NOTES

Macaque Model for Severe Acute Respiratory Syndrome

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Rhesus and cynomolgus macaques were challenged with 10⁷ PFU of a clinical isolate of the severe acute respiratory syndrome (SARS) coronavirus. Some of the animals developed a mild self-limited respiratory infection very different from that observed in humans with SARS. The macaque model as it currently exists will have limited utility in the study of SARS and the evaluation of therapies.

A novel coronavirus was recently identified as the causative agent of severe acute respiratory syndrome (SARS) (3, 7). One factor that led to this conclusion was the demonstration by Fouchier et al. and Kuiken et al. that exposure of the SARS coronavirus (SARS CoV) to a related host (i.e., cynomolgus macaques) led to the development of a comparable disease (1, 4). In their studies, SARS CoV administered intratracheally to effect delivery to the lower respiratory tract and conjunctiva of cynomolgus macaques resulted in respiratory and constitutional symptoms, shedding of virus, and pulmonary pathology. The availability of an authentic animal model for SARS is essential for a greater understanding of pathogenesis as well as the development and evaluation of effective vaccines and pharmacologic therapies.

Our studies included a 12- to 14-day life phase of evaluation with sequential clinical and virologic analyses, followed by a thorough evaluation of tissues harvested at necropsy. This differed from the previous studies, which emphasized short-term necropsies (i.e., 4 to 6 days) and end points of pathology. Individually housed macaques were acclimated in a biosafety level 3 facility prior to initiation of the experiment.

Cynomolgus macaques (Philippine origin and captive bred, 1.5 to 2.0 kg) and rhesus macaques (Indian origin and captive bred, 2.9 to 4.9 kg) were administered 10⁷ PFU of Tor2 SARS CoV by direct instillation into the trachea via an endotracheal tube or intravenous infusion via a saphenous vein. These experiments were approved by the Animal Care and Use Committees of the University of Pennsylvania and the Southern Research Institute. All in vivo and in vitro experiments with SARS CoV were performed under biosafety level 3 with investigators using respirators. The challenge stock was 1 passage removed from the passage 2 stock provided to us from Heinz

Feldmann (Winnipeg, Canada). An aliquot of the passage stock was converted to DNA with reverse transcriptase (RT), and open reading frames spanning N, M, E, and S were PCR amplified and subcloned, and one clone from each open reading frame was sequenced (both strands to 99.9% confidence level). The nucleotide sequences of the cloned open reading frames were 99.9% identical to the published Tor2 sequence (5). The abundance of SARS CoV RNA was quantitated in tissues by TaqMan PCR using oligonucleotides or probe to N. To minimize cross-contamination between samples during the necropsy, disposable tools were used for tissue and sample processing. In the TaqMan assay, cloned SARS CoV gene fragments were used to establish standard curves. Samples not treated with RT were incorporated into each experiment and always shown to be negative. Efficiency of RNA recovery was assessed by spiking known copies of SARS CoV RNA into negative tissue homogenates and was shown to be 85 to 100%. Sensitivity, linearity, and reproducibility of the assay were determined by mixing SARS CoV from lysates of infected Vero cells with tissue homogenates. Variation between different assays of the same samples was usually less than 20%.

Following anesthesia with ketamine (30 mg/kg of body weight intramuscularly), 10⁷ PFU of SARS CoV was injected intravenously (1 ml) or instilled into the trachea via an endotracheal tube (1 ml with a saline flush). Two rhesus macaques and two cynomolgus macaques each received SARS CoV from either the intratracheal (rhesus/IT and cyno/IT animals) or intravenous (rhesus/IV and cyno/IV animals) routes of administration. The challenge virus was an early passage preparation of a clinical isolate from a Canadian patient (called Tor2). The animals were observed several times a day, and biological samples were harvested prior to initiation of the study (days –5 and 0) and 3, 5, 7, and 12 to 14 days after challenge. These samples included blood for clinical pathology and blood, rectal, oropharyngeal, and nasal swabs for recovery of SARS CoV by cultivation on Vero cells and SARS CoV genomes by TaqMan

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TABLE 1. Summary of key findings at necropsy following SARS CoV challenge in macaques^a

Species ^b	ID	Route	Viral RNA (copies/100 ng) in:						Platelets (10 ³ /μl)		WBC (10 ³ /μl)/% lymphocytes		NAB (1/dilution)
			Li	Lu	LN (MD)	LN (MS)	Sp	Tr	Pre	D3	Pre	D3	
Cyno (F)	17087	IT	ND	8	130	ND	ND	ND	206	103	7.3/15	32/77	160
Cyno (F)	17104	IT	ND	ND	2	ND	ND	ND	158	83	7.5/23	7.2/42	320
Cyno (F)	17103	IV	22	ND	2.5	ND	110	ND	179	236	11.7/19	11.0/24	80
Cyno (M)	17179X	IV	ND	8.9	ND	110	170	ND	231	195	18.5/69	12.2/35	80
Rhesus (M)	RQ4364	IT	ND	ND	150	7,120	ND	42	244	136	8.6/30	6.4/40	80
Rhesus (M)	RQ4369	IT	ND	ND	ND	ND	ND	ND	233	90	15.3/60	9.6/58	20
Rhesus (M)	RQ4348	IV	ND	ND	ND	ND	ND	ND	185	119	11.2/50	9.2/58	10
Rhesus (M)	RQ4354	IV	ND	ND	2	ND	62	ND	227	138	10/33	7.6/40	20

^a The following tissues were analyzed: Li, liver; Lu, lung; LN (MD), mediastinal lymph node; LN (MS), mesenteric lymph node; Sp, spleen; Tr, trachea. The level of sensitivity was 2 copies/100 ng of RNA. Blood platelets and white blood cells (WBC) (with percent lymphocytes) are shown at baseline (Pre) and day 3 (D3) levels. Normal levels of white blood cells, total lymphocytes, and platelets are 4×10^3 to 15×10^3 , 1×10^3 to 5×10^3 , and 100×10^3 to 700×10^3 , respectively. ND, not detected. NAB to SARS CoV was determined by incubating serum with SARS CoV prior to analyses on Vero cells for formation of plaques. The highest dilution of serum that inhibited plaque formation was determined to be the neutralizing titer. The indicated NAB titers are from serum harvested at necropsy (days 12 to 14). No animals had SARS CoV NAB prior to challenge. The lower limit of the assay for NAB was 1:10.

^b Cyno, cynomolgus; F, female; M, male.

analysis. Animals were necropsied 12 to 14 days after challenge, and tissues were evaluated for histopathology and recovery of SARS CoV genomes and/or virus.

Clinical sequelae of SARS CoV in our study were minimal and were localized to the upper airway. The cyno/IT animals developed a mild cough and slightly decreased activity on days 2 and 3 after virus challenge; these findings quickly resolved and the animals were asymptomatic until days 8 to 10 when sneezing was noted. The other groups were free of symptoms during the period of observation except for one rhesus/IT animal (RQ4364), which demonstrated a noticeable change in disposition towards the end of the study; this otherwise docile animal became agitated and aggressive beginning on day 10. Body temperature measured with an implantable chip remained within normal limits for all animals. No animals demonstrated signs of respiratory distress. Blood chemistries and hematologies were unremarkable except for lymphocytosis in one animal (cyno/IT 17087) and thrombocytopenia in 7 of 8 animals; these abnormalities peaked at day 3 and were largely resolved by the time of necropsy (Table 1). Cultures of rectal, oropharyngeal, and nasal swabs were negative, although low

levels of viral RNA were detected in nasal swabs of both cyno/IT animals and in oral swabs of one cyno/IT animal (Table 2). Higher levels of neutralizing antibodies (NAB) to SARS CoV developed in the cynomolgus macaques than in the rhesus macaques (Table 1). It is likely these titers would have continued to increase beyond the 2-week time point when the animals were necropsied. Sera were also analyzed for the inflammatory cytokines gamma interferon, IP-10, RANTES, and interleukin-8 and were found to be unchanged from baseline in all rhesus and most cynomolgus macaques. The cyno/IT animal 17104 demonstrated 5- to 10-fold elevations at day 14 compared to prechallenge levels in gamma interferon, MCP-1, and IP-10 (data not shown).

Gross examination of the lungs of cyno/IT animal 17087 revealed a few scattered pleural adhesions. Upon sectioning the pulmonary parenchyma, there were a few small foci of consolidation in a rhesus/IT animal (RQ4369) that appeared as red lesions that were firm and much less crepitant than the surrounding normal tissue. The vast majority of lung tissue appeared normal in gross appearance.

Microscopic examination of the lung in animal RQ4369 revealed patchy areas of mild interstitial edema and alveolar inflammation interspersed with normal lung histology. Occasional areas of intra-alveolar edema were observed (Fig. 1A). The inflammation was mononuclear, with rare foci of macrophage accumulation (Fig. 1B). There was no evidence of giant cells or of diffuse alveolar damage, as has been observed in humans with SARS (6). Analysis of these sections for expression of SARS CoV antigens by immunohistochemistry was negative (data not shown). Gross and histological analyses of other organs from this animal and all tissues from the other animals were within normal limits. We detected substantial levels of viral RNA in a number of tissues including lymph nodes (2/4) and trachea and lung (2/4) from the intratracheally administered animals and spleen (3/4), lymph nodes (1/4), and liver (1/4) from the intravenously challenged animals (Table 1).

Our data indicate that high challenge doses of the Tor2 strain of SARS CoV administered into the lung cause a mild clinical syndrome in macaques that differs from human SARS in a number of important ways. The animals remained afebrile,

TABLE 2. RT-PCR analysis of SARS CoV genomes in the nonhuman primates infected with SARS CoV^a

ID (species)	Route	Nasal swabs (copies/reaction) at day:					Oral swabs (copies/reaction) at day:				
		0	3	5	7	12	0	3	5	7	12
17087 (cy)	IT	ND	ND	ND	8.4	NS	ND	ND	NS	ND	NS
17104 (cy)	IT	ND	4.6	ND	ND	NS	ND	2.9	5.4	ND	20
17103 (cy)	IV	ND	ND	ND	ND	NS	ND	ND	ND	ND	NS
17179X (cy)	IV	ND	ND	10	4.8	ND	ND	ND	ND	ND	NS
RQ4364 (rh)	IT	ND	ND	ND	ND	880	ND	2.3	ND	ND	ND
RQ4369 (rh)	IT	ND	ND	ND	ND	NS	ND	8.3	3.8	ND	NS
RQ4348 (rh)	IV	ND	ND	ND	ND	NS	ND	ND	ND	ND	NS
RQ4354 (rh)	IV	ND	ND	ND	ND	NS	ND	ND	ND	ND	NS

^a We systematically evaluated the impact of swab materials used for sample collection and carrier reagents. For swab samples, total RNA for each sample was coprecipitated with either tRNA or linear acrylamide, and all of the sample was used for RT reaction (100-μl reaction). An aliquot of each RT reaction (10 μl) was used for TaqMan PCR. cy, cynomolgus macaque; rh, rhesus macaque; NS, no sample available; ND, not detected.

FIG. 1. Histology of lung lesions of rhesus IT animal RQ4369. (A) In this area of consolidation, there is extensive edema fluid in the alveolar lumina. (B) This area illustrates an interstitial infiltrate that consists of chronic inflammatory cells.

and the clinical and histological consequences of intrapulmonary administration of virus were mild. There was no evidence at the time of necropsy that the animals were progressing to a fatal pulmonary syndrome. It is interesting that thrombocytopenia developed in almost all animals and has been described in 40% of infected patients (7).

While the numbers of animals per group in our study are insufficient to demonstrate statistically significant differences in the subtle findings described above, several trends emerged. In terms of clinical findings and recovery of virus, cynomolgus macaques appear more affected by infection with SARS CoV than rhesus macaques. Furthermore, evidence for sequelae of infection was more convincing following intratracheal administration than it was following intravenous administration.

The observation in our experiments of only mild sequelae of SARS CoV challenge in macaques differs from the studies of Fouchier et al. and Kuiken et al., who suggest a more severe syndrome that resembles SARS (1, 4). Clinical findings in our study were mild and localized to the upper respiratory tract (i.e., mild cough and sneezing), while the previous studies described respiratory distress in some animals, consistent with diffuse pneumonic processes, although objective confirmation of pulmonary compromise was not provided (1, 4). It is unclear what the clinical progression would have been in their studies, since those animals challenged with high-dose virus were necropsied at day 6. Discrepancies in pathology were also noted between the two studies. The only finding in our study was focal interstitial alveolar inflammation and edema in one animal at day 14, while the previous studies described gross foci of pulmonary consolidation in four of six animals at day 6 that reflected severe damage to alveolar and bronchiolar epithelia (1, 4). A third study of SARS CoV challenge in macaques was recently completed by investigators at the U.S. Army Medical Research Institute for Infectious Diseases (J. Paragas, personal communication). These investigators challenged both rhesus and cynomolgus macaques with the Urbani strain of

SARS CoV in a study that evaluated clinical sequelae for at least 28 days. Their animals demonstrated only minimal evidence of disease.

Reconciling apparent differences in the macaque experiments in which SARS CoV was administered into the lung is important when assessing the utility of the model. The source of the clinical viral isolate used to generate the challenge virus varied with each study as follows: a Canadian patient for our study, the Urbani strain for the study by Paragas and coworkers (personal communication), and patient 5688 for the studies by Fouchier et al. and Kuiken et al. (1, 4). The differences cannot be ascribed to insufficient challenge virus since the dose administered by Paragas and coworkers and by us was 10- to 50-fold higher than that used by Fouchier et al. and Kuiken et al. It is unlikely that our challenge stock of SARS CoV had attenuated during its expansion *in vitro*, since the same stock caused severe pathology in Stat-1-deficient mice and ferrets (unpublished data) and sequence analysis of a number of open reading frames failed to detect significant differences in nucleotide sequence compared to the clinical isolate (data not shown). The routes of SARS CoV administration differed between the studies (i.e., intratracheal for our study and intratracheal, conjunctival, and intranasal for Fouchier et al. and Kuiken et al. [1, 4]); it is hard to envision how this impacted on pulmonary disease.

We propose a model of disease in SARS CoV-challenged macaques that is consistent with most of the data generated by the National Institutes of Health, Fouchier et al., Kuiken et al., and us. Intrapulmonary exposure of SARS CoV to macaques does lead to low-level virus replication, although the clinical consequences, which are limited to the respiratory tract, are mild and self-limited. Lung pathology and recovery of virus is more evident when animals are necropsied at earlier time points, as was the case with the Fouchier et al. and Kuiken et al. experiments (i.e., days 4 to 6), than at later time points after the infection resolved, as was done in our study (i.e., days 12 to

14). We do not believe that the clinical manifestations are sufficiently robust to be useful in evaluating pathogenesis of the disease or assessing therapeutic efficacy. In fact, the recent evaluation of pegylated alpha interferon in SARS CoV-challenged macaques focused exclusively on short-term (i.e., day 4) nonclinical end points such as recovery of virus, infection of pulmonary epithelial cells, and inflammatory foci in lung (2).

How strongly the macaque model supports the conclusion that SARS is caused by the novel coronavirus, SARS CoV, is a matter of debate. Indeed, the extensive epidemiologic data that links the virus to the disease are quite compelling. It seems prudent, however, to explore other species of animals, as well as adapted strains of SARS CoV, in the development of animal models of SARS.

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REFERENCES

1. Fouchier, R. A., T. Kuiken, M. Schutten, G. van Amerongen, G. J. van Doornum, B. G. van den Hoogen, M. Peiris, W. Lim, K. Stohr, and A. D. Osterhaus. 2003. Aetiology: Koch's postulates fulfilled for SARS virus. *Nature* **423**:240.
2. Haagmans, B. L., T. Kuiken, B. E. Martina, R. A. Fouchier, G. F. Rimmelzwaan, G. van Amerongen, D. van Riel, T. de Jong, S. Itamura, K. H. Chan, M. Tashiro, and A. D. Osterhaus. 2004. Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nat. Med.* **10**:290–293.
3. Ksiazek, T. G., D. Erdman, C. S. Goldsmith, S. R. Zaki, T. Peret, S. Emery, S. Tong, C. Urbani, J. A. Comer, W. Lim, P. E. Rollin, S. F. Dowell, A. E. Ling, C. D. Humphrey, W. J. Shieh, J. Guarner, C. D. Paddock, P. Rota, B. Fields, J. DeRisi, J. Y. Yang, N. Cox, J. M. Hughes, J. W. LeDuc, W. J. Bellini, and L. J. Anderson. 2003. A novel coronavirus associated with severe acute respiratory syndrome. *N. Engl. J. Med.* **348**:1953–1966.
4. Kuiken, T., R. A. Fouchier, M. Schutten, G. F. Rimmelzwaan, G. van Amerongen, D. van Riel, J. D. Laman, T. de Jong, G. van Doornum, W. Lim, A. E. Ling, P. K. Chan, J. S. Tam, M. C. Zambon, R. Gopal, C. Drosten, S. van der Werf, N. Escriou, J. C. Manuguerra, K. Stohr, J. S. Peiris, and A. D. Osterhaus. 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**:263–270.
5. Marra, M. A., S. J. Jones, C. R. Astell, R. A. Holt, A. Brooks-Wilson, Y. S. Butterfield, J. Khattra, J. K. Asano, S. A. Barber, S. Y. Chan, A. Cloutier, S. M. Coughlin, D. Freeman, N. Girn, O. L. Griffith, S. R. Leach, M. Mayo, H. McDonald, S. B. Montgomery, P. K. Pandoh, A. S. Petrescu, A. G. Robertson, J. E. Schein, A. Siddiqui, D. E. Smailus, J. M. Stott, G. S. Yang, F. Plummer, A. Andonov, H. Artsob, N. Bastien, K. Bernard, T. F. Booth, D. Bowness, M. Czub, M. Drebot, L. Fernando, R. Flick, M. Garbutt, M. Gray, A. Grolla, S. Jones, H. Feldmann, A. Meyers, A. Kabani, Y. Li, S. Normand, U. Stroher, G. A. Tipples, S. Tyler, R. Vogrig, D. Ward, B. Watson, R. C. Brunham, M. Krajden, M. Petric, D. M. Skowronski, C. Upton, and R. L. Roper. 2003. The genome sequence of the SARS-associated coronavirus. *Science* **300**:1399–1404.
6. Nicholls, J. M., L. L. Poon, K. C. Lee, W. F. Ng, S. T. Lai, C. Y. Leung, C. M. Chu, P. K. Hui, K. L. Mak, W. Lim, K. W. Yan, K. H. Chan, N. C. Tsang, Y. Guan, K. Y. Yuen, and J. S. Peiris. 2003. Lung pathology of fatal severe acute respiratory syndrome. *Lancet* **361**:1773–1778.
7. Peiris, J. S., C. M. Chu, V. C. Cheng, K. S. Chan, I. F. Hung, L. L. Poon, K. I. Law, B. S. Tang, T. Y. Hon, C. S. Chan, K. H. Chan, J. S. Ng, B. J. Zheng, W. L. Ng, R. W. Lai, Y. Guan, and K. Y. Yuen. 2003. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* **361**:1767–1772.