(Hc 0 4078

Journal of General Virology (2004), **85**, 3109–3113 DOI 10.1099/vir.0.80111-0

Short Communication

Protective humoral responses to severe acute respiratory syndrome-associated coronavirus: implications for the design of an effective protein-based vaccine

Hai Pang,[1,2] Yinggang Liu,[3] Xueqing Han,[1,2] Yanhui Xu,[1,2] Fuguo Jiang,[1,2] Donglai Wu,[3] Xiangang Kong,[3] Mark Bartlam[1,2] and Zihe Rao[1,2]

Correspondence
Zihe Rao
raozh@xtal.tsinghua.edu.cn

[1]Laboratory of Structural Biology, Tsinghua University, Beijing 100084, China

[2]National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China

[3]Harbin Veterinary Research Institute, Chinese Academy of Agriculture, 427 Maduan Street, Harbin 150001, China

Some of the structural proteins of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) carry major epitopes involved in virus neutralization and are essential for the induction of protective humoral responses and the development of an effective vaccine. Rabbit antisera were prepared using full-length N and M proteins and eight expressed fragments covering the S protein. Antisera to S and M proteins were found to have different neutralizing titres towards SARS-CoV infection *in vivo*, ranging from 1 : 35 to 1 : 128. Antiserum to the N protein did not contain neutralizing antibodies. Epitopes inducing protective humoral responses to virus infection were located mainly in the M protein and a region spanning residues 13–877 of the S protein. The neutralizing ability of antisera directed against the expressed structural proteins was greater than that of convalescent patient antisera, confirming that, as immunogens, the former induce strong, SARS-CoV-specific neutralizing antibody responses. The *in vitro* neutralization assay has important implications for the design of an effective, protein-based vaccine preventing SARS-CoV infection.

Received 15 March 2004
Accepted 2 July 2004

Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) is the causative agent of an atypical, highly transmissible respiratory disease that struck in 32 countries in 2003 resulting in approximately 800 deaths (Drosten *et al.*, 2003; Lee *et al.*, 2003; Poutanen *et al.*, 2003; Rota *et al.*, 2003). Development of a safe and effective vaccine to prevent SARS-CoV infection should be pursued with urgency as SARS is highly lethal and, if the virus does re-emerge, is likely to become endemic in regions of the world. SARS-CoV belongs to a diverse family of large, enveloped, single-stranded, positive-sense RNA viruses (Rota *et al.*, 2003). The full-length viral genome, which also serves as an mRNA, may initiate a SARS-CoV infection in transfected cells (Yount *et al.*, 2003). Therefore, the use of killed, recombinant or live-attenuated virus as a vaccine to immunize humans against SARS-CoV infection may pose serious risks (Martin *et al.*, 1999). To evaluate the potential of expressed structural proteins of SARS-CoV for the development of a safe and effective vaccine against virus infection, we investigated the neutralizing ability of antibodies against SARS-CoV derived from sera of animals that had been immunized with the expressed structural proteins.

Eight truncated fragments covering the S protein (Fig. 1a) and the full-length N protein were expressed in *Escherichia coli*, using genes of the BJ01 strain (GenBank accession no. AY278488) of SARS-CoV isolated in China, while the M protein was expressed in *Pichia pastoris* as it could not be easily expressed in *E. coli*. All fragments of the S protein containing a six-histidine tag were expressed in *E. coli* as inclusion bodies. The proteins were purified by sonication and repeated washing, and then dissolved in Tris/HCl buffer with 8 M urea and further purified using Ni-NTA agarose (Invitrogen). Finally, the denatured proteins were refolded by dialysis in Tris/HCl buffer (pH 7·4). Soluble N and M proteins containing six-histidine tags were also purified using Ni-NTA agarose (Fig. 1b). It should be noted that, unlike our expression products, the native S protein is highly glycosylated and that oligosaccharides may affect the generation of neutralization antibodies. Consequently,

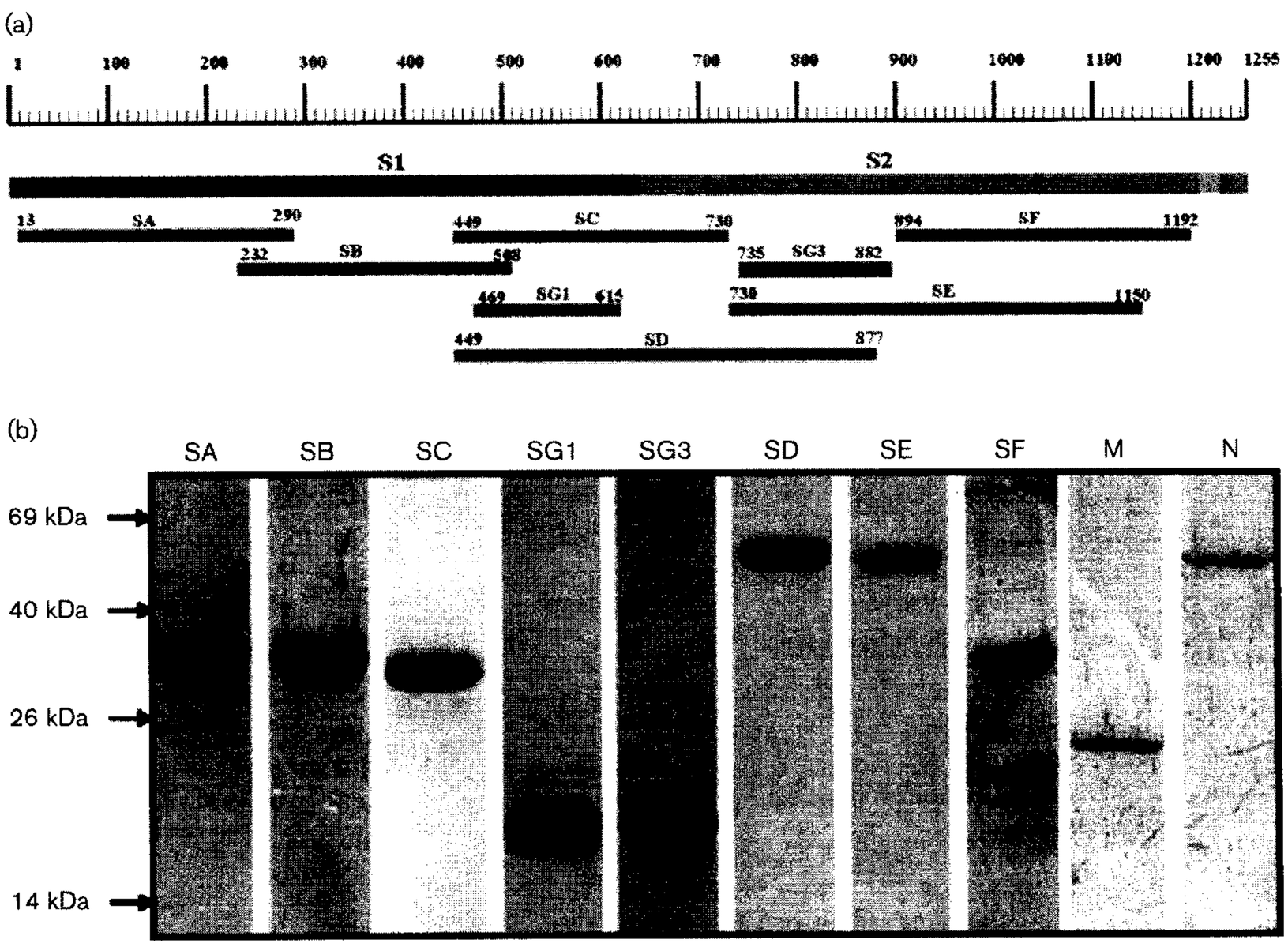

Fig. 1. Expression and purification of fragments of the SARS-CoV S protein. (a) Length and location of expressed fragments of the SARS-CoV S protein. Gene segments of the protein encoded by SARS-CoV were PCR amplified, cloned into the pET28a vector (Stratagene) and expressed in *E. coli* strain BL21 (DE3). The ruler indicates the size of the proteins in amino acids. Fragments are represented by red bars and the N- and C-terminal positions of each fragment are given. On the long bar, blue represents the N-terminal S1 region of the S protein; green represents the C-terminal region, S2; red within the N terminus represents the signal peptide; and orange within S2 represents the transmembrane region. (b) Purification of the expressed fragments of the S protein and the full-length N and M proteins. The fragments of the S protein, expressed as inclusion bodies, were purified by denaturation and refolding, whereas full-length N and M proteins (expressed in *P. pastoris* using the vector pPICZaA; Invitrogen) were purified using Ni-NTA agarose, separated by 12 % SDS-PAGE and stained with Coomassie brilliant blue to check the level of purification.

we cannot rule out the possibility that the lack of glycosylation in *E. coli* has influenced the outcome of our experiments. Nevertheless, expression of the proteins in *E. coli* could provide a simple and cheap method for large-scale production of vaccines protecting against SARS-CoV infection.

To generate antibodies to the purified proteins, we subcutaneously immunized 10 New Zealand white rabbits (male, 2–3 kg) each with one of the recombinant proteins. On day 0, each rabbit was vaccinated with 0·5 mg purified protein in Freund's complete adjuvant. Animals received further vaccinations on days 14 and 21 with 0·5 mg of each protein in Freund's incomplete adjuvant. Finally, the 10 rabbit sera were collected on day 28 after vaccination. Antibody titres were assessed by ELISA using the recombinant proteins. Using end-point dilution, high antibody titres were observed, ranging from 1 : 60 000 to 1 : 120 000 (Fig. 2a).

For neutralization tests, Vero E6 cells were grown in MEM medium supplemented with 10 % fetal bovine serum (FBS) and antibiotics in 96-well plates. To assay each serum, 200 $TCID_{50}$ of virus was added at each step of a serial twofold dilution of heat-inactivated antisera (for 45 min at 56 °C) in triplicate wells. After incubation for 1 h at 37 °C, these mixtures containing 100 $TCID_{50}$ of virus were added to the rinsed Vero E6 monolayers. Using a microscope, the plates were monitored daily for the presence of cytopathic effect until day 4–5 after incubation. Rabbit pre-immune sera were included in each test as controls. In addition, dilutions of each serum without virus were added to control wells to assay for toxicity of the sera. The rabbit sera were highly toxic to Vero E6 cells, with all cells being killed at dilutions of 1 : 2 to 1 : 8 and approximately 20 % of cells being killed at a 1 : 16 dilution. The neutralizing capacity of the sera directed against the SF fragment and the N protein could not be detected at dilutions below 1 : 16. Neutralizing titres were expressed as the reciprocal of the highest serum

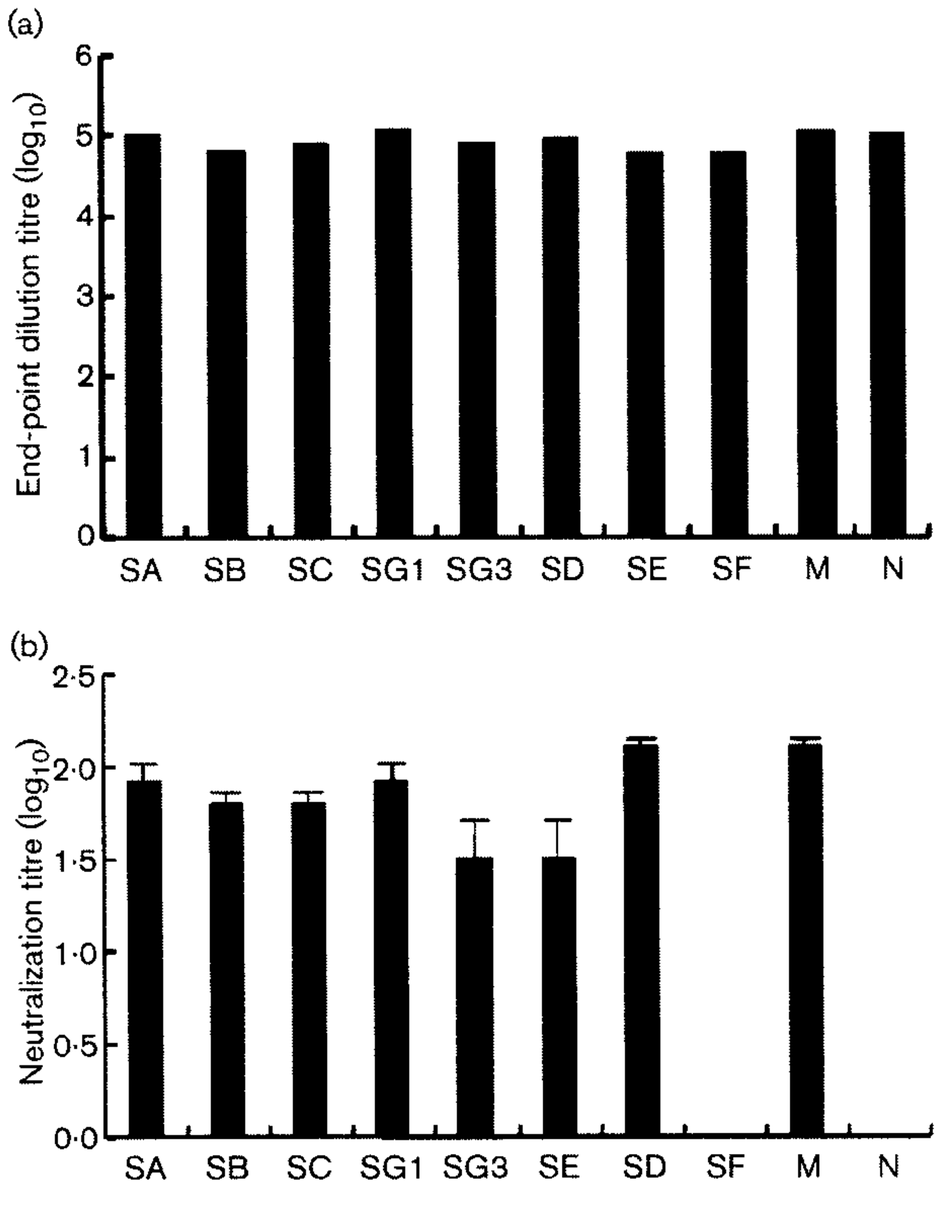

Fig. 2. Neutralization titres of antisera to the expressed structural proteins of SARS-CoV. (a) The titre of SARS-CoV-specific antibodies in the serum of immunized rabbits was determined and quantified by end-point dilution ELISA assay using the expressed proteins. Briefly, purified proteins (100 μl per well of 1 μg ml^{-1} solution, incubated overnight at 4 °C) were used to capture antibodies in the sera (incubated for 1 h at 37 °C), which were then detected with 100 μl horseradish peroxidase-conjugated goat anti-rabbit IgG (Jingmei Biotech) per well (diluted 1 : 5000 in PBS containing 0·5% Tween 20 and 10% FBS), followed by 100 μl *o*-phenylenediamine dihydrochloride solution (Sigma) per well for 30 min at room temperature in the dark. End-point titres were defined as the highest plasma dilution that resulted in an absorbance value (A_{450}) two times higher than that of non-immune plasma with a cut-off value of 0·05. Data are presented as log_{10} values. (b) Detection of virus-specific neutralizing antibodies in each serum by inhibition of Vero E6 cells lysis by SARS-CoV strain BJ01 in a micro-neutralization assay. Data are presented as the mean ± SE for four sets of tests each carried out in triplicate. (c) Evaluation of neutralizing capacity in pooled human convalescent sera. Equal volumes of antisera were mixed with several individual rabbit antisera raised using the fragments of the S protein, and their neutralizing capacity was determined under the same conditions described above. Data are presented as the mean ± SE.

dilution that gave full protection against the standard virus dose used in these experiments. The neutralizing capacity of the antisera to each protein was characterized and validated *in vitro* and the results of the neutralization assay are shown in Fig. 2(b) as log_{10} values. The neutralizing capacity of antibodies to SA (at a 1 : 89 serum dilution), SB (1 : 79), SC (1 : 79) and SG1 (1 : 89) derived from the S1 domain of S protein was higher than that of SG3 (1 : 35) and SE (1 : 35) from the S2 region. No neutralization could be detected by antibodies to fragment SF from the S2 region, suggesting that the S1 domain of the S protein may be the major target of neutralizing antibodies preventing SARS-CoV infection. A recent report showed that an adenovirus-based vaccine with the SARS-CoV S1 fragment could induce strong neutralizing antibody responses to SARS-CoV infection in monkeys (Gao *et al.*, 2003). Generally, a fragment with a domain (ligand) in the envelope protein that can bind to viral receptors on target cells may induce a higher neutralizing antibody titre than other fragments that do not contain such a ligand. Our SB fragment (residues 232–508) overlapped a 193 aa fragment of the S protein (residues 318–510) that has been shown to bind to angiotensin-converting enzyme 2, a functional receptor of SARS-CoV (Li *et al.*, 2003; Wong *et al.*, 2003). The neutralization titre of the antiserum to SB was almost equal to that of antisera to the SA, SC and SG1 fragments (Fig. 2b), suggesting that antibodies to some domains of the S protein of SARS-CoV may influence receptor binding by a steric hindrance effect, as described for reoviruses (Nason *et al.*, 2001). In addition to this steric hindrance effect, our observations suggested that SARS-CoV may also utilize more than one receptor for fusion with target cells during virus entry. It is known that human immunodeficiency virus type 1 (HIV-1) and HIV-2 require at least two receptors, CD4 and a member of the CXC-chemokines, to mediate virus entry into target cells (Endres *et al.*, 1996; Oberlin *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996).

Notably, the neutralizing capacity of antiserum to SD (1 : 128), which overlapped both the SG1 and SG3 fragments, was higher than that of either SG1 or SG3

individually (Fig. 1a and 2b). To confirm this synergistic effect of neutralizing capacity between antibodies to epitopes in SG1 and SG3, we mixed the two antisera in equal volumes to measure the neutralizing titre. The neutralizing capacity of the mixed antisera against SG1 and SG3 was equal to that of SD (Fig. 2b and c). Neutralization of coronaviruses is a specific event that requires the presentation of the epitope involved in neutralization in the appropriate structural context. Work with transmissible gastroenteritis coronavirus (TGEV) has shown that the S glycoprotein domain recognized by the receptor on ST cells is located in the globular domain of the protein close to the antigenic sites A and D (Sune *et al.*, 1990). In TGEV neutralization experiments using binary combinations of mAbs specific for different antigenic sites, a synergistic effect was also observed (Sune *et al.*, 1990).

To investigate synergistic effects in more detail, we measured the neutralizing capacity of a mixture of antisera against SA, SB and SD, which covered residues 13–877 of the S protein. As expected, the mixed sera showed a higher neutralizing titre of 1 : 342 (Fig. 2c). Similarly, the mixed sera to SA, SB and SC, covering residues 13–730 of the S protein, had a titre of 1 : 297. The mixture of antisera against SA, SB, SC and SG3, covering residues 13–882 of the S protein, had a titre of 1 : 342, which was the same as for the mixture of sera to SA, SB and SD (Fig. 2c). Generally, the humoral response against a virus is directed towards many different envelope protein epitopes. The results suggest that the region from residues 13–877 of the S protein, including an important epitope in fragment SG3 (residues 735–882), is essential for the development of an effective protein-based vaccine preventing SARS-CoV infection.

It has been reported that the M protein of TGEV is required for virus assembly and budding, that it is immunodominant and that M-specific antibodies both neutralize TGEV and mediate the complement-dependent lysis of TGEV-infected cells (Delmas *et al.*, 1986; Risco *et al.*, 1995; Woods *et al.*, 1987). To investigate the neutralizing capacity of antiserum to the M protein of SARS-CoV, we examined its neutralizing titre using serum of heat-inactivated complement. The neutralization titre of the M protein antiserum was higher than 1 : 128 (Fig. 2b), suggesting that the M protein is important in development of an effective protein-based vaccine preventing SARS-CoV infection. To evaluate the synergistic effect between antisera to different epitopes, the M antiserum was mixed with antisera against the SA, SB and SD fragments (covering residues 13–877 of the S protein) and a neutralization assay was performed. The neutralizing capacity of the mixed SA + SB + SD + M antisera showed a considerable synergistic effect, with a neutralizing titre of 1 : 396 (Fig. 2c). This suggested that sera to the M and S proteins can effectively enhance each other's activity.

We also assayed the neutralizing capacity of antiserum to the N protein, but no neutralizing ability was detected *in vitro* (Fig. 2c). However, recent evidence indicates that the N protein can induce a T-cell response to SARS-CoV infection (Gao *et al.*, 2003). An effective vaccine may also aim to elicit a vigorous cellular immunity. Therefore, the N protein is also important in development of an effective vaccine preventing SARS-CoV infection.

Neutralizing antibodies that bind efficiently to the envelope spikes of SARS-CoV can offer protection or may be beneficial if present at appropriate concentrations before exposure to the virus. To define the neutralizing capacity of convalescent antisera from SARS-CoV patients, we determined the neutralization titres for sera from three convalescent patients under the same experimental conditions, by pooling together equal volumes of antisera from each patient. The resulting neutralization titre was found to be 1 : 342. Clearly, the neutralization titre of mixed antisera to fragments SA, SB and SD (covering residues 13–877 of the S protein) and the M protein was higher than that of antisera from convalescent patients (Fig. 2c).

Our results have confirmed that, as major antigens, the expressed unglycosylated structural proteins can induce a strong, SARS-CoV-specific, neutralizing antibody response in rabbits. These results provide crucial information for a rational approach towards maximizing antibody responses elicited by potential vaccines. It will also be essential to determine whether humans and animals generate similar responses to SARS-CoV (Cyranoski, 2004; Fouchier *et al.*, 2003; Guan *et al.*, 2003; Martina *et al.*, 2003). Virus-challenge tests in animal models will be required for different SARS-CoV isolates to assess the protective capacity of the expressed proteins as vaccines. Furthermore, before any potential vaccine can be tested in humans, a non-human primate challenge model must be used to evaluate its effectiveness against SARS-CoV infection and to exclude any potential side effects (Fouchier *et al.*, 2003), such as those observed for candidate vaccines against feline infectious peritonitis virus (Vennema *et al.*, 1990). In addition, the development of a vaccine against SARS-CoV infection in animals also needs to prevent the virus being carried and transmitted to humans by animals, such as masked palm civet, ferret and cat, and causing infection in humans (Cyranoski, 2004; Guan *et al.*, 2003; Martina *et al.*, 2003).

Acknowledgements

This work was supported by the Ministry of Science and Technology (MOST), the Chinese Academy of Sciences (CAS) and the National Natural Science Foundation of China (NSFC) [grant nos 2002BA711A12, G1999075600, GZ236 (202/9) and 2003CB514103].

References

Cyranoski, D. (2004). Swift response greets return of SARS in China. *Nature* **427**, 89.

Delmas, B., Gelfi, J. & Laude, H. (1986). Antigenic structure of transmissible gastroenteritis virus. II. Domains in the peplomer glycoprotein. *J Gen Virol* **67**, 1405–1418.

Drosten, C., Gunther, S., Preiser, W. & 23 other authors (2003). Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* **348**, 1967–1976.

Endres, M. J., Clapham, P. R., Marsh, M. & 13 other authors (1996). CD4-independent infection by HIV-2 is mediated by fusin/CXCR4. *Cell* **87**, 745–756.

Fouchier, R. A., Kuiken, T., Schutten, M. & 7 other authors (2003). Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* **423**, 240.

Gao, W., Tamin, A., Soloff, A., D'Aiuto, L., Nwanegbo, E., Robbins, P. D., Bellini, W. J., Barratt-Boyes, S. & Gambotto, A. (2003). Effects of a SARS associated coronavirus vaccine in monkeys. *Lancet* **362**, 1895–1896.

Guan, Y., Zheng, B. J., He, Y. Q. & 15 other authors (2003). Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* **302**, 276–278.

Lee, N., Hui, D., Wu, A. & 11 other authors (2003). A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* **348**, 1986–1994.

Li, W., Moore, M. J., Vasilieva, N. & 9 other authors (2003). Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454.

Martin, T., Parker, S. E., Hedstrom, R., Le, T., Hoffman, S. L., Norman, J., Hobart, P. & Lew, D. (1999). Plasmid DNA malaria vaccine: the potential for genomic integration after intramuscular injection. *Hum Gene Ther* **10**, 759–768.

Martina, B. E., Haagmans, B. L., Kuiken, T., Fouchier, R. A., Rimmelzwaan, G. F., Van Amerongen, G., Peiris, J. S., Lim, W. & Osterhaus, A. D. (2003). Virology: SARS virus infection of cats and ferrets. *Nature* **425**, 915.

Nason, E. L., Wetzel, J. D., Mukherjee, S. K., Barton, E. S., Prasad, B. V. & Dermody, T. S. (2001). A monoclonal antibody specific for reovirus outer-capsid protein $\sigma 3$ inhibits $\sigma 1$-mediated hemagglutination by steric hindrance. *J Virol* **75**, 6625–6634.

Oberlin, E., Amara, A., Bachelerie, F. & 10 other authors (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833–835.

Poutanen, S. M., Low, D. E., Henry, B. & 11 other authors (2003). Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* **348**, 1995–2005.

Risco, C., Anton, I. M., Sune, C., Pedregosa, A. M., Martin-Alonso, J. M., Parra, F., Carrascosa, J. L. & Enjuanes, L. (1995). Membrane protein molecules of transmissible gastroenteritis coronavirus also expose the carboxy-terminal region on the external surface of the virion. *J Virol* **69**, 5269–5277.

Rota, P. A., Oberste, M. S., Monroe, S. S. & 32 other authors (2003). Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300**, 1394–1399.

Sune, C., Jimenez, G., Correa, I., Bullido, M. J., Gebauer, F., Smerdou, C. & Enjuanes, L. (1990). Mechanisms of transmissible gastroenteritis coronavirus neutralization. *Virology* **177**, 559–569.

Trkola, A., Dragic, T., Arthos, J. & 7 other authors (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184–187.

Vennema, H., de Groot, R. J., Harbour, D. A., Dalderup, M., Gruffydd-Jones, T., Horzinek, M. C. & Spaan, W. J. (1990). Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. *J Virol* **64**, 1407–1409.

Wong, S. K., Li, W., Moore, M. J., Choe, H. & Farzan, M. (2003). A 193-amino-acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2. *J Biol Chem* **279**, 3197–3201.

Woods, R. D., Wesley, R. & Kapke, P. A. (1987). Complement-dependent neutralization of transmissible gastroenteritis virus by monoclonal antibodies. *Adv Exp Med Biol* **218**, 493–500.

Wu, L., Gerard, N. P., Wyatt, R. & 9 other authors (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor. *Nature* **384**, 179–183.

Yount, B., Curtis, K. M., Fritz, E. A., Hensley, L. E., Jahrling, P. B., Prentice, E., Denison, M. R., Geisbert, T. W. & Baric, R. S. (2003). Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci U S A* **100**, 12995–13000.