Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity

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We investigated the contributions of the structural proteins of severe acute respiratory syndrome (SARS) coronavirus (CoV) to protective immunity by expressing them individually and in combinations from a recombinant parainfluenza virus (PIV) type 3 vector called BHPIV3. This vector provided direct immunization of the respiratory tract, the major site of SARS transmission, replication, and disease. The BHPIV3/SARS recombinants were evaluated for immunogenicity and protective efficacy in hamsters, which support a high level of pulmonary SARS-CoV replication. A single intranasal administration of BHPIV3 expressing the SARS-CoV spike protein (S) induced a high titer of SARS-CoV-neutralizing serum antibodies, only 2-fold less than that induced by SARS-CoV infection. The expression of S with the two other putative virion envelope proteins, the matrix M and small envelope E proteins, did not augment the neutralizing antibody response. In absence of S, expression of M and E or the nucleocapsid protein N did not induce a detectable serum SARS-CoV-neutralizing antibody response. Immunization with BHPIV3 expressing S provided complete protection against SARS-CoV challenge in the lower respiratory tract and partial protection in the upper respiratory tract. This was augmented slightly by coexpression with M and E. Expression of M, E, or N in the absence of S did not confer detectable protection. These results identify S among the structural proteins as the only significant SARS-CoV neutralization antigen and protective antigen and show that a single mucosal immunization is highly protective in an experimental animal that supports efficient replication of SARS-CoV.

Severe acute respiratory syndrome (SARS) was first identified in November 2002 in China and spread internationally before being successfully contained in 2003 by classical public health measures (1). More recently, several cases were confirmed in 2004 in China. The etiologic agent of SARS is a previously unknown coronavirus (CoV), SARS-CoV (1). The emergence of SARS-CoV is not well understood, and its apparent presence in animal reservoirs provides the possibility of reemergence, possibly in forms with increased infectivity. Thus, a vaccine is needed, in particular for outbreak control and for immunizing medical personnel, who accounted for many of the cases of illness and death in the outbreak of 2002–2003.

The SARS-CoV genome is a single-strand positive sense RNA of ≈29,700 nucleotides that has been completely sequenced (2, 3) and contains 11 significant ORFs. By analogy with other known coronaviruses (4), the 5′-proximal two-thirds of the genome contain two ORFs, 1A and 1B, which encode polyproteins of the replicase complex. These are followed by ORFs encoding the structural proteins: the envelope spike protein S (1,255 aa), which mediates attachment to cellular receptors and entry by fusion with cell membranes; the small envelope protein E (76 aa), which acts as a scaffold protein to trigger assembly; the matrix protein M (221 aa), which is an integral membrane protein involved in budding and which interacts with the nucleocapsid and S proteins (5, 6); and the nucleocapsid protein N (422 aa). SARS-CoV lacks the envelope-associated hemagglutinin-esterase glycoprotein that is encoded by some coronaviruses.

Immunization with one or more SARS-CoV subunit antigens, either administered as purified protein or expressed from viral or DNA vaccine vectors, is one approach to designing a vaccine against SARS. This approach would be facilitated by knowledge of the relative importance of the various viral structural proteins in inducing protective immunity. It also is important to determine whether one or more vectored SARS-CoV antigens can induce protection against challenge in an experimental animal that supports a high level of SARS-CoV replication. This was investigated in the present study by using a parainfluenza virus (PIV) vaccine candidate, BHPIV3 (7), as a vector for the SARS-CoV structural proteins expressed individually or in combinations. BHPIV3 is a version of bovine PIV type 3 (BPIV3) in which the genes encoding the BPIV3 major protective antigens, the fusion F and hemagglutinin–neuraminidase (HN) glycoproteins, were replaced with their counterparts from human (H)PIV3. BPIV3 is attenuated in primates because of a natural host range restriction and is a promising candidate vaccine against HPIV3 because it is attenuated and immunogenic in infants and young children (8). BHPIV3 is an improved version as it bears major protective antigens that exactly match those of HPIV3 (7). BHPIV3 vectors expressing up to three SARS-CoV structural proteins were evaluated for immunogenicity and protective efficacy in hamsters, which support a high level of pulmonary replication of both SARS-CoV and BHPIV3.

Materials and Methods

Cells and Viruses. The Urbani strain of SARS-CoV was propagated in simian Vero cells and contained under approved biosafety level 3 conditions. Titration of SARS-CoV was performed by determination of the tissue culture 50% infectious dose ($TCID_{50}$) in Vero cells (9). The recombinant BHPIV3 viruses were propagated on simian LLC-MK2 cells at 32°C and titrated by determination of $TCID_{50}$ on LLC-MK2 cells (7).

Generation of Recombinant BHPIV3 Expressing SARS-CoV Proteins. The ORFs encoding the SARS-CoV S, N, M, and E proteins were individually amplified from SARS-CoV virion RNA by RT-PCR. The primers were designed to modify the upstream end of each ORF by the addition of a BPIV3 gene junction containing (in left-to-right order) a gene-end (AAGTAAGAAAAA), intergenic (CTT) and gene-start (AGGATTAAAG) motif, followed by sequence preceding the ORF (S ORF: GCTAGCGGATCCGCCACCATG; M, E, and N ORFs: GCCACCATG) that contains the initiation ATG (underlined) in a context favorable for translation initiation (10). The S cDNA was

Abbreviations: SARS, severe acute respiratory syndrome; CoV, coronavirus; PIV, parainfluenza virus; BPIV3, bovine PIV type 3; HN, hemagglutinin–neuraminidase; S, spike protein; $TCID_{50}$, tissue culture 50% infectious dose.

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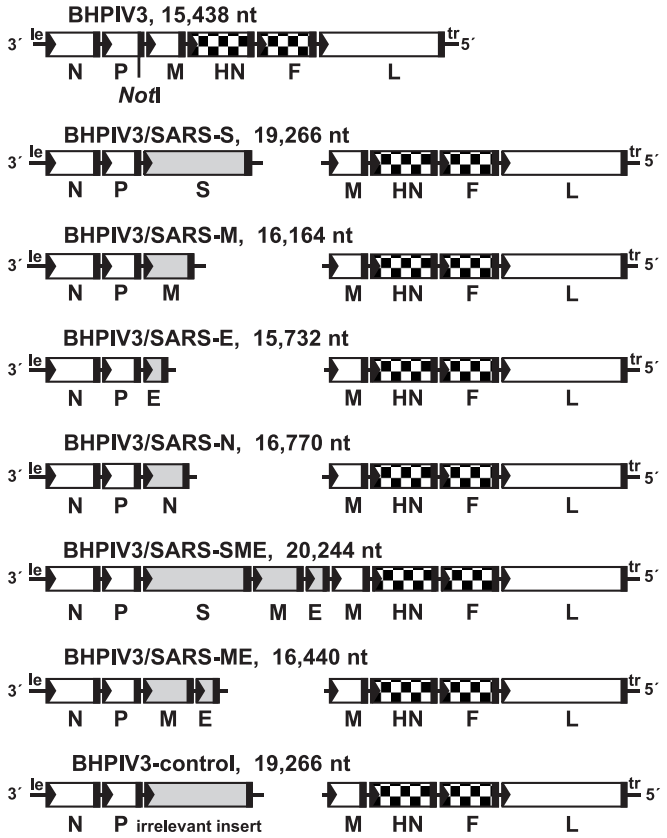

Fig. 1. Maps of the genomes of BHPIV3 vectors expressing the SARS-CoV S, M, E, and N ORFs or a heterologous control sequence equivalent in length to the S ORF. The BPIV3 backbone genes are shown as open rectangles, the HPIV3 surface protein genes are checked rectangles, and the SARS-CoV ORFs and control sequence are shaded rectangles. PIV3 gene-start and gene-end transcription signals are shown as black triangles and bars, respectively, flanking each rectangle. The figure is not drawn to scale. le, leader; tr, trailer.

designed to have a *Mlu*I site downstream of the ORF, whereas the M, E, and N cDNAs each had a *Mlu*I site upstream of the gene-end signal and a *Bss*HII site downstream of the ORF. Finally, *Not*I sites were placed flanking each cDNA. The sequence of each cloned cDNA was confirmed. The cloned ORFs were inserted individually into a unique *Not*I site present in the downstream noncoding region of the BHPIV3 P gene in a cloned cDNA of the complete BHPIV3 antigenome (Fig. 1). In addition, we inserted the M and E inserts in tandem in one antigenome and the S, M, and E inserts in another, which was possible because the *Mlu*I and *Bss*HII sites have compatible cohesive ends. In the single-, double-, or triple-insert constructions, each SARS-CoV ORF was flanked by the BPIV3 transcription signals necessary for expression by the BPIV3 polymerase. The total lengths of the inserts were as follows: S, 3,828 nt; M, 726 nt; E, 294 nt; N, 1,332 nt; ME, 1,002 nt; SME, 4,806 nt; compared to a genome length of 15,438 nt for the BHPIV3 vector. The construct encoding the S protein was prepared previously (11), together with a control construct in which the S ORF was replaced by a sequence of identical length that was derived from the antisense sequence of the polymerase L gene of respiratory syncytial virus and does not contain a significant ORF. The nucleotide length of each antigenomic cDNA was designed to be an even multiple of six, which is necessary for efficient PIV replication and likely represents a requirement for nucleocapsid organization (12). The recombinant viruses were recovered by transfecting BSR T7/5 baby hamster kidney cells (13), which constitutively express T7 RNA polymerase, with plasmids encoding the respective antigenome and the BPIV3 N, P, and L proteins.

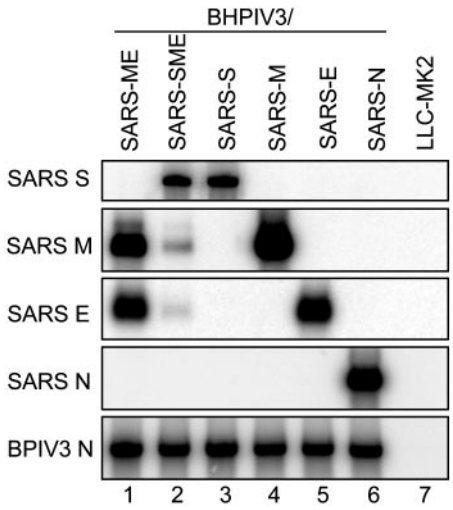

Fig. 2. Transcription of the SARS-CoV inserts into monocistronic mRNAs. LLC-MK2 cells were mock-infected (lane 7) or infected with the indicated BHPIV3 vector (lanes 1–6) at an input multiplicity of infection of 3 $TCID_{50}$ per cell. Total intracellular RNA was isolated 72 h after infection, electrophoresed on a 1% agarose gel in the presence of 0.44 M formaldehyde, transferred to nylon membrane, and hybridized with α-^{32}P-labeled double-stranded DNA probes specific for SARS S, M, E, and N, or BPIV3 N, as indicated on the left. The images were prepared with a PhosphorImager.

Serological Assays. Serial 2-fold dilutions of heat-inactivated serum were tested in a microneutralization assay for antibodies that neutralized 220 $TCID_{50}$ of SARS-CoV. The neutralizing antibody titer of serum was calculated as highest reciprocal serum dilution that neutralized infectivity in half of four parallel wells (14).

Results

Recombinant BHPIV3 Expressing the SARS-CoV S, M, E, and N Proteins. BHPIV3, a recombinant virus containing the surface proteins of HPIV3 and the internal proteins of BPIV3 (7, 15), was used as a vector to express the S, M, E, and N structural proteins of SARS-CoV individually or in the combination SME or ME (Fig. 1). In the recombinant BHPIV3 constructs, each SARS-CoV ORF was under the control of BPIV3 transcription signals and inserted between the P and M genes. When grown *in vitro*, virus stocks of each recombinant reached a final titer of 7.45–8.7 $\log_{10}$ $TCID_{50}$ per ml, showing that there was little difference in the *in vitro* growth efficiency of BHPIV3 bearing the single small E insert of 0.3 kb versus the three inserts SME of aggregate length 3.8 kb, representing 20% the length of the genome of BHPIV3. The recombinant viruses were subjected to RT-PCR to amplify the genome region containing the SARS-CoV insert(s), and sequence analysis showed that each insert was correct and free of mutation.

Each SARS-CoV insert was flanked by a set of BPIV3 gene-start and gene-end transcription signals and thus should be expressed as a separate mRNA. To confirm this, cells were infected with each recombinant virus, and total intracellular RNA was isolated and analyzed by Northern blot hybridization with DNA probes specific to the SARS-CoV S, M, E, or N gene or the BPIV3 N gene. As shown in Fig. 2, SARS-CoV S, M, E, and N monocistronic mRNAs were detected among the intracellular RNA from cells infected with the appropriate BHPIV3 virus. In the case of the triple insert virus BHPIV3/SARS-SME, the level of expression of the M and E genes, located downstream

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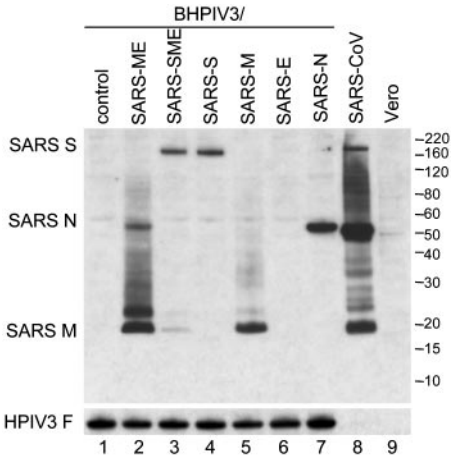

Fig. 3. Expression of SARS-CoV proteins by BHPIV3 vectors. LLC-MK2 cells were infected with the individual BHPIV3 vectors (lanes 1–7) at an input multiplicity of infection of 5 $TCID_{50}$ per cell. In addition, Vero cells were mock-infected (lane 9) or infected with 5 $TCID_{50}$ per cell of SARS-CoV (lane 8). Cell lysates were prepared 42 h after infection, in the case of SARS-CoV, or 72 h after infection, in the case of the BHPIV3 vectors, denatured under reducing conditions, subjected to SDS/PAGE in 4–12% gradient gels, and transferred to nitrocellulose. SARS-CoV proteins were detected by using convalescent serum from an African green monkey that had been infected with SARS-CoV. The BHPIV3 F protein was detected in a parallel blot by using HPIV3-specific rabbit hyperimmune serum (*Lower*). Detection of bound antibodies was done with horseradish peroxidase-conjugated goat anti-human (in the case of the anti-SARS-CoV serum) or anti-rabbit (in the case of the anti-HPIV3 serum) antibody, respectively, and visualized by chemiluminescence.

of S, was considerably lower than the levels found in the recombinants carrying single or double gene insertions. This finding indicates that the presence of the large S insert reduced the efficiency of transcription of the immediate downstream genes. Nonetheless, this had little or no apparent effect on the replication of the BHPIV3 vectors *in vitro*, as noted above, or *in vivo*, as described later. Also, accumulation of the BHPIV3-encoded N mRNA was similar among the various constructs (Fig. 2).

To investigate the synthesis of the SARS-CoV proteins, LLC-MK2 cells were infected with individual BHPIV3 vectors, and cell lysates were subjected to Western blot analysis in parallel with lysates from SARS-CoV-infected Vero cells (Fig. 3). The blots were probed individually with convalescent serum from four different SARS-CoV-infected African green monkeys. As shown by representative results in Fig. 3 for a single antiserum, this detected bands of the appropriate sizes to correspond to the SARS-CoV S (apparent size, 200 kDa), M (20 kDa), and N (55 kDa) proteins (Fig. 3, lanes 5 and 7). The gel profile of the M protein expressed from the BHPIV3 vectors also contained a heterodisperse smear of more slowly migrating material that might represent incomplete denaturation. Expression of the SARS-CoV E protein could not be detected in Western blots of lysates of cells infected with BHPIV3/SARS-E, -ME, or -SME or with SARS-CoV. Expression of HPIV3 F protein by the BHPIV3 vectors, which is encoded by a gene downstream of the SARS-CoV insert(s), was very similar for each vector. Thus, the overall level of expression of BHPIV3 vector protein was not greatly affected by the foreign inserts.

The expression of the SARS-CoV proteins also was investigated by indirect immunofluorescence of LLC-MK2 cells infected with the various BHPIV3 viruses (Fig. 4). To simultaneously visualize BHPIV3 infection and SARS protein expression, cells were stained with the SARS-CoV-specific convalescent monkey serum described above and with serum

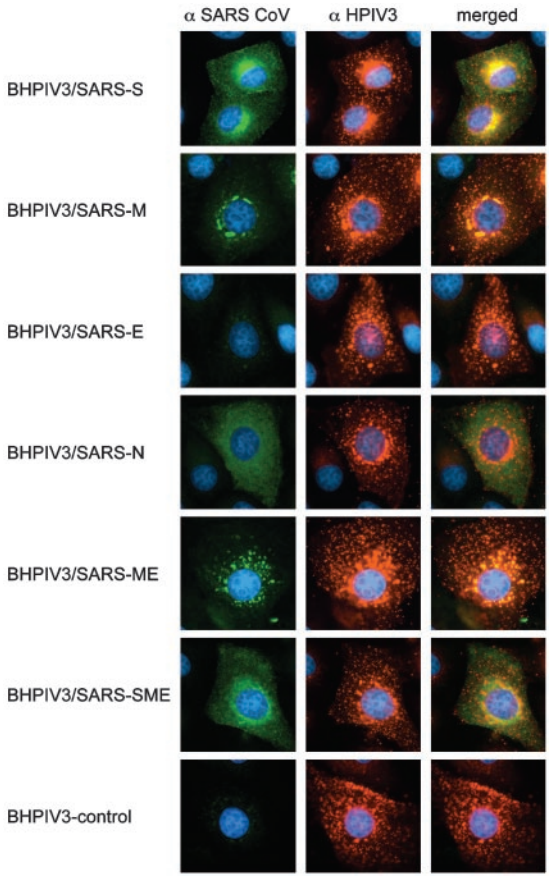

Fig. 4. Indirect immunofluorescence of LLC-MK2 cells infected with BHPIV3 vectors. Cells on cover slips were infected at an input multiplicity of infection of 0.05 $TCID_{50}$ with the BHPIV3 vectors listed to the left, incubated for 24 h, fixed with 4% paraformaldehyde, and permeabilized with 1% Triton X-100. SARS-CoV proteins were visualized (*Left*) by incubation with convalescent serum from a SARS-CoV-infected African green monkey followed by an Alexa488-conjugated goat anti-human antibody (Molecular Probes). BHPIV3 proteins were visualized (*Center*) by incubation with convalescence serum from HPIV3-infected hamsters, followed by Alexa594-conjugated goat anti-hamster antibody (Molecular Probes). Nuclear chromatin staining (blue) was performed with 4′,6-diamidino-2-phenylindole (Sigma).

from hamsters that had been infected with HPIV3 and would be primarily reactive with the HPIV3-derived HN and F proteins of BHPIV3. The HPIV3-specific serum yielded a strong perinuclear staining pattern, reflecting the abundant accumulation of the HN and F glycoproteins in the Golgi network, and a patchy pattern that would be typical for PIV HN and F proteins, present at the cell surface in lipid rafts (16–18). Upon staining with SARS-CoV-specific antibodies, a similarly intense perinuclear and punctate/diffuse staining was found in cells infected with BHPIV3/SARS-S, consistent with the expression of the glycoprotein in the Golgi network and its trafficking to more distal cell membranes. BHPIV3/SARS-N-infected cells exhibited staining distributed evenly in the cytoplasm, consistent with its status as an internal protein. The SARS-CoV matrix protein M, when expressed alone, seemed to be concentrated in the Golgi network. The coexpression of M and E yielded a strong perinuclear staining pattern and, in addition, a punctate pattern that was suggestive of expression at more distal membranes. This would be consistent with studies with model coronaviruses in which it was found that M is retained in the Golgi apparatus when expressed alone but is translocated to more distal cell membranes when expressed with E (19, 20). The SARS E protein

Table 1. Immunogenicity and protective efficacy in hamsters of BHPIV3 recombinants expressing SARS-CoV structural proteins

	Response to immunization				Response to challenge§			
	BHPIV3 replication, mean $\log_{10}$ $TCID_{50}$ per g ± SE†		Serum antibodies, mean reciprocal $\log_2$ ± SE‡		SARS-CoV replication, mean $\log_{10}$ $TCID_{50}$ per g ± SE		% with detectable SARS-CoV	
Immunizing virus*	Nasal turbinates	Lungs	HPIV3 HAI	SARS-CoV-neutralizing	Nasal turbinates	Lungs	Nasal turbinates	Lungs
SARS-CoV	ND	ND	ND	8.0 ± 0.2	1.6 ± 0.1	≤ 1.5 ± 0	17	0
BHPIV3/SARS-S	5.4 ± 0.2	4.3 ± 0.2	9.2 ± 0.5	6.8 ± 0.3	3.5 ± 0.1¶	≤ 1.5 ± 0	100‖	0
BHPIV3/SARS-M	5.7 ± 0.1	5.6 ± 0.2	11.0 ± 0.5	≤ 2 ± 0.0	6.1 ± 0.2	5.6 ± 0.1	100	100
BHPIV3/SARS-E	4.9 ± 0.2	5.0 ± 0.4	10.3 ± 0.5	≤ 2 ± 0.0	5.8 ± 0.3	6.5 ± 0.2	100	100
BHPIV3/SARS-N	6.9 ± 0.2	5.6 ± 0.3	10.2 ± 0.5	≤ 2 ± 0.0	5.8 ± 0.3	5.3 ± 0.2	100	100
BHPIV3/SARS-SME	5.5 ± 0.2	4.3 ± 0.1	8.2 ± 0.6	6.0 ± 0.3	2.9 ± 0.4¶	≤ 1.5 ± 0	67**	0
BHPIV3/SARS-ME	3.4 ± 0.1	3.9 ± 0.2	8.7 ± 0.5	≤ 2 ± 0.0	6.2 ± 0.2	6.0 ± 0.2	100	100
BHPIV3-control	4.5 ± 0.1	4.0 ± 0.2	7.7 ± 0.5	≤ 2 ± 0.0	6.2 ± 0.2	5.6 ± 0.2	100	100

ND, not done.

*Golden Syrian hamsters in groups of 12 were immunized on day 0 by intranasal inoculation with a 0.1-ml inoculum containing 10^6 $TCID_{50}$ of the indicated BHPIV3 recombinant or 10^3 $TCID_{50}$ of SARS-CoV.

†Six hamsters from each BHPIV3 group were killed on day 4 after immunization. Lungs and nasal turbinates were harvested, and BHPIV3 titers were determined.

‡Sera were collected from BHPIV3-immunized animals 25 days after immunization and assayed for hemagglutinin inhibition titers against HPIV3. Sera were negative (titers ≤ 1) prior to immunization. Sera were collected from SARS-CoV-infected animals 28 days after immunization and assayed for neutralizing antibodies against SARS-CoV. Sera were negative (titers ≤ 2) before immunization.

§Twenty-eight days after immunization, the animals were challenged by intranasal inoculation with 3 $\log_{10}$ $TCID_{50}$ of SARS-CoV in a 0.1-ml volume. Animals were killed on day 3 after challenge. Lungs and nasal turbinates were harvested, and SARS-CoV titers were determined. The lower limit of detection was 1.5 $\log_{10}$ $TCID_{50}$ per g of tissue.

¶These values were not significantly different from the SARS-CoV group by ANOVA and Tukey/Kramer post hoc test.

‖Significantly different from SARS-CoV group, $P = 0.015$, calculated by using Fisher's exact test.

**Not significantly different compared with the BHPIV3/SARS-S or SARS-CoV group.

expressed alone could not be detected above a low level of background autofluorescence that also was observed for BHPIV3-control infected cells. Upon the availability of SARS M- and E-specific antibodies, protein trafficking and the possible formation of SARS-CoV virus-like particles could be studied.

Immunization with the BHPIV3/SARS Viruses. Hamsters in groups of 12 were infected intranasally with one of the various BHPIV3 viruses, and an additional group of six animals was infected intranasally with SARS-CoV (Table 1). Six animals from each of the BHPIV3 groups were killed on day 4, and the nasal turbinates and lungs were harvested and virus titers determined. This confirmed that each of the BHPIV3 viruses replicated efficiently in the hamster respiratory tract (Table 1). Specifically, each of the single-insert viruses attained a titer of 4.9–6.9 $\log_{10}$ $TCID_{50}$ in the nasal turbinates (upper respiratory tract) and 4.3–5.6 $\log_{10}$ $TCID_{50}$ in the lungs (lower respiratory tract). The BHPIV3/SARS-SME triple-insert virus replicated as efficiently as the BHPIV3/SARS-S single-insert virus, indicating that the addition of the two extra small genes did not affect replication efficiency *in vivo*. The double-insert virus BHIPV3/SARS-ME replicated to a somewhat lower titer (3.4 and 3.9 $\log_{10}$ $TCID_{50}$, respectively) than the others, which might represent a slight deleterious effect of expressing the combination of M and E.

Sera were collected from the remaining animals on day 28 (BHPIV3 groups) or 25 (SARS-CoV group) after immunization and assayed for (*i*) antibodies against the HN protein of the vector itself, measured by a hemagglutination inhibition assay, and (*ii*) neutralizing antibodies against SARS-CoV (Table 1). The hemagglutination inhibition titers were highest for the single-insert viruses expressing the smaller SARS-CoV proteins, namely M, E, and N, and were somewhat reduced for viruses expressing the larger single inserts, namely S and the equivalent control insert, and for the double- and triple-inserts SME and ME. Thus, the immunogenicity of the vector decreased by a small amount with increased size and complexity of the insert(s), probably reflecting small effects on vector gene expression and replication. Nonetheless, each of the BHPIV3 vectors induced a strong systemic response against itself.

A strong neutralizing immune response against SARS-CoV was detected in the two groups immunized with BHPIV3 expressing the S protein, namely BHPIV3/SARS-S and BHPIV3/SARS-SME (Table 1). The mean neutralizing antibody titer for these two groups was, respectively, 2- and 4-fold lower than for the group that had been immunized by infection with SARS-CoV, differences that were not statistically significant. Neutralizing antibodies to SARS-CoV were not detected in any of the animals immunized with BHPIV3 expressing SARS-CoV N, M, or E, in absence of S. To investigate the possible induction of nonneutralizing antibodies, the individual hamster sera were used to analyze Western blots prepared with lysates of Vero cells that had been infected with SARS-CoV (5 $TCID_{50}$ per cell) and harvested 42 h after infection (not shown). Each of the sera of the animals immunized with BHPIV3/SARS-N showed a strong reaction to SARS-CoV N (not shown). Sera from animals immunized with BHPIV3/SARS-S and -M also yielded positive signals with the respective SARS-CoV protein. Sera from animals immunized with BHPIV3/SARS-S or -SME showed a reaction to SARS-CoV S, and both BHPIV3/SARS-SME and -ME induced detectable antibodies to SARS-CoV M. None of the animals immunized with BHPIV3 expressing E, ME, or SME yielded a signal specific to E.

Protective Efficacy of BHPIV3/SARS Viruses. The hamsters that had been immunized by infection with one of the various BHPIV3 viruses or SARS-CoV were challenged on day 28 after immunization with an intranasal inoculation of 10^3 $TCID_{50}$ of SARS-CoV. Three days later, the animals were killed, and the nasal

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turbinates and lungs were harvested and analyzed for replication of challenge SARS-CoV (Table 1). Animals that had been immunized with the BHPIV3 control virus lacking a SARS-CoV insert supported a high level of SARS-CoV replication in the upper and lower respiratory tract (6.2 and 5.6 $\log_{10}$ $TCID_{50}$, respectively). In contrast, animals that had been immunized by infection with SARS-CoV were highly resistant to challenge, such that challenge SARS-CoV was not detected in the lungs of any animal and only a low level was detected in the upper respiratory tract of one of six animals (Table 1). Immunization with two of the BHPIV3 viruses, namely the S and SME viruses, resulted in significant restriction of challenge virus. Specifically, both viruses conferred complete protection of the lower respiratory tract. In the upper respiratory tract, the S and SME viruses conferred a 500- or 2,000-fold reduction, respectively, in challenge SARS-CoV replication, values that were not significantly different from that of animals that had been immunized with SARS-CoV (Table 1). All six animals that had been immunized with the S virus had detectable challenge virus in the upper respiratory tract, a statistically significant difference from the SARS-CoV group, whereas four of six animals immunized with the SME virus had detectable challenge virus, which is not significantly different from the SARS-CoV group. There was no detectable resistance to SARS-CoV challenge in the upper or lower respiratory tract of animals that had been immunized with BHPIV3 vectors expressing N, M, E, or M plus E. Thus, the S protein was the only significant protective antigen among the SARS-CoV structural proteins, and its protective efficacy was increased slightly by coexpression of M and E.

Discussion

We used the respiratory virus BHPIV3 as a vector to express SARS-CoV structural proteins to evaluate immunogenicity and protective efficacy in hamsters, an experimental animal that supports a high level of SARS-CoV replication. The use of a respiratory virus as vector provided for direct, efficient immunization of the respiratory tract, the principal site of SARS transmission, replication, and disease in humans. Among the viral structural proteins, the S glycoprotein proved to be the only significant neutralization and protective antigen of SARS-CoV, as evaluated in the permissive hamster model.

BHPIV3 is being developed as an attenuated vaccine candidate for HPIV3 (14, 21), but its attenuation phenotype is specific for primates. In hamsters, the vector replicated to a higher titer and therefore provided a good test of the immunogenicity of the expressed SARS-CoV antigens. The vector itself induced a high titer of serum antibodies specific for its HN protein. Furthermore, each SARS-CoV ORF was engineered to be in a context favorable for translation, and each was placed under the control of a separate set of BPIV3 transcription signals between the P and M genes of the vector for efficient expression as a separate mRNA. Abundant mRNA accumulation was confirmed, although accumulation of the M and E mRNAs was reduced for the SME virus. We were able to confirm expression of the relevant SARS-CoV protein in cell culture in each case except for the small E protein, which could not be detected in cell culture whether infected with the relevant BHPIV3 vector or SARS-CoV, or whether probed with antibodies from animals infected with the relevant BHPIV3 vector or SARS-CoV. Coexpression of M and E did alter the pattern of intracellular accumulation of M, as assayed by immunofluorescence. This provided indirect evidence that E was expressed. Although we could not formally prove that the BHPIV3/SARS-E, -ME, and -SME vectors expressed E protein, it seems likely that the failure to detect E expressed from these vectors or SARS-CoV reflects a deficiency in the available antibodies, reduced immunogenicity or antigenicity of E, or perhaps all of these factors.

Serum antibody responses were observed by Western blot for the S, M, and N proteins expressed by the BHPIV3 vectors, but, as noted above, we lacked a method for detecting a response to E. Neutralizing activity was detected only in the case of BHPIV3 expressing S and SME, thus identifying S as the only significant neutralization antigen. The neutralization titer induced by the BHPIV3/SARS-S and -SME recombinants was 2- and 4-fold lower, respectively, than that induced by SARS-CoV. However, the level of replication by the BHPIV3/SARS-S and -SME vectors in the hamster was ≈5-fold lower in the upper respiratory tract and 20-fold lower in the lower respiratory tract compared to SARS-CoV. Given this substantially lower level of replication, the vectors compared well with SARS-CoV for the ability to induce neutralizing antibodies.

Animals immunized with a single dose of BHPIV3/SARS-S or -SME were completely protected against SARS-CoV replication in the lower respiratory tract, as also was the case for animals immunized by infection with SARS-CoV itself. In the upper respiratory tract, immunization with the S vector reduced challenge virus replication 500-fold, with challenge virus detected in all six animals, whereas the SME vector reduced challenge virus replication 2,000-fold, with challenge virus detected in four of the six animals. The number of animals that were completely protected from challenge virus replication for the groups immunized with BHPIV3/SARS-SME versus SARS-CoV was not significantly different, whereas the difference between the BHPIV3/SARS-S and SARS CoV group was significant.

In contrast, the N, M, E, and ME vectors did not induce detectable resistance to SARS-CoV challenge, and thus these proteins were not significant protective antigens. Although these antigens did not induce neutralizing antibodies, they were potential antigens for antiviral cytotoxic T cells (CTLs), as has been shown to be the case for the N protein of the coronavirus mouse hepatitis virus (4) and infectious bronchitis virus (22). CTLs can confer short-term protection against challenge with a respiratory virus. For respiratory syncytial virus, the N and M2–1 proteins induced short-term resistance in mice against virus challenge in the absence of neutralizing antibodies (23), and in the case of M2–1, protection was confirmed to be mediated by CD8+ CTLs (24). As another example, the chimeric virus rHPIV3–1, in which the HN and F neutralization antigens of HPIV3 had been replaced by their counterparts from PIV type 1, conferred short-term resistance in hamsters against HPIV3 challenge in the absence of neutralizing antibodies against HPIV3 (25). The likely explanation was that the HPIV3-derived internal proteins induced a cell-mediated protective response against HPIV3 challenge. Whereas CTLs can confer pulmonary resistance in both the upper and lower respiratory tract to challenge with a respiratory virus, the protective effect observed in hamsters waned over 3–4 months, and there was no evidence of an anamnestic protective response upon challenge (25). In the present study, the use of outbred animals and a 28-day time interval between immunization and challenge should have permitted the detection of any significant protective response mediated by CTLs, but none was observed. Any protective response of shorter duration that might have occurred likely would be of limited practical value for immunoprophylaxis. This does not rule out a role for CTLs in protective immunity, but suggests that any contribution is minor. The CTL response was not examined directly because this cannot readily be done in outbred hamsters.

We constructed a single virus expressing two (ME) or three (SME) SARS-CoV proteins to investigate effects of possible protein interaction on immunogenicity and efficacy. It has been shown for several coronaviruses that coexpression of S, M, and E results in the formation of virus-like particles that bud from infected cells (19, 26–28). Also, whereas M expressed alone was

retained in the Golgi network, coexpression of M and E resulted in expression at more distal cell membranes (19, 20, 27, 29). Thus, the expression of these combinations in addition to the single inserts might increase the opportunity for producing the proteins in an immunogenic form. Coexpression of SARS-CoV M and E did appear to mobilize M from the Golgi network to distal cellular membranes, as judged by immunofluorescence, but there was no observed change in immunogenicity or protective efficacy. The SME virus did appear to have slightly greater immunogenicity compared to the S virus, based on the marginally greater reduction in the frequency of detectable virus in the upper respiratory tract as noted above. Increased immunogenicity could reflect the formation of particles. A more detailed examination of the possible effects of coexpression of the SME and ME combinations of proteins awaits further work.

We recently examined the immunogenicity and protective efficacy of the BHPIV3/SARS-S virus in African green monkeys (11). That study had the limitation that SARS-CoV replication in the respiratory tract was examined by nasal swab and tracheal lavage, and the amount of virus shed was low and sporadic. In that study, a single immunization with BHPIV3/SARS-S completely prevented shedding of SARS-CoV challenge virus. However, the formal possibility existed that there might have been considerable replication of the challenge SARS-CoV that was not reflected by shedding. In the present study, a single intranasal administration of the same virus in hamsters provided complete protection in the lungs and a strong reduction in the nasal turbinates, as measured directly in tissue homogenates. Thus, a single intranasal immunization with a recombinant vector expressing the S protein was highly immunogenic and protective against SARS-CoV challenge. In contrast, the N, M, and E proteins, singly or in two combinations, did not make a significant contribution to a neutralizing antibody response or protective immunity. Finally, there was no evidence that immunization with any of the SARS-CoV antigens, involving the induction of either neutralizing or nonneutralizing antibodies, led to antibody-mediated enhancement of infection, as described for feline infectious peritonitis virus (30–32).

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