

# Mucosal immunisation of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS

Alexander Bukreyev, Elaine W Lamirande, Ursula J Buchholz, Leatrice N Vogel, William R Elkins, Marisa St Claire, Brian R Murphy, Kanta Subbarao, Peter L Collins

## Summary

**Background** The outbreak of severe acute respiratory syndrome (SARS) in 2002 was caused by a previously unknown coronavirus—SARS coronavirus (SARS-CoV). We have developed an experimental SARS vaccine for direct immunisation of the respiratory tract, the major site of SARS-coronavirus transmission and disease.

**Methods** We expressed the complete SARS coronavirus envelope spike (S) protein from a recombinant attenuated parainfluenza virus (BHPIV3) that is being developed as a live attenuated, intranasal paediatric vaccine against human parainfluenza virus type 3 (HPIV3). We immunised eight African green monkeys, four with a single dose of BHPIV3/SARS-S and four with a control, BHPIV3/Ctrl, administered via the respiratory tract. A SARS-coronavirus challenge was given to all monkeys 28 days after immunisation.

**Findings** Immunisation of animals with BHPIV3/SARS-S induced the production of SARS-coronavirus-neutralising serum antibodies, indicating that a systemic immune response resulted from mucosal immunisation. After challenge with SARS coronavirus, all monkeys in the control group shed SARS coronavirus, with shedding lasting 5–8 days. No viral shedding occurred in the group immunised with BHPIV3/SARS-S.

**Interpretation** A vectored mucosal vaccine expressing the SARS-coronavirus S protein alone may be highly effective in a single-dose format for the prevention of SARS.

*Lancet* 2004; **363**: 2122–27  
See Commentary page 2102

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892, USA (A Bukreyev PhD, E W Lamirande BS, U J Buchholz PhD, L N Vogel MS, W R Elkins DVM, B R Murphy MD, K Subbarao MBBS, P L Collins PhD); and Bioqual, Rockville, MD, 20850 (M St Claire DVM)

**Correspondence to:** Alexander Bukreyev, Building 50, Room 6505, NIAID, NIH, 50 South Drive, Bethesda, MD 20892 (e-mail: AB176v@nih.gov)

## Introduction

Severe acute respiratory syndrome (SARS) emerged in southeast Asia in late 2002 and subsequently spread internationally. To date, it has resulted in more than 8000 cases and 774 deaths.<sup>1</sup> The causative agent was quickly identified as a previously unknown member of the *Coronaviridae* family.<sup>2–8</sup> SARS infection of human beings has since been contained through infection-control measures. However, resurgence is still a threat because the causative agent remains in animal reservoirs that are not fully understood and sporadic cases continue to be reported. We have an incomplete understanding of the genesis of SARS coronavirus (SARS-CoV), and there may be the potential for the emergence of variants capable of greater transmissibility. Coronaviruses are enveloped viruses with a genome that is a single strand of positive sense RNA of 30 kilobases or more in length. Sequence analysis of the RNA genome of the SARS coronavirus identified 11 open reading frames encoding proteins typical of coronaviruses, including the envelope spike (S) protein of the virus particle.<sup>5–7</sup>

SARS illness in humans is chiefly a pneumonia, notwithstanding the occurrence of systemic disease signs and detection of virus or viral RNA in other organs.<sup>4,9,10</sup> The primary mode of transmission of SARS seems to be through mucosal membranes of the eyes, nose, or mouth;<sup>1,9</sup> faecal-oral transmission has also been suggested but its occurrence and relative importance have not yet been documented. The prominent role of the respiratory tract in SARS transmission and disease suggest that direct immunisation of the respiratory mucosa would be an effective strategy for immunoprophylaxis against SARS. Furthermore, mucosal immunisation of the respiratory tract with live attenuated respiratory virus vaccines efficiently induces systemic as well as local immunity.<sup>11–16</sup>

As an approach for the development of a vaccine against SARS coronavirus, we took advantage of an existing live attenuated vaccine virus, BHPIV3, that is being developed for intranasal paediatric immunisation against HPIV3 infection and disease.<sup>11,14</sup> BHPIV3 was derived from bovine (B)PIV3,<sup>14,16</sup> a closely-related bovine counterpart of HPIV3 that is attenuated in primates because of a natural host range-restriction. It has also been shown to be attenuated and immunogenic in humans, and is a candidate vaccine against HPIV3.<sup>14,16</sup> BPIV3 was modified previously with recombinant DNA methods to replace its F and HN protective surface antigen genes with their HPIV3 counterparts, yielding BHPIV3.<sup>11</sup> BHPIV3 was an improved HPIV3 vaccine, since it bears protective antigens that exactly match HPIV3.<sup>11</sup>

In our study, BHPIV3 was further modified by the insertion of a transcriptional cassette containing the coding sequence of the full-length S protein of SARS coronavirus. The S protein was chosen because studies with other coronaviruses showed that it is a major viral surface protein important in initiating infection.

Additionally, immunisation of experimental animals with the S protein of other coronaviruses induced virus-specific immunity and, in some cases, conferred protection against subsequent challenge.<sup>17–19</sup> We investigated the replication, immunogenicity, and protective effects of this experimental BHPIV3/SARS-S vaccine in African green monkeys (*Cercopithecus aethiops*).

## Methods

### Construction of BHPIV3/SARS-S and BHPIV3/Ctrl viruses

The Urbani strain of SARS coronavirus was provided by L J Anderson and T G Ksiazek of the Centers for Disease Control and Prevention, Atlanta, GA, USA, and propagated in Vero cells.<sup>20</sup> All experiments involving infectious SARS coronavirus were done under approved biosafety level 3 conditions.<sup>20</sup> Viral genomic RNA was isolated and used in RT-PCR to synthesise and amplify a 3768 bp cDNA containing the complete SARS-coronavirus S coding sequence. The cDNA was designed so that the SARS coding sequence was flanked by short HPIV3-specific transcription signals that are necessary for the foreign gene to be expressed by the transcriptional program of the BHPIV3 vector (sequences of the oligonucleotide primers and details of RT-PCR and DNA construction are available from A Bukreyev).<sup>11,12</sup>

A PCR product of the expected length was purified and inserted into a *NotI* restriction endonuclease site that had previously been introduced into a complete cloned cDNA of BHPIV3,<sup>11</sup> and the sequence of the insert and flanking regions was confirmed. In this configuration, the SARS-coronavirus S insert is present as an added gene located between the BHPIV3 P and M genes (figure 1) and would be expressed as a separate mRNA by the BHPIV3 polymerase. Certain idiosyncratic features of HPIV3 molecular genetics were also accommodated: the insert was designed so that the BHPIV3 genome length remained an even multiple of six, which is required for efficient HPIV3 replication and is thought to reflect a nucleocapsid spacing requirement.<sup>21</sup> In addition, the insert was designed to maintain the spacing of the transcriptional units within this hexamer organisation.<sup>21</sup> The recombinant BHPIV3/SARS-S virus was recovered and propagated in cell culture as described elsewhere.<sup>11,12</sup>

We constructed and recovered the control virus using the same methods as those described for BHPIV3/SARS-S, except that the S open reading frame of the insert was replaced by a foreign sequence of identical length (derived from a complementary copy of the genome of human

respiratory syncytial virus) that did not have any significant open reading frame. Thus, this control gene would be expressed as a separate mRNA, but would not encode a significant foreign protein (details of this construction available from A Bukreyev).

### Virus growth and virological and serological assays

BHPIV3/SARS-S and BHPIV3/Ctrl were propagated on LLC-MK2 monkey kidney cells, and viral titres were determined by limiting dilution on the same cells using haemadsorption with guinea pig erythrocytes (a property of the HPIV3 HN protein) to detect virus infection.<sup>11,12</sup> Titres are expressed in units of tissue culture 50% infectious dose (TCID<sub>50</sub>), which are similar in magnitude to a plaque forming unit.

Serum antibodies specific to HPIV3 were quantified by an haemagglutination inhibition (HAI) assay in which dilutions of serum were tested for the ability to block agglutination of guinea pig erythrocytes in vitro by HPIV3, in parallel with known positive and negative control standards.<sup>11,12</sup> SARS coronavirus was propagated in Vero monkey kidney cells, and viral titres were measured by limiting dilution in the same cells scored by a 50% end point of visible cytopathology.<sup>20</sup>

Serum antibodies specific to SARS coronavirus were quantified in Vero cells by testing dilutions for the ability to neutralise 100 TCID<sub>50</sub> units of SARS coronavirus per well, in parallel with known positive and negative control standards.<sup>20</sup> Each sample involved four wells per dilution in a 96 well plate, and viral cytopathic effect was read on day 3 and day 4 and expressed as a 50% endpoint.

### Detection of the S protein

LLC-MK2 cells were infected with BHPIV3/SARS-S or the control virus at multiples of 5 TCID<sub>50</sub> units of infection per cell, and cells were harvested 18 h post-infection.<sup>12</sup> In addition, the medium overlying other infected cells was harvested at 48 h post-infection, and virus was concentrated by centrifugation at 8000 g at 4°C for 18 h. The viral pellets were resuspended and subjected to centrifugation on a 30–60% w/v discontinuous sucrose gradient at 130 000 g at 4°C for 90 min, after which the banded virus was harvested. Western blot analysis was done with NuPage protein electrophoresis system and WesternBreeze immunodetection kit (Invitrogen, Carlsbad, CA, USA). We detected S protein with serum samples from mice infected with SARS coronavirus<sup>20</sup> and a second antibody of alkaline-phosphatase-conjugated goat anti-mouse IgG (Invitrogen).

### Infection and challenge of African green monkeys

We used eight young adult African green monkeys of either sex (bodyweight 3.6–5.9 kg) with a confirmed absence of detectable serum antibodies against HPIV3 or SARS coronavirus. We immunised four animals with BHPIV3/SARS-S and four with BHPIV3/Ctrl. We used a single combined intranasal and intratracheal inoculation with 10<sup>6</sup> TCID<sub>50</sub> units of virus in a 1 mL inoculum per site (combined dose of 10<sup>6.3</sup> TCID<sub>50</sub> units).

Animals were first anaesthetised with ketamine hydrochloride given intramuscularly at a dose of 10 mg/kg, and then placed in dorsal recumbency. Through each nostril, we administered

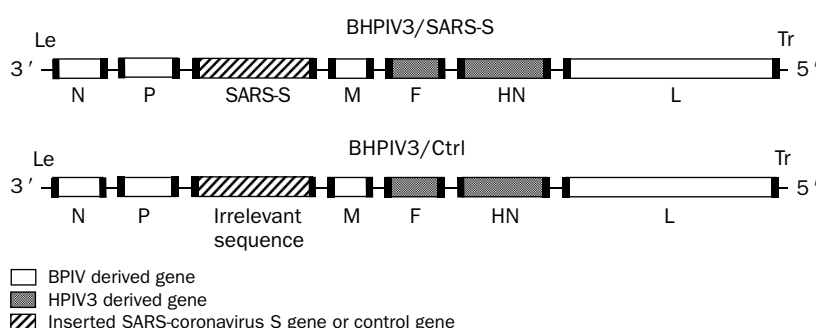
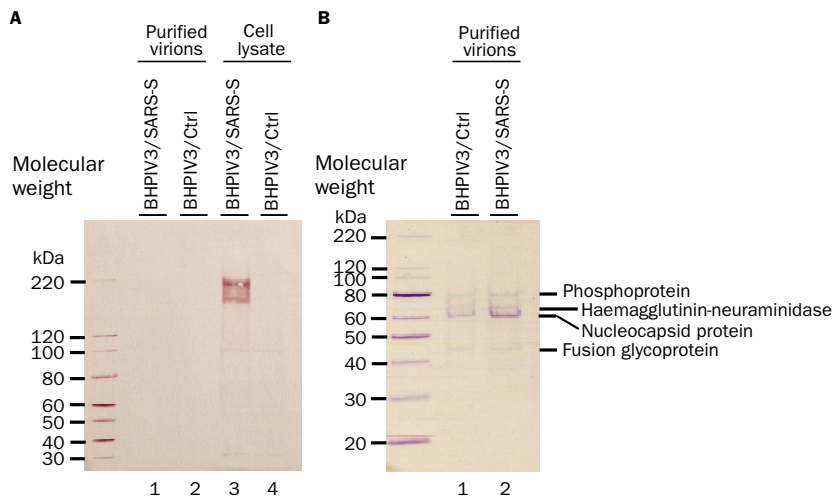


Figure 1: RNA genome maps for BHPIV3/SARS-S and control BHPIV3/Ctrl viruses

N=nucleocapsid protein. P=phosphoprotein. F=fusion glycoprotein. HN=haemagglutinin-neuraminidase glycoprotein. L=polymerase protein. Black bars at beginning and end of each gene represent PIV3-specific transcription signals, and gap between rectangles represents the PIV3 intergenic trinucleotide. Leader (Le) and trailer (Tr) sequences at 3' and 5' ends of the PIV3 genome are short extragenic regions containing promoter sequences.



**Figure 2: Expression of SARS-coronavirus S protein by BHPIV3/SARS-S in cell culture and its absence in the BHPIV3 virus particle**

**A:** western blot analysis shows presence of the SARS-coronavirus S protein in lysates of cells infected with BHPIV3/SARS-S (lane 3) and its absence in lysates from cells infected with BHPIV3/Ctrl (lane 4). S protein was not detectable in purified, concentrated BHPIV3/SARS-S virus particles (lane 1) or in the negative control BHPIV3/Ctrl (lane 2). **B:** direct Coomassie staining of a gel loaded with replicate samples of purified, concentrated BHPIV3/Ctrl (lane 1) and BHPIV3/SARS-S (lane 2) shows viral protein was present in excess of what should be necessary for detection by western blot analysis.

0.5 mL of inoculum with a sterile Luer syringe introduced about 3–5 mm into each nostril. For intratracheal inoculation, a sterile stainless steel laryngoscope was used to observe the epiglottis, and the 1 mL inoculum was delivered through a sterile flexible catheter that had been inserted about 3 cm past the epiglottal opening. We used separate sterilised instruments for every animal. Nasopharyngeal swabs were taken on days 0–10, 12, and 14, and tracheal lavages were done on days 2, 4, 6, 8, 10, and 14,<sup>22</sup> and titration of BHPIV3/SARS-S and BHPIV3/Ctrl was done as described previously. For tracheal lavage, we used a laryngoscope and catheter placed as described above, with a 2 mL wash volume of phosphate-buffered saline that was instilled and aspirated back. For the SARS-coronavirus challenge, the animals were given a similar combined intranasal and intratracheal inoculation with  $10^6$  TCID<sub>50</sub> units of virus in a 1 mL inoculum at each site. Nasopharyngeal swabs and tracheal lavages were obtained on the same schedule as that used after the initial immunisation. These experiments were

done in an approved animal biosafety level 3 facility. Virus titration was done as described earlier. We took serum samples 1 day before immunisation (day -1), 1 day before the challenge (day 27), and 28 days post challenge (day 56), which were analysed for virus-specific antibodies as described above.

The primate study was approved by the National Institutes of Health (USA) Animal Care and Use Committee and was done in a laboratory approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

### Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or in the writing of the report.

### Results

BHPIV3 was modified to express the envelope spike S protein of SARS coronavirus (figure 1). The recombinant BHPIV3/SARS-S virus was recovered and propagated in cell culture in parallel with the control virus, BHPIV3/Ctrl. The presence of foreign inserts did not affect the efficiency of replication of the vector in vitro. The BHPIV3 genome is a single strand of negative sense RNA of 15.5 kilobases that contains six non-overlapping genes (figure 1). The virally-encoded polymerase initiates at the left end of the genome and transcribes each gene in turn, including any inserted foreign gene, into a separate mRNA. The correct expression of the S sequence as a separate mRNA was confirmed by northern blot analysis of intracellular RNA isolated from LLC-MK2 cells infected with BHPIV3/SARS-S (data not shown).

Expression of the SARS-coronavirus S protein was confirmed by western blot analysis of lysates of BHPIV3/SARS-S-infected cells with serum of mice that had been infected with SARS coronavirus<sup>20</sup> (figure 2A, lane 3). This showed that the S protein migrated as two major, diffuse bands of about 170 and 200 kDa. The two forms of the S protein probably differ by the extent of glycosylation; based on the nucleotide sequence, the

	BHPIV3 shedding				Serum antibodies	
	Nasal swab		Tracheal lavage		HPIV3 HAI titre (reciprocal log <sub>2</sub> )	SARS-coronavirus neutralising titre (reciprocal log <sub>2</sub> )
	Duration (days)	Peak titre (log <sub>10</sub> TCID <sub>50</sub> /mL)	Duration (days)	Peak titre (log <sub>10</sub> TCID <sub>50</sub> /mL)		
<b>BHPIV3/SARS-S</b>						
Monkey						
V101 (F)	3	3.7	0	≤0.5*	9	4.1
V104 (F)	5	3.5	0	≤0.5	8	3.2
V117 (F)	7	3.2	1	2.0	8	4.2
V191 (M)	8	5.0	3	2.5	9	4.2
<b>BHPIV3/Ctrl</b>						
Monkey						
V099 (F)	9	4.5	9	3.5	8	≤2†
V103 (F)	6	5.0	5	6.5	9	≤2
V107 (F)	6	3.5	3	3.5	9	≤2
122 (F)	10	4.2	5	2.7	10	≤2

F=female. M=male. Serum samples taken 1 day before immunisation did not have detectable HAI antibodies (detection limit 1.0 reciprocal log<sub>2</sub> dilution units) or detectable SARS-coronavirus-neutralising antibodies (lower limit of detection was ≤2 reciprocal log<sub>2</sub>). \*Lower limit of detection of virus. †Lower limit of detection of serum neutralising antibodies.

**Table 1: Responses to mucosal immunisation with BHPIV3/SARS-S and BHPIV3/Ctrl**

	SARS-coronavirus shedding				Serum antibodies
	Nasal swab		Tracheal lavage		SARS-coronavirus neutralising titre (reciprocal log <sub>2</sub> )
	Duration (days)	Peak titre (log <sub>10</sub> TCID <sub>50</sub> /mL)	Duration (days)	Peak titre (log <sub>10</sub> TCID <sub>50</sub> /mL)	
<b>BHPIV3/SARS-S</b>					
Monkey					
V101 (F)	0	≤0.5*	0	≤0.5*	7.2
V104 (F)	0	≤0.5	0	≤0.5	8.2
V117 (F)	0	≤0.5	0	≤0.5	7.2
V191 (M)	0	≤0.5	0	≤0.5	7.2
<b>BHPIV3/Ctrl</b>					
Monkey					
V099 (F)	7	1.5	3	3.5	7.1
V103 (F)	5	1.7	0	≤0.5	6.2
V107 (F)	7	3.0	3	2.5	7.0
V122 (F)	7	1.5	1	3.0	7.2

F=Female. M=male. \*Lower limit of detection of virus.

Table 2: Responses to challenge with SARS coronavirus in monkeys immunised with BHPIV3/SARS-S or BHPIV3/Ctrl

predicted full length unmodified S protein is 138 kDa and has 23 potential glycosylation sites.<sup>5-7</sup> There was no evidence of proteolytic cleavage of the SARS S protein into two subunits, as occurs with some coronaviruses. Since the SARS-coronavirus S protein is a transmembrane virion component and BHPIV3 also is an enveloped virus, we wanted to determine whether the S protein was incorporated into BHPIV3 particles. Western blot analysis did not detect the S protein in purified, concentrated BHPIV3/SARS-S virus particles (figure 2A, lane 1) under conditions where direct Coomassie staining (figure 2B, lane 2) showed that viral protein was present in quantities greater than that required for detection by western blot. The absence of significant incorporation of S protein into virions suggests that its expression would be unlikely to affect the tropism of the vector and is a safety factor.

Table 1 shows the monkeys' responses to immunisation. Analysis of the nasal swab samples taken after immunisation showed that the peak titre (3.9 vs 4.3 log<sub>10</sub> TCID<sub>50</sub>/mL) and duration of shedding (5.8 vs 7.8 days) from the upper respiratory tract did not differ significantly between BHPIV3/SARS-S and BHPIV3/Ctrl. Analysis of the tracheal lavage samples, a measure of shedding from the lower respiratory tract, showed that two of the four animals infected with BHPIV3/SARS-S did not have detectable virus shedding, whereas the other two showed some shedding at 2 days but this was less than for the BHPIV3/Ctrl-immunised animals (table 1). Thus, expression of the SARS S protein had an attenuating effect on the replication of the vector, particularly in the lower respiratory tract. Nevertheless, BHPIV3/SARS-S and BHPIV3/Ctrl each induced a moderate titre of HPIV3-specific antibodies as measured by an HAI assay (table 1). In addition, BHPIV3/SARS-S induced a detectable level of serum antibodies that neutralised SARS-S coronavirus in vitro (mean reciprocal titre of 3.9 log<sub>2</sub>).

Table 2 shows responses to challenges with SARS coronavirus. All four monkeys immunised with BHPIV3/Ctrl shed challenge SARS coronavirus from the upper and lower respiratory tract. By contrast, none of the animals immunised with BHPIV3/SARS-S shed SARS coronavirus on any day from either the upper or lower respiratory tract. SARS-coronavirus-neutralising serum antibody titres 28 days after the challenge are shown in table 2. The increase in titre in the BHPIV3/SARS-S group after the challenge could be the result of an immune response to the antigen present in the virus inoculum or to a low level of replication of SARS coronavirus.

## Discussion

We have developed an experimental SARS vaccine using an existing live attenuated HPIV3 vaccine candidate, BHPIV3, as a vector to express the SARS-coronavirus S protein. The use of this respiratory virus as a vector provides for direct immunisation of the respiratory tract, the main site of SARS coronavirus transmission and disease. The monkeys that were vaccinated with BHPIV3/SARS-S were highly protected against the challenge infection. This finding identified the S protein as a major protective antigen of SARS coronavirus and indicates that vaccines against SARS should include this protein.

Topical immunisation of the respiratory tract also induced detectable SARS-coronavirus-neutralising serum antibodies, evidence of a systemic immune response. However, the post-immunisation titre induced by BHPIV3/SARS-S was almost eight-fold lower than that achieved by SARS-coronavirus infection of the BHPIV3/Ctrl-immunised animals. This difference in immunogenicity might reflect the natural restriction of HPIV3 to the respiratory tract, whereas SARS coronavirus disseminates systemically, at least in non-human primates.<sup>8,9</sup> There was no evidence of immune-mediated enhancement of infection or disease, which occurs for one coronavirus, feline infectious peritonitis virus.<sup>23</sup> Thus, our results show that one mucosal immunisation with vectored SARS S protein was sufficient to protect against shedding after a large challenge dose of SARS coronavirus.

Some cynomolgus monkeys (*Macaca fascicularis*) infected with SARS coronavirus have been reported to develop clinical disease signs including a transient rash, respiratory distress, and lethargy.<sup>24,8</sup> We have investigated three species of monkeys, namely cynomolgus, rhesus (*M. mulatta*), and African green monkeys, for permissiveness for SARS-coronavirus replication and possible disease (unpublished data). Of the three, African green monkeys supported the highest levels of replication as measured by virus shedding and hence were used in this study. None of the 12 animals in any of the three species developed overt disease signs, and we did not note disease signs in this study. Mice, ferrets, and cats have been shown to support high levels of pulmonary SARS-coronavirus replication, and some of the infected ferrets became lethargic.<sup>25, 20</sup> We did not assess BHPIV3/SARS-S in mice because the HPIV3 vaccine vector is severely restricted for replication in this animal, and the cat and ferret models were only recently reported. Thus, although our study showed complete protection against shedding of challenge virus, we could not assess whether protection would be afforded

against clinical disease signs. This study limitation is not unusual. With respect to human viruses, experimental animals rarely provide faithful models of the infection and disease that is observed in humans, and disease signs often are minimal, altered, or absent. The measurement of infectious challenge virus in secretions or other samples from experimental animals is a general standard for measuring the efficacy of viral vaccines. The assessment of an experimental vaccine in a non-human primate is especially important in view of the phylogenetic and anatomical similarity to humans, and is an appropriate last step before clinical trials.

Attenuated versions of HPIV3 are under active development as vaccines for intranasal immunisation of infants and young children, as are HPIV3-based vectored vaccines that also express protective antigens of respiratory pathogens such as measles virus, respiratory syncytial virus, and metapneumovirus.<sup>12,13,15</sup> HPIV3 efficiently infects the respiratory tract but does not spread far beyond it, which is an important safety factor. HPIV3-based vectors have proven effective in inducing local and systemic immunity against a number of foreign antigens.<sup>12,13,15</sup> Furthermore, safe intranasal administration of attenuated HPIV3 and related viruses has been shown possible.<sup>16,26,27</sup> An additional safety feature is that RNA recombination is almost non-existent in nature for the family of viruses represented by HPIV3, whereas recombination is extremely frequent in coronaviruses, and the potential for recombination with circulating human coronavirus would be a concern for a live-attenuated SARS-coronavirus vaccine virus.

As currently constructed, the BHPIV3/SARS-S vector is an excellent candidate for clinical testing as a vaccine that is likely to be highly attenuated, safe, and effective against both HPIV3 and SARS for infants and young children, in whom the vector would replicate efficiently. This vaccine would be especially useful if a more transmissible version of SARS coronavirus emerges and immunisation of infants and children is needed. However, any replicating viral vector bearing the protective antigens of a common human pathogen, such as adenovirus or HPIV3, is unlikely to replicate sufficiently well in adults to be immunogenic because of a prevalence of neutralising antibodies to such pathogens.<sup>26,27</sup> Fortunately, parainfluenza viruses have been amenable to swapping of the surface proteins without loss of infectivity *in vitro* or *in vivo*.<sup>28,29</sup> Therefore, it should be possible to replace the HPIV3 HN and F surface proteins with those of an antigenically-distinct parainfluenza virus for which the general population lacks immunity—in particular one of the many avian parainfluenza viruses such as an attenuated strain of Newcastle disease virus. The resulting vector would be a useful SARS vaccine for mucosal immunisation of the entire human population.

#### Contributors

A Bukreyev participated in the design of the constructs, construction and *in-vitro* characterisation of the recombinant viruses, the design of the monkey experiment, SARS-coronavirus titrations, and interpretation of the results. E Lamirande participated in the construction and *in-vitro* characterisation of the recombinant viruses, SARS-coronavirus titrations, and serum neutralisation assays. U Buchholz gave useful recommendations on BHPIV3 recovery, did northern blot analysis, and participated in SARS-coronavirus titrations. L Vogel prepared challenge virus and participated in serum neutralisation assays. W R Elkins and M St Claire supervised the animal experiments. B Murphy participated in planning of the experiments and interpretation of the results. K Subbarao prepared SARS-coronavirus RNA and mouse SARS-coronavirus-immune serum, established the African green monkey model for SARS coronavirus, and participated in the planning of the experiments and interpretation of the results. P Collins participated in design of the constructs, planning of the experiments and interpretation of the results, and providing general guidance for the project.

#### Conflict of interest statement

None declared.

#### Acknowledgments

We thank Kim-Chi Tran for technical assistance and Kathryn Hanley for assistance with statistical analysis. This project was funded as part of the NIAID Intramural Program.

#### References

- 1 WHO. Communicable disease surveillance and response: severe acute respiratory syndrome (SARS) <http://www.who.int/csr/sars/en>. (accessed Dec 31, 2003).
- 2 Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003; **348**: 1953–66.
- 3 Poutanen SM, Low DE, Henry B, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* 2003; **348**: 1995–2005.
- 4 Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; **348**: 1967–76.
- 5 Rota PA, Oberste MS, Monroe SS, et al. Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003; **300**: 1394–99.
- 6 Zeng FY, Chan CW, Chan MN, et al. The complete genome sequence of severe acute respiratory syndrome coronavirus strain HKU-39849 (HK-39). *Exp Biol Med (Maywood)* 2003; **228**: 866–73.
- 7 The complete genome of the SARS associated Coronavirus. *Gene Bank sequence AY274119* 2003.
- 8 Kuiken T, Fouchier RA, Schutten M, et al. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* 2003; **362**: 263–70.
- 9 Peiris JS, Yuen KY, Osterhaus AD, Stohr K. The severe acute respiratory syndrome. *N Engl J Med* 2003; **349**: 2431–41.
- 10 Peiris JS, Chu CM, Cheng VC, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* 2003; **361**: 1767–72.
- 11 Schmidt A, McAuliffe J, Huang A, et al. Bovine parainfluenza virus type 3 (BPIV3) fusion and hemagglutinin-neuraminidase glycoproteins make an important contribution to the restricted replication of BPIV3 in primates. *J Virol* 2000; **74**: 8922–29.
- 12 Schmidt AC, McAuliffe JM, Murphy BR, Collins PL. Recombinant bovine/human parainfluenza virus type 3 (B/HPIV3) expressing the respiratory syncytial virus (RSV) G and F proteins can be used to achieve simultaneous mucosal immunization against RSV and HPIV3. *J Virol* 2001; **2001**: 4594–603.
- 13 Durbin AP, Skiadopoulos MH, McAuliffe JM, Riggs JM, Surman SR, Collins PL, Murphy BR. Human parainfluenza virus type 3 (PIV3) expressing the hemagglutinin protein of measles virus provides a potential method for immunization against measles virus and PIV3 in early infancy. *J Virol* 2000; **74**: 6821–31.
- 14 Murphy BR, Collins PL. Live-attenuated virus vaccines for respiratory infection and parainfluenza viruses: applications of reverse genetics. *J Clin Invest* 2002; **110**: 21–27.
- 15 Tang RS, Schickli JH, MacPhail M, et al. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3' proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. *J Virol* 2003; **77**: 10819–28.
- 16 Karon RA, Makhene M, Gay K, Wilson MH, Clements ML, Murphy BR. Evaluation of a live attenuated bovine parainfluenza type 3 vaccine in two- to six-month-old infants. *Pediatr Infect Dis J* 1996; **15**: 650–54.
- 17 Callebaut P, Pensaert M. Expression and immunogenicity of the spike glycoprotein of porcine respiratory coronavirus encoded in the E3 region of adenovirus. *Adv Exp Med Biol* 1995; **380**: 265–70.
- 18 Daniel C, Talbot PJ. Protection from lethal coronavirus infection by affinity-purified spike glycoprotein of murine hepatitis virus, strain A59. *Virology* 1990; **174**: 87–94.
- 19 Tuboly T, Nagy E, Dennis JR, Derbyshire JB. Immunogenicity of the S protein of transmissible gastroenteritis virus expressed in baculovirus. *Arch Virol* 1994; **137**: 55–67.
- 20 Subbarao K, McAuliffe J, Vogel L, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J Virol* 2004; **78**: 3572–77.
- 21 Kolakofsky D, Pelet T, Garcin D, Hausmann S, Curran J, Roux L. Paramyxovirus RNA synthesis and the requirement for hexamer genome length: the rule of six revisited. *J Virol* 1998; **72**: 891–99.
- 22 Crowe JE, Jr, Collins PL, London WT, Chanock RM, Murphy BR. A comparison in chimpanzees of the immunogenicity and efficacy of

- live attenuated respiratory syncytial virus (RSV) temperature-sensitive mutant vaccines and vaccinia virus recombinants that express the surface glycoproteins of RSV. *Vaccine* 1993; **11**: 1395–1404.
- 23 Weiss RC, Scott FW. Antibody-mediated enhancement of disease in feline infectious peritonitis: comparisons with dengue hemorrhagic fever. *Comp Immunol Microbiol Infect Dis* 1981; **4**: 175–89.
- 24 Fouchier RA, Kuiken T, Schutten M, et al. Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* 2003; **423**: 240.
- 25 Martina BE, Haagmans BL, Kuiken T, et al. Virology: SARS virus infection of cats and ferrets. *Nature* 2003; **425**: 915.
- 26 Clements ML, Belshe RB, King J, et al. Evaluation of bovine, cold-adapted human, and wild-type human parainfluenza type 3 viruses in adult volunteers and in chimpanzees. *J Clin Microbiol* 1991; **29**: 1175–82.
- 27 Karron RA, Wright PF, Newman FK, et al. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in healthy infants and children. *J Infect Dis* 1995; **172**: 1445–50.
- 28 Tao T, Skiadopoulos MH, Davoodi F, Riggs JM, Collins PL, Murphy BR. Replacement of the ectodomains of the hemagglutinin-neuraminidase and fusion glycoproteins of recombinant parainfluenza virus type 3 (PIV3) with their counterparts from PIV2 yields attenuated PIV2 vaccine candidates. *J Virol* 2000; **74**: 6448–58.
- 29 Tao T, Durbin AP, Whitehead SS, Davoodi F, Collins PL, Murphy BR. Recovery of a fully viable chimeric human parainfluenza virus (PIV) type 3 in which the hemagglutinin-neuraminidase and fusion glycoproteins have been replaced by those of PIV type 1. *J Virol* 1998; **72**: 2955–61.

## Uses of error

### Immediate and delayed learning

Seth Powsner

Mistakes made during training are easier for me to acknowledge. An implicit excuse is provided by the training role. Memories of three mistakes remain particularly vivid.

Internship. A general medicine ward. A geriatric patient, almost recovered. Little work remained. However, custom mandated a lumbar puncture (LP) for any patient who had a positive blood test for syphilis without recorded treatment. Positive spinal fluid meant more penicillin. I had come to regard LPs as benign, bedside procedures: just check for bulging optic discs. I mastered a technique for one handed opening and closing of screw-top specimen tubes. I could do LPs on my own, with no senior resident or nurse. So, I was working alone in my patient's room at the end of the hallway. I manoeuvred a long needle into the vertebral interspace. I removed the stylet. Out flowed bright red blood. Undiluted. Freely flowing. I fought my panic, replaced the stylet, fought my panic, removed the needle, fought my panic, pressed the site. No call button in reach. Too embarrassing to yell. Much time passed. A nurse finally happened along. Lesson learnt: Beware of overconfidence and always arrange backup.

Residency. A modern psychiatric ward. A depressed grandmother. This was an opportunity to do daily psychotherapy. My attempt comprised questions such as "Tell me your feelings. What is on your mind? What do you remember?" During the sixth session, she backed away in her chair. She was trying to get away even though her chair was stuck in a corner. There was no place to go. She literally began pushing herself up the wall. I stopped.

In retrospect, her children's suicides were clear markers of inherited affective illness. I was rubbing salt in old wounds. Electroconvulsive treatments worked perfectly. Lesson learnt: Beware of preconceived notions of illness and treatment, and shift paradigms as needed.

Internship. A general pediatrics ward. A pretty, young teenager admitted for an excision. Her family was pleasant, unsophisticated, of modest means, grateful that neurofibromatosis had only required small excisions over the years. Unfortunately, this procedure revealed sarcoma in her thigh. Off to our library. Small chances for 5-year survival, assuming hip disarticulation, according to case series of the time. Attending physicians proposed amputation. Mother and patient balked. They seemed to have known neurofibromatosis might take her. They wanted my opinion. I almost suggested travel to the Bahamas and Brompton's cocktails. I stifled myself, promised discussion later, went to a trusted consultant. This oncologist sided with the others: "try for the best chance." I closed ranks. I endorsed treatment—only to have a sudden, private, visceral sense of wrong. They accepted amputation. She died within a year, not well. I never asked how she wanted to die. Lesson learnt: Beware of assumptions about a patient's life and preferences; ask.

It has been said that you can learn for your entire life from mistakes you make in your first year, or you can make first year mistakes for your entire life. For me, these cases are illustrations in a long course on therapeutic humility. I have more errors to relate, but the invitation requested a short article, not a full issue.

Department of Psychiatry, Yale-New Haven Hospital, New Haven, CT 06504-8900, USA (S Powsner MD)