Severe acute respiratory syndrome coronavirus persistence in Vero cells

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Keywords: severe acute respiratory syndrome · coronavirus · infection · microbiological techniques

Background Several coronaviruses establish persistent infections in vitro and in vivo, however it is unknown whether persistence is a feature of the severe acute respiratory syndrome coronavirus (SARS-CoV) life cycle. This study was conducted to investigate viral persistence.

Methods We inoculated confluent monolayers of Vero cells with SARS-CoV at a multiplicity of infection of 0.1 TCID50 and passaged the remaining cells every 4 to 8 days for a total of 11 passages. Virus was titrated at each passage by limited dilution assay and nucleocapsid antigen was detected by Western blot and immunofluorescence assays. The presence of viral particles in passage 11 cells was assessed by electron microscopy. Changes in viral genomic sequences during persistent infection were examined by DNA sequencing.

Results Cytopathic effect was extensive after initial inoculation but diminished with serial passages. Infectious virus was detected after each passage and viral growth curves were identical for parental virus stock and virus obtained from passage 11 cells. Nucleocapsid antigen was detected in the majority of cells after initial inoculation but in only 10%–40% of cells at passages 2–11. Electron microscopy confirmed the presence of viral particles in passage 11 cells. Sequence analysis at passage 11 revealed fixed mutations in the spike (S) gene and ORFs 7a–8b but not in the nucleocapsid (N) gene.

Conclusions SARS-CoV can establish a persistent infection in vitro. The mechanism for viral persistence is consistent with the formation of a carrier culture whereby a limited number of cells are infected with each round of virus replication and release. Persistence is associated with selected mutations in the SARS-CoV genome. This model may provide insight into SARS-related lung pathology and mechanisms by which humans and animals can serve as reservoirs for infection.

Severe acute respiratory syndrome (SARS) is a transmissible, febrile illness that is characterized by rapid progressive respiratory compromise with an incubation period of 6 days. The causative agent, SARS-coronavirus (SARS-CoV), has been isolated from clinical material obtained from SARS patients, and this virus produces a SARS-like illness following experimental inoculation into non-human primates, ferrets and domestic cats. Recent cases of SARS in Singapore, Guangzhou, and Taiwan, China, months after the abatement of the SARS epidemic, raise concerns about the possibility of persistent or recurrent infections.

Persistent coronavirus infections are recognized in vitro and in vivo. The human coronaviruses OC43 and 229E persistently infect human neural cell lines. A murine coronavirus, mouse hepatitis virus (MHV), establishes persistent infection in neonatal or immunosuppressed mice, and has been detected in the central nervous system up to one year after experimental infection. Persistence of MHV in vivo is associated with deletions in the
genes that encode the spike (S) and nucleocapsid (N) proteins. Here we partially characterize cellular and viral aspects of persistent SARS-CoV infection in Vero cells and discuss the implications of these findings for SARS pathogenesis and epidemiology.

**METHODS**

**Virus and cells**
SARS-CoV (strain TOR2) was a gift from Heinz Feldmann and Frank Plummer (National Microbiology Laboratory, Science Centre for Human and Animal Health, Winnipeg, Canada). First passage SARS-CoV, with a titer of 6 × 10⁵ 50% tissue culture infectious doses (TCID₅₀/ml), was used in all experiments. The Vero E6 cell line was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle medium (GIBCO Laboratories, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine (GIBCO Laboratories), and penicillin streptomycin solution (each 100 U/ml; GIBCO Laboratories).

**Antibodies**
Human SARS-convalescent serum was a gift from Tony Mazzulli (Mount Sinai Hospital, Toronto, Canada). Murine polyclonal antiserum to recombinant SARS-CoV N protein was a gift from Luis Villarreal (University of California Irvine, CA, USA) and Ilya Trakht (Columbia University, NY, USA).

**Acute infection**
Naïve Vero cells (1 × 10⁵ cells/well) were plated on an 8-well chamber slides (Labtek, Nunc, USA), infected at a multiplicity of infection (MOI) of 0.1 TCID₅₀ and observed for 6 days (acute infection). Cells and supernatants were collected at 24-hour intervals for virus titration and RNA quantification by real-time PCR. Cells were fixed in 3.7% formaldehyde at parallel time points for immunocytochemistry.

**Persistent infection**
Vero cells, at 60% to 80% confluence in 25-cm² flasks, were infected at a MOI of 0.1 TCID₅₀ and passaged every 4 to 8 days for a total of eleven passages. At each passage, aliquots of cells were seeded in 6-well plates (Nunclon, Nunc, USA) at a density of 5 × 10⁵ cells/well for titration of virus and into 8-well chamber slides at a density of 1 × 10⁵ cells/well for immunofluorescence. Supernatants and cell homogenates, obtained by freeze-thawing, were harvested from the 6-well plates after incubation periods of 2 and 48 hours at 37°C. Cells on chamber slides were fixed in 3.7% formaldehyde (Fisher Scientific, USA) at 24-hour intervals.

**Comparison of virus production during acute and persistent infection**
Vero cells, either acutely infected with SARS-CoV TOR2 (MOI 0.1) or persistently infected with SARS-CoV TOR2 for 11 passages (infected at MOI 0.1 in passage 1), were analyzed by virus titration (supernatant) and real-time PCR (total cell RNA) over an interval of 148 hours. Cells were distributed in 6-well plates and supernatant and cells for analysis of virus titer and synthesis of nucleocapsid mRNA, respectively, was harvested from one well each after 0, 24, 48, 72, 96, or 148 hours of incubation. In addition, a growth curve for the virus obtained from persistently infected Vero cells at passage 11 (passage-11 virus) was determined. Naïve Vero cells were infected with passage-11 virus at an MOI of 0.1 and samples were taken after 0, 24, 48, 72, 96, or 120 hours.

**Detection of viral antigens by immunocytochemistry**
Vero cells were fixed in 3.7% formaldehyde, blocked for 30 minutes using 10% normal goat serum (NGS) (Sigma, USA), then permeabilized with 0.4% Triton X-100 in phosphate buffered saline (PBS), and incubated for 30 minutes with either mouse antiserum to SARS-CoV nucleocapsid (diluted 1:1000) or human SARS convalescent serum (diluted 1:500) in 1% NGS, 0.4% Triton X-100 in PBS. After three washes in PBS, the cells were incubated for 30 minutes with either goat anti-human IgG-fluorescein isothiocyanate conjugate (FITC) (diluted 1:100; Sigma) or goat anti-mouse IgG-tetramethyl rhodamine isothiocyanate conjugate (TRITC) (diluted 1:500; Sigma) in 1% NGS, 0.4% Triton X-100 in PBS. The cells were washed three times in PBS, incubated for 20 minutes with Hoechst nuclear stain (Sigma), washed again, and examined by fluorescence microscopy. Fluorescent images were taken with a Nikon E600 microscope, Spot Insight Color digital camera (Diagnostic Instruments Inc., USA) and Spot software at 40 x magnification.

**Western blot assay for detection of nucleocapsid antigen**
Cell homogenates were boiled in SDS reducing
buffer (5% beta-mercaptoethanol (v/v), 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.0625 mol/L Tris-HCl, pH 6.8) and resolved on 5%/15% discontinuous SDS-PAGE gels. Separated proteins were transferred to nitrocellulose membranes (Trans-Blot transfer medium, BioRad, USA). Membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) for 1 hour at room temperature. Mouse antiserum to SARS N protein was diluted 1:5000 in 1% nonfat dry milk in PBS and incubated with the membrane overnight at 4°C. Membranes were washed 5 times with 0.1% Tween-20 in PBS, and subsequently incubated with a 1:2000 dilution of anti-mouse IgG conjugated to horseradish peroxidase (BioRad) for 1 hour at 37°C. Immunolabeled bands were visualized by chemiluminescence (Amersham ECL kit) and exposed to film (Kodak BIOMAX ML, USA).

Limited dilution assay for virus titration
Vero cells (10^4 cells/well) in 96-well plates (Nunc) were inoculated with serial 10-fold dilutions of sample and incubated for 4 days at 37°C. Cells were fixed in 3.7% formaldehyde and stained with 40% methanol containing 0.1% crystal violet. Non-infected and mock-infected Vero cells served as negative controls.

TaqMan real-time PCR assay
SARS CoV RNA was quantitated by real-time PCR using a primer/probe set that amplified a fragment of the N gene. The primer set shown in Table 1 amplified a 229 bp fragment and was used in combination with the internal probe CIID-290747, labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) (TIB MolBiol, USA).

DNA and RNA standards were generated by cloning a 1277 nt fragment comprising portions of the N gene and the 3’ non-coding region (Table 1) into pGEM-T Easy vector (Promega, USA). DNA standards were obtained by serially diluting linearized plasmid. RNA standards were generated by in vitro transcription of linearized plasmid template using the mMESSAGE mMACHINE T7 kit (Ambion, USA).

Five microliters of total RNA were used for reverse transcription (SuperScript II, Invitrogen, USA). Five microliters of cDNA solution were used for real-time PCR, performed using a TaqMan Universal Master Mix kit (PE Applied Biosystems, USA) on an ABI PrismTM 7700 sequence detection system (PE Applied Biosystems). Thermocycling conditions were as follows: 2 minutes at 50°C (Amperase UNG, USA), 10 minutes at 95°C (polymerase activation); 45 cycles of 15 seconds at 95°C (denaturation), and 1 minute at 60°C (annealing and extension).

Detection of virus by electron microscopy
Vero cell monolayers persistently infected with SARS-CoV were grown until confluent, washed twice in PBS, and fixed with 2.5% glutaraldehyde for 20 minutes. Following two washes with distilled water, cells were treated for 15 minutes with 1% tannic acid, washed twice, treated for one hour with 1% uranyl acetate, dehydrated in ascending concentrations of ethanol, and embedded in low-viscosity epoxy resin.
[hydroxypropyl methacrylate (EMS), Epon mix Lx112; Ladd Research, USA]. Ultrathin sections were collected on a Hacker-Bright Cryostat Safecut 7000 Microtome, stained with 2% uranyl acetate and post-fixed with 2% lead citrate. Micrographs were obtained using a JEOL 1200EXII electron microscope (Jeol Ltd., Tokyo, Japan).

RT-PCR and DNA sequence analysis of S, N and ORF 7a-8b genes
Total RNA from acutely infected, persistently infected, or control cells was extracted using Trizol Reagent (MRC Biotech, USA). After reverse transcription with Superscript II (Invitrogen, USA) and random hexamer primers (Pharmacia, USA), cDNA was amplified (Expand High Fidelity System, Roche Biochemicals, USA) with the primer set described in Table 1. Regions selected for sequence analysis included the amino-terminus of the S protein, the N protein, and the genomic region from ORF 7a to ORF 8b in which 29 and 45 bp deletions that disrupt ORFs 8a and 7b, respectively, have previously been described. Products were cloned using the TOPO TA cloning kit (Invitrogen). Five clones from each amplification reaction were analyzed by dyeoxy sequencing.

RESULTS

Cell culture characteristics during acute and persistent infection
Sixty to eighty percent confluent monolayers of Vero cells were infected with SARS-CoV TOR2 strain at an MOI of 0.1 TCID_{50}/cell and observed daily. At ninety-six hours post infection, approximately half of the cells had detached from the monolayer. The surviving cells repopulated the culture such that the monolayer was unremarkable by day 7. These cells were continuously passaged 11 times and exhibited only limited cytopathic effect at early time points after each subsequent passage. Acute infection of Vero cells with virus obtained from persistently infected cells passage 11 behaved identically to the acute infection described above for the TOR2 strain (Fig. 1) however, the cytopathic effect observed was less prominent and appeared later in the growth curve.

Comparison of virus production in acute and persistent infection
Viral growth curves obtained from passage 11 persistently infected cells (Fig. 1a) were similar to those obtained from cells acutely infected with SARS-CoV at an MOI of 0.1 (Fig. 1b). Virus obtained after passage 11 was used to infect naïve Vero cells at an MOI of 0.1 TCID_{50} and the viral titers over a 6-day time course were identical to those seen in acute infection with passage 1 viral stock (Fig. 1c). SARS-CoV RNA was measured by real-time PCR as an independent indicator of viral replication and the findings paralleled the titration results (Fig. 1).

At each passage, an aliquot of the persistent cells was transferred to 6-well plates, and after 2 or 48 hours of incubation, supernatant and cell homogenate from one well was used for virus titration, respectively. At 2 hours no virus was detected in supernatant; the titer in cell extract was approximately 10^{3} TCID_{50}/ml. At 48 hours the titer in both supernatant and cell extract was approximately 10^{6} TCID_{50}/ml.

Detection of viral antigens in acute and persistent infection
Nucleocapsid protein was detected by Western blot assay in cell homogenates obtained 24, 48, and 72 hours after acute infection and at the same timepoints in passage 11 cells (after passage). A second band of lower size, recognized by the anti-N antibody, was also observed (Fig. 2).

Fig. 1. Time course of virus production and viral nucleocapsid RNA synthesis in (A) persistently infected Vero cells, (B) naïve Vero cells inoculated with original virus SARS-CoV Tor2 strain, or (C) naïve Vero cells inoculated with virus isolated from persistently infected Vero cells. Infectious virus was measured by limited dilution titration assay. The experiments were repeated twice. An MOI of 0.1 TCID_{50}/cell was used in the time course. Viral nucleocapsid RNA was measured by a TaqMan real-time PCR assay.
Fig. 2. Nucleocapsid protein expression in acutely and persistently infected Vero cells. Proteins from acutely infected and passage 11 persistently infected Vero cells were size fractionated by SDS-PAGE, transferred to nitrocellulose and examined for the presence of nucleocapsid protein using mouse antisera to nucleocapsid protein. N protein was observed as early as 24 hours after acute infection and in persistently infected cells. A truncated protein of approximately 40 kD was visible also 24 hours after acute infection, and in persistently infected cells.

Nucleocapsid antigen was detected by immunofluorescence in greater than 90% of the cells 24 hours after infection; however, in passages 2 to 11, the persistent cells showed only 10% to 40% of cells expressing nucleocapsid protein 24 hours after passage; identical percentages of infected cells were labeled using anti-N antibody and human SARS convalescent serum (Fig. 3). The nucleocapsid protein staining had a punctate appearance and was found in a perinuclear and cytoplasmic distribution in acute (Fig. 3d) and persistently infected cells (Fig. 3a); localization was similar when using human convalescent sera (Fig. 3b). No staining was observed in non-infected cells (Fig. 3f).

Detection of virus by electron microscopy in persistently infected Vero cells
Electron microscopy confirmed the presence of viral particles in Vero cells at passage 11. Viral particles were found in vesicles classically associated with virus budding (Fig. 4).

DNA sequence analysis of viral genes in acute or persistent infection
Regions of the SARS-CoV genome corresponding to

Fig. 3. Demonstration of viral proteins in acutely and persistently infected Vero cells by indirect immunofluorescence. Fig. 3, a through c; persistently infected Vero cells at passage 11; a; mouse antinucleocapsid antisera; b; goat anti-mouse TRITC; c; Hoechst counterstain; d and e; acutely infected Vero cells; d; goat anti-human FITC, e; Hoechst counterstain; f; negative control; non infected Vero cells. Magnification is 40 x.
Table 2. Sequence changes in the SARS-CoV genome during persistent infection

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<th>Fragment sequenced</th>
<th>SARS genome region</th>
<th>Position of mutation</th>
<th>Type of mutation</th>
<th>Codon change</th>
<th>Amino acid change</th>
<th>Number of clones observed</th>
<th>Rate of substitution for that region</th>
<th>Rate of fixed mutations for that region</th>
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<td>Spike S1</td>
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<td>GCC → GCT</td>
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The nucleotide positions are from strain TAR2 (Genbank accession number: AY274119).

Fig. 4. Electron micrograph of SARS-CoV persistently infected Vero cells. Virus particles in the cytoplasm (Fig. 4a and b); Vesicles classically associated with virus budding (Fig. 4c).
S1, N and ORFs 7a-8b were amplified by RT-PCR from the original virus stock and from passage 11 cells for sequence analysis. These regions were selected because deletions or mutations in the S and N genes have been detected in persistent infection with MHV, HCoV OC43, and HCoV 229E, and because the region encompassing ORFs 7a-8b has been implicated in interspecies transfer of SARS-CoV. Five independent clones were analyzed from each gene region, except for the nucleocapsid region where 9 clones were analyzed. Mutations were defined as fixed when they appeared in 3 or more clones. Fixed mutations were more common in the S gene (5/10 mutations) than in the region comprising ORFs 7a-8b (2/6), or in the N gene (0/7) (Table 2). Fixed mutations in the S gene were nonsynonymous (5/5). One of nine N gene clones contained an 8 nucleotide (nt) insertion that resulted in a frameshift and created a premature stop codon at position 885 of the N gene.

**DISCUSSION**

Here we demonstrate that SARS-CoV can establish a persistent infection in cell culture; immunofluorescence and Western blot assays indicated the presence of viral antigen in persistently infected Vero cells, and infectious virus was detected after each passage for a total of 11 passages. Encapsulated viral particles were seen by electron microscopy in small and large cytoplasmic vacuoles in passage 11 Vero cells, resembling changes described in acute SARS-CoV infection. Interestingly, multinucleated giant cells were a feature of acute and persistent SARS-CoV infection and are commonly seen in other coronavirus infections, as well as in SARS-related lung pathology.

Mechanisms proposed to account for persistent coronavirus infection include the production of mutant or interfering viruses, or the establishment of a carrier culture in which subpopulations of acutely infected cells release progeny virions that infect additional subpopulations to maintain a low level of infection within the culture system.

In an experimental model of coronavirus infection, MHV persistence in vitro is mediated by selection of resistant host cells that regulate expression of virus-specific carcinoembryonic antigen cell adhesion molecule (CEACAM) receptors. Down regulation of the CEACAM receptor is associated with coevolution of variant coronaviruses that display altered affinity for this receptor or bind to other members of the CEACAM superfamily. These binding events are mediated by the S protein and persistence is postulated to be accompanied by mutations that alter the ability of this protein to bind to its receptor. MHV persistence in vivo is similarly associated with S gene mutations.

The observation that only a small subset of the persistent cells were infected with SARS-CoV immediately after passage and that this percentage decreased with serial passages indicates that SARS-CoV can persist by establishing a carrier culture. The similarity of the growth curves and viral nucleocapsid RNA synthesis in naive Vero cells infected with either the original virus or virus taken from persistently infected passage 11 cells indicates that there is no significant change in virus replication. It remains to be seen whether the recently identified receptor for SARS-CoV, angiotensin converting enzyme II, is involved in this cellular adaptation.

The mutations in SARS-CoV that we observed in persistently infected Vero cells are consistent with the MHV model of coronavirus persistence. The fixed mutation rate in the S gene was 3.9 nt/kb and all these mutations were non-synonymous. This high mutation rate is not a result of an intrinsic assay error rate (estimated at 4 x 10⁻⁴), but on an error rate of retrovirus reverse transcriptase enzymes of 10⁻⁴ for complex RNA templates. The observed mutation rate in the S gene of SARS-CoV may indicate a positive selection for these variants during persistent Vero cell infection; it is similar to previously reported viral RNA mutation rates in persistently CoV infected mice (0.5 x 10⁻³ to 5 x 10⁻³ mutations per nt in the N and S genes respectively). These viral genomic changes do not appear to affect their replicative ability. An elevated mutation rate of 3.2 nt/kb was observed in ORFs 7a-8b with 18 of 22 mutations being non-synonymous (all of them fixed mutations also). This region of the genome contains a 29 nt insertion in a SARS-CoV variant recently identified in civet cats or raccoon dogs in China, which gives rise to a fused, longer ORF in this virus. It is currently unclear whether ORFs 7a-8b encode proteins important to the SARS-CoV life cycle, and whether the observed elevated mutation rate in virus
obtained from passage 11 Vero cells may reflect a lack of selective pressure on these products, possibly restricted to the cell culture system. It should be noted that no fixed mutations were found in the N gene.

The persistence of SARS-CoV in culture, most likely through the formation of a carrier culture, has implications for the understanding of SARS pathogenesis. A carrier culture mechanism could explain both contiguous and distant spread of virus within the respiratory and gastrointestinal tracts, facilitating shedding of virus from mucosal surfaces and resulting in spatial and temporal heterogeneity of SARS-related pathology. Viral genomic changes seen in persistent infection may also influence the course of disease; however, a more detailed analysis of virus-cell interaction is required. Whether SARS-CoV produces persistent infection in vivo in either humans or as-yet-to-be-identified animal reservoirs remains to be determined; nonetheless, analogies to other coronavirus systems, findings of SARS-CoV nucleic acid in feces weeks after resolution of respiratory symptoms and observations in the Vero cells described here indicate the plausibility of this model.

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Medical news

Malignant tumor becomes China’s No. 1 killer

Professor SUN Yan, a well-known oncologist and a fellow of the Chinese Academy of Engineering, said on March 4 that malignant tumor had currently become the No. 1 killer of Chinese citizens living in urban areas, as well as the No. 2 cause of death for rural residents.

According to information provided by Professor SUN, the number of malignant tumor cases averaged 1.6 million per year, while the number of resulting deaths totaled 1.3 million. One in every five dead patients died of malignant tumor; one in every two hundred families suffered from the shock of the disease. In Beijing, about 12,000 people would be newly diagnosed of having the killer disease each year, and approximately 10,000 would die.

(Source: Chinanews)