

A Live Attenuated Severe Acute Respiratory Syndrome Coronavirus Is Immunogenic and Efficacious in Golden Syrian Hamsters[▽]

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The immunogenicity and protective efficacy of a live attenuated vaccine consisting of a recombinant severe acute respiratory syndrome (SARS) coronavirus lacking the E gene (rSARS-CoV-ΔE) were studied using hamsters. Hamsters immunized with rSARS-CoV-ΔE developed high serum-neutralizing antibody titers and were protected from replication of homologous (SARS-CoV Urbani) and heterologous (GD03) SARS-CoV in the upper and lower respiratory tract. rSARS-CoV-ΔE-immunized hamsters remained active following wild-type virus challenge, while mock-immunized hamsters displayed decreased activity. Despite being attenuated in replication in the respiratory tract, rSARS-CoV-ΔE is an immunogenic and efficacious vaccine in hamsters.

Severe acute respiratory syndrome (SARS) is a respiratory illness caused by a novel coronavirus, SARS-CoV, which emerged in late 2002 and spread globally to infect 8,096 people, resulting in 774 deaths (World Health Organization; http://www.who.int/csr/sars/country/table2004_04_21/en/index.html). Since the initial outbreak, there have been few sporadic cases of community-acquired and laboratory-acquired infections. Masked palm civets were identified as carriers of SARS-CoV (5) and horseshoe bats carry a SARS-CoV-like virus (6, 7), suggesting that a future outbreak would likely originate from an animal reservoir. The threat of another SARS-CoV outbreak emphasizes the need for vaccines and continued research on the prevention and treatment of SARS-CoV. In the absence of ongoing human infections, these experiments must be conducted with relevant animal models. Several vaccine strategies are in development, including inactivated virus, subunit, virus-like particles, DNA, vectored, and reverse genetics-engineered vaccines (4, 8). In this study, we examine the immunogenicity and efficacy of a live-virus vaccine by use of an engineered SARS-CoV with the structural E gene deleted in the Golden Syrian hamster model. This model supports viral replication and associated pathology in the lungs, and infected animals display reduced activity.

An infectious cDNA clone of SARS-CoV (Urbani) was assembled as a bacterial artificial chromosome (1) and a virus lacking the E gene (ΔE) was engineered as previously described (2). Recombinant SARS-CoV-ΔE (rSARS-CoV-ΔE) was restricted in replication in vitro and in vivo (2), prompting us to evaluate the immunogenicity and efficacy of this virus as a live attenuated vaccine in hamsters. The attenuated rSARS-CoV-ΔE vaccine was compared with mock infection and rSARS-CoV infection by use of 7-week-old male Golden Syr-

ian hamsters [LVG (SYR); Charles River Laboratories, Wilmington, MA] that were intranasally inoculated with 100 μl of 10³ × the 50% tissue culture infectious dose (TCID₅₀) of rSARS-CoV or rSARS-CoV-ΔE or with medium only as previously described (2). Sera were collected from the hamsters before immunization and on day 28 after immunization; two-fold dilutions of heat-inactivated sera were tested for the presence of antibodies that neutralized the infectivity of 100 TCID₅₀ of SARS-CoV in Vero cell monolayers as described previously (10). The immunogenicity and efficacy of the rSARS-CoV-ΔE vaccine were evaluated using the homologous virus, SARS-CoV Urbani, as well as a heterologous rSARS-CoV bearing the spike (S) protein gene of the GD03 virus (3). Similar titers of neutralizing antibodies were elicited by rSARS-CoV and rSARS-CoV-ΔE against the homologous and heterologous strains of SARS-CoV (Table 1). In both cases, neutralizing antibody titers against the homologous virus were higher (five- to eightfold) than those against the heterologous virus, as reported earlier (3).

About 4 weeks after immunization, hamsters were challenged intranasally with 100 μl of 10³ TCID₅₀ of the homologous SARS-CoV Urbani or the heterologous rSARS-CoV GD03 strain. Four hamsters per group were sacrificed at two time points after challenge (2 and 5 days) and their lungs and nasal turbinates (NT) were harvested to determine the level of virus replication and for histopathological examination. These time points were selected because peak SARS-CoV titers in the lungs of hamsters occur on day 2 postinfection and histopathological findings are most prominent on day 5 postinfection (9). Virus titers for 10% (wt/vol) tissue homogenates were determined for Vero cell monolayers as described previously (10), and virus titers were expressed as TCID₅₀/g of tissue, with a lower limit of detection of 10^{1.5} TCID₅₀/g.

Intranasal immunization with rSARS-CoV-ΔE and rSARS-CoV provided complete protection from pulmonary replication of homologous challenge virus, while this virus replicated

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TABLE 1. Neutralizing antibody titers in sera of hamsters immunized with rSARS-CoV or rSARS-CoV-ΔE against homologous and heterologous strains of SARS-CoV

Immunogen	Mean (\pm SE) reciprocal neutralizing antibody titer against:	
	Urbani (homologous)	GD03 (heterologous)
L15 (mock)	$\leq 5.7 \pm 0^a$	$\leq 5.7 \pm 0^a$
rSARS-CoV	367 ± 97^b	45 ± 10^b
rSARS-CoV-ΔE	280 ± 73^b	52 ± 9^b

^a Lower limit of detection is 5.7.

^b $P < 0.05$ compared to titers in mock-immunized animals.

to titers of $10^{7.1}$ and $10^{7.2}$ TCID₅₀/g in the lungs of mock-immunized hamsters on days 2 and 5 postchallenge, respectively (Fig. 1). The challenge virus replicated to titers of $10^{8.7}$ and $10^{6.6}$ TCID₅₀/g on days 2 and 5 postchallenge, respectively, in the NT of mock-immunized hamsters (Fig. 1A). In contrast, virus titers of between 10^3 and 10^5 TCID₅₀/g were observed for the NT of the rSARS-CoV- and rSARS-CoV-ΔE-immunized hamsters on day 2 postchallenge, a significant reduction (10,000-fold) compared to the titers for mock-immunized hamsters ($P < 0.05$); moreover, challenge virus was not recovered from the NT of the rSARS-CoV- and rSARS-CoV-ΔE-immunized hamsters on day 5 postchallenge, indicating that the virus replicated to low titer and was cleared quickly from the upper respiratory tract of immunized hamsters compared to what was seen for mock-immunized hamsters.

The heterologous GD03 virus replicated to high titers of $10^{7.3}$ and $10^{5.6}$ TCID₅₀/g in the NT and $10^{5.8}$ and $10^{5.5}$ TCID₅₀/g in the lungs of mock-immunized hamsters on days 2 and 5 postchallenge, respectively. In contrast, the lungs of hamsters immunized with rSARS-CoV-ΔE and rSARS-CoV were completely protected from replication of the GD03 virus, and the challenge virus was cleared by day 5 postchallenge from the NT (Fig. 1B).

The lungs of two hamsters per group harvested on days 2 and 5 postchallenge were fixed in 10% formalin and processed for histopathological examination and immunohistochemical analysis as described previously (10). Mock-immunized hamsters had focal antigen staining in the trachea (not shown) and mild to moderate, focal to diffuse inflammatory infiltrates in the lungs on day 2 and focal intense inflammatory infiltrates (Fig. 2D) and scattered antigen staining on day 5 following challenge with SARS-CoV Urbani (not shown). In contrast, hamsters immunized with rSARS-CoV or rSARS-CoV-ΔE had only focal mild inflammatory infiltrates on days 2 and 5 following challenge with homologous virus (Fig. 2), and viral antigen was not detected in the lungs (not shown). On days 2 and 5 following challenge with the heterologous virus GD03, focal to diffuse, mild to moderate infiltrates were noted in the lungs of mock-immunized hamsters. Focal moderate infiltrates were seen in the lungs of one hamster immunized with rSARS-CoV-ΔE on day 2, but significant pulmonary inflammatory infiltrates were not seen on day 5 postchallenge. Pulmonary inflammation was not seen on day 2 or 5 when hamsters immunized with rSARS-CoV were challenged with the GD03 virus (not shown).

In order to determine whether hamsters became less active

following infection with SARS-CoV, a Nalgene activity wheel (Nalge Nunc International, Rochester, NY) equipped with a magnetic switch with an LCD counter that records revolutions was placed in their cages overnight, with water and food available ad libitum. The time the hamsters spent in the cage with the activity wheel and the number of revolutions were recorded, and the latter was reported as an average number of revolutions per hour. Four hamsters were observed each night for five consecutive nights before immunization (days -7 to -3) and after immunization. Activity was recorded again before and after challenge with SARS-CoV Urbani. The statistical significance of the change in hamster activity following immunization and challenge was compared as least-squares means contrasts from a repeated-measures analysis of variance with a Bonferroni correction.

The baseline activity level for hamsters prior to immunization was between 700 and 1,000 revolutions/h (Fig. 3A). The activity level of the rSARS-CoV-ΔE-immunized hamsters did not change after immunization ($P = 0.32$), but the activity level of the hamsters immunized with rSARS-CoV decreased to ~ 300 revolutions/h ($P < 0.005$). These data are consistent with our previous observation that rSARS-CoV-ΔE was attenuated compared to rSARS-CoV (2) and that activity was markedly reduced following SARS-CoV infection (8). Three weeks after immunization, the baseline activity level for all of the groups

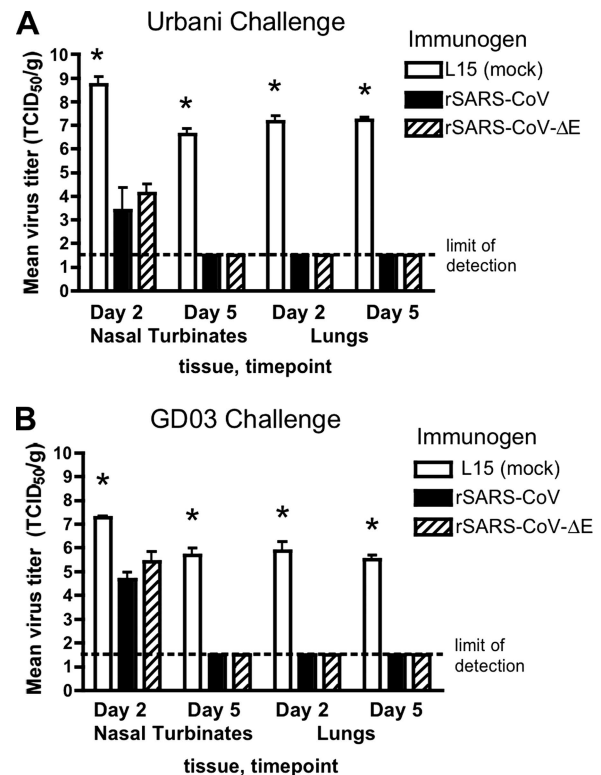


FIG. 1. Replication of SARS-CoV Urbani (A) and GD03 (B) in the NT and lungs of mock-immunized hamsters and hamsters immunized with rSARS-CoV-ΔE or rSARS-CoV on days 2 and 5 postchallenge. Virus titers represent the mean from four hamsters per day. Error bars indicate standard errors. *, $P < 0.05$ (Kruskal-Wallis, Mann-Whitney U test). The lower limit of detection is $10^{1.5}$ TCID₅₀/g tissue.

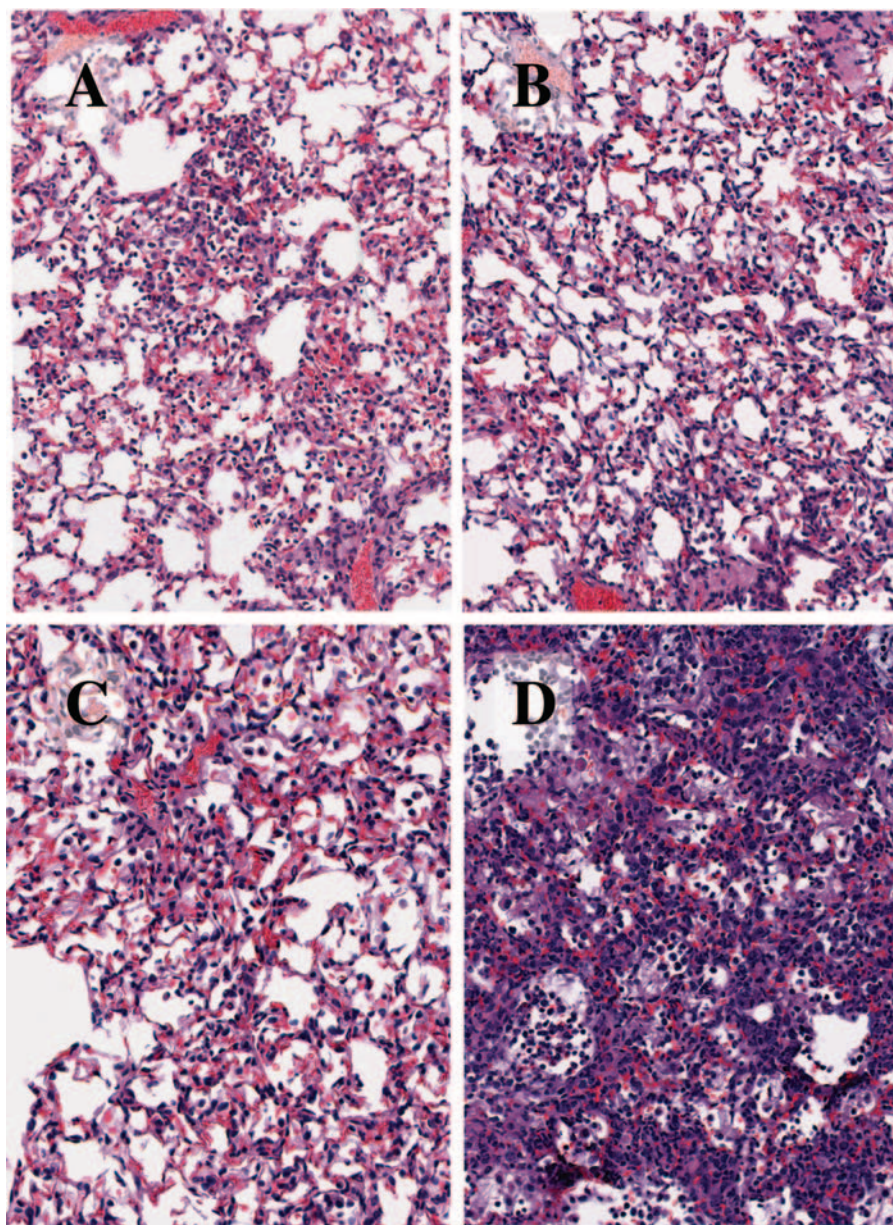


FIG. 2. Lung histopathology of normal (uninfected) (A), rSARS-CoV-immunized (B), rSARS-CoV-ΔE-immunized (C), and mock-immunized (D) hamsters 5 days after challenge with SARS-CoV Urbani. Hematoxylin and eosin staining. Magnification, $\times 20$.

returned to the preimmunization level (Fig. 3B). The activity of the mock-immunized hamsters decreased to less than 100 revolutions/h ($P < 0.005$) following challenge with SARS-CoV Urbani, while the activities of the hamsters immunized with rSARS-CoV (564 revolutions/h; $P < 0.005$) and rSARS-CoV-ΔE (608 revolutions/h; $P = 0.23$) decreased only slightly. The decreased activity of the rSARS-CoV-immunized hamsters following challenge does not appear to be biologically significant, though it was statistically significant, presumably because of the variance around the mean.

Our data indicate that an engineered rSARS-CoV-ΔE strain did not cause clinical illness in hamsters, as measured by use of an activity wheel. We have previously shown that rSARS-CoV-ΔE is attenuated 20- to 200-fold in vitro and 100- to

1,000-fold in hamster lung or NT (2). The lower pulmonary viral load was accompanied by less inflammation, consistent with the difference we observed with the activity wheel (2). rSARS-CoV-ΔE elicited serum-neutralizing antibodies against both the homologous and heterologous viruses at levels comparable to those seen for rSARS-CoV. Neutralizing titers were about eightfold higher against the homologous virus than against the heterologous virus, reflecting the antigenic differences between the viruses (3).

Interestingly, immunization with rSARS-CoV-ΔE reduced the replication of the wild-type challenge viruses in the upper respiratory tract of hamsters and completely protected lungs against homologous and heterologous challenge. The observation of complete protection in the lower respiratory tract and

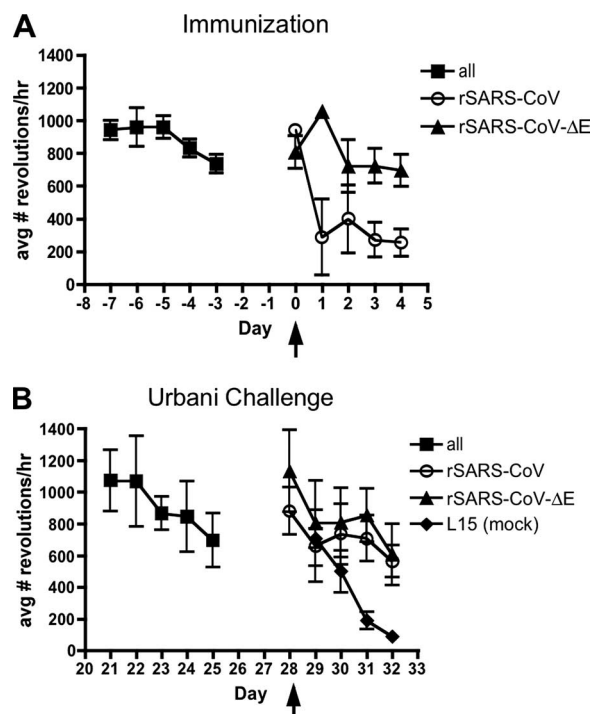


FIG. 3. Hamster activity wheel use. (A) Activity of hamsters recorded on indicated days before (squares) and after intranasal immunization (arrow) for hamsters immunized with rSARS-CoV or rSARS-CoV- Δ E. $n = 4$ per group. Error bars indicate standard errors. The difference in the activity of hamsters that received rSARS-CoV- Δ E and the preimmunization activity of all hamsters was not significant ($P = 0.32$). The difference in the activity of hamsters that received rSARS-CoV and the preimmunization activity of all hamsters was significant ($P < 0.005$). (B) Activity of hamsters immunized with rSARS-CoV, rSARS-CoV- Δ E, or medium alone (L15; mock) recorded on indicated days before (squares) and after intranasal challenge with SARS-CoV Urbani (arrow). $n = 4$ per group. Error bars indicate standard error. The difference in the activity of hamsters that received rSARS-CoV- Δ E and the prechallenge activity of all hamsters was not significant ($P = 0.23$). The difference in the activity of hamsters that received rSARS-CoV and the prechallenge activity of all hamsters was significant ($P < 0.005$). The difference in the activity of hamsters that were mock immunized and the prechallenge activity of all hamsters was significant ($P < 0.005$).

partial protection in the upper respiratory tract is consistent with protection mediated by serum antibodies (10, 11). Widespread eosinophilic pulmonary infiltrates described following challenge to mice vaccinated with an alphavirus expressing the SARS-CoV N protein (3) were not prominent in hamsters vaccinated with rSARS-CoV- Δ E. This is reassuring, because this live attenuated vaccine virus expresses the N protein in the context of the other SARS-CoV proteins. Following challenge with homologous or heterologous virus, the lungs of mock-immunized hamsters had focal intense inflammatory infiltrates and viral antigen was present. However, hamsters immunized with rSARS-CoV- Δ E or rSARS-CoV showed only mild focal infiltrates in the lungs and viral antigen was not detected.

These results correlated with the activity of the hamsters and the quantitative virological data.

The ability of the rSARS-CoV- Δ E vaccine to protect against challenge with the heterologous virus GD03 is significant, because this virus strain is antigenically one of the most divergent strains from SARS-CoV Urbani; it clusters phylogenetically with the animal SARS-CoV isolates (3), and was selected as a representative of an animal SARS-CoV because if SARS were to reemerge, it would likely come from an animal source. Our data indicate that the rSARS-CoV- Δ E shows promise as a live attenuated vaccine. In addition, it could be used to produce an inactivated vaccine that may be safer to handle than virulent wild-type viruses. Evaluation of this vaccine candidate in other animal models is in progress.

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REFERENCES

- Almazan, F., M. L. Dediego, C. Galan, D. Escors, E. Alvarez, J. Ortego, I. Sola, S. Zuniga, S. Alonso, J. L. Moreno, A. Nogales, C. Capiscol, and L. Enjuanes. 2006. Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. *J. Virol.* **80**:10900–10906.
- DeDiego, M. L., E. Alvarez, F. Almazan, M. T. Rojas, E. Lamirande, A. Roberts, W. J. Shieh, S. R. Zaki, K. Subbarao, and L. Enjuanes. 2007. A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. *J. Virol.* **81**:1701–1713.
- Deming, D., T. Sheahan, M. Heise, B. Yount, N. Davis, A. Sims, M. Suthar, J. Harkema, A. Whitmore, R. Pickles, A. West, E. Donaldson, K. Curtis, R. Johnston, and R. Baric. 2006. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. *PLoS Med.* **3**:e525.
- Enjuanes, L., M. L. Dediego, E. Alvarez, D. Deming, T. Sheahan, and R. Baric. 2008. Vaccines to prevent severe acute respiratory syndrome coronavirus-induced disease. *Virus Res.* **133**:45–62.
- Guan, Y., B. J. Zheng, Y. Q. He, X. L. Liu, Z. X. Zhuang, C. L. Cheung, S. W. Luo, P. H. Li, L. J. Zhang, Y. J. Guan, K. M. Butt, K. L. Wong, K. W. Chan, W. Lim, K. F. Shortridge, K. Y. Yuen, J. S. Peiris, and L. L. Poon. 2003. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science* **302**:276–278.
- Lau, S. K., P. C. Woo, K. S. Li, Y. Huang, H. W. Tsoi, B. H. Wong, S. S. Wong, S. Y. Leung, K. H. Chan, and K. Y. Yuen. 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc. Natl. Acad. Sci. USA* **102**:14040–14045.
- Li, W., Z. Shi, M. Yu, W. Ren, C. Smith, J. H. Epstein, H. Wang, G. Crameri, Z. Hu, H. Zhang, J. Zhang, J. McEachern, H. Field, P. Daszak, B. T. Eaton, S. Zhang, and L. F. Wang. 2005. Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**:676–679.
- Roberts, A., E. W. Lamirande, L. Vogel, J. P. Jackson, C. D. Paddock, J. Guarner, S. R. Zaki, T. Sheahan, R. Baric, and K. Subbarao. 2008. Animal models and vaccines for SARS-CoV infection. *Virus Res.* **133**:20–32.
- Roberts, A., L. Vogel, J. Guarner, N. Hayes, B. Murphy, S. Zaki, and K. Subbarao. 2005. Severe acute respiratory syndrome coronavirus infection of Golden Syrian hamsters. *J. Virol.* **79**:503–511.
- Subbarao, K., J. McAuliffe, L. Vogel, G. Fahle, S. Fischer, K. Tatti, M. Packard, W. J. Shieh, S. Zaki, and B. Murphy. 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. *J. Virol.* **78**:3572–3577.
- Traggiai, E., S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M. R. Gismondo, B. R. Murphy, R. Rappuoli, and A. Lanzavecchia. 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat. Med.* **10**:871–875.