

# Early Events of SARS Coronavirus Infection in Vero Cells

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An isolate from a patient in the recent severe acute respiratory syndrome (SARS) outbreak in Singapore was used to infect Vero E6 cells. This study concentrated on the first 30 min of infection. It was discovered that the SARS coronavirus attached, entered, and uncoated the nucleocapsids, all within a 30-min period. At 5 min after infection, several virus particles lined the Vero cell plasma membrane. Virus particles were at various stages of fusion at the cell surface, since entry was not a synchronised process. After entry (10 and 15 min), spherical core particles moved into the cytoplasm within large vacuoles. Quite surprising at such early stages of infection (20 min), a virus-induced change in the infected cells was evident. The induction of myelin-like membrane whorls was obvious within the same vacuoles as the core particles. The significance of this virus-induced change is unknown at this stage. By 25–30 min postinfection (p.i.), the spherical core particles appeared to be disassociating and, in their place, doughnut-shaped electron-dense structures were observed. These could be the virus genomes together with the helical nucleocapsids. They were no longer in large vacuoles but packaged into smaller vacuoles in the cytoplasm, and occasionally in small groups. *J. Med. Virol.* 71:323–331, 2003.

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**KEY WORDS:** virus entry; SARS infection; early events

## INTRODUCTION

The recent outbreak of severe acute respiratory syndrome (SARS), starting in southern China, that then spread to Hong Kong, Vietnam, Canada, and Singapore, is caused by a newly discovered coronavirus, now called the SARS coronavirus. Sequence analyses of various isolates have indicated that although this virus shares many similarities with coronaviruses, it is genetically

distinct from all known coronaviruses [Marra et al., 2003; Rota et al., 2003; Ruan et al., 2003]. The data also suggest that this virus is likely to be an animal coronavirus, whose probable host could be civet cats, badgers, or some rodents. This virus may have been present for sometime before deletions in the genome occurs that enable it to cross from its natural host to humans [Marra et al., 2003; Rota et al., 2003; Ruan et al., 2003].

The SARS coronavirus was isolated using Vero E6 cells [Drosten et al., 2003; Ksiazek et al., 2003]. This cell line is also preferred for in vitro culture of SARS coronavirus. This virus does not grow in many other commonly used cell lines, especially those routinely used to isolate respiratory viruses, such as HeLa, rhabdomyosarcoma, Madin-Darby cell kidney (MDCK), and LLC MK2 [Peiris et al., 2003]. In our hands, virus titre of  $10^7$  plaque-forming units (PFU) per ml of cell culture supernatant can easily be attained within 24 hr of inoculation, with a starting multiplicity of infection (MOI) of  $\sim 10$ . Furthermore, no reduction of virus titre has so far been observed despite multiple passages (unpublished data, E.E. Ooi & E.E. See). Since the SARS coronavirus appeared to favour Vero E6 cells compared to the other cell lines, we sought to examine the early microscopic events of SARS coronavirus infection in Vero E6 cells.

## MATERIALS AND METHODS

### Cell and Virus

The SARS coronavirus (2003VA2774) was isolated from a SARS patient in Singapore by the Department of Pathology, Singapore General Hospital. The virus was grown up in Vero E6 cells (ATCC: C1008) in the Environmental Health Institute, Singapore. The virus was

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harvested at 24 hr p.i. The titre of this stock virus was  $1 \times 10^7$  PFU/ml and was used to infect new cell monolayers for this study.

### Electron Microscopy

The Vero cell monolayers were infected with the SARS coronavirus for 5, 10, 15, 20, 25, and 30 min. At the end of each period, the cells were fixed with 2% glutaraldehyde and 2.5% paraformaldehyde for 4 hr. At the end of this fixation period, the infected monolayer was washed with cold phosphate-buffered saline (PBS). This was followed by postfixation with 1% osmium tetroxide before the cells were dehydrated with a series of ethanol of ascending percentages. The cells were embedded in low-viscosity epoxy resin, polymerised before ultramicrotomy was performed. The ultrathin sections were stained with 2% uranyl acetate and were postfixed with 2% lead citrate before viewing under electron microscopy (CM 120 BioTwin, Philips, The Netherlands). The images were captured digitally with a MultiScan 792 digital camera (Gatan, USA). The ultrastructural studies were performed in the Electron Microscopy Unit, Faculty of Medicine, National University of Singapore.

## RESULTS

### Virus Entry

At 5 min after the SARS coronavirus was exposed to Vero E6 cells, several virus particles were seen attaching onto the plasma membrane (Fig. 1). In Figure 1a, two virus particles (arrow) were in the process of fusing with the cell plasma membrane. The virus envelope has fused with the plasma membrane (arrowhead). At the surface of another cell, three virus particles at different stages of attachment and fusion were observed (Fig. 1b). There were regions of thickened membrane (Fig. 1c, arrow) indicating areas in which the virus envelope had fused with the plasma membrane.

Within the first 10 min, some virus particles were internalised into vacuoles (arrow) that were just below the plasma membrane surface (Fig. 2, arrows). The particles were without envelopes and most likely were the virus cores. Unlike the pleomorphic appearance of the enveloped form, these core particles were of consistent size (50–70 nm in diameter). They were clearly seen (arrowhead) within the vacuoles (arrow) at higher magnification in Figure 2b. Since entry was not a synchronised process, other virus particles (Fig. 2a, arrowheads) were still at the fusing stage at the plasma membrane.

In some cells, numerous virus particles were lined up next to each other and in the process of fusing, along a segment of the plasma membrane (Fig. 3a, arrows).

At higher magnification (Fig. 3b), structures resembling empty envelopes were seen lining the plasma membrane (arrows). In addition, many virus spherical core particles enclosed in large vacuoles (arrowhead)

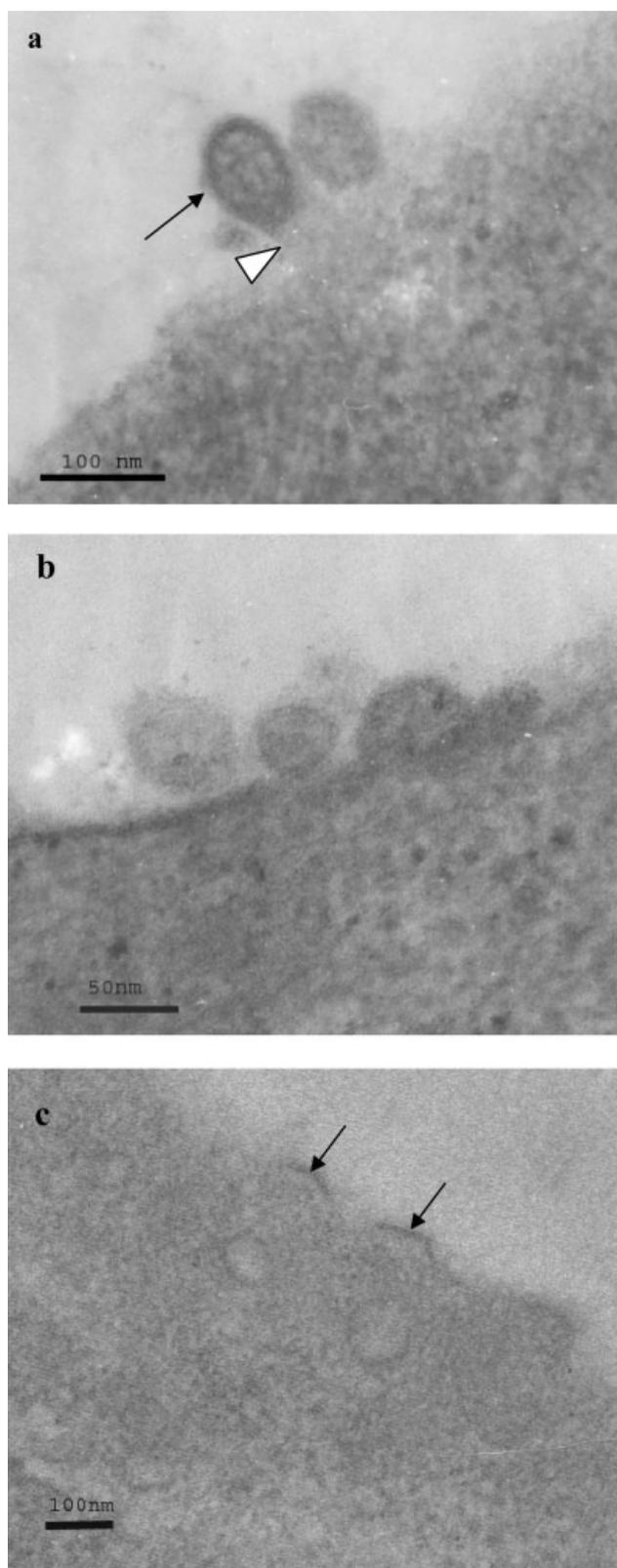


Fig. 1. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 5 min. **a:** Two virus particles (arrow) are in the process of attaching and fusing with the cell plasma membrane. The region of fusion is indicated with an arrowhead. **b:** In another cell, three virus particles all at different stages of fusion can be seen. **c:** In some occasions, the fused virus envelopes are seen as thickened regions (arrow) in the plasma membrane.

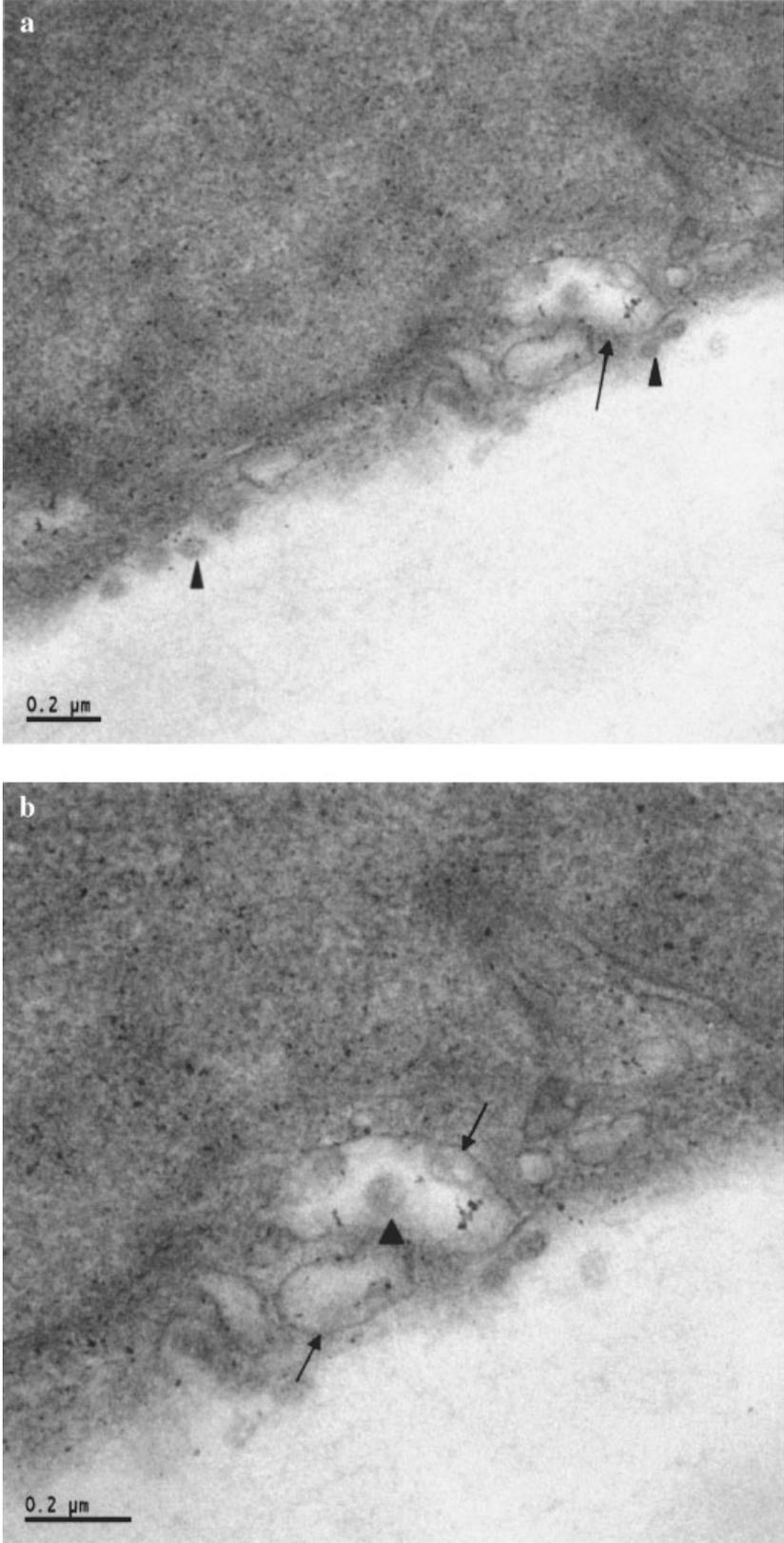


Fig. 2. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 10 min. **a:** Some virus particles are internalised in large vacuoles (arrow), whereas virus particles remain (arrowheads) at the attachment and fusion stages on the cell surface. **b:** At higher magnification, the internalised particles no longer have envelopes but are 50–70-nm spherical core particles (arrowhead).

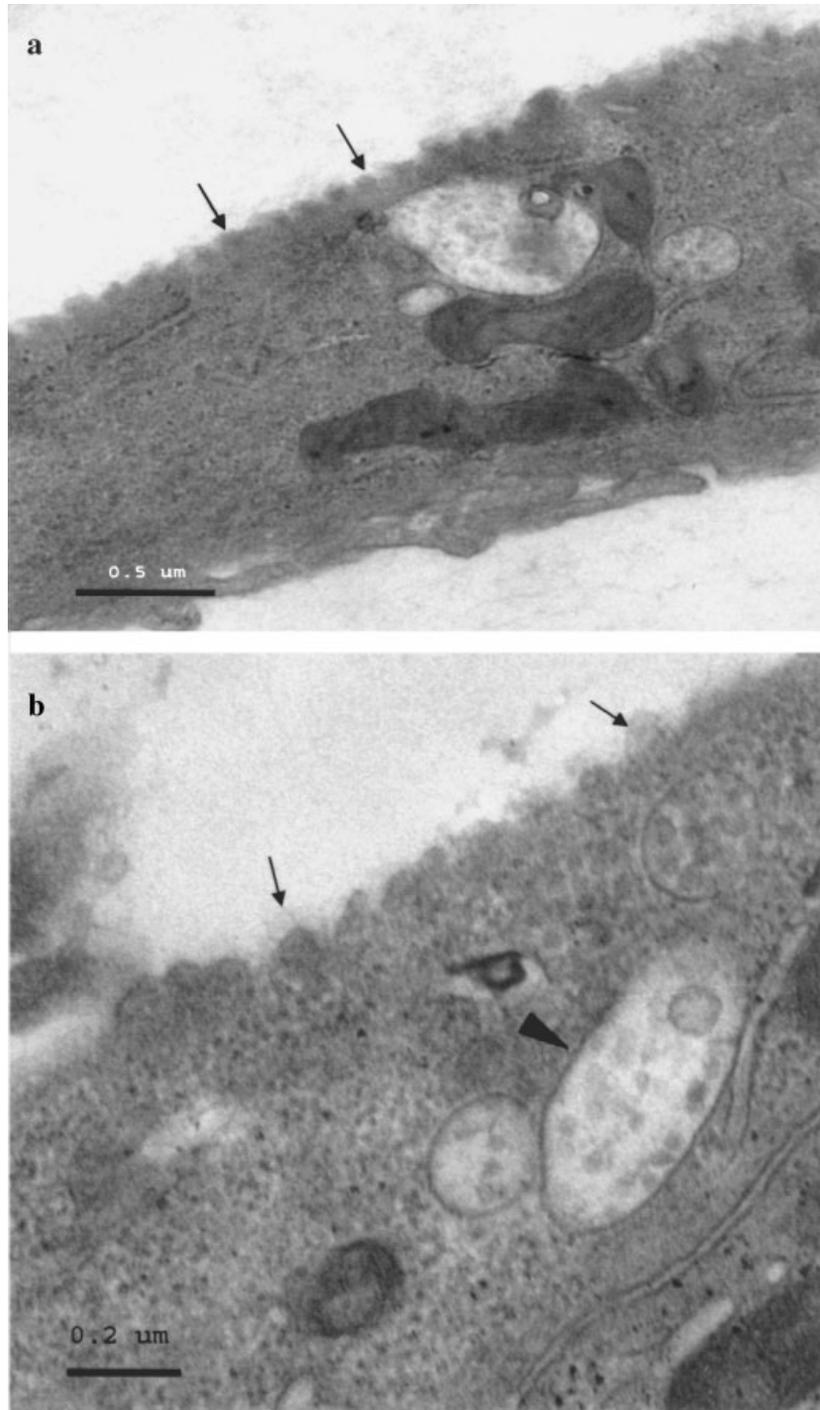


Fig. 3. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 10 min. **a:** Many virus particles (arrows) are able to infect one cell. These virus particles line up close to each other on the cell surface. **b:** Electron micrograph at higher magnification clearly shows the virus particles (arrows) lining the cell surface. Large vacuoles (arrowheads) containing the numerous internalised-core particles have moved deeper into the cytoplasm.

were at the cell periphery (within the cytoplasm). When compared to Figure 2, these vacuoles had moved deeper into the cytoplasm.

The observation at 15 min postinfection (p.i.), did not differ much from 10 min p.i. (Fig. 4a). At this magni-

fication, the empty envelopes (arrow) left behind at the plasma membrane after virus internalisation were very obvious. Again, a vacuole containing core particles (arrowhead) was seen at the cell periphery. Some of the spherical core particles appeared to have disassociating

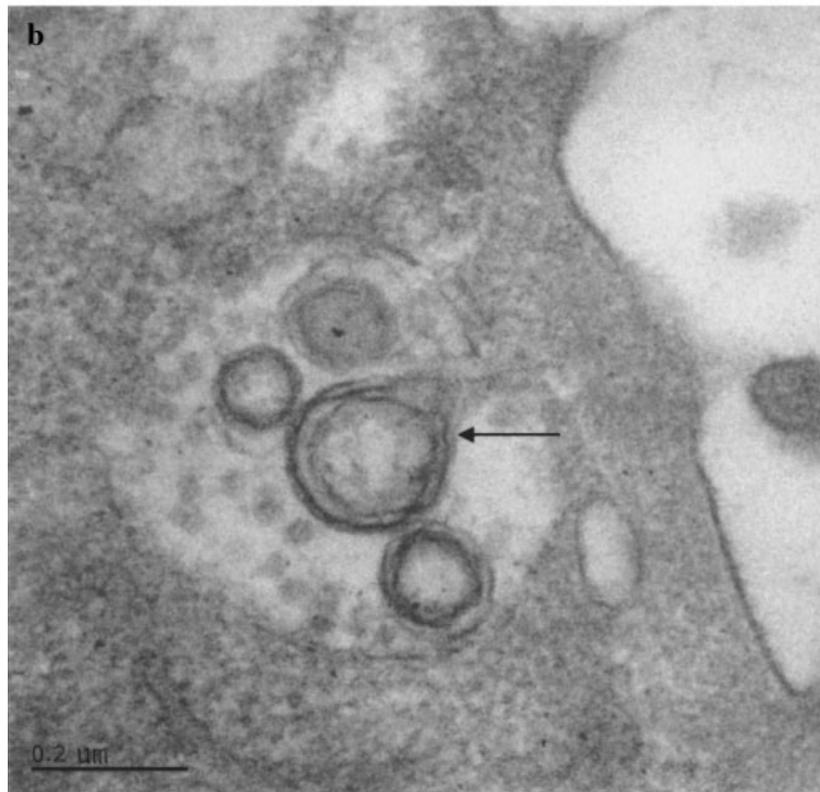
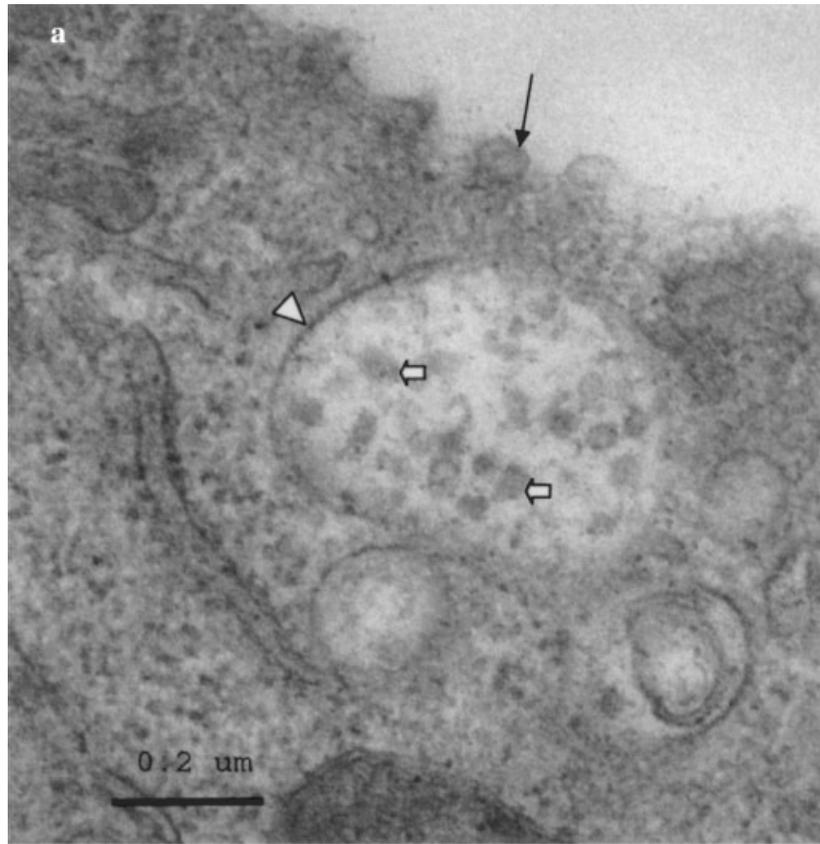


Fig. 4. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 15 min. **a:** Empty virus envelopes (arrow) are often seen still attached to the cell surface. A large vacuole (arrowhead) containing the spherical cores, shows that some of these cores appear to be disassociating (thick arrows). **b:** A consistent virus-induced change is the presence of myelin-like membrane whorls (arrow) within the same vacuole as the spherical core particles.

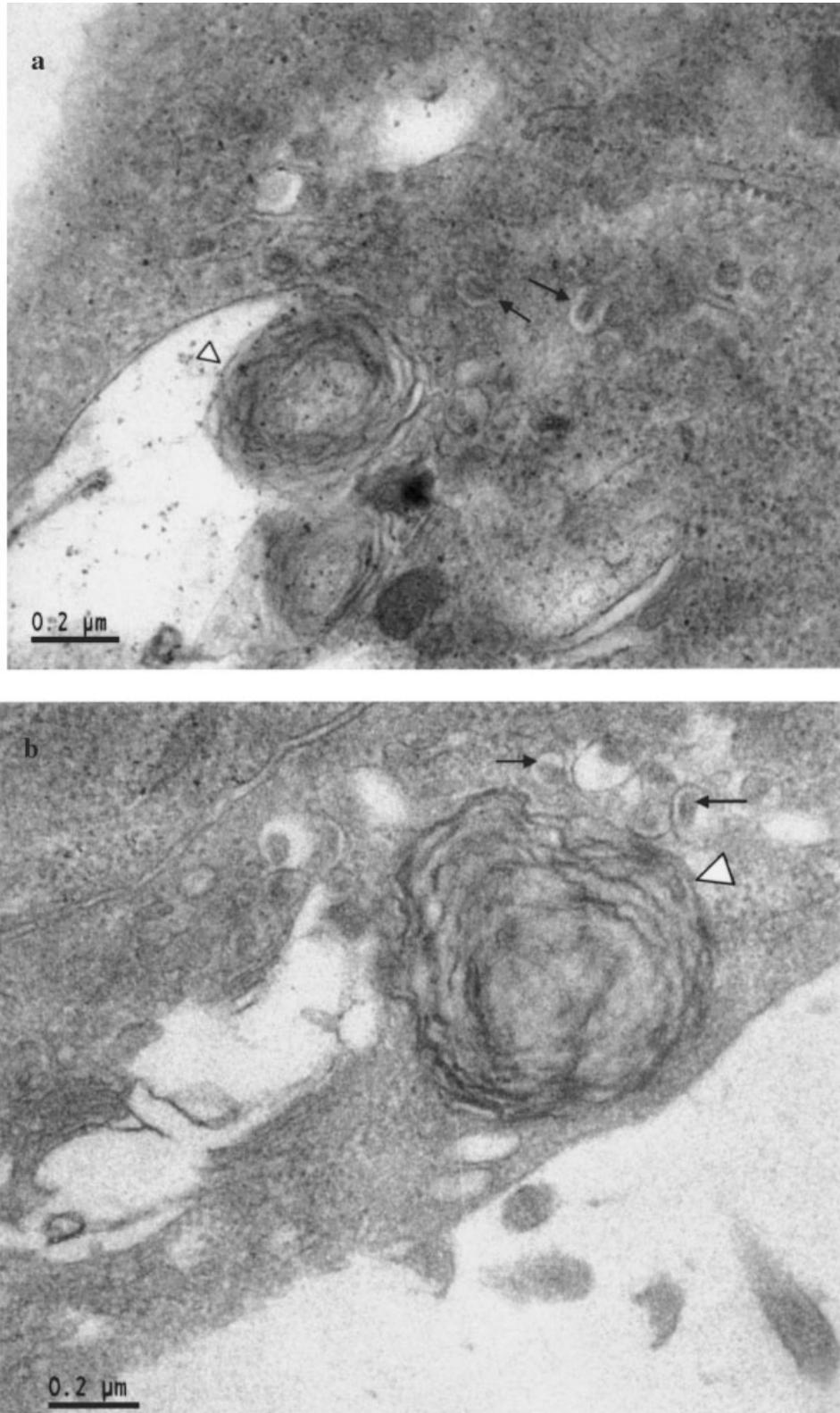


Fig. 5. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 25 min. **a:** At this stage, the spherical core particles are not obvious but instead doughnut-shaped electron-dense structures (arrows) are present. These could be the virus genome with the associated proteins. They now appear to be single and enclosed

in small vacuoles scatter in the cytoplasm, but still in close association with the membrane whorls (arrowhead). **b:** Higher-magnification electron micrograph showing the doughnut-shaped electron-dense virus genomes (arrows) clearly within small vacuoles. These vacuoles localise around the membrane whorls (arrowhead).

(thick arrows). At 20 min, an interesting myelin-like membrane structures developed within the same vacuoles as the core particles (Fig. 4b, arrow). However, the significance of this is currently unknown.

The virus-induced ultrastructure changes in the infected cells at 25 min (Fig. 5) and at 30 min (Figs. 6 and 7) were very similar. The noticeable difference to the earlier timing was, most of the core particles have

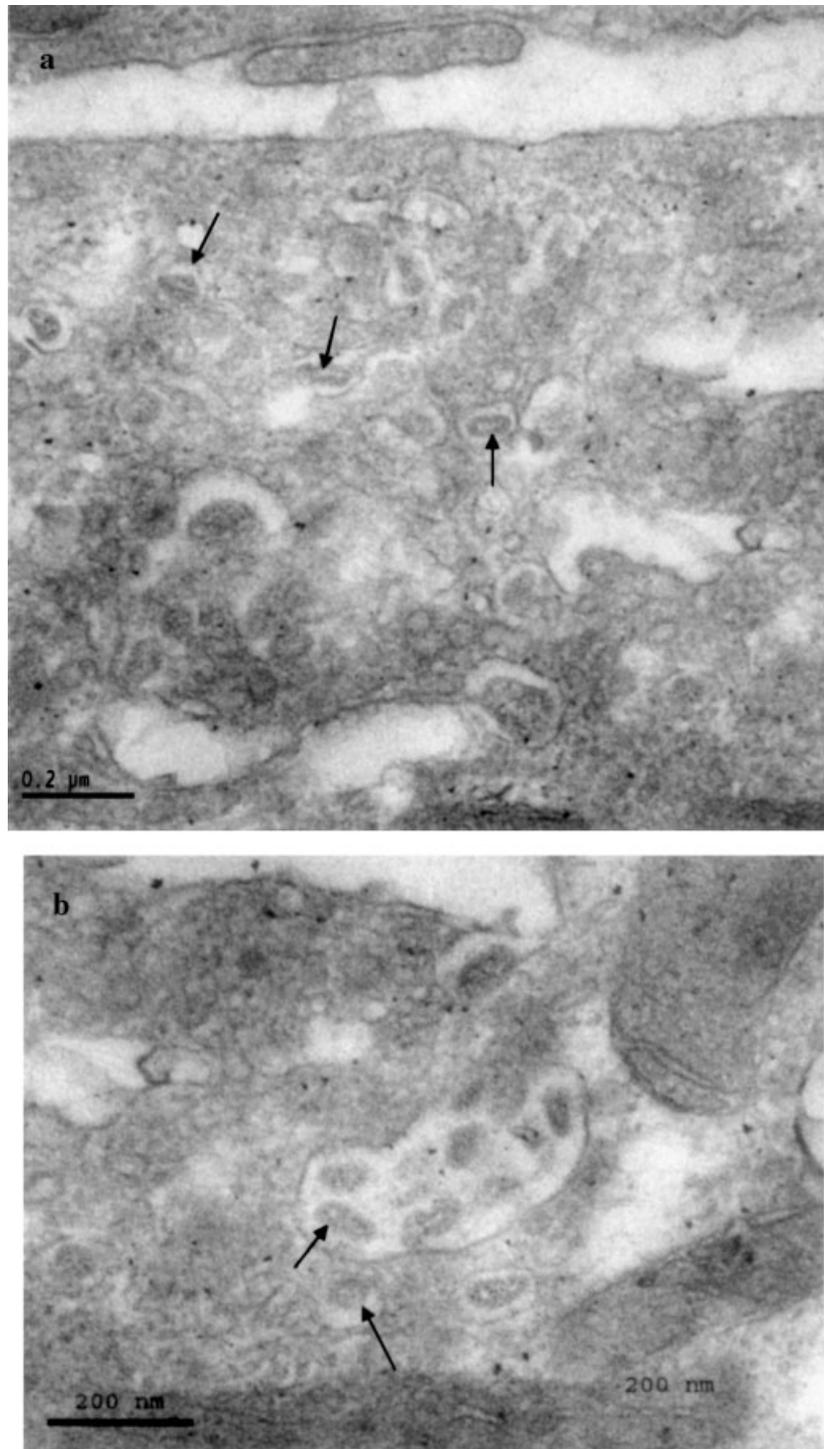


Fig. 6. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 30 min. **a:** The ultrastructure of an infected cell shows similar observations as in the 25-min infection. Individual doughnut-shaped electron-dense genomes (arrows), in small vacuoles are commonly seen in the infected cytoplasm. **b:** Occasionally, a small group of these electron-dense structures (arrows) can be seen within one vacuole as well.

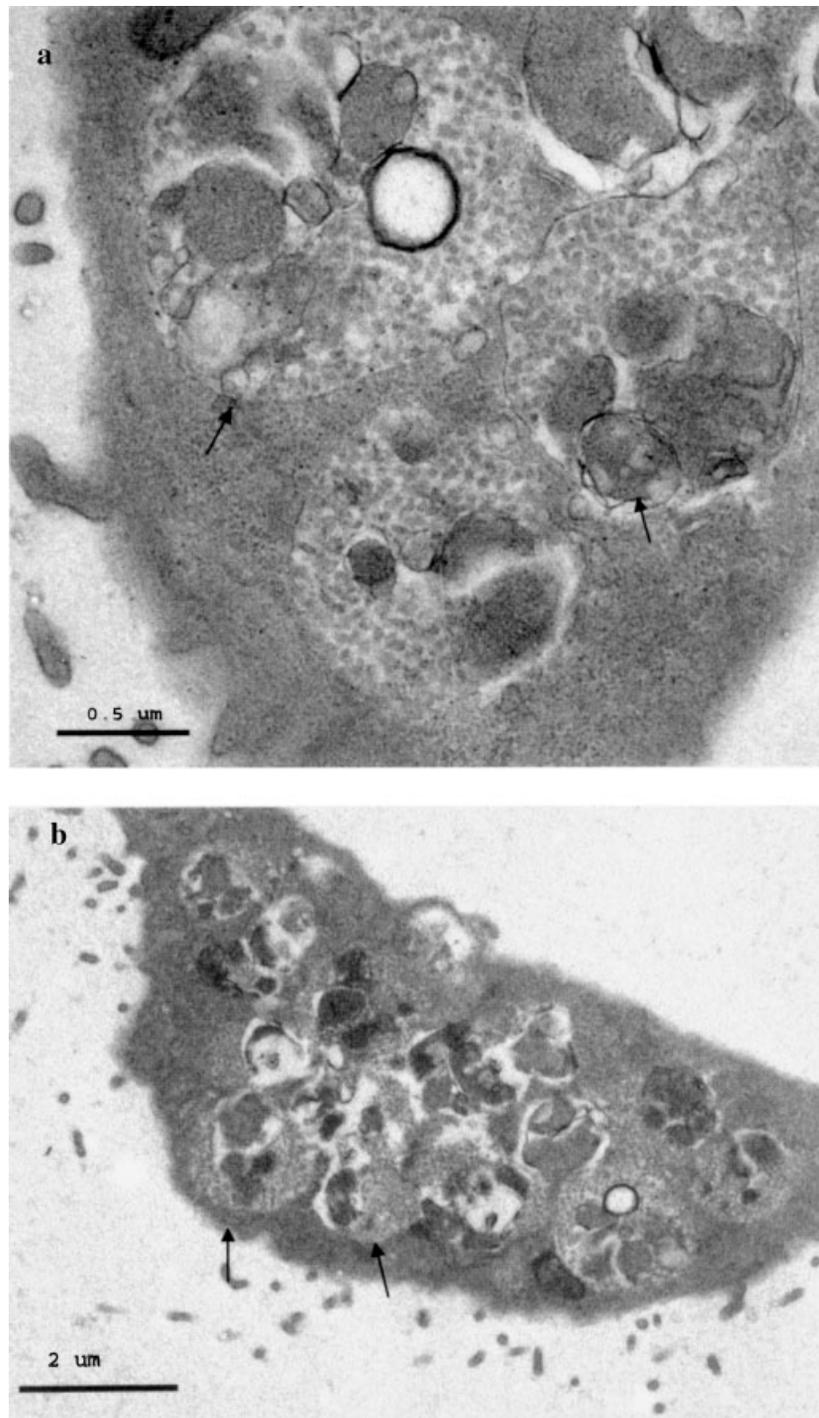


Fig. 7. Vero cells infected with recent severe acute respiratory syndrome (SARS) coronavirus for 30 min. **a,b:** In some cells, the cytoplasm contains several of the large vacuoles (arrows) with spherical core virus particles and membrane whorls.

discarded the spherical coats revealing doughnut-shaped electron-dense structures (Fig. 5a,b, arrows). These structures are most likely the virus genomes with the associated helical nucleocapsids. They were no longer seen in large vacuoles as in the earlier timing (Figs. 2–4). Instead, most of these electron-dense

circular structures were seen singly in small vesicles in the cytoplasm (arrows). They were also closely associated with the myelin-like membrane whorls (arrowheads).

The doughnut-shaped electron-dense structures (Fig. 6, arrows) were clearly scattered in the cytoplasm.

Most of these structures were now individually enclosed. However, at times, small vacuoles enclosing a few such structures could be seen (Fig. 6b, arrows).

Just after 30 min into infection, the dramatic ultra-structural changes seen in a few of the infected cells (Fig. 7, arrows) were surprising, as this was still very early in the replication cycle of the virus. Large vacuoles containing spherical core particles in association with myelin-like structures were common sights. A low magnification (Fig. 7b) micrograph gave an overview of a cell that the cytoplasm was filled with several of such vacuoles.

## DISCUSSION

Electron microscopy with negative staining was used to determine the identity of this causative agent of SARS [Drosten et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003], but there are no reports on the early cellular events with SARS coronavirus infection. This is the first detailed documentation on the entry events of SARS coronavirus in Vero cells. Samples were collected at 5-min intervals over a period of 30 min. This study demonstrated that the process of virus attachment, internalisation, and uncoating of the core particles occurred within a 30-min period. The virus entered the cells by fusing part of the virus envelope protein with the plasma membrane (Fig. 1). Numerous virus particles were able to infect the same cell, although internalisation was not synchronised (Figs. 2 and 3).

The internalised core particles were packed into large vacuoles and subsequently were transported into the cytoplasm (Figs. 2–4). These core structures then cast off the spherical cores revealing doughnut-shaped electron-dense genomes with their helical nucleocapsids (Figs. 5 and 6). At this stage, the genomes were no longer found in the large vacuoles, but most were seen singly within smaller vacuoles (Fig. 6), scattered within the cytoplasm.

An interesting observation is that this entry process was closely associated with the induction of a series of myelin-like membrane whorls within the same large vacuoles. This membrane structure was seen as early as 20 min after infection (Fig. 4). The close association would indicate that the membrane whorls could have some role in the very early event of virus replication

(Figs. 4 and 5). However, in the present microscopic study, the role was not deciphered.

Perhaps the infection process should be followed beyond 30 min, in an effort to understand the functional role of the membrane whorls in SARS coronavirus infection. A longer period of study would also be beneficial to follow the assembly process of the progeny virus particles.

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