Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry

Heike Hofmann*, Krzysztof Pyrc†, Lia van der Hoek†, Martina Geier*, Ben Berkhout†, and Stefan Pöhlmann*‡

*Institute for Clinical and Molecular Virology and Nikolaus Fiebiger Center, University Erlangen–Nürnberg, 91054 Erlangen, Germany; and †Department of Human Retrovirology, Academic Medical Center, University of Amsterdam, 1105 AZ, Amsterdam, The Netherlands

Coronavirus (CoV) infection of humans is usually not associated with severe disease. However, discovery of the severe acute respiratory syndrome (SARS) CoV revealed that highly pathogenic human CoVs (HCoVs) can evolve. The identification and characterization of new HCoVs is, therefore, an important task. Recently, a HCoV termed NL63 was discovered in patients with respiratory tract illness. Here, cell tropism and receptor usage of HCoV-NL63 were analyzed. The NL63 spike (S) protein mediated infection of different target cells compared with the closely related 229E-S protein but facilitated entry into cells known to be permissive to SARS-CoV-S-driven infection. An analysis of receptor engagement revealed that NL63-S binds angiotensin-converting enzyme (ACE) 2, the receptor for SARS-CoV, and HCoV-NL63 uses ACE2 as a receptor for infection of target cells. Potent neutralizing activity directed against NL63- but not 229E-S protein was detected in virtually all sera from patients 8 years of age or older, suggesting that HCoV-NL63 infection of humans is common and usually acquired during childhood. Here, we show that SARS-CoV shares its receptor ACE2 with HCoV-NL63. Because the two viruses differ dramatically in their ability to induce disease, analysis of HCoV-NL63 might unravel pathogenicity factors in SARS-CoV. The frequent HCoV-NL63 infection of humans suggests that highly pathogenic variants have ample opportunity to evolve, underlining the need for vaccines against HCoVs.

Coronaviruses (CoVs) are enveloped RNA viruses that are grouped according to genome sequence and serology (1). Human CoVs (HCoVs) 229E and OC43 are members of groups I and II, respectively, and infection with these viruses is thought to be responsible for ≈30% of common-cold cases (1). In contrast, infection with severe acute respiratory syndrome (SARS)-CoV causes a severe respiratory tract illness (RTI) that is fatal in ≈10% of infected individuals (2, 3). The factors that determine the pathogenicity of CoVs are incompletely understood; however, a role for the spike (S) protein has been suggested (4). The S proteins of CoVs, which provide virions with a corona-like appearance, mediate infection of target cells and play a central role in viral replication (4). The interaction of CoV S proteins with specific cellular receptors determines, to a large extent, which cells can be infected (5), and the entry process is an attractive target for antiviral therapy (6).

Recently, a HCoV termed NL63 was discovered in infants and immunocompromised adults with RTI (7, 8). HCoV-NL63 is a group I CoV and is most closely related to HCoV-229E (7–9). HCoV-229E employs CD13 (aminopeptidase N) as a receptor for infection of target cells (10, 11). Because the NL63- and 229E-S proteins share 56% amino acid identity (7), it is conceivable that HCoV-NL63 also engages CD13 for infectious cellular entry. However, the HCoV-NL63-S protein contains a unique, 179-aa sequence at its N terminus that does not share homology with other known CoV proteins and that might alter the receptor specificity of NL63-S relative to 229E-S (7).

In general, the functional organization of CoV S proteins is similar to that of glycoproteins from several unrelated viruses (12), such as retroviruses, and the SARS-CoV-S protein can be incorporated into the membrane of retroviral particles (13). These so called pseudovirions ("pseudotypes") accurately mimic receptor engagement and membrane fusion of SARS-CoV and can be used to study S function (13–18). Here, we used retroviral pseudotypes to analyze cell tropism and receptor engagement of HCoV-NL63. We report that the NL63-S protein engages the SARS-CoV receptor angiotensin-converting enzyme (ACE) 2, but not CD13, for cellular entry and that replication of HCoV-NL63 in cell lines depends on ACE2. Moreover, analysis of neutralizing activity in human sera revealed that HCoV-NL63 infection of humans is more frequent than infection with HCoV-229E and is usually acquired during childhood.

Methods

Plasmids. Eukaryotic expression vectors for murine hepatitis virus (MHV) and feline infectious peritonitis virus (FIPV) S proteins were constructed by isolation of the respective fragments from pTUG31-MHV-S and pTUG31-FIPV-S by using *Bam*HI followed by insertion into the *Bgl*II site of pCAGGS. A pCAGGS-based plasmid for expression of the 229E-S protein was constructed by PCR amplification with a fragment comprising the 229E-S gene as template followed by insertion into pCAGGS by using *Kpn*I and *Xho*I. For expression of NL63-S, RNA from HCoV-NL63-infected cells was isolated and reverse-transcribed, and the NL63-S coding region was amplified and cloned into the pCAGGS vector by using *Eco*RI and *Xho*I. The eukaryotic expression plasmid for SARS-CoV-S is described in ref. 16. For construction of soluble Fc fusion proteins, HCoV-S1 subunits were amplified by PCR and inserted into plasmid pAB61 (19).

Cell Culture, Infection, and Reporter Assays. The lymphatic cell lines CEMx174 and B-THP were cultured in RPMI medium 1640 supplemented with 10% FCS. 293T, Huh-7, Vero E6, HOS (human osteosarcoma), MRC-5, U373, and FCWF (*Felis catus*) cells were maintained in Dulbecco's MEM (GIBCO/BRL) supplemented with 10% FCS; HeLa cells were cultured in MEM (GIBCO/BRL) supplemented with 5% FCS; LLC-MK2 cells were grown in a 2:1 mixture of MEM/Hanks' solution and MEM/Earle's salts (Invitrogen) with 10% FCS. HCoV-NL63
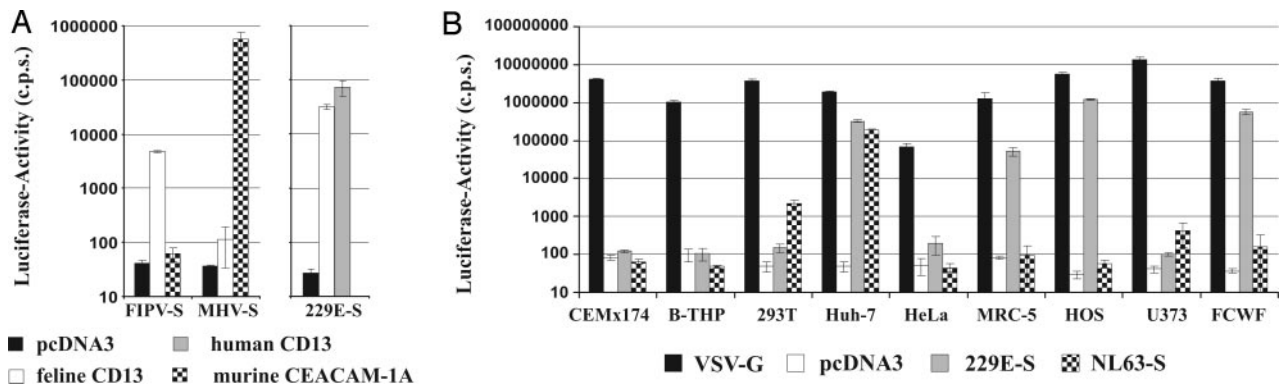

Fig. 1. Cellular tropism of NL63-S- and 229E-S-bearing pseudotypes. (*A*) 293T cells were transfected with CoV receptors or pcDNA3 and infected with the indicated viral pseudotypes, and luciferase activities in the cell lysates were determined. c.p.s., counts per sec. A representative experiment is shown. Comparable results were obtained in an independent experiment. Error bars indicate SD. (*B*) Cell lines were infected with the indicated p24-normalized viral pseudotypes, and luciferase activities in the cell lysates were determined. The results were confirmed in three independent experiments. Error bars indicate SD.

was cultured on LLC-MK2 cells as described in ref. 7. Tissue culture 50% infectious dose was measured by cytopathic effect (CPE) development in LLC-MK2 cells after inoculation with serial dilutions of viral supernatant. HIV-based pseudotypes were produced as described in ref. 16. Briefly, the pNL4-E$^-$R$^-$Luc plasmid and expression vectors for CoV-S proteins or control glycoproteins [vesicular stomatitis virus G protein (VSV-G) and murine leukemia virus glycoprotein (MLV-GP)] were cotransfected into 293T cells. The supernatant was used for infection of target cells followed by determination of luciferase activity 72 h after infection by using a commercially available kit (Promega). For analyses of receptor engagement, 293T cells were transiently transfected with expression vectors encoding murine carcinoembryonic antigen (CEACAM)-1a, human or feline CD13, human ACE1 or ACE2, seeded into 96-well plates, and infected with pseudovirions normalized for comparable infection of Huh-7 cells.

Antibodies and FACS Analysis. Surface expression of ACE1 or ACE2 on transfected 293T cells was detected by FACS analysis with purified polyclonal antibodies directed against the respective proteins (R & D Systems) in combination with a FITC-labeled secondary antibody (Dianova, Hamburg, Germany). Soluble Fc fusion proteins were transiently expressed in 293T cells, concentrated from culture supernatant by using Centri-conPlus ultrafilters (Millipore), and incubated for 30 min on ice with 293T cells expressing either pcDNA3 or ACE2. Bound Fc fusion proteins were subsequently detected by using an anti-human Cy5-coupled secondary antibody (Dianova).

Inhibition of S-Mediated Entry into Target Cells by Soluble ACE2. Soluble ACE2 ectodomain was concentrated from the supernatant of transiently transfected 293T cells as described in ref. 20. For inhibition of S-mediated infection, NL63-, SARS-CoV-, or 229E-S pseudotypes standardized for equal luciferase activity upon infection of Huh-7 cells were preincubated with various dilutions of soluble ACE2 for 1 h at 37°C followed by infection of Huh-7 target cells. Luciferase activity in the cell extracts was determined after 72 h.

Neutralization Assays. NL63-S, 229E-S, or control pseudotypes standardized for equal luciferase activity upon infection of Huh-7 cells were preincubated with a 1:50 dilution of human serum samples for 1 h at 37°C. Alternatively, cells were incubated with purified polyclonal antibodies directed against ACE1 or ACE2. Thereafter, Huh-7 target cells were infected, and luciferase activity in the cell extracts was determined after 72 h. For

inhibition of HCoV-NL63 replication, LLC-MK2 or Huh-7 cells were incubated with ACE1 or ACE2 antibodies for 30 min before infection with HCoV-NL63 at a multiplicity of infection of 1.6×10^{-2} (LLC-MK2) or 3×10^{-2} (Huh-7). Virus replication in the culture was assessed on day 4 or 5 after infection by scoring development of a CPE.

Results

Cell Tropism of NL63- and 229E-S-Bearing Pseudotypes. We first confirmed that the pseudotyping system is, indeed, an adequate tool to analyze receptor engagement by S proteins of widely different CoVs. Retroviral pseudotypes bearing the S proteins of HCoV-229E, FIPV, and MHV were used for infection of 293T cells transiently expressing CD13 or murine CEACAM-1, the receptors for HCoV-229E, FIPV, and MHV, respectively (Fig. 1*A*). All viruses encode the luciferase reporter gene, which is expressed only upon successful integration of the proviral genome into the genome of host cells. Expression of human and feline CD13 rendered the cells permissive to infection driven by 229E-S and FIPV-S, respectively, and expression of CEACAM-1 allowed entry of MHV-S-bearing pseudotypes, whereas control-transfected cells were not infected (Fig. 1*A*). Thus, receptor engagement of the pseudovirions bearing different CoV S proteins is identical to that of the CoVs from which the S proteins were derived, underlining the fact that pseudoparticles are adequate tools to determine receptor binding by CoV S proteins.

Because the sequence of the S protein of HCoV-NL63 is 56% identical to that of HCoV-229E, we first analyzed the range of target cells susceptible to infection driven by these S proteins. Viruses bearing the G protein of vesicular stomatitis virus (VSV) served as positive control, whereas pseudovirions bearing no viral glycoprotein were used as negative control. All cell lines tested were highly susceptible to infection driven by VSV-G but were resistant to infection by control viruses bearing no glycoprotein (Fig. 1*B*). Pseudovirions harboring the 229E-S protein infected MRC-5, HOS, and FCWF cells with appreciable efficiency, whereas none of these cells was susceptible to infection driven by NL63-S, indicating that the two S proteins interact with different cellular receptors. NL63-S mediated entry into Huh-7 and, with variable efficiency, into 293T cells (Fig. 1*B*). These cells should, therefore, express the HCoV-NL63 receptor, and at least Huh-7 should be permissive to HCoV-NL63 entry.

The fusion activity of glycoproteins of enveloped viruses is activated by either receptor binding or protonation in endosomal vesicles (21). Viruses that use the latter entry route can be inhibited by lysosomotropic agents such as bafilomycin A. Bafilomycin A and NH$_4$Cl treatment of Huh-7 cells revealed that
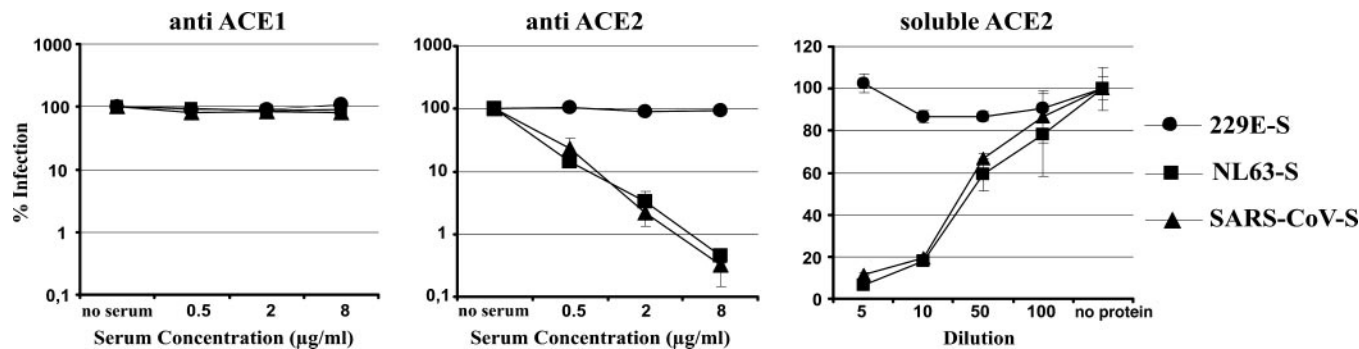
Fig. 2. Inhibition of NL63-S-driven infection by ACE2-specific antibodies and soluble ACE2. Huh-7 cells were preincubated with ACE1- (*Left*) or ACE2- (*Center*) specific polyclonal antibodies, or the pseudotyped virions were preincubated with the ACE2 ectodomain (*Right*). Subsequently, the Huh-7 cells were infected with the indicated pseudovirions, and luciferase activities in the cell lysates were quantified. The results are shown as the percent of infection in the absence of inhibitor and were confirmed in two independent experiments. Error bars indicate SD.

infectious entry driven by NL63-S protein depends on the low-pH environment in intracellular vesicles (data not shown). Similar results were obtained for 229E-S-dependent infection, which is in agreement with published data (22). Thus, the S proteins of HCoV-229E and HCoV-NL63 employ the same route of entry but likely interact with different receptors.

HCoV-NL63 Engages ACE2 as a Receptor for Infectious Cellular Entry. Comparison of the cell tropism of NL63-S-bearing pseudovirions with that documented for replication-competent SARS-CoV and SARS-CoV-S-harboring pseudotypes revealed striking similarities. Thus, Huh-7 and 293T cells are permissive to both NL63- and SARS-CoV-S-driven infection (13, 16, 18) and express the SARS-CoV receptor ACE2 (20, 23–25), whereas CEMx174, HeLa, and HOS cells are not permissive (13, 16, 18) and do not express ACE2 (20, 25). We therefore determined whether ACE2 plays a role in HCoV-NL63 infection. Purified antibodies against the ectodomain of ACE1 did not modulate infection of Huh-7 cells by pseudotypes bearing 229E-, NL63-, or SARS-CoV-S (Fig. 2 *Left*). In contrast, purified antibodies against the ectodomain of ACE2 (Fig. 2 *Center*) or preincubation

of pseudovirions with soluble ACE2 ectodomain (Fig. 2 *Right*) potently blocked infection driven by NL63- and SARS-CoV- but not 229E-S protein, indicating that NL63-S employs ACE2 for infectious cellular entry. To further investigate interactions of NL63-S with CoV receptors, CD13, ACE2, and the controls ACE1 and empty vector were overexpressed in 293T cells followed by infection with pseudotyped virions normalized for comparable infection of Huh-7 cells. Expression of CD13 rendered 293T cells highly permissive to infection driven by the S protein of HCoV-229E but not HCoV-NL63 or SARS-CoV (Fig. 3*A*). The reverse observation was made for cells expressing ACE2 (Fig. 3*A*), confirming that, despite the similarity between 229E- and NL63-S proteins, the latter engages ACE2 and not CD13 for cellular entry. FACS analysis employing soluble S1 domains of NL63- and SARS-CoV-S revealed binding of NL63-S1 to cells expressing ACE2 but not empty vector (Fig. 3*B*), indicating that ACE2 and NL63-S protein directly interact. Of note, SARS-CoV-S bound more efficiently to ACE2-expressing cells than NL63-S (Fig. 3*B*), which could be indicative of a higher binding affinity. Finally, replication of HCoV-NL63 in LLC-MK2 and Huh-7 cells (Fig. 4*A*), both of which express
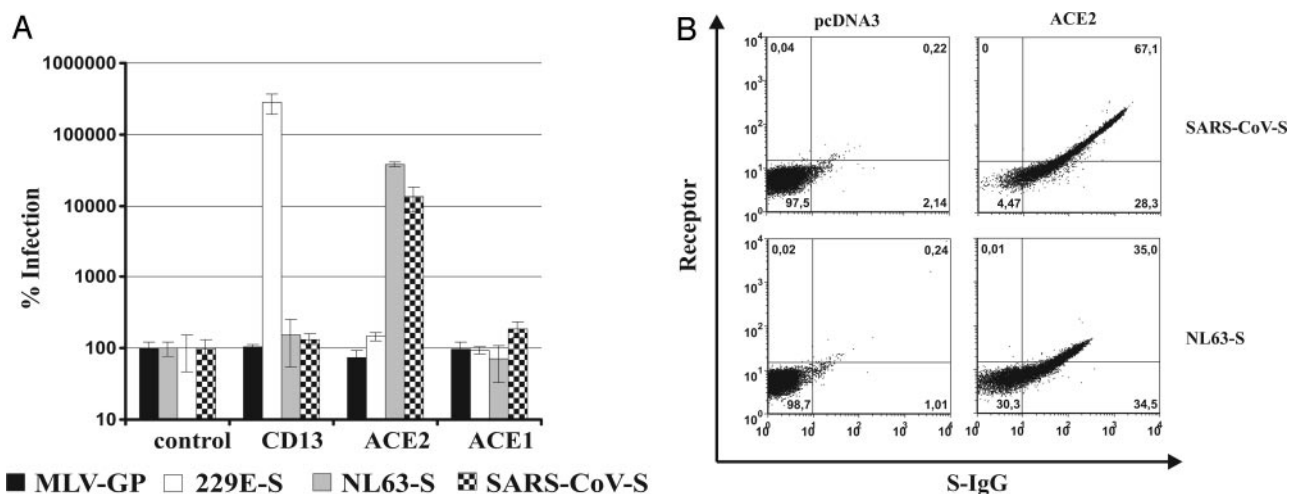
Fig. 3. Expression of ACE2 potentiates NL63-S-driven infection, and soluble NL63-S protein binds to ACE2-positive cells. (*A*) 293T cells expressing CD13, ACE2, ACE1, or pcDNA3 were infected with the indicated pseudotypes, and luciferase activities in the cell lysates were determined. The results are shown as the percent of infection of pcDNA3-transfected cells. Similar results were obtained in three independent experiments. Error bars indicate SD. (*B*) ACE2 or pcDNA3 were transiently expressed on 293T cells, the cells were incubated with the S1 subunit of NL63-S or SARS-CoV-S fused to the Fc portion of human immunoglobulin, and receptor expression and S-Fc-fusion-protein binding were analyzed by FACS. Two independent experiments yielded similar results. NL63- and SARS-CoV-S binding was assessed with the same batch of transfected cells. Differences in ACE2 signal might be due to the masking of different ACE2 epitopes by the two S proteins, resulting in differential recognition of ACE2 by the polyclonal serum.
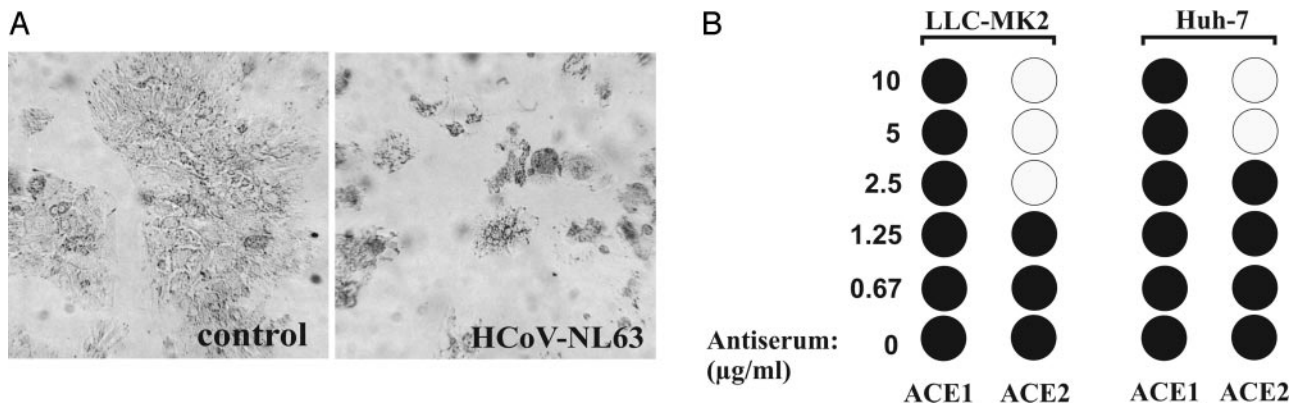
Fig. 4. Inhibition of HCoV-NL63 replication by ACE2-specific antibodies. (*A*) Huh-7 cells were infected with HCoV-NL63 or mock infected, and CPE development was assessed 5 d after infection. Comparable results were obtained in several independent experiments. (*B*) LLC-MK2 and Huh-7 cells were preincubated with the indicated concentrations of ACE1- or ACE2-specific polyclonal antibodies and infected with HCoV-NL63, and the development of CPE was assessed. ●, CPE development; ○, absence of CPE. Similar results were obtained in an independent experiment.

ACE2 (18, 20), was inhibited by antibodies against ACE2 but not ACE1 (Fig. 4*B*), demonstrating that replication-competent HCoV-NL63 engages ACE2 as a receptor for spread in target cells. In summary, these data suggest a major role for ACE2 in HCoV-NL63 infection.

Neutralizing Activity Directed Against NL63-S Is Common in Sera from Adults. HCoV-NL63 had initially been isolated from infants and was also detected in immunocompromised adults (7, 8). To evaluate the frequency of HCoV-NL63 in comparison with HCoV-229E infection, we first analyzed the neutralizing activity of sera obtained from adults with RTI, healthy adults, and infants between 3 and 6 months of age. Virtually all sera from adults with RTI or from healthy adults neutralized NL63-S-bearing pseudotypes with high efficiency (Fig. 5*A*). In contrast, strong neutralization of 229E-S-harboring pseudotypes was observed with only a minority of the sera analyzed (Fig. 5*A*), and sera from infants poorly neutralized infection driven by both NL63-S and 229E-S (Fig. 5). None of the sera analyzed neutralized infection by MLV-GP-bearing pseudotypes (Fig. 5*B*), demonstrating that the neutralization of NL63-S and 229E-S-bearing pseudotypes was, indeed, due to antibodies directed against the respective viral S proteins. Several sera strongly neutralized NL63-S-driven infection but had no effect on 229E-S-mediated infection (Fig. 5*A* and data not shown), suggesting that a neutralizing humoral immune response directed against HCoV-NL63 does not necessarily confer protection against infection by the closely related HCoV-229E. In turn, these data also indicate that neutralization of NL63-S-dependent infection was, in most cases, not due to crossreactivity of antibodies directed against 229E-S, although the presence of crossneutralizing antibodies cannot be excluded. Moreover, all sera from healthy adults recognized the N-terminal, unique sequence in NL63-S and inhibited HCoV-NL63 replication in LLC-MK2 cells (data not shown), further underlining that neutralization of NL63-S-driven infection was due to NL63-S-specific antibodies and not to crossneutralization.

Because sera from infants between 3 and 6 months of age did not modulate NL63-S-driven infection (Fig. 5*A*), we investigated at which age a neutralizing antibody response against HCoV-NL63 becomes detectable. Analysis of sera from children of five different age groups revealed that neutralizing antibodies directed against NL63-S are first detectable in individuals with an average age of ≈1.5 years and are found in most individuals with an average age of ≈8 years (Fig. 5*B*). Thus, HCoV-NL63 infection is frequent and is usually acquired during childhood.

Discussion

We demonstrated that HCoV-NL63 engages the SARS-CoV receptor ACE2 for infectious entry. Smith and colleagues independently obtained the same result (M. K. Smith, S. Tusell, B. B., L.v.d.H., and K. Holmes, unpublished data). The carboxypeptidase ACE2 is an important component of the renin–angiotensin system, which controls blood pressure (27, 28), and ACE2 is required for cardiac function in mice (29). ACE2 expression in lung and intestine (30) explains important aspects of SARS-CoV tropism, and the protein likely plays a central role in SARS-CoV spread (12). However, it is unclear how the virus induces disease and whether the way it engages ACE2 contributes to this process. Analysis of HCoV-NL63, a related but less pathogenic virus that shares the receptor, and thus, a major feature of its replication strategy, with SARS-CoV might yield important insights into this question.

The receptor specificity of viral glycoproteins determines, at least in part, which cell types can be infected, and the range of permissive cells has important implications for viral pathogenicity. HCoV-NL63 engages the same receptor and, consequently, infects the same target cells as SARS-CoV, but, in contrast to SARS-CoV, the virus usually induces only mild or moderate respiratory disease (31–34). However, HCoV-NL63 was also detected in infants and immunocompromised adults with relatively severe RTI (7, 8, 35), suggesting that infection might have more profound pathogenic effects in individuals with reduced immune defenses. It is therefore conceivable that HCoV-NL63 lacks a specific pathogenicity factor present in SARS-CoV. Such a factor could be encoded by one or several of the accessory genes, nine of which are found in the SARS-CoV genome (36, 37). In stark contrast, the HCoV-NL63 genome harbors only a single accessory gene (7, 8). Whereas the function of the SARS-CoV accessory genes is largely unknown, the accessory genes of MHV were found to be dispensable for replication but required for full viral pathogenicity (38). Some of the SARS-CoV accessory genes might, therefore, encode proteins that promote the development of SARS. The contribution of the accessory genes to the replication and pathogenesis of HCoV-NL63 and SARS-CoV must ultimately be assessed in infected animals. The establishment of HCoV-NL63 reverse genetics systems and small-animal models, both already described for SARS-CoV (39, 40), are important prerequisites for these studies.

Another explanation for the apparent differences in HCoV-NL63 and SARS-CoV pathogenicity could be differences in interactions with ACE2. The SARS-CoV-S protein binds to

Fig. 5. Neutralization of NL63-S-driven infection by human sera. (*A*) The indicated pseudovirions were incubated with 50-fold-diluted sera from healthy adults, adults with RTI, or infants and added onto Huh-7 cells, and luciferase activities in the cell lysates were determined. The results were confirmed in an independent experiment. Error bars indicate SD. (*B*) The indicated pseudovirions were incubated with 50-fold-diluted sera from a total of 25 infants of defined age groups and used for infection of Huh-7 cells as described for *A*. Sera from four individuals were analyzed per age group. Within age groups, bars indicate results obtained with serum from single individuals. The black bars indicate infection in the absence of patient serum. An independent experiment yielded similar results. Error bars indicate SD.

human ACE2 with high efficiency (24), and amino acid residues in SARS-CoV-S, which are crucial for the interaction, have been identified (41). However, the binding of SARS-CoV-S to murine ACE2 is clearly less efficient (42) and might account for the limited replication of the virus upon inoculation into mice (39, 42). Soluble NL63-S protein bound less robustly to ACE2-expressing cells compared with SARS-CoV-S, perhaps reflecting

reduced affinity. If so, the S proteins might, at least partially, account for the differential pathogenicity of HCoV-NL63 and SARS-CoV. In this scenario, HCoV-NL63 variants, which bind to ACE2 with high affinity and induce severe disease, should have ample opportunity to evolve, considering the frequent HCoV-NL63 infection of humans and high mutation rate of CoVs.

The interaction of NL63-S with ACE2 is most puzzling when taking into account that NL63-S is closely related to 229E-S, which binds CD13, but shares no appreciable amino acid identity with SARS-CoV-S, which binds ACE2. Similarly, the amino acid sequence of the CD13-binding site in 229E-S is 57% conserved in NL63-S, whereas the alignment of the ACE2-binding site of SARS-CoV-S with the most closely related sequence in NL63-S reveals only 14% amino acid identity. These data suggest that NL63- and SARS-CoV-S might have evolved different strategies to contact ACE2 or bind to different regions in ACE2. In fact, NL63-S harbors a 179-aa insertion at its N terminus (7, 8), which does not share homology with any CoV sequences and which might be involved in ACE2 recognition. The latter speculation is in agreement with our observation that the bacterially purified unique region of NL63-S is recognized by human sera (data not shown), which exhibit strong neutralizing activity directed against NL63-S. Mutagenic analysis could be used to address the role of the unique region in NL63-S-mediated cellular entry. One of many possible ways to test whether SARS-CoV- and NL63-S bind to ACE2 differentially is to assess the impact of ACE2 inhibitors on SARS-CoV and HCoV-NL63 infection. Inhibitory compounds that bind to the active site in ACE2 and induce substantial conformational changes have been described in ref. 43, but their antiviral activity remains to be determined.

Analysis of the NL63-S protein interactions with ACE2 might also reveal important insights into the evolution of this virus. One could imagine that HCoV-NL63 and HCoV-229E have a common ancestor and, over time, NL63-S acquired ACE2 usage, whereas 229E-S evolved the use of CD13 as a receptor. Alternatively, HCoV-NL63 might have acquired the unique N-terminal sequence in its S protein by recombination with cellular or viral sequences, and these sequences might have conferred ACE2 binding to NL63-S. If so, this recombination event probably occurred early in HCoV-NL63 evolution because the unique sequence exhibits the same low G+C content, which is characteristic for the entire HCoV-NL63 genome (9). However, the unique region in NL63-S does not share appreciable homology with any sequences in the database (7). A viral or cellular donor of this sequence is, therefore, not obvious.

Infection with HCoV-229E and HCoV-OC43 is thought to be frequent and to account for a substantial amount of common-cold cases (1). Our observation that virtually all sera from adults potently neutralized NL63-S- but not 229E-S-mediated infection suggests that HCoV-NL63 infection is more prevalent than infection with HCoV-229E. Indeed, recent reports demonstrate that HCoV-NL63 is globally distributed and that infection is associated with RTI in children (31–35). Intriguingly, HCoV-NL63 infection was also found to be associated with Kawasaki disease (26), which can affect the coronary arteries and is a major cause for acquired heart disease in young children. The expression of ACE2 in coronary vessels (28) further supports a possible role of HCoV-NL63 in Kawasaki disease. In any event, diagnostic tests to detect HCoV-NL63 infection need to be developed, especially when considering the frequency of infection and the apparent similarities in HCoV-NL63 and SARS-CoV replication. In this regard, it is of interest that several patient sera potently neutralized NL63-S-driven infection but did not inhibit infection driven by 229E-S or SARS-CoV-S (data not shown), suggesting that neutralizing antibodies directed against HCoV-NL63 might not protect against infection with other HCoVs.

In summary, comparative analysis of HCoV-NL63 and SARS-CoV might reveal important aspects of SARS pathogenesis. It will be especially interesting to investigate whether the mode of ACE2 engagement by the viral S proteins impacts viral replication and pathogenesis. The establishment of reverse genetics systems and animal models for HCoV-NL63 replication are indispensable for these studies. The characterization of NL63 and SARS-CoV-S interactions with ACE2 might also have important implications for inhibitor development, because the S–ACE2 interface is a major target for therapeutic intervention. Finally, the apparent similarities between HCoV-NL63 and SARS-CoV replication and the frequent HCoV-NL63 infection of humans suggest that pathogenic HCoVs can evolve, highlighting the need for efficient vaccines against HCoVs.

1. Holmes, K. V. (2001) in *Fields Virology*, eds. Fields, B. N., Knipe, D. M., Howley, P. M., Griffin, D. E., Lamb, R. A., Martin, M. A., Roizman, B. & Straus, S. E. (Lippincott, Williams & Wilkins, Philadelphia), pp. 1187–1203.
2. Stadler, K., Masignani, V., Eickmann, M., Becker, S., Abrignani, S., Klenk, H. D. & Rappuoli, R. (2003) *Nat. Rev. Microbiol.* **1,** 209–218.
3. Peiris, J. S., Yuen, K. Y., Osterhaus, A. D. & Stohr, K. (2003) *N. Engl. J. Med.* **349,** 2431–2441.
4. Gallagher, T. M. & Buchmeier, M. J. (2001) *Virology* **279,** 371–374.
5. Kuo, L., Godeke, G. J., Raamsman, M. J., Masters, P. S. & Rottier, P. J. (2000) *J. Virol.* **74,** 1393–1406.
6. Bosch, B. J., Van Der Zee, R., de Haan, C. A. & Rottier, P. J. (2003) *J. Virol.* **77,** 8801–8811.
7. van der Hoek, L., Pyrc, K., Jebbink, M. F., Vermeulen-Oost, W., Berkhout, R. J. M., Wolthers, K. C., Wertheim-van Dillen, P. M. E., Kaandorp, J., Spaargaren, J. & Berkhout, B. (2004) *Nat. Med.* **10,** 368–373.
8. Fouchier, R. A., Hartwig, N. G., Bestebroer, T. M., Niemeyer, B., de Jong, J. C., Simon, J. H. & Osterhaus, A. D. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 6212–6216.
9. Pyrc, K., Jebbink, M. F., Berkhout, B. & van der Hoek, L. (2004) *Virol. J.* **1,** 7.
10. Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjostrom, H., Noren, O. & Laude, H. (1992) *Nature* **357,** 417–420.
11. Yeager, C. L., Ashmun, R. A., Williams, R. K., Cardellichio, C. B., Shapiro, L. H., Look, A. T. & Holmes, K. V. (1992) *Nature* **357,** 420–422.
12. Hofmann, H. & Pöhlmann, S. (2004) *Trends Microbiol.* **12,** 466–472.
13. Simmons, G., Reeves, J. D., Rennekamp, A. J., Amberg, S. M., Piefer, A. J. & Bates, P. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 4240–4245.
14. Giroglou, T., Cinatl, J., Jr., Rabenau, H., Drosten, C., Schwalbe, H., Doerr, H. W. & von Laer, D. (2004) *J. Virol.* **78,** 9007–9015.
15. Han, D. P., Kim, H. G., Kim, Y. B., Poon, L. L. & Cho, M. W. (2004) *Virology* **326,** 140–149.
16. Hofmann, H., Hattermann, K., Marzi, A., Gramberg, T., Geier, M., Krumbiegel, M., Kuate, S., Überla, K., Niedrig, M. & Pöhlmann, S. (2004) *J. Virol.* **78,** 6134–6142.
17. Moore, M. J., Dorfman, T., Li, W., Wong, S. K., Li, Y., Kuhn, J. H., Coderre, J., Vasilieva, N., Han, Z., Greenough, T. C., *et al.* (2004) *J. Virol.* **78,** 10628–10635.
18. Yang, Z. Y., Huang, Y., Ganesh, L., Leung, K., Kong, W. P., Schwartz, O., Subbarao, K. & Nabel, G. J. (2004) *J. Virol.* **78,** 5642–5650.
19. Birkmann, A., Mahr, K., Ensser, A., Yaguboglu, S., Titgemeyer, F., Fleckenstein, B. & Neipel, F. (2001) *J. Virol.* **75,** 11583–11593.
20. Hofmann, H., Geier, M., Marzi, A., Krumbiegel, M., Peipp, M., Fey, G. H., Gramberg, T. & Pöhlmann, S. (2004) *Biochem. Biophys. Res. Commun.* **319,** 1216–1221.
21. Smith, A. E. & Helenius, A. (2004) *Science* **304,** 237–242.
22. Blau, D. M. & Holmes, K. V. (2001) *Adv. Exp. Med. Biol.* **494,** 193–198.
23. Wang, P., Chen, J., Zheng, A., Nie, Y., Shi, X., Wang, W., Wang, G., Luo, M., Liu, H., Tan, L., *et al.* (2004) *Biochem. Biophys. Res. Commun.* **315,** 439–444.
24. Li, W., Moore, M. J., Vasilieva, N., Sui, J., Wong, S. K., Berne, M. A., Somasundaran, M., Sullivan, J. L., Luzuriaga, K., Greenough, T. C., *et al.* (2003) *Nature* **426,** 450–454.
25. Nie, Y., Wang, P., Shi, X., Wang, G., Chen, J., Zheng, A., Wang, W., Wang, Z., Qu, J., Luo, M., *et al.* (2004) *Biochem. Biophys. Res. Commun.* **321,** 994–1000.
26. Esper, F., Shapiro, E. D., Weibel, C., Ferguson, D., Landry, M. L. & Kahn, J. S. (2005) *J. Infect. Dis.* **191,** 499–502.
27. Rice, G. I., Thomas, D. A., Grant, P. J., Turner, A. J. & Hooper, N. M. (2004) *Biochem. J.* **383,** 45–51.
28. Danilczyk, U., Eriksson, U., Crackower, M. A. & Penninger, J. M. (2003) *J. Mol. Med.* **81,** 227–234.
29. Crackower, M. A., Sarao, R., Oudit, G. Y., Yagil, C., Kozieradzki, I., Scanga, S. E., Oliveira-dos-Santos, A. J., da Costa, J., Zhang, L., Pei, Y., *et al.* (2002) *Nature* **417,** 822–828.
30. Hamming, I., Timens, W., Bulthuis, M. L., Lely, A. T., Navis, G. J. & van Goor, H. (2004) *J. Pathol.* **203,** 631–637.
31. Bastien, N., Anderson, K., Hart, L., Van Caeseele, P., Brandt, K., Milley, D., Hatchette, T., Weiss, E. C. & Li, Y. (2005) *J. Infect. Dis.* **191,** 503–506.
32. Ebihara, T., Endo, R., Ma, X., Ishiguro, N. & Kikuta, H. (2005) *J. Med. Virol.* **75,** 463–465.
33. Esper, F., Weibel, C., Ferguson, D., Landry, M. L. & Kahn, J. S. (2005) *J. Infect. Dis.* **191,** 492–498.
34. Moes, E., Vijgen, L., Keyaerts, E., Zlateva, K., Li, S., Maes, P., Pyrc, K., Berkhout, B., van der Hoek, L. & Van Ranst, M. (2005) *BMC Infect. Dis.* **5,** 6.
35. Arden, K. E., Nissen, M. D., Sloots, T. P. & Mackay, I. M. (2005) *J. Med. Virol.* **75,** 455–462.
36. Marra, M. A., Jones, S. J., Astell, C. R., Holt, R. A., Brooks-Wilson, A., Butterfield, Y. S., Khattra, J., Asano, J. K., Barber, S. A., Chan, S. Y., *et al.* (2003) *Science* **300,** 1399–1404.
37. Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Penaranda, S., Bankamp, B., Maher, K., Chen, M. H., *et al.* (2003) *Science* **300,** 1394–1399.
38. de Haan, C. A., Masters, P. S., Shen, X., Weiss, S. & Rottier, P. J. (2002) *Virology* **296,** 177–189.
39. Subbarao, K., McAuliffe, J., Vogel, L., Fahle, G., Fischer, S., Tatti, K., Packard, M., Shieh, W. J., Zaki, S. & Murphy, B. (2004) *J. Virol.* **78,** 3572–3577.
40. Yount, B., Curtis, K. M., Fritz, E. A., Hensley, L. E., Jahrling, P. B., Prentice, E., Denison, M. R., Geisbert, T. W. & Baric, R. S. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 12995–13000.
41. Wong, S. K., Li, W., Moore, M. J., Choe, H. & Farzan, M. (2004) *J. Biol. Chem.* **279,** 3197–3201.
42. Li, W., Greenough, T. C., Moore, M. J., Vasilieva, N., Somasundaran, M., Sullivan, J. L., Farzan, M. & Choe, H. (2004) *J. Virol.* **78,** 11429–11433.
43. Towler, P., Staker, B., Prasad, S. G., Menon, S., Tang, J., Parsons, T., Ryan, D., Fisher, M., Williams, D., Dales, N. A., *et al.* (2004) *J. Biol. Chem.* **279,** 17996–18007.

MICROBIOLOGY