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Soluble recombinant human metapneumovirus G protein is immunogenic but not protective

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Summary

Human metapneumovirus (HMPV) expresses the major surface glycoproteins F and G. We evaluated the protective efficacy of immunization with G. We generated a recombinant form of G ectodomain (GΔTM) that was secreted from mammalian cells and purified by affinity chromatography. We tested the immunogenicity of GΔTM in cotton rats. Animals were immunized with PBS, GΔTM alone or adjuvanted, or were infected once with HMPV, and challenged with live HMPV at 28 days. Animals vaccinated with adjuvanted and non-adjuvanted GΔTM developed high levels of serum antibodies to both recombinant and native G protein; however, vaccinated animals did not develop neutralizing antibodies and were not protected against virus challenge. Unlike the analogous non-fusion glycoproteins of other human paramyxoviruses, HMPV G does not appear to be a protective antigen. This represents a unusual feature of HMPV.

Keywords

paramyxovirus; metapneumovirus; vaccine; glycoprotein

1. Introduction

The recently discovered human metapneumovirus (HMPV) is a major cause of upper and lower respiratory tract infections in children and adults [1–11]. HMPV is a member of the paramyxovirus family, along with the related respiratory syncytial virus (RSV) and parainfluenza virus (PIV). HMPV and RSV share a number of clinical and genetic similarities [2,3,6,12,13]. HMPV infection leads to significant morbidity in infants and other special populations, including immunocompromised, high-risk, and elderly patients [9,14–21], and hospitalization rates are similar to those of RSV, PIV, influenza virus and other significant viral pathogens of the upper and lower respiratory tracts. HMPV has a major impact on human

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health and safe, effective vaccines could decrease the burden of disease associated with this novel pathogen. Studies using recombinant live-attenuated viruses or soluble HMPV F protein have shown that F is a major protective antigen [22–25]; however, the contribution of G to protective immunity is not clear.

The fusion (F) and attachment glycoproteins (HN/H/G) are common to all paramyxoviruses. The *Paramyxoviridae* attachment proteins are all type II transmembrane proteins containing multiple glycosylation sites. Conversely, paramyxovirus attachment proteins vary significantly in both target receptors and biologic activity. While receptors and functional activities have been identified for many (but not all) paramyxovirus attachment proteins, the role of HMPV G protein in viral replication is unknown. Viruses of the *Respirovirus* and *Rubulavirus* genres possess HN attachment proteins that have both hemagglutination and neuraminidase activity, while those of the *Morbillivirus* and *Henipavirus* genres exhibit only hemagglutination activity. HMPV G protein has not been shown to possess either hemagglutination or neuraminidase activity. Recombinant HMPV lacking the G protein replicates at levels similar to wild-type virus in vitro, but exhibits reduced titers in vivo [26,27]. One report suggested that HMPV G serves as an attachment protein and binds to cell surface glycosaminoglycans and thus may have a true attachment function [28]. However, F protein interacts with RGD-binding integrins as a putative receptor [29]. In vitro data indicate that G may inhibit innate immune responses [30], suggesting an alternate possible biologic role for G. There is no CX3C chemokine motif in the HMPV G protein, distinguishing it from that of RSV, which interacts with the CX3CR1 receptor [31]. The role of HMPV G protein in establishing infection in susceptible hosts thus has not been established.

We have engineered a construct to express soluble, epitope-tagged HMPV G protein (GΔTM) lacking the transmembrane domain of the native protein in mammalian cells. We show here that soluble GΔTM protein retains elements of native HMPV G protein. GΔTM is expressed as a highly-glycosylated multimeric protein that is recognized by anti-HMPV serum. GΔTM is immunogenic in a cotton rat model, but does not elicit a neutralizing or protective antibody response.

2. Methods

2.1. Cloning of HMPV G full-length and HMPV G ectodomain

We used RT-PCR to amplify a full-length G sequence from isolate TN/96-12, a prototype A1 lineage strain according to the proposed nomenclature [32,33]. Primers were 5'-AACATTCGRRCAATAGAYATGYTCAAAGC-3' and 5'-GTTAACTAATTTGGTTTTATATTGTTGGTG-3'. The full TN/96-12 G sequence was sequence-optimized by a commercial source (GeneArt, GmbH, Regensburg, Germany) to alter suboptimal codon usage for mammalian tRNA bias, improve secondary mRNA structure, and remove AT-rich regions, increasing mRNA stability. The optimized full-length G sequence was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen) to generate the construct pcDNA3.1-G (DNA-G). This construct was amplified with primers 5'-GGTACCACAGGAGAACACCAGCGAG-3' and 5'-GATATCAGCTGGTCTGGTTGTAGGTGG-3' (*Kpn I/Eco RV* restriction sites underlined in primer sequences). PCR product was digested and ligated into vector pcDNA3.1/HisA (Invitrogen) using the same sites to generate the HMPV G ectodomain construct with an N-terminal hexahistidine sequence. This G ectodomain construct was subsequently digested with *HindIII* and *EcoRV* and cloned into vector pSecTag2B (Invitrogen) to introduce the Ig-κ signal sequence N-terminal to the hexahistidine tag and generate the plasmid DNA-GΔTM. All PCR products and plasmid constructs were confirmed by sequencing both strands on an ABI 3730xl DNA Analyzer in the Vanderbilt DNA Sequencing Core Facility.

2.2 HMPV G expression in adherent mammalian cells

Full-length G expression constructs were transfected into LLC-MK2 cell monolayers for immunofluorescent and immunoblot studies using Effectene (Qiagen) according to the manufacturer's protocol. For SDS-PAGE and immunoblots, cells were lysed in buffer consisting of 50 mM Tris-Cl, 140 mM NaCl, 1.5 mM MgCl₂, 0.5% NP-40 and PMSF protease inhibitor cocktail (Sigma).

2.3. Purification of his-tagged G

G ectodomain expression constructs were transfected into suspension 293-Freestyle cells for protein production using 293Fectin (Invitrogen) according to the manufacturer's protocol. Cells were centrifuged for 5 min at $100 \times g$ at room temperature, the supernatant harvested and filtered through 0.2 μ m filters before purification. Protein purification was performed on an ÄKTA FPLC system controlled by UNICORN 4.12 software (GE Healthcare). The his-tagged G ectodomain G Δ TM was purified by immobilized metal ion affinity chromatography using pre-packed HisTrap Ni-Sepharose columns (GE Healthcare). Sample was diluted with concentrated binding buffer stock to adjust pH, salt, and imidazole concentration before purification. Protein was loaded on a 5 ml HisTrap column with a loading flow rate of 5.0 ml/min, and the binding buffer contained 20 mM sodium phosphate, 0.5 M NaCl, 30 mM imidazole (pH 7.4). Wash and elution protocols were optimized extensively for imidazole concentration and wash/elution column volumes (data not shown). Unrelated proteins were washed out with four column volumes of 8% elution buffer, and the his-tagged G protein was eluted with four column volumes of 25% elution buffer containing 20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole (pH 7.4). Purified protein was concentrated and dialyzed against PBS through Amicon Ultra centrifugal filters with 30,000 and 100,000 MWCO (Millipore). Final protein was quantitated by a commercial modified Bradford assay [34,35] according to the manufacturer's instructions for the microassay procedure (Bio-Rad Protein Assay, Bio-Rad).

2.4. Protein electrophoresis and immunoblotting

Purified protein fractions were loaded on NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and run at 200 V in MES-SDS running buffer (Invitrogen). Gels were stained with Simply Blue SafeStain (Invitrogen) or Silver Stain Plus (Bio-Rad) to visualize protein bands. For Western blot analysis, separated protein bands were transferred to Invitrolon PVDF membrane (Invitrogen) at 30 V for 1 h. After blocking with 5% milk in Tris-buffered saline with 0.05% Tween-20 (TBS-T), membranes were incubated with anti-histidine mouse monoclonal antibody (Sigma) or anti-HMPV polyclonal guinea pig serum at a 1:500 dilution. Membranes were washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-guinea pig secondary antibodies diluted 1:1000 (Southern Biotech). Membranes were washed again with TBS-T and developed with TMB Membrane Peroxidase Substrate (KPL). Recombinant G Δ TM protein was subjected to enzymatic deglycosylation using the E-Degly kit (Sigma) according to the manufacturer's instructions.

2.5. Immunofluorescent microscopy

HMPV-infected or G-transfected LLC-MK2 cell culture monolayers were fixed with 10% formalin, washed with PBS-T and incubated with primary antibody diluted in PBS-T/milk for 1 h at 37 °C. After washing with PBS-T, cells were stained with AlexaFluor568-conjugated goat anti-guinea pig Ig (Molecular Probes) diluted 1:1000 in PBS-T/milk for 1 h at 37 °C. Cell monolayers were examined on an inverted Nikon Diaphot microscope and images captured with a Nikon D100 digital camera. Images were cropped and figures constructed using Adobe Photoshop and Illustrator. Contrast was increased by 50% in all images identically and symmetrically across the entire image for clarity of the printed image.

2.6. G protein specific ELISA

Guinea pig or cotton rat sera were tested for the presence of G protein antibodies. Briefly, 150 ng/well of purified HMPV G protein was adsorbed onto Immulon 2B plates overnight in carbonate buffer (pH 9.8) at 4 °C. The plates were blocked with 5% nonfat dried milk in PBS-T for 2 hours at room temperature. After washing with PBS-T, serial dilutions of serum samples were added to the plate and incubated for an hour at room temperature. The plates were washed, horseradish peroxidase (HRP)-conjugated anti-guinea pig IgG (1:1000 dilution) added (Southern Biotech) and incubated for one hour. Finally, the plates were washed and One-Step Turbo TMB peroxidase substrate (Pierce) added. The reactions were stopped by adding 50 µL of 1M HCl and the absorbance read at 450 nm. The ELISA titers were expressed as the reciprocal dilution of serum in which the absorbance was twice the background absorbance of HMPV-naïve serum.

2.7. Animal studies

Guinea pigs and cotton rats were purchased at 5–6 weeks of age from a commercial breeder (Harlan), fed standard diet and water *ad libitum* and kept in microisolator cages. Animals were anesthetized by isoflurane inhalation prior to immunization, blood sampling, or virus inoculation. To generate G-specific antiserum, guinea pigs were immunized with DNA-G plasmid in PBS (25 µg i.m. and 2.5 µg i.d.) at 0, 14, 28, and 120 days. Guinea pig serum was tested 14 days after each immunization for anti-HMPV G antibodies by immunofluorescence and immunoblot of virus-infected cells. Guinea pigs were terminally exsanguinated 30 days after the final immunization. The study was approved by the Vanderbilt Animal Care and Use Committee.

For GΔTM protein vaccination studies, cotton rats in groups of six were immunized intramuscularly twice on days 0 and 14 with PBS, 25 µg GΔTM protein alone, or 25 µg GΔTM adjuvanted 1:1 with TiterMax Gold (Sigma), or were infected intranasally once on day 0 with 2×10^5 PFU of HMPV in a volume of 200 µl. Serum was collected from cotton rats on day 0 and day 28 (pre-challenge) by retro-orbital bleed. Serum HMPV-neutralizing titers were determined by a plaque reduction assay as described [32]. The virus strain used was the same pathogenic subgroup A1 clinical isolate TN/96-12 that the G gene had been cloned from [33]. The stock used had been passaged seven times since the primary isolation. Virus was grown in LLC-MK2 cells and purified over a 20%/60% discontinuous sucrose gradient as previously described [32]. Cotton rats were inoculated on day 28 intranasally with 2×10^5 pfu in a volume of 200 µl. Four days after virus challenge, the animals were euthanized by CO₂ asphyxiation, and exsanguinated. Nasal and right lung tissues were collected and processed for virus titration, pathology and RNA extraction as described [32]. Lung sections from all animals were stained with hematoxylin and eosin and examined by light microscopy in a group-blinded fashion, and scored for location, intensity, and composition of inflammatory infiltrates, as well as the presence and extent of intraluminal mucus in airways of all sizes [32]. Virus yields were measured by plaque titration and cytokine gene expression levels were determined as described [32]. Briefly, lung tissue was harvested from infected rats at the time of necropsy and mRNA was extracted and stored at –80 °C until testing. Extracted mRNA was subjected to quantitative RT-PCR for cotton rat genes using the Quantitect Probe RT-PCR kit (Qiagen) and a Smart Cycler II (Cepheid). Primers and probes for cotton rat genes were designed using Primer Express (ABI) based on GenBank sequences for GAPDH, IL-4, IL-5, and IFN-γ (Accession numbers AF512009, AF421390, AF148211, and AF167349). Cycle threshold (Ct) data for each run were normalized to GAPDH and uninfected rats were used as the calibrator data set for the comparative $2^{-\Delta\Delta C_t}$ method of relative quantitation of gene expression [36]. Data were expressed as mean fold change from the calibrator group of uninfected cotton rats. The Vanderbilt Institutional Animal Care and Use Committee approved the study.

2.8. Statistical analysis

Mean viral titers, serum virus-neutralizing titers and gene expression levels were compared between groups with a 2-tailed t test assuming unequal variance. Cytokine levels were compared using the Mann-Whitney *U* test.

3. Results

3.1. Sequence-optimized HMPV G is expressed in mammalian cells

The native viral G sequence was cloned into a mammalian expression plasmid and transfected into LLC-MK2 cells. This construct did not appear to express, as HMPV G protein could not be detected by immunofluorescent or Western blot assays (data not shown). In contrast, the sequence-optimized construct DNA-G was expressed when transfected into LLC-MK2 cells and was readily detectable by anti-HMPV serum (Figure 1B); this anti-HMPV serum did not react with mock-transfected cells (Figure 1A). The predicted molecular weight of HMPV G protein monomer based on the primary sequence is ~26 kD; however, G is heavily glycosylated and mature G protein has been reported to migrate at apparent molecular weights of 80–100 kD [27, 28, 30, 37]. Immunoblots of DNA-G transfected LLC-MK2 cell lysates and sucrose-purified virus detected a band at ~75 kD, consistent with glycosylated G protein (Figure 1C, lanes 2 and 3). This band was the same size in both purified virus and DNA-G transfected cells.

3.2. DNA-G is immunogenic in guinea pigs but does not induce neutralizing antibodies

We chose to use guinea pigs for DNA-G construct immunization experiments, since this species mounted a high-titer neutralizing antibody response to a similar HMPV DNA-F construct in previous work [22]. After DNA-G immunization, the animals did develop serum antibodies that reacted with HMPV-infected cells by immunofluorescence, detecting antigen in a membrane distribution (Figure 1F), but did not react with mock-infected cells (Figure 1E). The guinea pig antisera also detected a ~75 kD band in immunoblots of HMPV-infected LLC-MK2 cell lysates and bound to HMPV by ELISA (data not shown); however, none of the DNA-G immunized guinea pigs developed a serum plaque reduction neutralizing titer >1:20 (data not shown). We hypothesized that this was due to poor antibody responses induced by DNA immunization and thus elected to generate recombinant G protein as an alternate approach.

3.3. Expression and purification of recombinant G protein

Soluble his-tagged G protein lacking the transmembrane domain and cytoplasmic tail expressed and secreted very low amounts of G ectodomain (data not shown), presumably due to loss of the native N-terminal sequence. The his-labeled coding sequence was cloned into plasmid DNA-GΔTM containing an Ig-κ leader sequence for secretion of the expressed protein product (Figure 2A). SDS-PAGE and immunoblot analysis of DNA-GΔTM-transfected 293-F cells demonstrated that the recombinant protein was secreted into the culture medium and not retained in cells (data not shown). Yields were in the range of 0.2–0.5mg/30 ml of culture medium. The predicted molecular weight of the secreted HMPV GΔTM based on the primary amino acid sequence is 25 kD, but reducing SDS-PAGE electrophoresis showed that mature GΔTM protein migrated as a single band of approximately 75 kD and was pure (Figure 2B, lane 1). GΔTM protein was detected by anti-HMPV serum in immunoblots (Figure 2C, lane 2). Electrophoresis and immunoblotting under native conditions demonstrated that GΔTM formed a multimeric form (Figure 2D, lane 3). While native gel electrophoretic migration is affected by factors other than mass and thus does not always accurately reflect molecular weight, GΔTM multimers migrated at a higher molecular weight than HMPV FΔTM, which exists in a trimeric form of ~180 kD (Figure 2D, lane 4)[22]. Native gel electrophoresis and immunoblot of HMPV-infected LLC-MK2 cell lysates showed that HMPV G wt migrated as a multimeric form (not shown).

3.4. N- and O-linked glycosylation contribute to the molecular weight of GΔTM protein

We demonstrated that N-linked glycosylation contributed modestly to the apparent molecular weight of GΔTM by digestion with PNGase F, which removes sugar moieties at N-linked glycosylation sites (Figure 2E, lane 5). Sialic acid did not show a significant contribution, as evidenced by additional digestion of GΔTM by α-neuraminidase (Figure 2E, lane 6). O-linked glycosylation did contribute to the apparent molecular weight of GΔTM, as enzymatic deglycosylation of GΔTM with PNGase F, α-neuraminidase, and O-glycosidase demonstrated a band at ~35 kD, which more closely approximates the predicted molecular weight of the soluble protein (Figure 2E, lane 7).

3.5. GΔTM protein is immunogenic in cotton rats but does not induce neutralizing antibodies

We used the cotton rat model to test protective efficacy of GΔTM, as this species is permissive for high levels of HMPV replication [22,32,38]. Mature, fully glycosylated, purified GΔTM was used as the immunizing antigen for these experiments. Animals were immunized intramuscularly twice on days 0 and 14 with PBS, GΔTM alone, or GΔTM in TiterMax Gold adjuvant; control animals were infected intranasally once on day 0 with HMPV (Figure 3). TiterMax Gold is a commercial water-in-oil emulsion containing the block copolymer CRL-8300, squalene and a sorbitan monooleate, analogous to Freund's adjuvant but less toxic. This adjuvant was chosen based on successful use for similar proteins by our group and others [22,39,40]. Animals were bled prior to challenge on day 28 and antibody responses determined by ELISA against recombinant GΔTM protein and immunofluorescence against HMPV-infected cell monolayers. HMPV-infected animals developed a mean anti-HMPV G IgG titer of 1:960 (Figure 4A). GΔTM was highly immunogenic; cotton rats immunized with GΔTM protein alone or with adjuvant mounted significantly higher anti-HMPV G titers compared to HMPV-infected rats (1:2560, $p = 0.03$ and 1:12,800, $p < 0.02$, respectively) (Figure 4A). Additionally, serum of GΔTM-immunized cotton rats reacted with HMPV-infected cells by indirect immunofluorescence (Figure 6 B–E) at titers similar to HMPV-infected animals (Figure 6 F–G). These data showed that GΔTM induced potent antibody responses in immunized cotton rats; however, neither the unadjuvanted nor the adjuvanted GΔTM-immunized cotton rats developed any significant in vitro plaque reduction neutralizing titer (all $< 1:20$, data not shown), similar to the results of DNA-G immunization of guinea pigs.

3.6. GΔTM protein is not protective against virus challenge in cotton rats

The four groups of cotton rats previously immunized twice on days 0 and 14 with PBS, GΔTM alone, or GΔTM adjuvant, or infected intranasally once on day 0 with HMPV were challenged intranasally with live HMPV on day 28 (Figure 3). None of the challenged animals exhibited respiratory symptoms (data not shown). Rats were euthanized and tissues collected for virus titration, histopathology and cytokine mRNA determination on day 32, 4 days post-infection. Neither unadjuvanted GΔTM or adjuvanted GΔTM induced any reduction in virus titer in either nasal or lung tissues, with all immunized animals shedding virus at levels similar to placebo-immunized rats (Figure 5 A, B). These three groups exhibited tissue virus titers similar to levels we had observed previously in HMPV infection of naïve animals [32]. In contrast, cotton rats that were previously infected with live HMPV demonstrated a statistically significant reduction in nasal turbinate and lung viral titers (Figure 5 A, B). Virus titers in all previously HMPV-infected animals were below the limit of detection (5 pfu/g). Thus, viral shedding was not reduced by GΔTM immunization.

3.7. GΔTM immunization does not enhance lung histopathology upon virus challenge

Lung tissues were collected from all four groups on day 32, four days following virus challenge. Lung sections were analyzed in a group-blinded fashion by a pathologist experienced with HMPV infection of rodent models (J.E.J.). There were no striking differences in the

histopathology among the four groups. However, subtle differences in histopathology were noted. Animals that were PBS-immunized and thus experiencing primary HMPV infection had very mild peribronchiolar mononuclear infiltrates, with a few intraluminal neutrophils and associated mucus plugging in the larger bronchioles. The interstitium contained a sparse mononuclear infiltrate. The features in this group were similar to those we previously described in cotton rats with primary HMPV infection [22,32,38]. All animals immunized with GΔTM, without or with adjuvant, exhibited mild to moderate interstitial and circumferential peribronchiolar mononuclear infiltrates, along with a few intraluminal neutrophils and occasional mucus plugs in large bronchioles. Cotton rats previously infected with HMPV and thus experiencing secondary infection showed minimal inflammation of any kind, notably less than in any of the other groups, consistent with the lack of detectable viral shedding in this group.

3.8. GΔTM immunization does not induce aberrant cytokine responses upon virus challenge

We determined the cytokine profiles in cotton rat lungs collected on day 32, four days following virus challenge, in order to assess for aberrant cytokine expression in response to viral challenge following GΔTM immunization. We measured interferon- γ and IL-2 mRNA as representative Th1 cytokines, and IL-5 and IL-10 mRNA as representative Th2 cytokines. Cytokine gene expression levels were upregulated in all infected groups compared with uninfected animals (Figure 5C); however, the only significant difference between groups was the higher level of IL-2 mRNA in cotton rats previously infected with HMPV prior to challenge. Thus, prior HMPV infection induced higher levels of this Th1 cytokine upon virus challenge. Importantly, no significant increase in levels of Th2 cytokines IL-5 and IL-10 was detected after virus challenge of the GΔTM-immunized animals.

Discussion

We describe here a method for the expression of soluble HMPV G protein. The expression of soluble GΔTM in a mammalian cell culture system was facilitated by the optimization of the gene coding sequence and the addition of a cleavable Igk leader sequence. Whether GΔTM possesses a native conformation is not proven by immunofluorescent detection of HMPV-infected cells by GΔTM-immunized cotton rat antibodies and detection of GΔTM by HMPV immune sera in several different assays. However, these findings suggest that at least some elements of native conformation are retained, and it is likely that recombinant GΔTM has similar immunological properties to the native HMPV G protein.

Our findings indicate that GΔTM protein is highly glycosylated, though enzymatic deglycosylation is not very efficient and metabolic inhibitors may be more effective tools to study this phenomenon. Liu et al. showed that G expressed in HMPV-infected cells was both N- and O-glycosylated, and similarly that this contributed the majority of the G protein's apparent molecular weight [28]. Liu found that the apparent molecular weight of G in PAGE was ~97 KD, while Skiadopolous et al reported an apparent molecular weight of ~80 KD [37] and Bao observed 80–90 kD [30]. The differences between these groups and our data may be due to different viral strains or cell types used. Other groups have studied HMPV G proteins from the A2 subgroup, while the strain we used was from an A1 virus. The number of amino acid residues, potential O- and N-linked glycosylation sites, and potential basic cleavage sites all differ between A1 and A2 virus subgroups.

We immunized animals with mature, glycosylated GΔTM and failed to induce either neutralizing antibodies or *in vivo* protection, despite very high serum levels of G-specific IgG. GΔTM retains at least some structural and biochemical characteristics and some immunogenic epitopes of native HMPV G protein, based on the results of protein analysis and immunogenicity experiments. It is possible that truncation of the transmembrane domain

altered the epitope presentation of the antigen; however, DNA vaccination with full-length G also did not induce neutralizing antibodies, whereas DNA vaccination with full-length F does [22]. A possible explanation for the lack of protective immunity induced by GΔTM is that glycosylation may impact antibody recognition of neutralizing epitopes; this has been well described for HIV [30]. However, several other lines of evidence support our finding that HMPV G does not elicit a protective response. Reverse-genetics engineered HPIV1 expressing HMPV G was not protective in a hamster model, despite substantial levels of HMPV G expression [37]. A recombinant alphavirus construct encoding HMPV G was not protective in mice or cotton rats, though the characteristics of this construct were not explored fully [41]. Taken together, these data lead us to conclude that HMPV G is not a protective antigen. This is unusual, since the analogous attachment proteins of other paramyxoviruses (measles H, mumps HN, PIV HN, RSV G) are all targets of protective, virus-neutralizing antibodies.

What then is the biological function of HMPV G? Recombinant HMPV lacking G protein is replication-competent *in vitro* and *in vivo*, though attenuated in hamsters and non-human primates [26,27]. Thus, HMPV G is nonessential for replication but contributes to disease. HMPV G is not well conserved between isolates from different subgroups, with only 34% amino acid identity between major and 65–68% between minor subgroups [13,33]. Analysis of G sequences collected over several years suggested mutation in response to selective pressure, presumably immune pressure [42]; however, functional T cell epitopes have not been identified in HMPV G [43,44]. Our data show that G does not induce neutralizing antibodies, as determined by several different experimental approaches. A putative mechanism for immune pressure is thus lacking. Further studies are needed to determine the contribution of HMPV G to pathogenesis and immunity.

In summary, we present a method for generating soluble HMPV G protein. Recombinant GΔTM was heavily glycosylated and formed oligomers. HMPV GΔTM was immunogenic and a potent inducer of antibodies, but these antibodies appear to be neither neutralizing nor protective. It is possible that the high degree of glycosylation contributes to the apparent immune avoidance of HMPV G protein. These data confirm the first instance of a paramyxovirus G protein that is not a protective antigen.

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References

1. Williams JV, Wang CK, Yang CF, Tollefson SJ, House FS, Heck JM, et al. The role of human metapneumovirus in upper respiratory tract infections in children: a 20-year experience. *J Infect Dis* 2006 Feb 1;193(3):387–395. [PubMed: 16388486]
2. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, et al. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N Engl J Med* 2004 Jan 29;350(5):443–450. [PubMed: 14749452]
3. van den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, et al. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. *J Infect Dis* 2003 Nov 15;188(10):1571–1577. [PubMed: 14624384]
4. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med* 2001 Jun; 7(6):719–724. [PubMed: 11385510]

5. Sloots TP, Mackay IM, Bialasiewicz S, Jacob KC, McQueen E, Harnett GB, et al. Human metapneumovirus, Australia, 2001–2004. *Emerg Infect Dis* 2006 Aug;12(8):1263–1266. [PubMed: 16965711]
6. Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, et al. Children with respiratory disease associated with metapneumovirus in Hong Kong. *Emerg Infect Dis* 2003 Jun;9(6):628–633. [PubMed: 12781000]
7. Mullins JA, Erdman DD, Weinberg GA, Edwards K, Hall CB, Walker FJ, et al. Human metapneumovirus infection among children hospitalized with acute respiratory illness. *Emerg Infect Dis* 2004 Apr;10(4):700–705. [PubMed: 15200863]
8. Martinello RA, Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Human metapneumovirus and exacerbations of chronic obstructive pulmonary disease. *J Infect* 2006 Oct;53(4):248–254. [PubMed: 16412516]
9. Falsey AR, Erdman D, Anderson LJ, Walsh EE. Human metapneumovirus infections in young and elderly adults. *J Infect Dis* 2003 Mar 1;187(5):785–790. [PubMed: 12599052]
10. Esper F, Martinello RA, Boucher D, Weibel C, Ferguson D, Landry ML, et al. A 1-year experience with human metapneumovirus in children aged <5 years. *J Infect Dis* 2004 Apr 15;189(8):1388–1396. [PubMed: 15073675]
11. Boivin G, De Serres G, Cote S, Gilca R, Abed Y, Rochette L, et al. Human metapneumovirus infections in hospitalized children. *Emerg Infect Dis* 2003 Jun;9(6):634–640. [PubMed: 12781001]
12. McAdam AJ, Hasenbein ME, Feldman HA, Cole SE, Offermann JT, Riley AM, et al. Human metapneumovirus in children tested at a tertiary-care hospital. *J Infect Dis* 2004 Jul 1;190(1):20–26. [PubMed: 15195239]
13. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. Analysis of the genomic sequence of a human metapneumovirus. *Virology* 2002 Mar 30;295(1):119–132. [PubMed: 12033771]
14. Englund JA, Boeckh M, Kuypers J, Nichols WG, Hackman RC, Morrow RA, et al. Brief communication: fatal human metapneumovirus infection in stem-cell transplant recipients. *Ann Intern Med* 2006 Mar 7;144(5):344–349. [PubMed: 16520475]
15. Larcher C, Geltner C, Fischer H, Nachbaur D, Muller LC, Huemer HP. Human metapneumovirus infection in lung transplant recipients: clinical presentation and epidemiology. *J Heart Lung Transplant* 2005 Nov;24(11):1891–1901. [PubMed: 16297797]
16. Boivin G, De Serres G, Hamelin ME, Cote S, Argouin M, Tremblay G, et al. An outbreak of severe respiratory tract infection due to human metapneumovirus in a long-term care facility. *Clin Infect Dis* 2007 May 1;44(9):1152–1158. [PubMed: 17407031]
17. Hamelin ME, Cote S, Laforge J, Lampron N, Bourbeau J, Weiss K, et al. Human metapneumovirus infection in adults with community-acquired pneumonia and exacerbation of chronic obstructive pulmonary disease. *Clin Infect Dis* 2005 Aug 15;41(4):498–502. [PubMed: 16028158]
18. van den Hoogen BG. Respiratory tract infection due to human metapneumovirus among elderly patients. *Clin Infect Dis* 2007 May 1;44(9):1159–1160. [PubMed: 17407032]
19. Vicente D, Montes M, Cilla G, Perez-Trallero E. Human metapneumovirus and chronic obstructive pulmonary disease. *Emerg Infect Dis* 2004 Jul;10(7):1338–1339. [PubMed: 15338546]
20. Williams JV, Crowe JE Jr, Enriquez R, Minton P, Peebles RS Jr, Hamilton RG, et al. Human metapneumovirus infection plays an etiologic role in acute asthma exacerbations requiring hospitalization in adults. *J Infect Dis* 2005 Oct 1;192(7):1149–1153. [PubMed: 16136455]
21. Williams JV, Martino R, Rabella N, Otegui M, Parody R, Heck JM, et al. A prospective study comparing human metapneumovirus with other respiratory viruses in adults with hematologic malignancies and respiratory tract infections. *J Infect Dis* 2005 Sep 15;192(6):1061–1065. [PubMed: 16107960]
22. Cseke G, Wright DW, Tollefson SJ, Johnson JE, Crowe JE Jr, Williams JV. Human metapneumovirus fusion protein vaccines that are immunogenic and protective in cotton rats. *J Virol* 2007 Jan;81(2):698–707. [PubMed: 17050599]
23. Herfst S, de Graaf M, Schrauwen EJ, Ulbrandt ND, Barnes AS, Senthil K, et al. Immunization of Syrian golden hamsters with F subunit vaccine of human metapneumovirus induces protection against challenge with homologous or heterologous strains. *J Gen Virol* 2007 Oct;88(Pt 10):2702–2709. [PubMed: 17872522]

24. Skiadopoulos MH, Biacchesi S, Buchholz UJ, Riggs JM, Surman SR, Amaro-Carambot E, et al. The two major human metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is a major contributor to this antigenic relatedness. *J Virol* 2004 Jul;78(13):6927–6937. [PubMed: 15194769]
25. Tang RS, Mahmood K, Macphail M, Guzzetta JM, Haller AA, Liu H, et al. A host-range restricted parainfluenza virus type 3 (PIV3) expressing the human metapneumovirus (hMPV) fusion protein elicits protective immunity in African green monkeys. *Vaccine* 2005 Feb 25;23(14):1657–1667. [PubMed: 15705469]
26. Biacchesi S, Pham QN, Skiadopoulos MH, Murphy BR, Collins PL, Buchholz UJ. Infection of nonhuman primates with recombinant human metapneumovirus lacking the SH, G, or M2-2 protein categorizes each as a nonessential accessory protein and identifies vaccine candidates. *J Virol* 2005 Oct;79(19):12608–12613. [PubMed: 16160190]
27. Biacchesi S, Skiadopoulos MH, Yang L, Lamirande EW, Tran KC, Murphy BR, et al. Recombinant human Metapneumovirus lacking the small hydrophobic SH and/or attachment G glycoprotein: deletion of G yields a promising vaccine candidate. *J Virol* 2004 Dec;78(23):12877–12887. [PubMed: 15542640]
28. Liu L, Bastien N, Li Y. Intracellular Processing, Glycosylation, and Cell Surface Expression of Human Metapneumovirus Attachment Glycoprotein. *J Virol*. 2007 Oct 3;
29. Cseke G, Maginnis MS, Cox RG, Tollefson SJ, Podsiad AB, Wright DW, et al. Integrin {alpha}v{beta}1 promotes infection by human metapneumovirus. *Proc Natl Acad Sci U S A*. 2009 Jan 21;
30. Bao X, Liu T, Shan Y, Li K, Garofalo RP, Casola A. Human metapneumovirus glycoprotein G inhibits innate immune responses. *PLoS Pathog* 2008;4(5):e1000077. [PubMed: 18516301]
31. Tripp RA, Jones LP, Haynes LM, Zheng H, Murphy PM, Anderson LJ. CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nat Immunol* 2001 Aug;2(8):732–738. [PubMed: 11477410]
32. Williams JV, Tollefson SJ, Johnson JE, Crowe JE Jr. The cotton rat (*Sigmodon hispidus*) is a permissive small animal model of human metapneumovirus infection, pathogenesis, and protective immunity. *J Virol* 2005 Sep;79(17):10944–10951. [PubMed: 16103146]
33. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, et al. Antigenic and genetic variability of human metapneumoviruses. *Emerg Infect Dis* 2004 Apr;10(4):658–666. [PubMed: 15200856]
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976 May 7;72:248–254. [PubMed: 942051]
35. Spector T. Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for less than or equal to 0.5 to 50 microgram of protein. *Anal Biochem* 1978 May;86(1):142–146. [PubMed: 655375]
36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001 Dec;25(4):402–408. [PubMed: 11846609]
37. Skiadopoulos MH, Biacchesi S, Buchholz UJ, Amaro-Carambot E, Surman SR, Collins PL, et al. Individual contributions of the human metapneumovirus F, G, and SH surface glycoproteins to the induction of neutralizing antibodies and protective immunity. *Virology* 2006 Feb 20;345(2):492–501. [PubMed: 16300813]
38. Williams JV, Chen Z, Cseke G, Wright DW, Keefer CJ, Tollefson SJ, et al. A recombinant human monoclonal antibody to human metapneumovirus fusion protein that neutralizes virus in vitro and is effective therapeutically in vivo. *J Virol* 2007 Aug;81(15):8315–8324. [PubMed: 17522220]
39. Beck I, Gerlach H, Burkhardt E, Kaleta EF. Investigation of several selected adjuvants regarding their efficacy and side effects for the production of a vaccine for parakeets to prevent a disease caused by a paramyxovirus type 3. *Vaccine* 2003 Feb 14;21(9–10):1006–1022. [PubMed: 12547615]
40. Berhanu A, Wilson RL, Kirkwood-Watts DL, King DS, Warren TK, Lund SA, et al. Vaccination of BALB/c mice with *Escherichia coli*-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. *J Virol* 2008 Apr;82(7):3517–3529. [PubMed: 18199639]

41. Mok H, Tollefson SJ, Podsiad AB, Shepherd BE, Polosukhin VV, Johnston RE, et al. An alphavirus replicon-based human metapneumovirus vaccine is immunogenic and protective in mice and cotton rats. *J Virol* 2008 Nov;82(22):11410–11418. [PubMed: 18786987]
42. Padhi A, Verghese B. Positive natural selection in the evolution of human metapneumovirus attachment glycoprotein. *Virus Res.* 2007 Oct 9;
43. Melendi GA, Zavala F, Buchholz UJ, Boivin G, Collins PL, Kleeberger SR, et al. Mapping and characterization of the primary and anamnestic H-2(d)-restricted cytotoxic T-lymphocyte response in mice against human metapneumovirus. *J Virol* 2007 Oct;81(20):11461–11467. [PubMed: 17670840]
44. Herd KA, Mahalingam S, Mackay IM, Nissen M, Sloots TP, Tindle RW. Cytotoxic T-lymphocyte epitope vaccination protects against human metapneumovirus infection and disease in mice. *J Virol* 2006 Feb;80(4):2034–2044. [PubMed: 16439559]

Figure 1. Expression of HMPV DNA-G and immunogenicity in guinea pigs

A. Mock-transfected LLC-MK2 cells stained with guinea pig (GP) anti-HMPV serum and AlexaFluor568-goat anti-GP Ig. **B.** DNA-G transfected LLC-MK2 cells stained as in A. **C.** Denaturing, nonreducing SDS-PAGE immunoblotted with anti-HMPV serum. Lane 1, mock-infected LLC-MK2 cell lysate. Lane 2, sucrose-purified HMPV; HMPV proteins indicated on right of lane. Lane 3, DNA-G transfected cell lysate. **D.** Light microscopic image of mock-infected LLC-MK2 cells. **E.** Mock-infected LLC-MK2 cells stained with DNA-G immunized GP serum at 1:160 dilution and AlexaFluor568-goat anti-GP Ig. **F.** HMPV-infected LLC-MK2 cells stained as in B. All photomicrographs images at 20× original magnification. Contrast for all images increased by 50% for clarity in printed image.

Figure 2. Recombinant G Δ TM construct expression

A. Schematic representation of human metapneumovirus G protein wild type (wt) and G Δ TM expression construct. CT = cytoplasmic tail and TM = transmembrane domain. Bar indicates scale in amino acids. **B.** Lane 1: denaturing, reducing SDS-PAGE of purified G Δ TM stained with Coomassie blue. **C.** Lane 2: denaturing, reducing SDS-PAGE of purified G Δ TM immunoblotted with anti-HMPV serum. **D.** Native SDS-PAGE of recombinant HMPV proteins, blotted with anti-HMPV serum. Lane 3: G Δ TM. Lane 4: HMPV F Δ TM. **E.** Enzymatic deglycosylation of G Δ TM, denaturing SDS-PAGE and immunoblotted with anti-HMPV serum. Lane 5: G Δ TM + PNGase F. Lane 6: G Δ TM + PNGase F + neuraminidase. Lane 7: G Δ TM + PNGase F + neuraminidase + O-glycosidase. Molecular weight markers in kilodaltons shown to left of each image.

Figure 3. Schematic of vaccine experimental design

Four groups of animals were immunized twice on days 0 and 14 with PBS, GΔTM alone (GΔTM), or adjuvanted GΔTM (GΔTMadj), or infected once intranasally with HMPV on day 0. Days shown on horizontal axis.

Figure 4. Antibody responses to GΔTM in cotton rats

A. Cotton rat serum IgG titers to HMPV GΔTM protein; y-axis scale represents reciprocal serum dilutions (log₂) and data points indicate endpoint dilution positive by ELISA. **B–G.** Light microscopic and immunofluorescent images of HMPV-infected LLC-MK2 cell monolayers stained with GΔTM-immunized or HMPV-infected cotton rat (CR) serum and AlexaFluor568-goat anti-GP IgG. **B, C.** GΔTM-immunized CR, 1:40. **D, E.** Adjuvanted GΔTM-immunized CR, 1:160. **F, G.** HMPV-infected CR, 1:160. All images at 20× original magnification. Contrast for all images increased by 50% for clarity in printed image.

Figure 5. Virus titers and cytokine responses following virus challenge of immunized animals
Groups were immunized twice on days 0 and 14 with PBS, GΔTM alone (GΔTM), or adjuvanted GΔTM (GΔTMadj), or infected once intranasally with HMPV on day 0. All animals were challenged with HMPV intranasally on day 28 and euthanized for virus titration, histological analysis, and cytokine mRNA measurement on day 32. **A.** Nasal titers of HMPV expressed as pfu/g of nasal turbinate (NT). **B.** Lung titers of HMPV expressed as pfu/g of lung tissue. Comparisons between groups were made by t test, 2-tailed, assuming unequal variance. **C.** Cytokine gene mRNA expression levels in lung homogenates of HMPV-infected naive and immunized cotton rats expressed as 2^(-ΔΔCt) compared to PBS-immunized animals.

Comparisons between groups were made by t test, 2-tailed, assuming unequal variance; bars represent mean $\pm$ SEM. * $p = 0.02$ G Δ TM vs. HMPV group.