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REVIEW ARTICLE

Human Coronaviruses 229E and NL63: Close Yet Still So Far

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HCoV-NL63 and HCoV-229E are two of the four human coronaviruses that circulate worldwide. These two viruses are unique in their relationship towards each other. Phylogenetically, the viruses are more closely related to each other than to any other human coronavirus, yet they only share 65% sequence identity. Moreover, the viruses use different receptors to enter their target cell. HCoV-NL63 is associated with croup in children, whereas all signs suggest that the virus probably causes the common cold in healthy adults. HCoV-229E is a proven common cold virus in healthy adults, so it is probable that both viruses induce comparable symptoms in adults, even though their mode of infection differs. Here, we present an overview of the current knowledge on both human coronaviruses, focusing on similarities and differences. [*J Formos Med Assoc* 2009;108(4):270–279]

Key Words: common cold, croup, human coronavirus 229E, human coronavirus NL63

Coronaviruses

Coronaviruses (CoVs), a genus of the *Coronaviridae* family, are positive-strand RNA viruses with the largest viral genome of all RNA viruses (27–32 kb).[1] The genomic RNA is capped, polyadenylated and covered with nucleocapsid proteins. The virus is enveloped and carries large spike glycoproteins. All CoVs have a common genome organization, in which the replicase gene encompasses the 5′ two-thirds of the genome and is comprised of two overlapping open reading frames (ORFs), ORF1a and ORF1b. The structural gene region, which covers the 3′ third of the genome, encodes the canonical set of structural protein genes in the order 5′ - spike (S) - envelope (E) - membrane (M) and nucleocapsid (N) - 3′ (Figure 1). Some group II CoVs carry an additional structural protein that encodes a hemagglutinin esterase. The gene is located between the ORF1b and S gene. Expression of the non-structural replicase proteins is mediated by translation of the genomic RNA that gives rise to the biosynthesis of two large polyproteins, pp1a (encoded by ORF1a) and pp1ab (encoded by ORF1a and ORF1b), which is facilitated by a ribosomal frameshift at the ORF1a/1b junction. In contrast, the structural proteins are translated from subgenomic mRNAs. These subgenomic mRNAs are the result of discontinuous transcription, a hallmark of CoV gene expression. The structural gene region also harbors several ORFs that are interspersed along the structural protein coding genes. The number and location of these accessory ORFs vary between the CoV species.

In animals, CoV infections can lead to a variety of syndromes, e.g. bronchitis, gastroenteritis, progressive demyelinating encephalitis, diarrhea,

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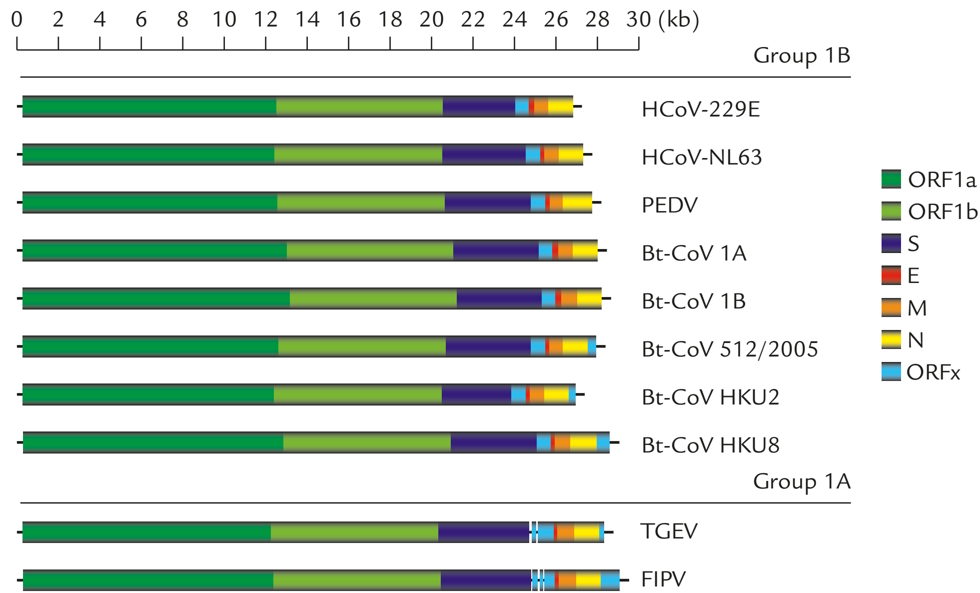

Figure 1. Schematic representation of the genomic organization of group 1 coronaviruses (CoVs). Group 1B CoVs HCoV-229E (NC002645), HCoV-NL63 (NC005831), porcine epidemic diarrhea virus (PEDV; NC003436), bat coronavirus 1A (Bt-CoV 1A; NC010437), Bt-CoV 1B (NC010436), Bt-CoV 512/2005 (NC009657), Bt-CoV HKU2 (NC009988) and Bt-CoV HKU8 (NC010438), and group 1A CoVs porcine transmissible gastroenteritis virus (TGEV; NC002306) and feline infectious peritonitis virus (FIPV; NC007025) genome organization. The open reading frames (ORFs) are denoted as replicase 1A (ORF1a), replicase 1B (ORF1b), S, E, M, N and accessory genes (ORFx).

peritonitis and respiratory tract disease.[1] The first reports on human CoVs (HCoV) appeared in the mid-1960s. The human viruses were isolated from persons with the common cold, and two species were detected: HCoV-229E and HCoV-OC43.[2,3] Almost 40 years later, a CoV was identified as the causative agent of the severe acute respiratory syndrome (SARS).[4,5] A highly effective global public health response prevented further spread of this virus, and as a result, SARS-CoV was eradicated from the human population. Soon thereafter, it became clear that there are more HCoVs. HCoV-NL63 was identified in 2004 and HCoV-HKU1 in 2005.[6,7] Both viruses are not emerging viruses like SARS-CoV but were previously unidentified. In fact, infections caused by these viruses are as common and widespread as HCoV-229E and HCoV-OC43 infections.[8]

The SARS outbreak intensified research on the unknown animal CoVs. As many as 16 new animal CoV species have been identified in the last 3 years.[9–16] There are currently 29 complete reference genome sequences available in GenBank of the various viruses, and three phylogenetically distinct groups exist (Figure 2).[17,18] HCoV-229E and HCoV-NL63 belong to the group 1 CoVs, together with various CoVs isolated from pigs, cats and bats. As shown in Figure 2, HCoV-229E and HCoV-NL63 are the only two human viruses that have a relatively close relationship. HCoV-OC43 is a group 2 virus and clusters tightly with bovine, porcine and equine CoVs. HCoV-HKU1 is not part of that cluster, although the virus clearly belongs to the group 2 CoVs. SARS-CoV is of animal origin, with civet cat SARS-CoV and bat SARS-CoV as very close relatives.[19]

Discovery of Group 1 CoVs

The first described CoV of group 1 was porcine transmissible gastroenteritis virus (TGEV), which was isolated in 1946 from pigs suffering from gastroenteritis.[20] Almost two decades later, one research group located in the UK identified a human respiratory tract pathogen from nasal washings of persons with the common cold.[2] This novel pathogen, HCoV-229E, was later characterized

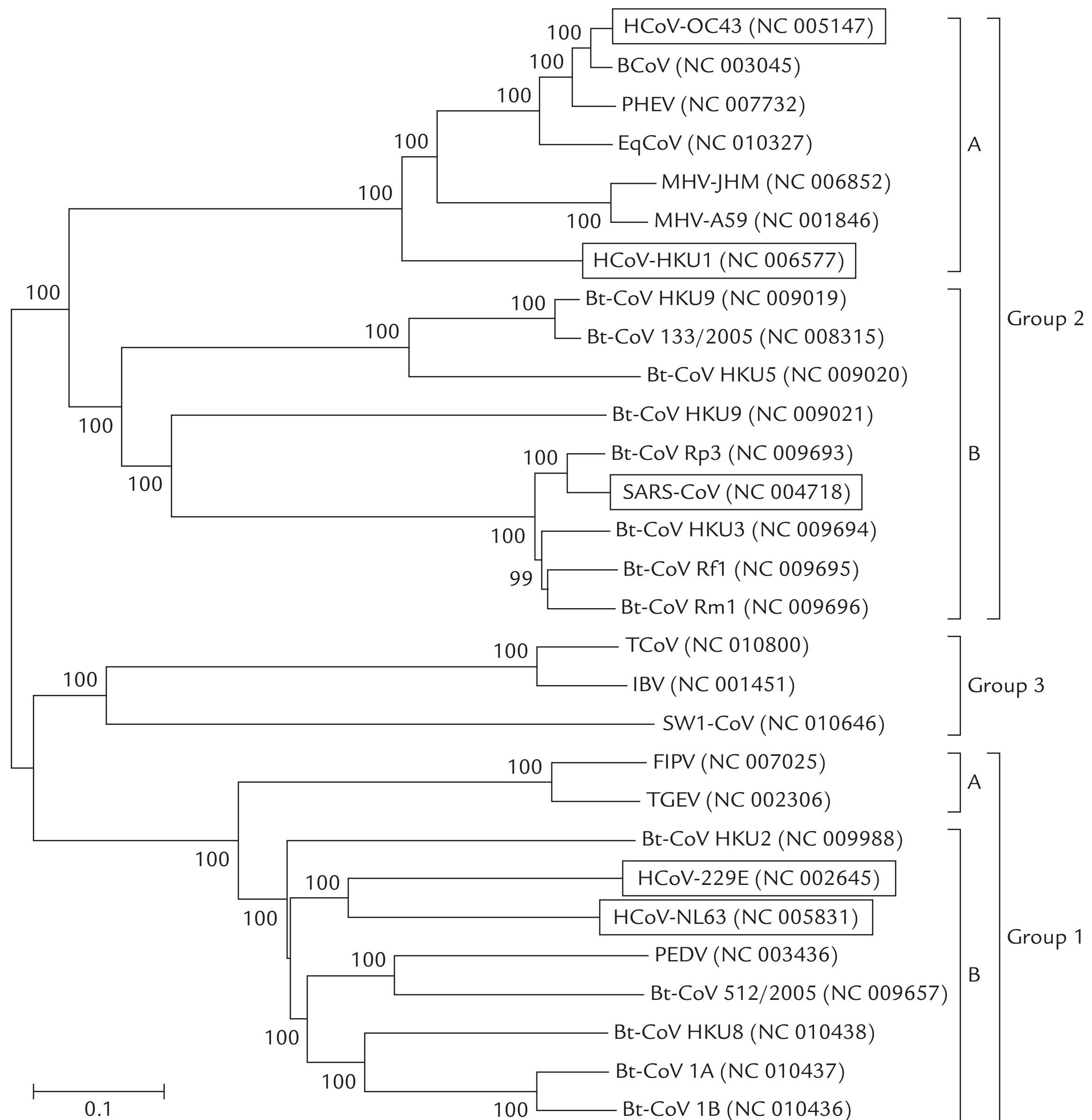

Figure 2. Phylogenetic tree of 29 full-genome coronavirus (CoV) reference strains. The full-genome CoV reference sequences were aligned with ClustalX v2.09.[17] Phylogenetic analysis was conducted with the neighbor-joining method, Kimura 2-parameter distances and a bootstrap of 1000 replicates, using MEGA version 4.01.[18] Bootstrap values below 75 are not shown. The five human CoV species are highlighted by a rectangle. The viruses are denoted as bovine coronavirus (BCoV), porcine hemagglutinating encephalomyelitis virus (PHEV), equine coronavirus (EqCoV), mouse hepatitis virus (MHV), severe acute respiratory syndrome CoV (SARS-CoV), Turkey coronavirus (TCoV), avian infectious bronchitis virus (IBV), porcine epidemic diarrhea virus (PEDV), bat CoV (Bt-CoV), porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and beluga whale coronavirus (SW1-CoV).

morphologically by electron microscopy and compared with the already-well-known avian infectious bronchitis virus.[21] The viruses exhibited a typical crown-like appearance (from Latin *corona*). During the following years, another group 1 member, canine coronavirus, was isolated from sentry dogs with diarrhea and mild gastroenteritis.[22] Similar clinical symptoms were later observed in pigs during a diarrheal outbreak in 1978 on four separate swine breeding farms.[23] The recovered pathogen, now known as porcine epidemic diarrhea virus (PEDV), was first mistyped as a

member of the rotavirus family, yet it soon became clear that the virus shared the morphological characteristics of CoV but was serologically distinct from TGEV.[23] Two cat-associated CoV species were identified in 1981. Feline enteric coronavirus and feline infectious peritonitis virus (FIPV) shared serological characteristics, but differed in clinical outcome.[24] In 1986, another porcine CoV was isolated, porcine respiratory coronavirus, a close relative of TGEV.[25] Hereafter, no new group 1 members were discovered for more than 15 years.

In 2004, we isolated HCoV-NL63 from a 7-month-old child with bronchiolitis.[6] Shortly thereafter, Fouchier et al independently described the same virus from a clinical sample collected in 1988.[26] In 2005, it became clear that several bat species can harbor CoVs that belong to group 1.[10,14,16] Most of these viruses cluster with HCoV-229E, HCoV-NL63 and PEDV, although none of them is a very close relative to any of these viruses.

There are notable differences in the genome composition that divides the group 1 viruses into two separate branches, named 1A and 1B (Figure 1). All group 1A members contain several short accessory protein-coding genes between the S and E genes and one or two accessory protein genes on the 3′ side of the N gene. In contrast, all group 1B members carry only one accessory protein gene, between the S and E genes, with the exception of some bat CoVs. Three bat CoVs carry an additional ORF at the 3′-side of the N gene. The function of the accessory proteins from the group 1 CoVs is unknown. Reverse genetic analyses of FIPV and extensive cell culture adaptation of PEDV, TGEV and HCoV-229E suggest that they are not required for *in vitro* virus replication.[27–31] Moreover, deletion of FIPV, PEDV and TGEV accessory genes results in attenuation of the virus, which indicates that the group 1 accessory proteins represent pathogenicity factors.[27–30]

The discovery timeline of the group 1 CoVs illustrates that this group has grown only recently into a more mature form in which its members can infect a diversity of mammalian hosts. It is not unlikely that additional members will be identified in the near future.

Evolution and Variability of HCoV-229E and HCoV-NL63

HCoV-229E was the first HCoV to be fully sequenced;[32] however, it is striking that the sequence information of circulating strains is very poor. Only one study has described the variability of the S and N genes over time, which suggests that genetic drift shapes HCoV-229E evolution.[33] Fortunately, the sequence information allows calculation of the evolution rate of the virus. With this evolutionary rate, the time to the most common recent ancestor of HCoV-NL63 and HCoV-229E could be calculated.[34] As many as 1000 years ago, the viruses evolved from a common ancestor.[34]

For HCoV-NL63, many more sequences of circulating strains are now available. Four full genomes have been sequenced, and 312 sequences of other regions are available in GenBank (compared to 123 for HCoV-229E). The full-length HCoV-NL63 sequences have shown that two types of viruses exist, but recombination between HCoV-NL63 strains occurs frequently.[34]

Unfortunately, it is unknown whether different types of HCoV-229E strains exist and recombination occurs, since only the first full-length sequence of the laboratory-adapted strain VR-740 is available thus far.[32] Full-length sequences of clinical isolates are urgently needed to address this question. The limitation of having just one laboratory-adapted strain sequence is exemplified by our analysis of the ORF4 region of HCoV-229E.[31] The laboratory-adapted VR-740 strain contains ORF4a and ORF4b genes, and it was assumed that clinical isolates would follow the same gene order. We have sequenced the region from several clinical samples and revealed that HCoV-229E in patients always contains an intact ORF4 gene that encodes one putative ORF4 accessory protein, whereas laboratory-adapted strains are very prone to mutations in this region.[31]

Cell Tropism of HCoV-229E and HCoV-NL63

The S glycoproteins of HCoV-229E and HCoV-NL63 are both class I fusion proteins that mediate infection of target cells.[35,36] The proteins share 56% amino acid identity, but do not use the same receptor.[37] The receptor-recognition regions within S are, for both viruses, not well-defined linear binding sites.[38] For HCoV-NL63, the region between amino acids 476 to 616 is important for binding, whereas for HCoV-229E, amino acids 417 to 547 are involved in receptor recognition.[39,40] HCoV-229E utilizes CD13 (also known as aminopeptidase N) as a receptor, whereas HCoV-NL63 uses angiotensin-converting enzyme 2 (ACE2) for cellular entry.[41–43]

CD13 is a zinc-binding metalloprotease that is ubiquitously expressed in various cell types, including small intestinal and renal tubular epithelial cells, the granulocytic and monocytic lineage, synaptic membranes from the central nervous system, and respiratory epithelial cells.[44–46] CD13 functions in digestion, angiogenesis and synaptic activity, and cleaves peptides bound to major histocompatibility complex molecules of antigen-presenting cells.[46] ACE2 belongs to the same protease family as CD13, and the protein is expressed in testicular, renal, cardiovascular, gastrointestinal and airway tissue.[47] Both metalloproteases are involved in the renin–angiotensin system, which regulates blood pressure. ACE2 plays a role in vasodilatation by C-terminal cleavage of angiotensin II into angiotensin 1–7, and angiotensin I into angiotensin 1–9, whereas CD13 functions at another level by N-terminal cleavage of angiotensin III into angiotensin I, and angiotensin IV into angiotensin 4–8.[48]

Besides HCoV-229E, CD13 is used by PEDV, TGEV and FIPV to enter the cell,[49–52] whereas HCoV-NL63 is the sole group 1 virus that uses ACE2. Only SARS-CoV uses the same protein for entry.[53] It has been suggested that SARS-CoV pathogenicity is related to the downregulation of ACE2 upon infection.[54] ACE2 protects against lung damage and the lack of ACE2 on the cell surface may account for the damage during infection.[54] Whether HCoV-NL63 induces a similar downregulation during infection is unknown.

HCoV-229E can be cultured on various types of cells derived from the human nervous system, cells of granulocytic and monocytic lineage, airway tract cells and hepatocytes.[44,45,55,56] HCoV-NL63 *in vitro* replication can be achieved by culturing upon monkey-kidney-derived cell lines, tertiary monkey kidney cells and hepatocytes.[6,26,43,57] On pseudostratified human primary lung epithelial cell cultures, CD13 and ACE2 proteins are expressed on the apical surface.[45,58] The release of newly produced HCoV-229E viral particles exhibits the same polarization as the receptor, and therefore, apical release, whereas for HCoV-NL63, this is still unknown.[45] Unfortunately, to date, no permissive animal models have been reported that can be utilized as *in vivo* models to further characterize HCoV-229E- or HCoV-NL63-induced pathogenicity.[59–61]

Prevalence of HCoV-NL63 and HCoV-229E

An accumulating number of reports has revealed that HCoV-229E and HCoV-NL63 infections occur without gender, age or geographic boundaries.[8,62–65] All children encounter their first HCoV-229E and HCoV-NL63 infection during early childhood.[43,66,67] In most children, these infections do not lead to severe clinical symptoms, but for some, the severity of the upper or lower respiratory tract infections can require hospitalization. HCoV-NL63 and HCoV-229E infections can account for 5% of all acute respiratory infections in the hospital, especially during the winter.[68,69] Very often, these severe infections are accompanied by a second respiratory virus infection.[70] At a later age, reinfection with the viruses occurs, but only in frail persons does the infection require hospital admission.[6,64,65] Studies with HCoV-229E infection of volunteers have shown that reinfection with common cold symptoms occurs when the level of antibodies directed

against the virus is low.[71] The decrease in titers of HCoV-229E antibodies is observed as soon as 1 year after infection, which indicates that every individual probably encounters numerous infections by HCoV-229E during a lifetime. Whether reinfection of HCoV-NL63 in healthy adults occurs is still unknown.

Disease Association of HCoV-NL63 and HCoV-229E

Until 1989, clinical infection trials with HCoV-229E in healthy volunteers were performed by researchers at the Medical Research Council (MRC) in Salisbury, UK. HCoV-229E was administered nasally to volunteers.[72] Among the infected volunteers, 50% developed the common cold. The observed symptoms included malaise, headache, nasal discharge, chills, cough and sore throat. One fifth of the volunteers developed fever. The incubation period ranged from 2 to 5 days, with a mean of just over 3 days. The duration of symptoms that were induced by HCoV-229E varied between 2 and 18 days, with a mean of 7 days. During the trials, researchers also noticed the high daily amount of disposable handkerchiefs used. From this, it was concluded that nasal discharge is one of the main symptoms of HCoV-229E infection. The number of handkerchiefs used ranged from 8 to 120, with a mean of 23 per day, a high number compared to other common cold viruses, such as rhinoviruses. In addition, the mean incubation period of HCoV-229E was significant longer than that of rhinoviruses, whereas the duration of the illness was somewhat shorter. Similar symptoms were observed with nine different HCoV-229E strains, thus, no indications that various strains of HCoV-229E induce different symptoms.[73]

The most frequently observed clinical manifestations in HCoV-NL63-infected patients are fever, cough, coryza, sore throat, bronchiolitis, bronchitis, pneumonia and croup.[8] As mentioned above, HCoV-NL63 infections in the hospital are frequently accompanied by infection with other respiratory viruses. Therefore, association of HCoV-NL63 with a certain disease remains difficult to establish. We investigated a large group of 949 children with lower respiratory tract infections and found that, among those infected with HCoV-NL63, a large percentage had croup (24%).[70] Focusing only on single HCoV-NL63 infections revealed a very strong association (43%, $p < 0.0001$). A second study confirmed this finding. Five hundred and thirty-nine Taiwanese children were tested and HCoV-NL63 was the most common pathogen (14.7%) in children who had croup.[74] Also, two Korean studies observed the association of HCoV-NL63 with croup.[62,75] One study found three (50%) cases of croup among HCoV-NL63-infected children, and the other found 64.2% of croup among 14 children with HCoV-NL63 infection. We hypothesize that HCoV-NL63 is responsible for croup, since in most studies, no other pathogen has been detected. Still, it cannot be ruled out that laryngotracheitis facilitates HCoV-NL63 replication, but the virus is not involved in causing the disease. Whether HCoV-229E is involved in croup is unknown. HCoV-229E testing of the above-mentioned 949 children (tested previously for HCoV-NL63) will shed more light on this matter. Therefore, it is of interest to determine the prevalence of HCoV-229E infection among children with croup.

There has been one study that has linked HCoV-NL63 infection to Kawasaki disease,[76] one of the most common forms of childhood vasculitis.[77] However, no subsequent study has been able to confirm this association.[78–82] HCoV-229E has been suggested as the causative agent of multiple sclerosis.[83–86] Some research groups have found a higher frequency of HCoV-229E in the brains of patients with multiple sclerosis compared to a control group. However, the high frequency might have been influenced by the increased susceptibility of these patients, as a result of damage to the blood–brain barrier.

Therapy

Common cold virus infections have a large impact on the economy because of the reduced

productivity of the working population. Therefore, effective viral treatment against the common cold may limit this economic impact. Additionally, effective treatment can modulate severe respiratory disease among children or elderly and immunocompromised patients. Currently, there are no treatments available for any of the HCoVs, including HCoV-NL63 and HCoV-229E. However, some candidate drugs have been investigated and might provide options for treatment in the future.

The viral replication cycle of HCoV-229E and HCoV-NL63 can be tackled theoretically by synthetic or natural antiviral compounds at various stages, including receptor binding, membrane fusion, transcription, RNA biosynthesis and post-translational processing. For HCoV-NL63 and HCoV-229E, there are no inhibitory neutralizing monoclonal antibodies available. However, HCoV-NL63 replication can be inhibited *in vitro* by pooled intravenous immunoglobulins from healthy adult donors, which probably contain neutralizing antibodies.[87] Whether this also relates to HCoV-229E remains to be investigated, although it is not unlikely since many healthy adults carry antibodies directed against HCoV-229E.[88] Treatment with intravenous immunoglobulins is beneficial in numerous (auto)immune diseases, such as multiple sclerosis, but also severe respiratory diseases and Kawasaki disease.[89]

Type I interferon (IFN-α and IFN-β) modulate the viral permissiveness and replication efficiency by toggling infected and neighboring cells into their antiviral state.[90] For HCoV-229E, it is known that IFN-α exhibits a potent antiviral activity towards HCoV-229E *in vitro* and *in vivo*.[91,92] However, prolonged intranasal administration of IFN-α to HCoV-229E-infected volunteers gave rise to blood-stained nasal discharge, a side effect which is perhaps worse than the common cold that is caused by HCoV-229E.[92]

Other novel means to inhibit viral replication are RNA interference[87,93] and broad-spectrum protease inhibitors.[87,91] Nevertheless, the *in vivo* efficacy and safety of these inhibitors remain to be established.

Concluding Remarks

To date, there is a lot known about HCoV-229E and HCoV-NL63, but there are several areas of research that are underrepresented. For instance, the sequence information on HCoV-229E is very limited, and an animal model for both HCoVs is urgently needed. Furthermore, the implication of the receptor usage of HCoV-229E and HCoV-NL63 on the renin–angiotensin system remains to be established. Future research will hopefully reveal the mechanism by which these viruses cause disease. Understanding of the pathogenesis may eventually lead to a simple, non-hazardous treatment that can be used with acute respiratory infections not only in the hospital, but also at home to cure common colds.

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