

Severe Acute Respiratory Syndrome Coronavirus Pathogenesis, Disease and Vaccines

An Update

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Background: A novel coronavirus has recently been identified as the cause of severe acute respiratory syndrome (SARS-CoV). The ability of this family of positive strand RNA viruses to move between species and cause severe disease in humans, with the potential for pandemic spread, has been confirmed.

Methods: An understanding of the disease and its pathogenesis and the genetics of coronavirus infections, as well as strategies to treat or prevent coronavirus infections, are essential. The history of coronavirus vaccines and the occurrence of laboratory-associated SARS-CoV infections underscore the need for stably attenuated strains of SARS-CoV and other coronaviruses.

Results: Rapid progress has been made in understanding the clinical disease of SARS in adults and children. In adults, systemic infection with clinical and biochemical abnormalities, as well as respiratory infection, may be the rule. SARS is much milder in children younger than 12 years old than it is in adolescents and adults. In children age 12 years and younger, symptoms are generally nonspecific and cold-like. Numerous approaches to the development of SARS-CoV vaccines have been undertaken, and there is evidence that antibodies to the spike protein may be protective from replication and pathology in animal models.

Conclusions: The availability of reverse genetic systems has made it possible to engineer and recover coronavirus variants that contain multiple genetically stable mutations that grow well in culture but are attenuated for replication, virulence or both. Such variants will be platforms for the safe growth of SARS-CoV and candidates for live attenuated vaccines.

Key Words: coronavirus, severe acute respiratory syndrome, mouse hepatitis virus, reverse genetics, children, replication, live attenuated vaccines, animal models, replicase proteins, attenuation

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Between November 2002 and July 2003, the coronavirus responsible for severe acute respiratory syndrome (SARS-CoV) infected >8500 people and caused >800 deaths in 32 countries. In addition to the dramatic and severe disease associated with this viral infection, the emergence and rapid transglobal spread of SARS-CoV caused tremendous social and economic disruption. Coronaviruses had previously been demonstrated to move between species in cell culture and were predicted to move readily between species in nature. The emergence of SARS-CoV has confirmed these laboratory predictions and has shown the capacity for adaptation, disease and transmission among humans. The SARS-CoV epidemic also represented perhaps the most extensive and coordinated public health response ever mounted against a human pathogen. It is likely that this public health intervention, along with perhaps some limitations on the transmissibility of the virus, were responsible for its control and the elimination of detectable transmission among humans.

Since the last case of SARS associated with the epidemic was reported in July 2003, there has been remarkable progress in many areas of research on the epidemiology, pathogenesis, replication, genetics and immune response of SARS-CoV. Together these studies have translated into significant progress in SARS-CoV vaccine development and animal testing. Nevertheless the dramatic emergence and seemingly just as rapid retreat of the epidemic has also left many unanswered questions. What was the original source of SARS-CoV, and is there a stable animal host? How was SARS-CoV able to enter human populations and establish a worldwide epidemic? What is the basis for transspecies movement and adaptation of coronaviruses? Will SARS re-emerge? Is it important to continue to pursue therapeutics and vaccines, and if so what kind? Are other coronaviruses capable of the same kind of transspecies movement? Although many of these questions cannot yet be answered, the rapid progress in research gives significant insights into many of them. This review will summarize advances in: (1) SARS clinical disease in adults and children; (2) coronavirus dis-

eases and vaccines relevant to SARS; and (3) progress in vaccine development for SARS-CoV.

SARS AS A SYSTEMIC AND RESPIRATORY DISEASE

The opportunity to define the basis for SARS transmission and the clinical disease of SARS-CoV was limited by the acute and severe nature of the epidemic and by the need to respond to the disruption of health and social systems at the epicenters of the epidemic. Nevertheless, despite the limited duration of the epidemic, review of multiple studies indicates that there were several features suggesting that SARS-CoV is significantly different in its transmission and pathogenesis than other viruses that cause lower respiratory tract disease and epidemics.¹⁻¹⁰ The pneumonia associated with SARS-CoV was more likely to result in dyspnea, hypoxemia and respiratory failure than that caused by other respiratory viruses, although none of these features is pathognomonic.

Interestingly, although the syndrome was named for these severe respiratory manifestations that lead to impressive overall mortality, the summary of cases suggests that SARS may be a systemic infection with severe respiratory disease as the major manifestation. First, the incubation period of SARS-CoV was 2–10 days, which is longer than the 1- to 4-day range of other viruses with disease limited to the respiratory tract. Second, although the manifestations of fever, chills/rigor, headache, myalgia and malaise associated with the onset of disease could not be distinguished from other respiratory illnesses, SARS-CoV-associated disease in adults demonstrated significantly less upper respiratory prodrome or illness, specifically rhinorrhea. Third, SARS was associated with both biochemical and clinical indicators of systemic infection. Diarrhea was observed and prominent in many cases of adult and pediatric disease, although, from the limited published data, it was not possible to precisely define the relationship of diarrhea to the respiratory illness. Biochemical studies demonstrated elevated transaminases and neutropenia/lymphopenia associated with illness before onset of severe respiratory signs and symptoms. Finally, there was evidence of virus replication in tissues other than the respiratory tract, with virus identified by culture, polymerase chain reaction (PCR) or both, from stool, blood and kidney. For the most part, virus was detectable only by means of PCR amplification of specific segments of the virus positive-strand RNA genome, which cannot be considered conclusive proof of systemic replication and spread. However, virus was detectable in many cases from stool by culture. In fact, the longest duration of virus detection and survival was in diarrheal stools. The duration and titer of virus were most consistent with replication and shedding rather than passive survival of infectious virus in the gastrointestinal tract.

Together these clinical, biochemical and diagnostic features support the conclusion that SARS-CoV is capable of

some form of systemic replication, dissemination and disease. This systemic model of infection is consistent with infection and disease caused by other mammalian coronaviruses that are capable of causing systemic infection but that have disease targeted to the respiratory, enteric or neurologic organs.

SARS IN CHILDREN

During the SARS epidemic, infection with SARS-CoV was documented in pregnant women, neonates, children and adolescents.¹¹⁻¹⁵ Overall the reported pediatric cases were a small minority of probable and confirmed cases, with fewer than 100 pediatric cases reported in any detail from around the world. In addition, because of the urgent need to describe pediatric disease during the epidemic, several of the initial reports relied on contact and epidemiologic and clinical parameters to diagnose probable or suspected cases. Finally the perceived need to intervene therapeutically with ribavirin, steroids or both with children in the setting of extreme morbidity and mortality of SARS in adults may have had an impact on the findings of the studies. Nevertheless valuable insights into SARS in children were obtained in the midst of the epidemic, and these have since been complemented with virologic, serologic, immunologic and radiologic studies of SARS in many of the children originally reported.¹⁶⁻¹⁸ A summary of SARS in children 2–18 years old in Table 1 is based on these multiple references, describing the likelihood of the finding or the severity of the clinical parameter on a qualitative – to ++++ scale. There was remarkable similarity in the findings of studies on different continents, demonstrating lack of mortality in children younger than 18 years of age and in general a much milder and shorter course of infection than in adults. Fever was present in all cases reported, cough was prominent and significant lymphopenia was present in a majority of cases. Further, there was less likely to be a positive test for SARS-CoV by reverse tran-

TABLE 1. Clinical Features of Pediatric SARS

	Fetus- Newborn	Child (1–12 yr)	Adolescent (12–18 yr)
Fever	0*	++++*	++++
Cough	0	++++	++++
Chills/rigors	0	0/+	+++
Myalgia	0	0/+	+++
Rhinorrhea	0	+++	0
Radiographic pneumonia	0	+	+++
Lymphopenia	0	++++	++++
Transaminase elevation	0	+++	+++
Hypoxemia	0	0	++
Mechanical ventilation	0	++++	++
Deaths	0	0	0
Virologic or serologic diagnosis	0	++	++

*Qualitative scale of severity or frequency based on consensus of references below: 0, none; +, rare or minimal severity; ++, up to one-half or mild severity; +++, majority of cases or prominent severity; +++++, present in all cases or identical with adult SARS in severity.^{1,6,13,19-25}

scription-PCR or culture. Later studies showed evidence of serologic conversion and positive reverse transcription-PCR of plasma in many cases, suggesting that the early lack of positive tests may have been caused by a lack of developed assays.

Perhaps most intriguing was the consistency of reports suggesting that SARS in children is likely to present as at least 2 very distinct diseases. In children older than 12 years old, SARS more closely approximated adult disease, with more common myalgia, dyspnea and clinical pneumonia with hypoxemia in addition to the findings above. The only cases of progression to confluent pneumonia and ventilatory support among children occurred in adolescents. Although the numbers of cases were insufficient for determinations of significance, there was a trend for severity to worsen with increasing age from 12 through 18 years. Nevertheless disease in adolescents was still less severe than in adults, with rare progression to ventilator requirements and lack of mortality. In contrast, SARS in children 1–12 years of age was more coldlike, with rhinorrhea being a fairly consistent finding, along with headache, chills, myalgia and rigors. As with adolescents, there was no reported mortality or severe morbidity associated with SARS-CoV infection in children younger than the age of 12 years.

The radiographic findings in probable and confirmed cases of SARS in children were predominantly focal alveolar infiltrate, atelectasis and patchy bronchiectasis.¹⁶ There were several interesting aspects to the radiographic features in children: (1) progression to multilobar disease was uncommon, occurring almost exclusively in adolescents with dyspnea and hypoxemia; (2) the radiographic changes resolved rapidly with improved clinical status; (3) hilar adenopathy or interstitial infiltrates such as seen with lower respiratory tract disease caused by other respiratory viruses was rarely noted; and (4) CT or x-ray changes consistent with pneumonia were detected in some children with very mild, nonspecific symptoms who lacked clinical pneumonia. Thus overall the radiographic changes in children were milder and of shorter duration than changes in adults, which is consistent with the milder clinical course.

Finally 2 reports of SARS in pregnant women and children did not find evidence of SARS clinical disease, characteristic laboratory changes or positive diagnostic tests.^{14,15} However, there were spontaneous abortions during the first trimester in 4 of 7 women with SARS in 1 study and in 5 women with SARS in the second or third trimester. Of these 5 women, 3 required emergency cesarean sections and the remaining 2 who carried to term developed oligohydramnios with intrauterine growth retardation of the infant.¹⁴ Because of the severity of the disease in the women and because they were on both intravenous ribavirin and steroids, and in the absence of virologic evidence, the findings may be

related either to hypoxemia and placental insufficiency or to direct toxic effects of the therapeutic interventions.

ANIMAL RESERVOIRS, TRANSSPECIES MOVEMENT AND ANIMAL MODELS

A remarkable amount of information was obtained over a very short period of time, demonstrating the ability of SARS-CoV to infect a wide number of animals and to move between animals and humans. SARS-CoV has been detected by culture, PCR or both in Himalayan palm civets and racoon dogs in the wet markets of Guangdong province. The epidemiologic and molecular phylogenetic studies suggested that these animals may have been a link in the transmission to humans or may have been the primary source of the virus.^{26,27} Subsequent failure to identify the virus in wild or farmed civets suggests that these animals may have been infected in the markets and served as amplifying or intermediate hosts for transmission to humans and that the primary or endemic animal reservoir remains to be identified.

Laboratory studies have shown that a wide variety of animals can be infected with SARS-CoV, including badgers, ferrets, mice, domestic cats and nonhuman primates such as cynomolgus macaques.^{19,28,29} This raises the possibility that SARS-CoV as isolated from humans may be a “generalist” with the capacity to infect a variety of animal species. This capacity may have resulted from a rare transspecies jump with subsequent adaptative changes that then allowed the virus to infect more widely. This model would be consistent both with the nucleotide sequence changes and deletions observed in SARS-CoV genomes isolated during the course of the epidemic and with laboratory observation on other coronaviruses that have been made over the past decade.^{30–32}

A study of 63 full length SARS-CoV sequences from isolates at different times during the epidemic demonstrated that there were both “adaptive” and “purifying” mutations in the coding sequences for both structural and nonstructural proteins suggesting continuous adaptation.²⁷ Although they were not associated with different outcomes, the virus was clearly modified. This phenomenon has been seen in the laboratory with other coronaviruses, where passage in culture over time or with cells of differing host origin results in a change from highly specific targeting to specific species to a more general capacity to infect cells from different types. For example, adaptation of mouse hepatitis virus to growth in hamster cells also results in an ability of the virus to grow in human cells.

Several animal models have been proposed for the study of SARS-CoV transmission, pathogenesis and disease and for use as the basis for studies of diagnostics, therapeutics and vaccines. Mice have provided a model for infection and replication, immune response and protection from challenge.¹⁹ Thus far, the model has been limited to these factors, because the duration of replication reported is brief and

evidence of pulmonary pathology or disease has not been reported. In contrast, SARS-CoV has been reported to cause infection and disease similar to human disease with pneumonia in cynomolgus macaques and ferrets.^{28,29} A primate model would be desirable and very useful for studies of SARS-CoV pathogenesis; however, reproducing such a model is a challenge and is expensive. Thus it is likely that small animal models will be pursued extensively with approaches such as adaptation of the virus to the animal, different routes of inoculation, the use of immunocompromised animals and further efforts to identify a natural host animal. Finally an important advance in understanding SARS-CoV and developing models has been the identification of a virus receptor, angiotensin-converting enzyme II (ACE-II).³³ Whether or not ACE-II represents the native, or only, receptor for SARS-CoV is unknown. Because the “original” SARS-CoV strain from the endemic reservoir is not known, it is possible that ACE-II was the receptor by which SARS-CoV adapted for infection of humans. The ability to express ACE-II on cells in culture and the development of transgenic animals expressing the human ACE-II will likely allow for more detailed studies of replication, pathogenesis and disease in animal models.

CORONAVIRUS DISEASES AND VACCINES

Whether or not SARS-CoV reemerges in humans as an epidemic or endemic disease, the SARS epidemic has focused attention on the capacity of coronaviruses to infect and cause severe disease in humans and on the need to develop strategies to protect against SARS-CoV and other coronaviruses. SARS-CoV is a member of the *Coronaviridae*, a family of positive-strand RNA viruses that have long been known to cause colds in humans and a wide variety of illnesses in many animal species. In general, the diseases caused by coronaviruses have been primarily gastrointestinal, respiratory or both (Table 2). In addition, some mammalian and avian coronaviruses cause central nervous system, renal

or hepatic disease. Coronaviruses have also shown the ability, both in the laboratory and in nature, to select for virus variants with changes in tropism and disease in the same animal species. For example, an emerged variant of the porcine transmissible gastroenteritis virus (TGEV) known as porcine respiratory coronavirus, causes respiratory infection rather than gastroenteritis, while inducing protective immunity against TGEV.²⁰

Before the SARS epidemic, vaccines for human coronaviruses were not pursued, due to the relatively mild nature of respiratory disease caused by the previously known human coronaviruses and because of challenges with both virus growth and with understanding the extent of coronavirus disease. However, the economically important, and often severe, endemic and epidemic coronaviral diseases of cattle, swine, cats and dogs have long been targets for development and use of vaccines. Multiple vaccine approaches have been tried for coronavirus diseases, including inactivated whole virus, recombinant protein, vectored subunit, heterologous virus and live attenuated virus vaccines.²¹ No single approach has been clearly useful for all of the different mammalian and avian coronaviruses and, in fact, animal coronavirus vaccines have been only variably successful. In general, durable immunity has required both humoral and cell-mediated responses, and live attenuated virus vaccines have been the most useful and licensed vaccines for infections of pigs, cattle and chickens. These vaccines have been adopted in part because of the economic consequences of the diseases and because better approaches were not available.

The history of vaccine development and use in coronaviruses also has provided cautionary tales in the quest to develop vaccines to prevent SARS. In addition to questions of efficacy in protection from disease, there has been a demonstrated enhancement of infection and disease in cats vaccinated with inactivated virus or protein vaccines against peritonitis virus (FIPV) that are subsequently challenged with virulent virus.^{22,34} It is likely that this enhancement is due to the fact that FIPV can replicate in macrophages and monocytes and that antibodies against the S glycoprotein of FIPV from inactivated or recombinant protein induces antibodies that are only partially neutralizing. At low levels, such antibodies may increase uptake of nonneutralized virus into macrophages and monocytes, leading to increased replication, increased immune response and more severe or fatal disease.

VACCINES FOR SARS

Together with the concerns about efficacy, enhancement of disease after experimental vaccination suggests that multiple approaches to vaccine development for SARS-CoV should be undertaken. Multiple animal models also will be essential, both to demonstrate safety and efficacy of the vaccine and to determine whether there is any evidence of disease

TABLE 2. Coronavirus Diseases of Animals and Humans

Virus	Host	Clinical Syndrome
HCoV-229E	Human	Colds, pneumonia
HCoV-OC43	Human	Colds, pneumonia
MHV	Mice	Hepatitis, encephalitis
TGEV/PRCV	Pigs	Gastroenteritis, pneumonia
BCoV	Cattle	Gastroenteritis, pneumonia
CCoV	Dogs	Gastroenteritis
FECV, FIPV	Cats	Peritonitis, enteritis
IBV	Chickens	Tracheitis, renal
SARS-CoV	Humans	Pneumonia, gastroenteritis
HCoV-NL, NL	Humans	Colds, pneumonia

HCoV indicates human coronavirus; PRCV, porcine respiratory coronavirus; BCoV, bovine coronavirus; CCoV, canine coronavirus; FECV, feline enteric coronavirus; IBV, infectious bronchitis virus; NL, NL63-Netherlands.

enhancement. The progress in research to develop a vaccine to protect against SARS disease has been quite rapid and has been helped by the description of pathogenesis and replication models in mice, ferrets, cynomolgus macaques, rhesus macaques and African green monkeys (Table 3).^{19,23,24,35,36,38,39} Several approaches have been reported to induce immune response, provide protection from replication and disease or both, including prior infection with SARS-CoV, passive antibodies and viral proteins expressed from DNA or from heterologous viruses such as adenovirus 5, modified vaccinia Ankara and parainfluenza virus type 3. The results have been encouraging in suggesting that antibodies against the S protein may be induced by a variety of approaches and that antibodies in animal models are protective from replication, pathology or both. In addition, passive polyclonal or monoclonal antibodies generated by different methods may be neutralizing and protective as well in more than 1 animal model. Furthermore there have been no reports of enhanced replication or disease in these studies to date. These results differ from other characterized coronaviruses in inducing a protective antibody response to virus or protein that may be sufficient for protection. In contrast, these animal models are new and vaccine approaches have mostly been in replication models for which the direct applicability to humans is unknown. Thus the concerns about protection in humans and of the possibility of enhanced disease must continue to be considered.

GENETIC APPROACHES TO ATTENUATION OF SARS-COV FOR SAFE USE AND VACCINE DEVELOPMENT

Live attenuated virus vaccine candidates for SARS have not yet been reported for several reasons, including: (1) lack of understanding of determinants of replication, pathogenesis and disease; (2) requirement for growth and passage of live SARS-CoV under biosafety level 3 conditions; (3) concerns for safety in testing; (4) concerns about reversion to virulence by mutation or homologous recombination; (5) concerns about recombination with other viruses in nature; and (6) the very complex molecular engineering of a 32-kb

RNA genome to allow targeted mutagenesis. Nevertheless several factors make it important to pursue research to stably attenuate SARS-CoV: (1) the demonstrated value of live attenuated vaccines in other coronaviruses suggests that such approaches for SARS might be necessary, especially in the setting of new variants; (2) other coronavirus inactivated or subunit vaccines have failed to protect during outbreaks; (3) inactivated vaccines are being considered for testing in China. Inactivated vaccines still require growth of large amounts of live virus. It was concluded that the laboratory-associated SARS cases in China in the winter of 2004 resulted from inadequately inactivated SARS-CoV, and thus biosafety is a concern in the laboratory and in possible manufacture of inactivated virus vaccines.⁴⁰

Recently systems for engineering of targeted mutations anywhere in the viral genome RNA have been established for both SARS-CoV and for the model coronavirus, mouse hepatitis virus.^{41,42} Because the engineering introduces changes in a complementary DNA (cDNA) copy of the RNA genome, the approach is known as “reverse genetics.” Analysis of the RNA genome of SARS-CoV and mouse hepatitis virus (MHV) shows many similarities in the organization and in the proteins responsible for viral replication. Thus the effect of engineered changes in highly conserved proteins, structures or amino acid residues can be tested in the MHV model system and applied to development of strategies for attenuation of SARS-CoV. The goal of such studies is to develop engineered SARS-CoV variants that: (1) contain multiple genetically stable mutations; (2) have wild-type growth in culture; (3) are attenuated for virulence in animals; (4) are protective against wild-type challenge; and (5) do not revert by mutation or recombination with other coronaviruses. Such virus variants would be platforms for safe use and study in the laboratory, would allow safe growth of large amounts of virus for inactivation and would be the platform to develop candidates for live attenuated virus vaccines.

Like other coronaviruses, the SARS-CoV genome is a positive-strand RNA molecule ~31 kb long (Fig. 1).^{25,37} All stages of viral replication occur in host cell cytoplasm. The genome is organized into 9 genes. Gene 1 (the replicase gene)

TABLE 3. Preclinical Testing of Immunogenicity and Efficacy of SARS-CoV Vaccines in Animals

Approach	Protein	Animal	Route	Humoral Response	Cellular Response	Protection R, P	Reference
Infection	Live virus	Mouse	IN	Yes	Yes	Yes-R	19
Ad5	S, N, M	Macaque	IM	Yes-S	Yes-N	NT	39
MVA	S	Mouse	IN, IM	Yes	NT	Yes-R	35
DNA	S	Mouse	IM	Yes	Yes	Yes-R	38
Passive Ab	Live virus	Mouse	IP	NT	NT	Yes-R	19
mAb-human-phage	Inactivated virus	Ferret	IP	mAb	NT	Yes-R,P	36
PIV3	S	AGM	IN, IT	Yes	NT	Yes-R	24
PIV3	S, N, M, E	Hamster	IN, IT	Yes	NT	Yes-R	33

Ad5 indicates adenovirus 5; MVA, modified vaccinia Ankara; PIV3, parainfluenza virus type 3; S, spike; N, nucleocapsid; M, membrane; E, small envelope; AGM, African green monkey; R, replication; P, pathology; NT, not tested; IN, intranasal; IT, intratracheal; IP, intraperitoneal; Ab, antibody; mAb, monoclonal antibody.

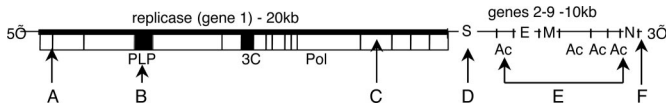


FIGURE 1. Organization, proteins and genetics of SARS-CoV genome. The SARS-CoV genome is a 32-kb, single-stranded, positive strand RNA molecule. The replicase gene comprises two-thirds of the genome coding capacity and expresses a replicase polyprotein (\square) that is processed by 2 viral proteinases, papain-like proteinase (PLP) and 3C-like proteinase (3C) (\blacksquare) into mature proteins of known and unknown function, such as an RNA-dependent RNA polymerase (Pol). Virus structural proteins (spike, S; nucleocapsid, N; membrane protein, M; and envelope protein, E) are expressed from downstream genes. Additional proteins of unknown function in replication and pathogenesis are referred to as accessory proteins (Ac). Arrows below the schematic indicate a design for reverse genetic introduction of multiple stable mutations, deletions or rearrangements in the genome to attenuate virulence. A indicates replicase polyprotein cleavage sites; B, proteinase activity or specificity; C, identified virulence determinants in replicase; D, mutations in structural or immune determinants; E, mutation, deletion or rearrangement of virulence genes; F, mutations in noncoding regions of the genome.

comprises two-thirds of the genome and contains all of the coding sequence for proteins responsible for viral RNA transcription and replication. The replicase gene is translated directly from the input genome RNA to yield the replicase polyprotein that is then processed into functional precursor and mature proteins by viral proteinases within the polyprotein designated PLP (papain-like proteinase) and 3CLpro (3C-like proteinase). It has been proposed that replicase gene products may serve roles in viral virulence and pathogenesis. Thus the proteins, their expression and their processing are attractive candidates for targeted mutagenesis and attenuation. Additional viral proteins are expressed from genes 2–9, including the structural proteins S, N (nucleocapsid), M (membrane protein) and E (small envelope protein), as well as several group-specific proteins. The structural proteins are found in the virion and may also serve other virus and cell-specific functions in modification of the intracellular environment, in cell signaling or in immune response. The group-specific proteins have been referred to as “accessory proteins,” because genetic deletion of the proteins does not affect virus growth in culture for several coronaviruses. The role of group-specific accessory proteins has only recently been studied in animals, and it is not known whether the accessory proteins serve specific roles in the animal in virus survival or virulence. Thus the accessory genes are also targets for engineered changes that might allow normal growth in culture but attenuate replication in animals.

The overall strategy for engineering multiple stable attenuating mutations into the SARS-CoV genome is shown

in Fig. 1. In collaborative projects in which the model virus MHV is used, experiments have targeted mutations, deletions and rearrangements to replicase polyprotein cleavage sites (site A), proteinase functions (site B), replicase enzymes (site C), structural proteins (site D), accessory proteins (site E) and nontranslated RNA structures (site F). Multiple independently attenuating changes will be combined across the genome to recover viruses that are both genetically stable and resistant to reversion and recombination. As an example, changes were introduced in the first cleavage site of the MHV replicase polyprotein (Fig. 1, site A), demonstrating that MHV tolerated changes that either retained or abolished cleavage.⁴³ These changes were stable to passage in culture, and several grew with wild-type growth in culture or with only minimally reduced titers. These viruses are being used to study replication, immune response and protection in mice. In addition, the same changes are being engineered in the SARS-CoV genome at the orthologous conserved sites with virus recovered for testing.

Such a comprehensive strategy for engineering multiple stable mutations that allow normal growth in culture but attenuate virulence and prevent reversion has not previously been attempted for any coronavirus. The capacity of the virus to recover in vitro replication competence after introduction of deleterious mutations, along with the power of targeted reverse genetic approaches, will allow rapid establishment and testing of mutants. These studies likely will yield critical insights into determinants of replication and virulence and will form the basis for attenuated SARS-CoV variants. Finally the strategies used will likely be applicable to development of vaccine for known or newly emerging coronaviruses of animals or humans.

SUMMARY

Since the SARS epidemic abated in July 2003, there has been substantial progress in many areas of research related to replication, pathogenesis, animal models and candidates for development as vaccine for SARS-CoV. Multiple strategies for potential vaccines have been described, with encouraging results for both immune response and protection in animal models. The development of reverse genetic approaches will allow targeted study of putative viral enzymes, structural proteins and determinants of replication and pathogenesis. The demonstrated ability to recover viruses with stable mutations that allow growth in culture but attenuate virulence in animals, along with the conservation of genome organization and protein sequence of model viruses, will make it possible to rapidly develop virus variants for safe use, growth for inactivation and as possible vaccine candidates. There still remain many unanswered questions concerning the origin, evolution and emergence of SARS-CoV, the differences between adult and pediatric disease and the determinants of pathogenesis and protection. However, the unprec-

edented support for research in these areas will likely yield answers to many, if not all, of these questions. In addition, the focus on coronavirus disease is likely to lead to identification and study of several new coronaviruses either that are endemic or epidemic in humans or that can be transmitted from animals to humans.

DISCUSSION

Question: Can you provide a reason for the milder disease seen in children infected with the SARS virus?

Mark Denison, MD: I don't know the reason. I can only offer an observation that if an infectious agent for which there is a vaccine (such as mumps or measles, rubella and varicella) is introduced into a population in which vaccination has lagged or that contains naive individuals, you will see milder disease in children and more severe disease in adults.

Fred Hayden, MD, University of Virginia: On the issue of aspartic proteinase inhibitors and their possible activities, a Hong Kong group has claimed activity in cell culture and also perhaps in the clinic with lopinavir/ritonavir/ribavirin combinations. Have you looked at that at all?

Mark Denison, MD: Yes, I did once look at aspartic acid proteinase and saw no activity against the MHV proteinases. I think, though, that there may not have to be a specific proteinase ligand interaction. For example, E64d shouldn't work really well against a 3C-like proteinase. But it may be that they just provide enough steric inhibition, or are capable of interacting around the proteinase, that they don't have to actually be an active-site targeted ligand to work. However, there is not yet a good explanation for why that should be the case, so the issue needs to be examined more carefully.

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