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The Life Cycle of SARS Coronavirus in Vero E6 Cells

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The aim of the study was to establish the life cycle of severe acute respiratory syndrome-associated coronavirus (SARS CoV) in host cells and determine the pathogenesis of SARS. Vero E6 cells (African green monkey kidney cells) were inoculated with SARS coronavirus for 3, 7, 24, 48, and 72 hr, respectively, and were observed under electron microscope. It was found that the SARS coronavirus entered the cells through membrane fusion instead of endocytosis, and then the nucleocapsids assembled in the RER and matured by budding into the smooth vesicles, which were derived from the Golgi apparatus. The smooth vesicles fused with the cell membrane, and the mature particles were released. A special phenomenon was that some virus-like particles appeared in the nucleus. We propose a scheme of the life cycle of SARS coronavirus and discuss the mechanism of its replication in Vero E6 cells. ***J. Med. Virol. 73:332–337, 2004.***

KEY WORDS: SARS coronavirus; life cycle; electron microscopy

INTRODUCTION

Early last spring outbreaks of the severe acute respiratory syndrome (SARS) occurred in China and many other countries. The World Health Organization (WHO) was alerted. The emerging disease is highly infectious and the mortality rate is high. As of June 27, 2003, a total of 8,456 SARS cases had been reported, and 809 patients died (available at the WHO [2003] website). A new human coronavirus had been identified that met the Koch's postulates and linked etiologically to the outbreak of SARS [Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003]. The complete sequence of two strains of the SARS-associated coronavirus (HCoV Tor2 isolate and SARS-CoV) had also been described [Marra et al., 2003; Rota Paul et al., 2003], and aspects of significance were outlined for "the postgenomic era" [Holmes Kathryn and Enjuanes, 2003]. Previously, some preliminary studies on SARS-associated coronavirus (CoV) morphology and morphogenesis were published [Ng et al., 2003; Zhang et al., 2003a]. In the present article, we describe further results on the life cycle of the SARS CoV in vitro by electron microscopy, and discuss the mechanism of its replication in Vero E6 cells.

MATERIALS AND METHODS

Virus and Cells

Vero E6 cells were obtained from Center for Disease Control of Guangdong Province, China. The virus was prepared as described previously [Zhang et al., 2003a]. The cells were grown to monolayer in 25 cm^2 culture flasks in Eagle's MEM supplemented with 10% fetal bovine serum (GIBCO, Invitrogen Corporation, USA) for 2∼3 days at 36.5°C. After removing the culture media, the cells were inoculated with SARS coronavirus at a multiplicity of 50 μl (250 μl for the 3-hr-sample) 6.75 log$TCID_{50}$/ml solution and were incubated in Eagle's MEM supplemented with 2% fetal bovine serum at 36.5°C. Then, samples were taken after 3, 7, 24, 48, and 72 hr, respectively. These samples were first treated with 0.02% versene and 0.25% chymotrypsin for 0.5–1 min, and then collected for further treatments.

Electron Microscopy

The collected cells were first fixed in 3.0% pH 7.2 glutaraldehyde for 1.5 hr, post-fixed in 1% osmium tetroxide for 1 hr followed by dehydration, and then embedded in Spurr (Sigma-Aldrich Co., USA). Sections were cut and stained with aqueous uranyl acetate and lead citrate.

Zhang Qinfen and Cui Jinming contributed equally to this work.

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RESULTS

Entry of the Virions Into Cells

Three hours post infecton (p.i.), it could be seen that the virions first attached themselves to the surface, and then their envelopes fused with the cell membrane and the nucleocapsids entered the cell (Fig. 1). The contours of the nucleocapsids were blurred after the virions lost their envelopes. Endocytosis was not found.

Virus Assembly and Maturation

Seven hours p.i., nucleocapsids first appeared in the swollen RER (Fig. 2a). Some ribosomes could be seen detached from RER. These nucleocapsids did not enclosed by envelope yet, and they were light-colored in the core. As the nucleocapsids increased in their number over time, the ribosomes attached to the surface of the swollen RER became fewer, and finally, disappeared completely (Fig. 2b). This type of structure and changes were consistent with the virus morphogenesis matrix vesicae (VMMV) reported previously [Zhang et al., 2003a].

Parallel to the above, the Golgi apparatus also swelled, forming smooth vesicles 7 hr p.i. (Fig. 3). As the infection developed, these smooth vesicles increased both in their number and size, resulting in severe vacuolization in the cells. Twenty four hours p.i., the nucleocapsids in VMMV budded into the smooth vesicles and acquired envelopes (Fig. 4). The particles inside smooth vesicles were about 100 nm in diameter, consistent with the size of mature virions. These smooth vesicles were identical with those reported previously [Zhang et al., 2003a].

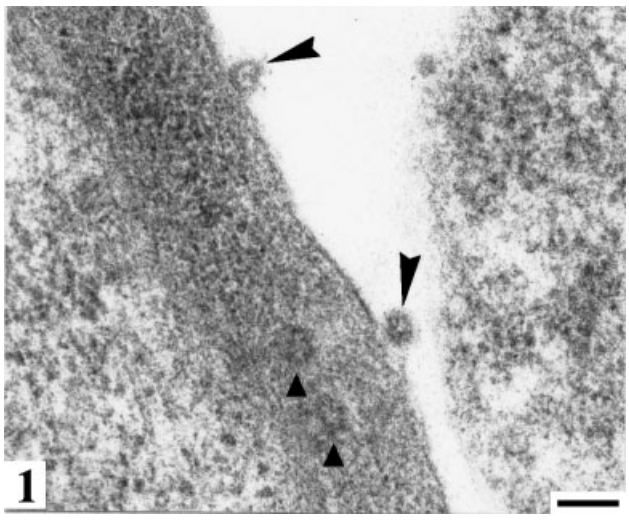
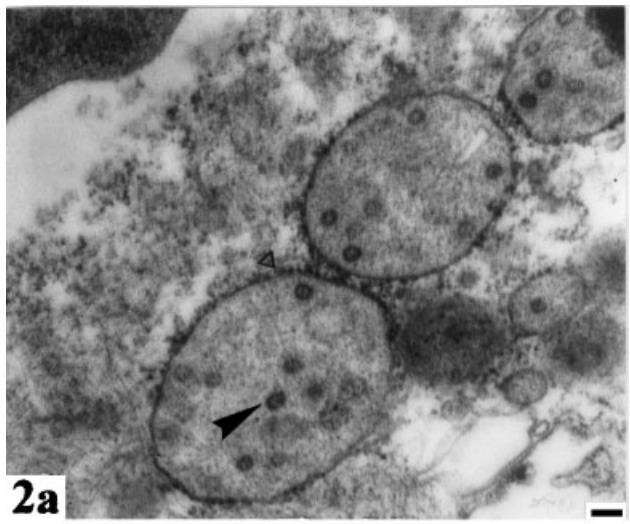


Fig. 1. Entry of severe acute respiratory syndrome-associated coronavirus (SARS CoV). The virions first attached themselves to the cell surface (►), and then their envelopes fused with cell membrane and the nucleocapsids entered the cell. The contours of these nucleocapsids were blurry after the virions lost their envelopes (Δ) (Bar = 100 nm).



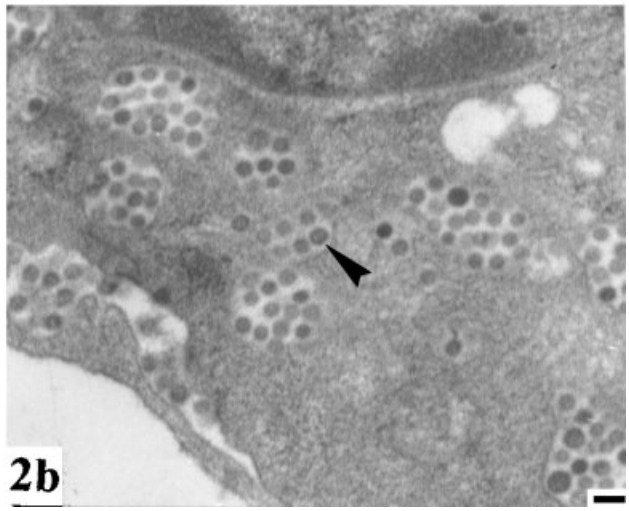


Fig. 2. The formation of the virus morphogenesis matrix vesicae (VMMV). **a**: Nucleocapsids assembled in the swollen RER (►). Some ribosomes attached on the membrane of the RER (Δ). **b**: As the number of the nucleocapsids increased, the ribosomes on the swollen RER disappeared. And this kind of swollen RER is named VMMV (►) (Bar = 100 nm).

Release of Virions

In the last phase of infection, smooth vesicles moved to the cell periphery and eventually fused with the cell membrane (Fig. 5a). Gaps appeared at the fusion sites and virions were released (Fig. 5b).

A Special Phenomenon During Morphogenesis

Forty eight hours p.i. virus-like particles were found in the nucleus of Vero E6 cells (Fig. 6). Their appearance is similar to that of the nucleocapsids in VMMV (Fig. 2b). Moreover, the nucleic membrane swelled to form blebs that contained nucleocapsids (Fig. 7a,b). These blebs were seen to detach from the nucleic membrane and turn into VMMV.

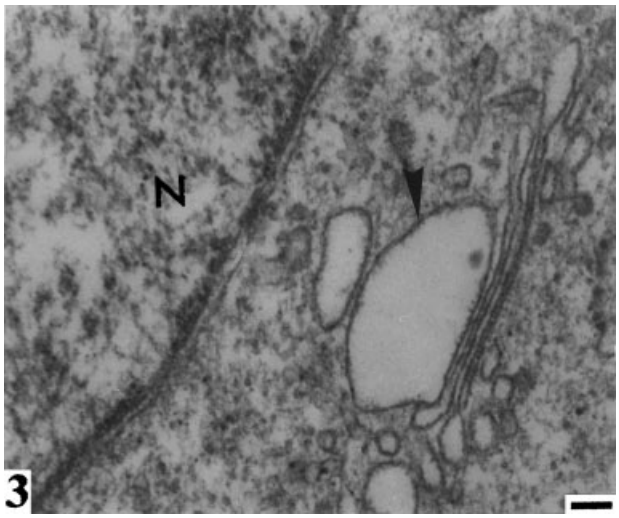


Fig. 3. The formation of the smooth vesicles. The Golgi apparatus swelled and formed the smooth vesicles (➤) (N, nucleus) (Bar = 100 nm).

A Proposed Scheme of the Life Cycle of SARS Coronavirus

A scheme of the life cycle of SARS coronavirus is proposed as follows (Fig. 8).

The SARS coronavirus first enters Vero E6 cells by membrane fusion, and is then followed by nucleic acid replication and protein synthesis. The N protein and genomic RNA are then assembled in the RER to form the nucleocapsid. As the infection progresses, the ribosomes become detached from the RER, and the RER gradually turn into VMMV. At the same time, the Golgi apparatus swells to form smooth vesicles. The nucleocapsids in VMMV bud into these smooth vesicles and acquire their envelopes and the assembly of the virions is completed.

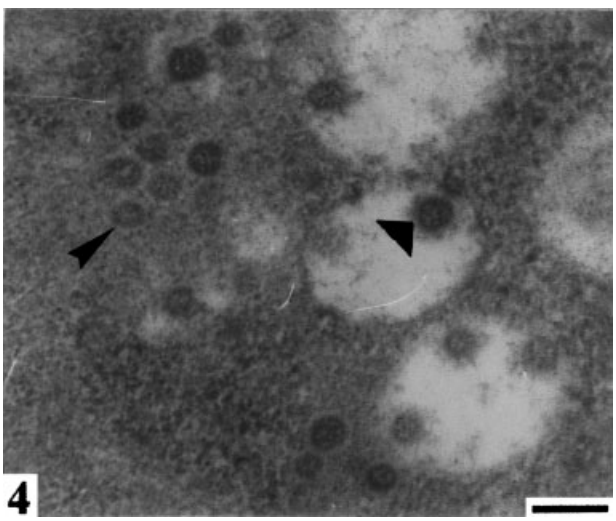
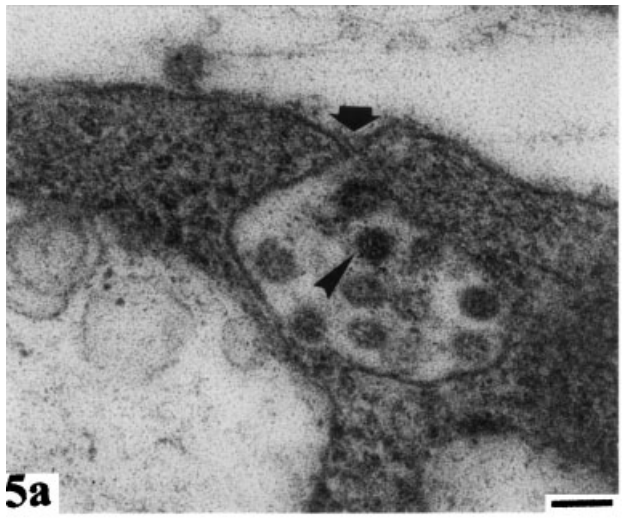


Fig. 4. Budding of the nucleocapsids from the VMMV to the smooth vesicles. The SARS CoV nucleocapsids budded from the VMMV (➤) into the smooth vesicles and obtained spikes and envelopes (▲) (Bar = 100 nm).



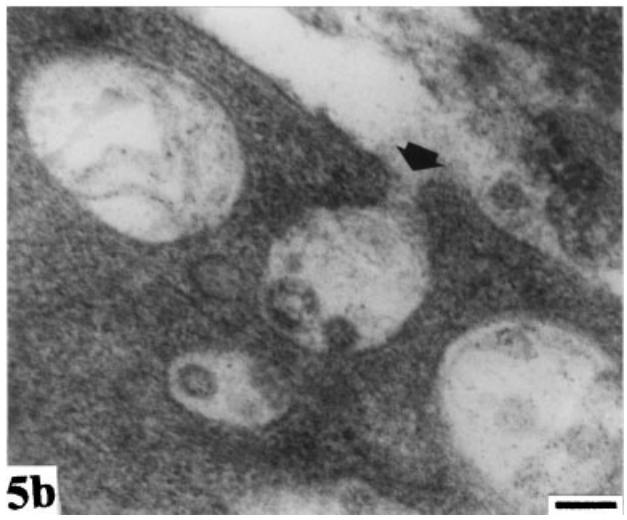


Fig. 5. Release of the virions. **a**: A smooth vesicle was fusing with the cell membrane (◗). Virions still located in the smooth vesicles (➤). **b**: A gap appeared at the fusion site and virions were released (◗) (Bar = 100 nm).

Finally, the smooth vesicles fuse with the cell membrane and virions are released.

DISCUSSION

Although many coronaviruses enter infected cells through endocytosis, the SARS coronaviruses were found to enter the cells through membrane fusion in our study, which was consistent with the results of other researchers [Ng et al., 2003]. According to a previous study, the S protein of coronavirus is the main component of the corona structure on the virus surface, and it binds to the receptor on the cell surface, inducing membrane fusion between virus envelope and cell membrane [Gallagher and Buchmeier, 2001]. Human aminopeptidase N (hAPN) is the receptor of 229E coronavirus [Yeager et al., 1992], whereas MHC I is the receptor of OC43 coronavirus [Collins, 1993]. The S

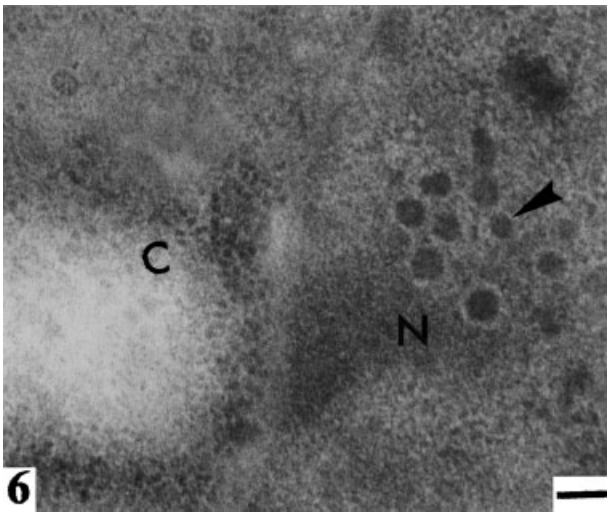
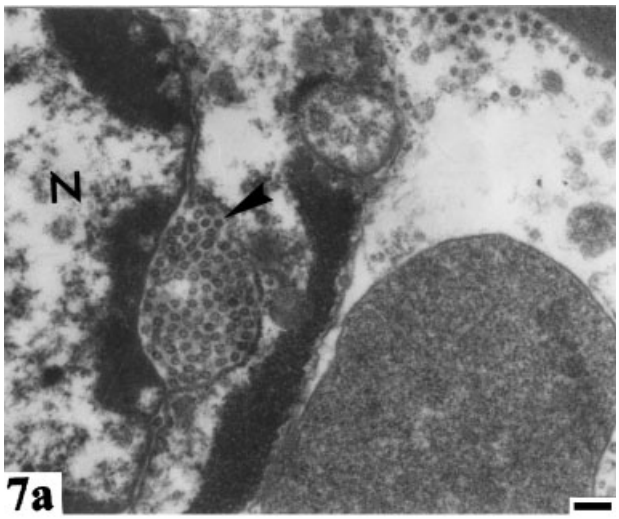


Fig. 6. Virus-like particles (►) inside the nucleus. Their appearance and size were similar to those of the nucleocapsids in the VMMV (see Fig. 2b) (N, nucleus; C, cytoplasm) (Bar = 100 nm).



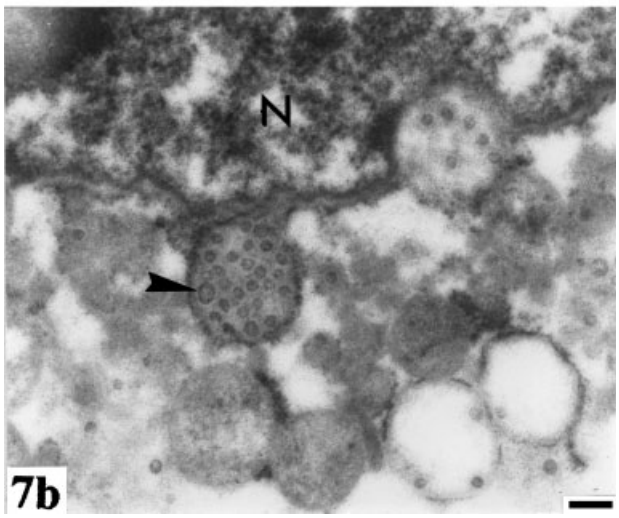


Fig. 7. The swollen nuclear membrane blebs. **a**: Many nucleocapsids assembled in a nuclear membrane bleb (►). **b**: One of the blebs detached from the nucleic membrane and turned into VMMV (►) (N, nucleus) (Bar = 200 nm).

protein of SARS coronavirus might react with the receptors from CEACAMs family [Krueger et al., 2001; Skubitz et al., 2001; Zhang et al., 2003b], inducing the membrane fusion.

After invasion, the virus replicates its genome and synthesizes its proteins by using the host cells' elements, and modifies the genomic RNA and protein precursors with the enzymes of host or its own. According to previous study, the S, M, E proteins of coronavirus congregate near RER and Golgi apparatus, and they are glycosylated in the Golgi apparatus [Nguyen and Hogue Brenda, 1997]. The N protein has RNA-binding site and ribosome-binding site, causing the virus RNA to bind with the ribosome and begin translation. It is of great importance for virus assembly that the N protein can recognize the genomic RNA and interact with other proteins [Nelson et al., 2000; Chen et al., 2002]. Furthermore, since the N protein has a nuclear localization signal (NLS), it may enter the nucleus and arrest the cell in G_2–M phase [Laude and Masters, 1995; Marra et al., 2003]. In the case of SARS coronavirus, its main structural proteins may have similar functions.

The core of SARS coronavirus is first assembled in the RER, where the N protein binds to the genomic RNA and forms the nucleocapsid. The RER gradually lose the ribosomes and swell to become VMMV [Zhang et al., 2003a]. The maturation of the viruses needs the M, E, S proteins, which always congregate near the Golgi apparatus [Krijnse-Locker et al., 1994]. Thus, after the formation of nucleocapsids, the viruses need to be transported to the Golgi apparatus and acquire envelopes with these proteins.

In our study, the SARS coronavirus matures by budding from the VMMV into the smooth vesicles, and these smooth vesicles are derived from the Golgi apparatus. This process is consistent with those of many other coronaviruses. The M and S proteins can form assembly-competent complexes, which provide a site for budding [Ducatelle et al., 1981; Massalski et al., 1982; Nguyen et al., 1997]. The fact that Golgi cisternae swell to become smooth vesicles might relate to the M protein located on the Golgi apparatus membrane.

Nucleocapsids of SARS coronavirus are found in the sample of 7 hr p.i. in our study; however, no coronavirus was found until 10 hr p.i. in a previous study [Stuart et al., 1983]. In some cases, the progeny coronavirus does not appear until 24 hr p.i. [Beesley and Hitchcock, 1982]. This phenomenon might cause the acute syndrome of SARS.

The smooth vesicles fuse with the cell membrane and release the mature SARS coronavirus particles. This process might be induced by the S protein [Garoff et al., 1998; Rossen et al., 2001]. In the end, the host cells are disrupted and the virions are released.

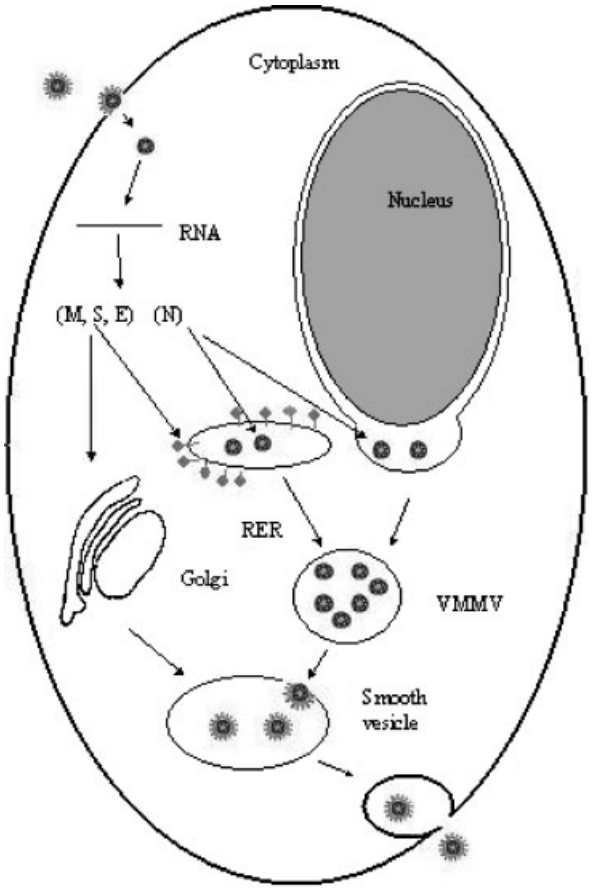


Fig. 8. Putative life cycle of SARS CoV in host cell.

It was thought that all the processes of coronavirus replication, assembly, and maturation took place in the cytoplasm [Beesley and Hitchcock, 1982; Stuart et al., 1983]; whereas, our study indicates that virus-like particles appear in the nucleus after a long time of infection. This phenomenon had not been reported previously in coronavirus studies, suggesting that the SARS coronavirus might have some morphogenetic peculiarities. The SARS coronavirus N protein (422 amino acids) aligns well with the N proteins from other representative coronaviruses. However, a short lysine rich region (KTFPPTEPKKDKKKKTDEAQ) appears to be unique to SARS. This region is suggestive of a NLS [Marra et al., 2003; Shi et al., 2003]. It is possible that the SARS CoV N protein has a novel nuclear function, which could play an important role in this phenomenon. Some other RNA viruses, for example, *Bombyx mori* cypovirus 1 has been found to exist in the nucleus [Tan et al., 2003]. Nevertheless, it is unclear whether these virus-like particles are SARS coronaviruses, since their appearance also resembles that of the perichromatin granules. To determine the nature of these particles, immune electron microscopy is essential to further study.

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