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Coronavirus Pandemic

Reducing persistent coronavirus infection in bats may lower the frequency of viral spillover to humans

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Abstract

Coronaviruses have been responsible for the emergence of pathogenic human diseases in recent decades, especially the coronavirus disease of 2019 (COVID-19). Phylogenetic studies of RNA (ribonucleic acid) viruses suggest that most human coronaviruses originated in bats, which are suitable reservoir hosts for many zoonotic viruses because of their unique biological and physiological features. The generation of human pathogenic coronaviruses is a result of genetic adaptation in bats and/or intermediate hosts, leading to spillover events. Therefore, we propose that specifically reducing or disrupting persistent coronavirus infection in bats may consequently decrease the frequency of human coronavirus diseases. We suggest several strategies to achieve the aforementioned goal in bats, including vaccination and targeted delivery of molecular inhibitors, such as monoclonal antibodies, aptamers, antisense oligonucleotides, and siRNA by use of viral nanoparticles. Advances in global bat research with the aim of controlling coronavirus infection in these mammals are pivotal in enhancing human health worldwide.

Key words: coronavirus; bats; spillover; vaccination; nanoparticles; aptamers.

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Introduction

Coronaviruses (CoVs) are enveloped, single-stranded, positive-sense RNA viruses that can cause severe respiratory and gastrointestinal diseases in mammals [1]. The human pathogenic CoVs were responsible for the emergence of severe acute respiratory syndrome (SARS) in 2003 [2], Middle East respiratory syndrome (MERS) in 2012 [3], and the recent COVID-19 pandemic in 2019 [4]. Phylogenetic studies of CoVs suggest that most human CoVs originated in bats. Bat CoVs primarily infect the animal's gastrointestinal (GI) cells but are also detected in the blood stream and other solid organs, including lung, liver, and kidneys [1,5]. However, unlike in humans, CoV-related disease of airway passages is seldom reported in bats [6].

The generation of most human pathogenic CoVs is a result of genetic adaptation in bats and/or intermediate hosts, such as civets and pangolins [1,5]. The mechanism of genetic adaptation is not yet fully understood, but likely occurs either through recombination events among coexisting CoVs, viral mutations, or a combination of both in animal hosts [1,5]. Bats constitute a highly diverse group of species, with distinct biological and physiological differences, not only among themselves but also when compared to other mammals. This has made them suitable reservoir hosts for many ancestral human pathogenic RNA viruses, including Nipah virus, Henipa virus, Lyssaviruses, and Ebola-like viruses (e.g., Marburg virus) [1,5].

To date, no direct progenitor of SARS-CoV-2 has been identified in bat populations, but several species of bat CoVs in Southeast Asia belonging to the genus *Rhinolophus* show a high degree of sequence homology across the entire viral genome to SARS-CoV-2. For example, Zhou *et al.* [4,7] reported two *Rhinolophus* viruses, RaTG13 and RpYN06, that share over 94% sequence homology with SARS-CoV-2. However, it should be noted that a lower sequence homology of the spike (S) gene was described for these two viruses as compared to SARS-CoV-2 [7]. Interestingly, some pangolin CoVs were shown to have identical amino acids at the six critical residues of the receptor binding domain (RBD) of the S protein to SARS-CoV-2, signifying the role of viral recombination as well as intermediate hosts for the spillover events [8,9]. Collectively, these reports strongly suggest that SARS-CoV-2-like viruses, circulate in bats and intermediate hosts prior to spill over to humans.

The mode of coronavirus transmission from bats and intermediate hosts to humans is an important junction at which mitigation can lower or prevent spillovers. While virions from coronavirus-infected bats have been detected in urine as well as saliva, infectious viral particles are primarily excreted in their feces (i.e., guano) [10,11]. The prevalent use of guano as a fertilizer in Southeast Asia has strongly been implicated in the rise of viral transmission, in addition to the direct interface of bats with intermediate hosts, in particular rats [12]. The problem of viral transmission has been further exacerbated within certain regions of China because of the proximity of farm livestock, bat roosting areas, wet markets, and human dwellings [13]. It is therefore reasonable to suggest that limiting the interface of bats and bats' excreta with intermediate hosts and humans would significantly reduce potential spillover events. To date, these preventive measures have been difficult to implement effectively in most regions of the world. Alternative strategies are thus warranted to curtail zoonotic human diseases.

Major efforts have been directed towards the prevention (e.g., vaccines), diagnosis (e.g., viral detection methodologies), and treatment (e.g., nucleoside analogs like remdesivir and monoclonal antibodies like sotrovimab) of human coronavirus-related diseases [13]. However, thus far, little attention has been given to eliminating or even limiting coronavirus spillovers from bats and intermediate hosts to humans. Here, we propose that specifically reducing or disrupting persistent CoV infection in bats may lower the frequency of spillover events, which are evidently implicated in the emergence of recent epidemic and pandemic viral outbreaks around the world.

The eradication of bats is not a solution

Total or regional eradication of bats in limiting coronavirus spillover is not a logical option because these mammals are indispensable in supporting and sustaining life for many other species on the planet, including humans. There are over 1,400 species of bats which account for nearly 20% of all mammals [12,14]. Bats significantly contribute to pollination and dispersal of seeds of numerous plants that are essential for various ecosystems around the world [14,15]. Their droppings (i.e. guano) are commonly used as agricultural fertilizer in Southeast Asia [16]. In addition, bats are essential for controlling the population of different insects on our planet, e.g., a single insectivorous bat consumes over 4,000 insects and arthropods daily [14,16]. Considering all the positive effects of bats on our planet, their absence will likely result in a major reduction of vegetation, shortage of food supplies, rampant insect-associated microbial diseases, and severe economic damages.

In recent years, climate change, human overpopulation, and global urbanization have been linked to the destruction of bats' natural habitats in forests, leading to their mass migration in search of food and new roosting areas [1,16]. Because of these forced relocations, bats now roost near populated animal farms, ranches, and markets where food is more abundant and accessible. Consequently, there is a breach of the normal boundaries between CoV-infected bats, intermediate host species, and humans, and therefore a higher frequency of zoonotic spillover and disease. Although reversing the negative effects of climate change and restoring natural habitats are the best long-term solutions to this problem, they may not be achievable in the next few decades. Therefore, it becomes imperative to find other, more timely and feasible strategies to limit the spillover events of pathogenic CoVs from bats to humans.

The unique biology, physiology, and immunology of bats

Bats have evolved several exceptional traits to survive myriad environments and coexist with commensal pathogens, including diverse RNA viruses. Some of their most unique characteristics include: (a) they are the only mammal capable of flying; (b) they are nocturnal and, therefore, hibernate during daylight (i.e. torpor); (c) they have the ability to dampen their inflammatory response to microbial organisms; (d) they live significantly longer than other animals of the same size and mass; (e) they have a sophisticated system for echolocation; (f) they have a lower incidence of cancer; (g) they have a lower birth rate; and, (h) a shorter GI tract to efficiently manage rapid energy needs [14,16,17]. Notably, certain unique features are shared among all species of bats (e.g., flight), whereas others are different based on species (e.g., diet).

Bats' metabolic rate increases approximately 30-fold during flight, concomitant with a significant increase in their body's temperature, from resting ~6 °C to ~41 °C, which is equivalent to a human with a high fever [16,18,19]. The temperature surge protects against resident microbial organisms by inhibiting their pathogenicity and thereby preventing serious disease. Another consequence of the metabolic surge during flight is a rise in the levels of oxidative stress, which is a result of increased respiration-dependent oxidative phosphorylation in the mitochondria [14]. As a result, bats have adapted to express stress mediators, such as

heat-shock proteins, to provide antioxidant protection and minimize tissue damage [14,20]. They also use cellular sensors, such as pattern recognition receptors (PRRs), to recognize oxidative-dependent damages (e.g., nucleic acid fragments) and activate the expression and release of interferons (IFNs) and other inflammatory mediators (e.g., interleukins) into the bloodstream [20,21]. This results in a baseline, sustained level of antiviral mediators, which combined with increased body temperature, keeps resident RNA viruses at bay [16,21,22]. This baseline immunity and temperature surge abate the CoV load without shifting the body into a hyper-active immune state (i.e., cytokines storm), unlike what has been observed in humans [16,17].

Recent progress in bat's innate and adaptive immune systems has revealed numerous genetic and physiological features that differ not only from other mammals such as humans, but are also distinct among bat species. Some of the unique, species-specific, molecular features of their immune system include: a. 2-5A-dependent endoribonuclease (RNase-L) that is directly induced by IFNs and functions to cleave viral mRNA, and therefore, is critical in reducing the bats' susceptibility to viral infection [23]; b. tumor necrosis factor alpha (TNF-□) as a potent inflammatory cytokine is suppressed at the level of gene expression [24]; c. the activity of the stimulator of interferon gene (i.e. *STING*), which functions as an intracellular adaptor in mediating IFN induction in response to DNA damage and oxidative stress, is weakened by an evolutionary mutation [25]; and d. *PHYIN* genes that encode for the components of inflammasomes, which are intracellular molecular complexes involved in augmenting host's inflammatory responses, are absent [26].

In summary, bats' unique immunological traits have evolved to cope with the flying phase in which increased DNA damage and oxidative stress can result in excessive inflammatory injuries and energy demand. To compensate for minimizing excessive immune activation, bats maintain a baseline antiviral response to protect themselves against persistent viral infection [16,17].

Interestingly, bats spend a large part of daylight in their roosts in a state of inactivity, also known as torpor or their hibernating phase [16,17]. During this inactive state, bats make a drastic physiologic shift that is expectedly opposite of the flying phase: their metabolic rate drastically decreases by approximately 30-fold, concomitant with a significant decrease in their body temperature and diminished antiviral immune mediators throughout their body [14,16]. Bats also evolved to have a smaller GI tract, which is about one-third in length in comparison to other mammals with an equivalent body mass and size (e.g., mice) [14]. The smaller GI tract allows for efficient food intake to comply with the rapid energy demand and increased metabolic rate discussed above. It is hypothesized that the enhanced ability to handle oxidative stress, the sustained low levels of immune activation during flight, and the periodic resting phase to repair damages and reset the entire body's physiology, all contribute to the bats' extended fitness and longevity [14,22]. Bats, on average, live 2-3 times longer than other mammals with a similar body mass or size (e.g., mice), ranging between 20 to 40 years [16]. However, other biological features may also play a role in longevity, including roosting in the safety of caves, being able to fly away from ground predators, lower energy demand during the torpor phase, and avoiding daylight predators by being nocturnal.

Hypothetical strategies to reduce persistent coronavirus infection in bats

Herein, we propose several molecular strategies to limit persistent CoV infection in bats, some of which are more amenable to implementation than others; however, to the best of our knowledge, none of these hypothetical approaches have been tested on bats in nature. Nevertheless, these proposed strategies are intended to stimulate further discussions on the feasibility of their effectiveness in interrupting persistent CoV infection in bats and to inspire simpler and better alternative ideas. As we consider these strategies below, one should be aware that, generally, only a small percentage of the bat population carries specific RNA viruses; for example, reports indicate that only about 6% of bats carry either rabies or SARS-CoV-1 viruses [6]. The other issue of paramount importance, for which there is no published report as of this writing, is the lack of an established and proven method, which can deliver therapeutic molecules (discussed below) to bats at the proximity of their targets with a high degree of efficacy. However, as we discuss below, viral nanoparticles (VNPs) are a set of promising technologies for therapeutic drug delivery to bats, and the efficacy of VNPs have already been shown *in vivo* in a variety of animal models [27][28].

Vaccination

Stimulation of the bat's own immune system against commensal RNA viruses can provide an effective and long-lasting strategy for reducing the spread of zoonotic viral infections without culling the

host population. Vaccination of reservoir hosts has been explored for other deadly viruses, including Ebola virus in *Rhesus macaques* [29], Hendra virus in ferrets [30], and Lyssavirus (i.e. rabies virus) in bats [31,32]. However, these studies were done with animals in captivity, so the efficacy and feasibility of vaccination in wild populations still remain unknown. Moreover, there are no published reports investigating vaccination against CoVs in bats.

Given CoVs reside in the bat's GI tract, oral delivery of immunogenic CoV epitopes and adjuvants for immune stimulation could provide long-lasting intestinal immunity as it has in the case of the oral polio vaccine in humans [33]. Transmission tracking in wild bats with fluorescently labeled topical gels has demonstrated high levels of coverage (>70% of adult bats), suggesting feasibility of vaccine delivery via orotopical transfer [31]. Another strategy is the infection of the host population with benign, transmissible viruses (discussed below) that express vaccine components, although more research on vaccine safety, efficacy, and coverage is required prior to any implementation.

Small molecules

With the advent of high-throughput screening technologies, it is now possible to discover high affinity compounds that interfere with or inhibit viral infection. This strategy might work best when the viral entry phase is targeted at inception in the bat GI tract. However, inhibitory compounds can also be designed to enter intestinal epithelial cells and target the intracellular stages of the CoV life cycle. As an example, using a broad selection of chemical compounds, Kao *et al.* [34] reported the discovery of several candidate molecules which effectively inhibited SARS-CoV-1 in a cultured monolayer of the Vero cell line at a median effective concentration (EC50) < 10 μM. Since bats require daily water intake to survive, water soluble molecules are likely to be more amenable for drug delivery to these mammals.

High-affinity biologics

Nucleic acids and proteins can be engineered to selectively bind and inhibit components of RNA viruses. Aptamers, which are short, stable oligonucleotides, and antibodies, which are large proteins with modifiable variable regions, are examples of biologics that can be designed to specifically target viral components through directed evolution techniques, such as SELEX (systematic evolution of ligands by exponential enrichment) and yeast surface display, respectively. For example, aptamers or antibodies targeted to the receptor binding domain (RBD) of the viral S protein to the angiotensin-converting enzyme 2 (ACE2) receptor are attractive candidates to block viral entry. In this way, these biologics would not only limit viral infection of host cells but also decrease viral transmission among bats.

Recently, neutralizing antibodies against S protein were trialed in humans and showed significant reduction in SARS-CoV-2 viral load in patients with mild to moderate COVID-19 [35]. Neutralizing aptamers have been shown to prevent mammalian host cell infection from SARS-CoV-2 by blocking the interaction between the coronavirus S protein and the host RBD/ACE2 receptor at a median inhibitory concentration (IC50) of ~5 nM [36]. Viral entry has also been shown to be inhibited by aptamers independently of the protein S and RBD/ACE2 receptor axis [37].

Given their smaller size and better access to targets compared to antibodies, aptamers can also be designed to target the CoV life cycle within a cell, e.g., viral replication, assembly, and release of progeny virions [38]. For example, Cho *et al.* [39] reported the discovery of a single-stranded (ss)-DNA aptamer with high affinity binding for the SARS-CoV-1 nucleocapsid that could interfere with viral progeny assembly and block infection. In addition to better tissue penetration, aptamers also carry the advantage of longer shelf-life and faster production time. Other engineered molecules to consider for targeting CoV in bats, include nanobodies, which are single-domain antibodies with significantly improved accessibility compared to larger antibodies, anti-sense oligomers, small interfering RNA (siRNA), short hairpin RNA (shRNA), and Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) vectors. These modalities have been shown to be effective in limiting microbial infections in certain animal models [40-42], but their application to inhibiting zoonotic RNA viruses has been limited.

Recombinant coronaviruses

Viral nanoparticles (VNPs) are a set of new technologies that make use of mammalian and plant viruses, and bacteriophages for therapeutic drug delivery and other medical interventions, such as diagnostic imaging [28]. A subset of VNPs is a technology that makes use of non-infectious, virus-like particles (VLPs) that are either devoid of viral genome or contain a non-infectious, viral genome with one or more therapeutic trans-gene(s) [28]. VLPs can be engineered for a variety of medicinal purposes,

including vaccine development and encapsidation of therapeutic molecules into virions in place of the viral genome for an effective payload delivery into cells and tissues. Alternatively, the exterior of the viral components (e.g., capsid) can be modified through chemical conjugation to a moiety, which can bind to a specific cell membrane receptor. This type of VLPs imparts targeted delivery of the therapeutic payloads to cancer cells, for example [28].

Interestingly, one can generate non-infectious, coronavirus-like particles (CVLPs) in a monolayer of 293K mammalian cell line [28]. These cell-based systems use an optimal co-expression of coronavirus structural components; including the matrix (M), envelop (E), nucleocapsid (N), and spike (S) protein [43,44]. Further advances in virus-based nanoparticles may enable scientists to develop non-infectious CVLPs in a cell-free system with or without modified genome. Because the anchoring of viral M and E proteins in the endoplasmic reticulum (ER) are essential for the assembly of coronavirus virions [43,45], isolation of a fractionated ER containing these anchored proteins would likely facilitate the *in vitro* assembly of virions in the presence of an optimal ratio of N and S proteins.

As emphasized above, one of the major obstacles for reducing CoV infection in bats is the use of an effective transport module to deliver a payload of inhibitory molecules to the bat's cells. CVLPs may potentially be amenable for this purpose in the following ways. For example, *in vitro*, genome-free CVLPs may allow packaging of small inhibitors, aptamers, antisense oligonucleotides, siRNAs, or CRISPR-Cas9-based molecules in the inner space of the virions. Alternatively, one could engineer CVLPs, containing one or more expression cassettes for inhibitory molecules targeted to a specific strain of bat coronavirus, such as monoclonal antibody, anti-sense RNA, and siRNA. It must be kept in mind that the generation of any CVLPs containing a modified genome requires a strict adherence to established safety guidelines and international laws. Since CVLPs can readily be transmitted to bats like wild-type coronaviruses, inhibitory molecules to a coronavirus strain can be delivered to the bat's GI tract and/or other organs.

Concluding remarks

We propose that reducing or disrupting persistent coronavirus infection in bats may lower the prevalence of human coronavirus diseases. To this aim, the authors have put forward several strategies to reduce persistent coronavirus infection in bats. Although the efficacy of the proposed inhibitory molecules has not yet been tested in bats, their efficacy in blocking viral infection in a variety of mammalian cell lines and in some animal models *in vivo* has been demonstrated [40,41]. Therefore, the first step is to investigate the efficacy of these inhibitory molecules in various cell lines derived from bats [46,47], as well as experimental bats *in vivo*, to select the most effective technology. The second step is to determine an effective technology to deliver therapeutic molecules to bats. Currently, VLPs are attractive programmable modules for efficient delivery of therapeutic molecules into mammalian cells. However, the production of CVLPs is at its infancy, and further research is needed in developing the *in vitro* production of CVLPs, in particular, the molecular art of conjugating or packaging the therapeutic molecules in the virions. After optimizing the production of CVLPs, one could then examine the efficacy of this system in both cell lines derived from bats and ultimately in experimental bats *in vivo*. Lastly, advances in understanding the biology of the immune system in bat species, especially the immune-linked tolerance to viral RNA infection, is essential to discover ways to harness bat's immune system to reduce persistent coronavirus infection. It is now clear that progress in global bat research is pivotal in enhancing human health worldwide.

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