot obstakel voor de ontwikkeling van vaccins tegen HMPV.

Virussen uit de *Paramyxoviridae* familie binden in de eerste stap van infectie aan het oppervlak van de gastheercel. Vervolgens fuseren de membranen van het virus en de gastheercel met elkaar bij neutrale pH (pH 7). Op deze manier komt het erfelijke materiaal van het virus in de gastheercel vrij en kan het virus zich gaan vermenigvuldigen. Echter, bij virussen behorende tot de *Orthomyxoviridae* familie, zoals het influenza virus, worden de virusdeeltjes door de gastheercel ingesnoerd in een compartiment (endosoom). Lokale pH veranderingen in het endosoom resulteren vervolgens in fusie van het virale membraan met het endosomale membraan, waarna het erfelijke materiaal van het virus vrij komt in de gastheercel. Onderzoekers uit Amerika beschreven recentelijk dat membraanfusie door HMPV, geïnitieerd door het F eiwit, afhankelijk is van blootstelling aan lage pH. Dit suggereerde dat HMPV, in tegenstelling tot alle andere paramyxovirussen, een fusie

mechanisme bezit dat overeenkomt met die van bijvoorbeeld influenza virussen. In hoofdstuk 8 beschrijven wij experimenten die zijn gedaan met F eiwitten van virussen uit elk van de 4 genetische HMPV subgroepen, A1, A2, B1 en B2. Uit deze experimenten bleek dat membraan fusie slechts voor enkele groep A virussen afhankelijk is van lage pH, maar voor de meeste virussen niet. Voor membraanfusie bij lage pH bleek het aminozuur (de bouwstenen van eiwitten) glycine, op aminozuur positie 294 van het F eiwit, verantwoordelijk te zijn. Uit gegevens uit publieke databases bleek dat er in de meeste F eiwitten een glutaminezuur zit op positie 294 en in slechts 4% van de F eiwitten een glycine. Wij bevestigden met ons onderzoek dat de F eiwitten die beschreven waren door de Amerikaanse onderzoekers, inderdaad afhankelijk zijn van een lage pH voor fusie. Maar aangezien de mutatie die daar verantwoordelijk voor is slechts in 4% van alle bekende HMPV F eiwitten voorkomt, blijkt dit dus geen algemene eigenschap van HMPV te zijn.

Rotterdam.

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Dankwoord

DANKWOORD

Het dankwoord. Slechts twee bladzijden. Ver weggestopt achter in het proefschrift. Maar toch de bladzijden die niet alleen als eerste, maar ook het meest gelezen worden.

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Curriculum vitae, publicaties

CURRICULUM VITAE

De auteur van dit proefschrift werd op 24 juli 1978 geboren te Rotterdam. In 1996 behaalde hij het V.W.O.diploma aan het IJsselcollege te Capelle aan den IJssel. In datzelfde jaar werd begonnen aan de opleiding biologie en medisch laboratoriumonderzoek aan het Hoger Laboratorium Onderwijs van de Hogeschool van Utrecht, te Utrecht. In januari 2000 werd deze opleiding in de biotechnologische afstudeerrichting afgerond. Na een half jaar gewerkt te hebben als analist op de afdeling virologie van het Erasmus MC, werd in september 2000 begonnen met de opleiding medische biologie aan de Faculteit der Aard- en Levenswetenschappen van de Vrije Universiteit Amsterdam. Na het behalen van het doctoraaldiploma in augustus 2002, werd begonnen aan het promotieonderzoek aan de afdeling virologie van het Erasmus MC te Rotterdam, onder begeleiding van Prof. Dr. Ron Fouchier en Prof. Dr. Ab Osterhaus, resulterend in dit proefschrift.

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