# Detection of Severe Acute Respiratory Syndrome–Associated Coronavirus in Pneumocytes of the Lung

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## Abstract

*Previous reports have indicated that patients with severe acute respiratory syndrome (SARS)–associated coronavirus infection could develop atypical pneumonia with fulminant pulmonary edema. However, the target cells of SARS viral infection have not been characterized in detail. We report the pathologic findings of the lung in 3 cases of SARS. Chest radiographs at 2 to 3 weeks of infection revealed an atypical pneumonia with pulmonary consolidation, a clinical characteristic of SARS infection. The presence of the SARS virus was determined by nested reverse transcription–polymerase chain reaction (RT-PCR), and the infected cells were identified by in situ hybridization in open-lung biopsy and postmortem necropsy specimens. Expression of SARS virus–encoded RNA was detected in all 3 cases by RT-PCR, and the SARS viral signal was localized in pneumocytes by using in situ hybridization.*

Severe acute respiratory syndrome (SARS) is an acute infectious disease that affects primarily the lower respiratory tract, with clinical manifestations of atypical pneumonia with dry cough, persistent fever, progressive dyspnea, and, sometimes, the abrupt deterioration of lung function[1-5] and the ensuing oxygen deprivation–associated systemic organ failures.[6,7] At this stage, the disease can be lethal.

Postmortem pathologic examination showed that fulminant pulmonary interstitial infiltrate, substantial pulmonary edema, and extensive pulmonary consolidation with alveolitis, formation of hyaline membrane, and the presence of desquamated alveolar epithelial cells, which corresponded well with the progression of clinical symptoms, frequently were observed.[4,7] An elegant study by Ksiazek et al[4] further demonstrated that in addition to the dispersed alveolar epithelial cells, foamy macrophages and multinucleated giant cells were abundant in the damaged alveolus as well. Inoculation with bronchoalveolar lavage fluid from these patients can induce not only cytopathic changes of Vero E6 cells, but also formation of multinucleated syncytial cells, an index of viral infection. The subsequent analysis of extracellular particles from the supernatant of cytopathically altered Vero E6 cells by negative-stain electron microscopy revealed the characteristic coronavirus particle.[4] However, the results of nucleotide sequence alignment indicated that these isolates were quite different from any known coronavirus,[1-5] a positive single-stranded RNA virus.[8]

Because the virus is SARS infection–specific, it was named SARS-associated coronavirus, or SARS virus. Nevertheless, the target cells of SARS viral infection and the essence of multinucleated giant cells are not well characterized.

In the present study, we used nested reverse transcription–polymerase chain reaction (RT-PCR) for the immediate determination of SARS viral infection, and the presence of the SARS viral signal was identified by using in situ hybridization.

## Materials and Methods

### Cases

Three patients with SARS infection were identified in the China Medical University Hospital, Taichung, and the National Taiwan University Hospital, Taipei, Taiwan, from March to May 2003.

Case 1 was a 55-year-old man, who had fever and dry cough 5 days after his return from Guangdong, China. Six days later, the symptoms of severe respiratory distress developed, and the man was admitted immediately to the hospital. Owing to his critical condition, endotracheal intubation was undertaken on admission, and open lung biopsy was performed to resolve the cause of pulmonary distress 7 days after there was no response to palliative treatment. The treatment did not include antiviral or steroid regimens. Interestingly, the patient's condition improved, and the endotracheal tube was removed 6 days after surgery. The patient recovered substantially and was discharged 3 weeks later.

Case 2 was a 56-year-old man in whom fever and dry cough developed 4 days after the visit by a relative from Hong Kong, China. Three days later, severe respiratory symptoms ensued, and he was admitted to the hospital. The patient responded poorly to combination therapy with steroids and ribavirin,[6] and his condition became critical 4 days after admission. Although an endotracheal tube was inserted when his condition became critical and the use of a mechanical ventilation device was initiated in a week, the patient died 3 days later.

Case 3 was a 36-year-old woman in whom fever and dry cough developed 5 to 6 days after accidental contact in a SARS-infested hospital. Two days later, the patient developed symptoms of severe respiratory distress and was transferred immediately to a SARS specialty hospital. Nevertheless, the patient did not respond to the combination therapy with steroids, ribavirin, and intravenous immunoglobulin, and her condition worsened 5 days after admission. Although endotracheal intubation and a mechanical ventilation device were used to attempt to improve the pulmonary condition, the patient died in 4 days.

### Methods

The presence of the SARS virus in biopsy samples was determined primarily by using nested RT-PCR.[1-5] The RNA in situ hybridization procedure was described previously.[9] Briefly, a 4-μm section from a paraffin-embedded tissue sample was deparaffinized in xylene, dehydrated, and predigested with 0.1 mg/mL of nuclease-free Proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 minutes. The slide was washed with distilled water, rinsed with 70% ethanol, and air dried. Fluorescein isothiocyanate–conjugated antisense probe (250 ng/mL in 50% formamide, 6× standard saline citrate, and 0.25% dry milk) to different regions of the SARS viral genome was placed over each tissue section. The probes ▌Table 1▐ were synthesized by Mission Biotech (Taipei, Taiwan), and the sequence was according to GenBank/AY278490.3.

The sample was denatured at 75°C on a thermal plate for 5 minutes and then moved to a moist chamber at 37°C for 2 hours. Following extensive washing with 2× standard saline citrate, the hybridization product was detected by using anti–fluorescein isothiocyanate antibodies conjugated with alkaline phosphatase (DAKO A/S, Copenhagen, Denmark). The chromogenic development was processed in a mixture of 4-nitroblue tetrazolium and

▌Table 1▐
**Probe Sequences for Localizing the Severe Acute Respiratory Syndrome Virus**

| Probe | Sequence | Nucleotides |
|-------|----------|-------------|
| REP1 | 5'FITC–AGTGTTATACCGCTACGACCGTACTGAATGCC–3' | 571–540 |
| REP2 | 5'FITC–CGAGTGAGTTCACGGAGTGCACCACTGCCATG–3' | 784–753 |
| REP3 | 5'FITC–TGCTCATGGTCACGGCAGCAGTAGACACCTCTCTTCG–3' | 967–931 |
| REP4 | 5'FITC–GTCTTGACAGGCAGGACATGGCATTTTCACTACAGCATT–3' | 1385–1347 |
| REP5 | 5'FITC–TTCACGACTCAGTATCTCAAGGAGATCCTCATTCAAGGTC–3' | 1631–1592 |
| M1 | 5'FITC–TTAAGCTCCTCAACGGTAATAGTACCGTTGTCTGC–3' | 26431–26397 |
| M2 | 5'FITC–AATCGCAATCCCGCCAGTCACCCAATTAATTCTGTAGACA–3' | 26636–26597 |
| M3 | 5'FITC–ATGACAAGTTCACTTTCCATGAGCGGTCTGGTCACAA–3' | 26809–26773 |
| N1 | 5'FITC–GTAGCCAATTTGGTCATCTGGACCACTATTGGTGTTG–3' | 28376–28340 |
| N2 | 5'FITC–CAGTTTCACCACCTCCGCTAGCCATTCGAGCAGGAGAA–3' | 28770–28733 |
| N3 | 5'FITC–TTGTGCAATTTGCGGCCAATGTTTGTAATCAGTTCCTTGT–3' | 29036–28997 |

FITC, fluorescein isothiocyanate; M, membrane protein-encoding area of the SARS viral genome; N, nucleic acid binding protein–encoding sequences of the SARS viral genome; REP, replicase-encoding region of the SARS viral genome.

5-bromo-4-chloro-3-indolphosphate (Boehringer Mannheim). The slide was counterstained with methyl green. Positive staining was recognized under the microscope as brownish purple granules. Probes to other viruses, eg, Epstein-Barr virus, type I human T-cell lymphotropic virus, cytomegalovirus, enterovirus 71, and parvovirus B19,[9-11] that were used in previous studies were included to determine the specificity of the SARS viral probes. A specimen of nasopharyngeal carcinoma with positive serologic results for the Epstein-Barr virus was used as a positive control for the in situ hybridization procedure. Paraffin-embedded sections of normal counterpart of the lung and lung specimens from patients with lung cancer or acute respiratory distress syndrome (ARDS) were used as negative controls.

Immunohistochemical characterization of CD68 was performed on paraffin-embedded sections of biopsy and necropsy tissues by using an LSAB method (DAKO, Carpinteria, CA).[9] The chromogenic reaction was visualized by peroxidase-conjugated streptavidin and aminoethylcarbazole (Sigma, St Louis, MO). Slides were counterstained with Mayer hematoxylin or methyl green, and positive staining was recognized under a microscope as crimson granules.

## Results

SARS viral infection was detected preliminarily by using RT-PCR in affected lung tissue samples from open lung biopsy (case 1) and necropsies (cases 2 and 3) ❚Table 2❚. SARS viral RNA, however, was not detected in peripheral blood samples. Histopathologically, in addition to the presence of various degrees of alveolar deformation and tissue fibrosis in the infected lung, cells with enlarged cytoplasm frequently were detected ❚Image 1A❚ and ❚Image 1B❚. The number of enlarged cells increased with the progression of disease severity. Moreover, in the acute phase (case 3), these enlarged cells (Image 1A) resembled alveolar macrophages and, possibly, activated type II pneumocytes ❚Image 1C❚.

In the chronic phase (case 2), the cytoplasm of these cells became highly vacuolated and, sometimes, laminated (Image 1B). The nuclei, however, became more convoluted. Further characterization showed that these cells were not immunoreactive to CD68, a bona fide marker of activated histiocytes ❚Image 2A❚, and suggested that, alternatively, they might be of pneumocyte origin, in particular, the type II pneumocyte. By using antisense oligonucleotide probes specific to the nucleic acid binding protein–encoding (N) and membrane protein-encoding (M) regions of the SARS viral genome, the SARS viral signal was detected in these enlarged cells ❚Image 2B❚. No signal to other viral probes was detected in these enlarged cells (data not shown). In

❚Table 2❚
**Clinical Features, RT-PCR Results, and Survival Conditions for Three Patients With SARS**

| Case No./Sex/Age (y) | Chest Radiographic Findings | RT-PCR Result | Condition |
|---|---|---|---|
| 1/M/55 | Atypical pneumonia | + | Alive |
| 2/M/56 | Atypical pneumonia | + | Died |
| 3/F/36 | Atypical pneumonia | + | Died |

RT-PCR, reverse transcription–polymerase chain reaction; SARS, severe acute respiratory syndrome; +, positive.

addition, the viral signal was not detected in the neighboring fibroblasts, capillary endothelial cells, or bronchial epithelial cells ❚Image 2C❚. Nevertheless, when antisense oligonucleotide probes specific to the replicase-encoding (REP) region of the SARS viral genome were used to determine the presence of virus, the intensity of the in situ hybridization signal decreased substantially compared with that detected by probes to the N and M regions ❚Image 2D❚ ❚Table 3❚.

Interestingly, liver cells were negative for the SARS viral signal; so were muscle cells, myocardial cells, endothelial cells, and splenocytes. The obvious local inflammation was found only in the lung tissues. Although marked inflammation was observed in the infected tissues, transudate and exudate were found mainly around epithelial cells that were positive for the SARS viral signal ❚Image 3A❚. No transudate or exudate was identified in the neighboring regions, which had abundant macrophages ❚Image 3B❚.

## Discussion

By detecting the viral signal in diseased lung tissue by combining RT-PCR and in situ hybridization, we found that these 3 patients had contracted SARS viral infection during the epidemic outbreak of SARS in Taiwan. Although an infection-associated pathologic lesion was shown basically in every part of the lung by radiographs, pathologic evaluations showed that mortality-related tissue damage was located mainly in the lower respiratory tract[4,7]; this finding corresponded well not only with our observation that the SARS virus infected mainly pneumocytes (in particular, type II pneumocytes that are major sources of surfactant, the essential element to maintain the normal function of the alveolus),[12] but also with the progression of clinical symptoms.

In fact, in an elegant review, Ware and Matthay described in detail the symptoms of ARDS,[12] which are quite similar to those found in patients with SARS viral infection, such as alveolar filling, pulmonary consolidation, and atelectasis revealed by radiographs and the presence of diffuse alveolar deformation, alveolar exudate, hyaline membrane, and tissue fibrosis detected by pathologic
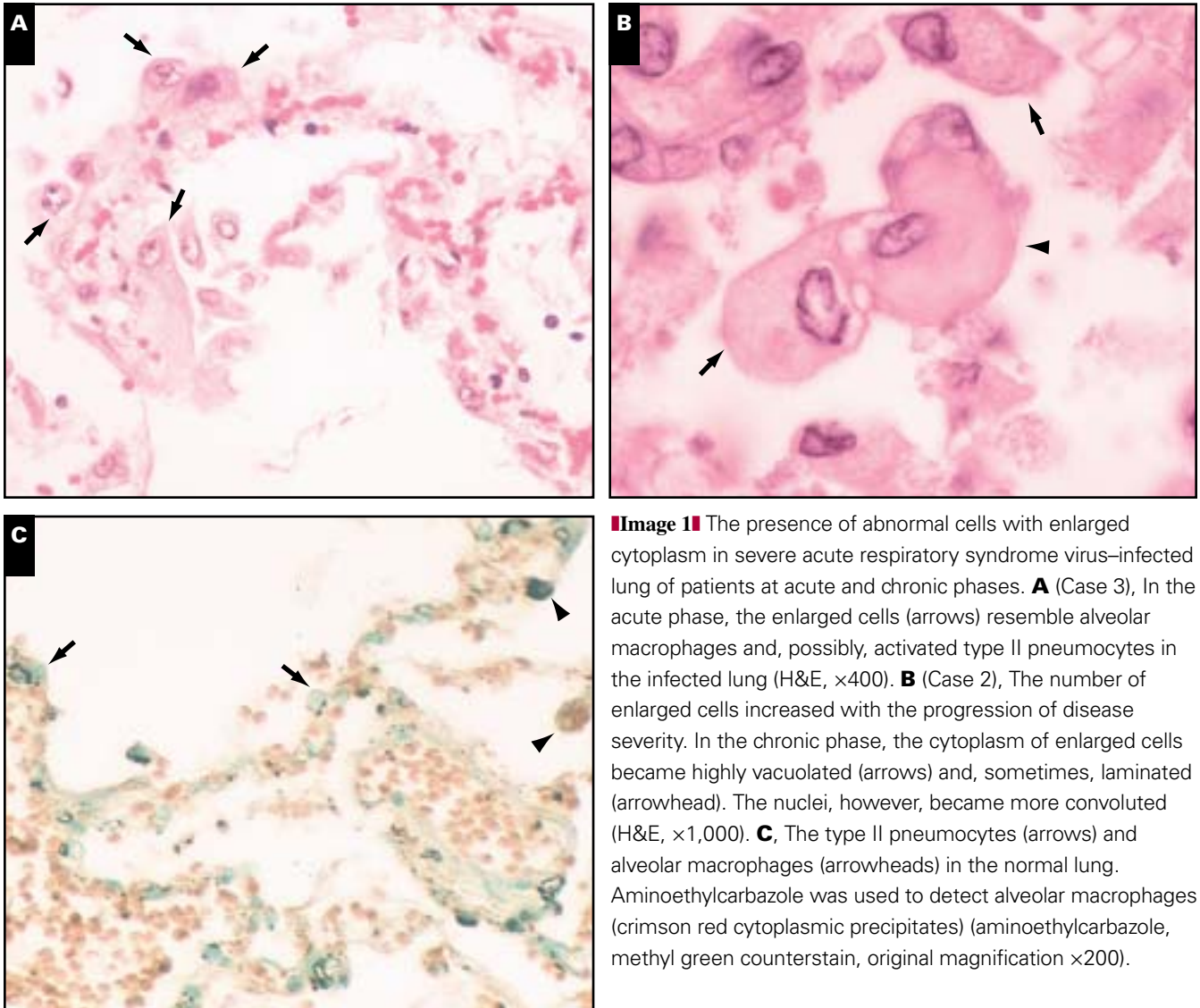
■**Image 1**■ The presence of abnormal cells with enlarged cytoplasm in severe acute respiratory syndrome virus–infected lung of patients at acute and chronic phases. **A** (Case 3), In the acute phase, the enlarged cells (arrows) resemble alveolar macrophages and, possibly, activated type II pneumocytes in the infected lung (H&E, ×400). **B** (Case 2), The number of enlarged cells increased with the progression of disease severity. In the chronic phase, the cytoplasm of enlarged cells became highly vacuolated (arrows) and, sometimes, laminated (arrowhead). The nuclei, however, became more convoluted (H&E, ×1,000). **C**, The type II pneumocytes (arrows) and alveolar macrophages (arrowheads) in the normal lung. Aminoethylcarbazole was used to detect alveolar macrophages (crimson red cytoplasmic precipitates) (aminoethylcarbazole, methyl green counterstain, original magnification ×200).

examination. Disease-related mortality, like that of patients with SARS, also correlated with advanced tissue fibrosis of the lung and, possibly, persistent hypoxemia and oxygen deprivation–related organ failure.[6,12]

It therefore is worth noting that abnormalities of the production and, probably, the composition of surfactant could be attributed mostly to the dysfunction of gas exchange, alveolar deformation, and oxygen deprivation–mediated systemic organ failure in patients with ARDS or SARS.[6,12] Moreover, based on the characteristics of positive-strand RNA genome, replication mode, and infection route,[4,8] the SARS virus would prefer infecting cells that have a membrane receptor for the virus and are ready for protein synthesis, ie, type II pneumocytes of the lung. Our pathologic findings supported such speculation by showing that the SARS virus could infect mainly type II cells and the infected type II cell might not only reduce the production of bona fide surfactant but also

increase the expression product of the viral protein.[8,13] Interestingly, amino acid sequences further indicated that the SARS viral protein might contain several potential chemokine-like motifs,[14] in particular, within the replicase polyprotein 1ab region. (The amino acid sequence was according to GenBank/P59641.) The potential chemokine-like motifs are listed in ■**Table 4**■.

As noted, chemokines, a superfamily of small (about 80% of the mature chemokines consist of only 66-78 amino acid residues) cytokines, are chemoattractants and activators of specific types of leukocytes.[14] Most of the family members have at least 4 conserved cysteine residues that can form 2 intramolecular disulfide bonds, and, based on the position of the first 2 cysteine residues, these chemokines are categorized into the CXC subfamily (α) that has 1 amino acid residue between the first 2 conserved cysteines and the CC subfamily (β), in which the first 2 cysteine residues are
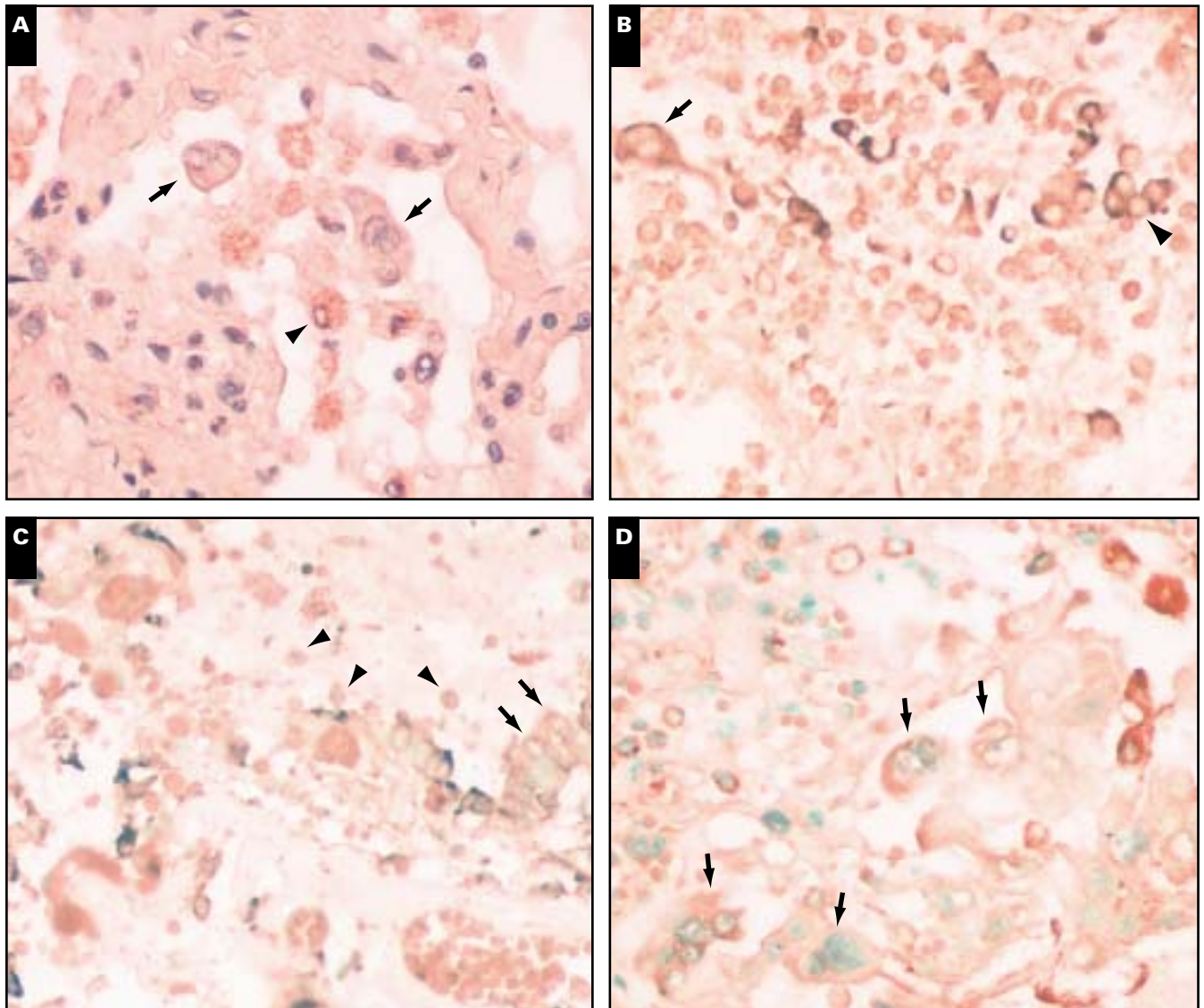
**▌Image 2▌** Characterization of the enlarged cells by immunohistochemical and in situ hybridization methods. **A**, The enlarged and multinucleated cells (arrows) were not immunoreactive to CD68, a bona fide marker of activated macrophage (a representative cell is indicated by the arrowhead). CD68 expression was detected by immunohistochemical analysis (CD68, hematoxylin counterstain, original magnification ×400). **B**, By using in situ hybridization and antisense oligonucleotide probes specific to the nucleic acid binding protein–encoding (N) and membrane protein–encoding (M) regions of the severe acute respiratory syndrome (SARS) viral genome, the SARS viral signal was detected in the enlarged and multinucleated cell (arrow, purple blue precipitates). The viral signal also was detected in a cluster of 4 oval-cuboidal cells (arrowhead) and suggested that they might be of pneumocyte origin, in particular, the type II pneumocyte. **C**, The viral signal was not detected in the neighboring fibroblasts, capillary endothelial cells, activated macrophages, foamy macrophages (arrowheads), or bronchial epithelial cells (arrows). **D**, When antisense oligonucleotide probes specific to the replicase-encoding region of the SARS viral genome were used to determine the presence of virus, the intensity of the in situ hybridization signal in enlarged and multinucleated cells (arrows) decreased substantially, to about 1/1,000 of that detected by the probes to the N and M regions (compared with the intensity of the viral signal in Image 2B). **B-D**, Following localization of the SARS viral signal by in situ hybridization, immunohistochemical analysis with CD68 was used to identify macrophages (**B-D**, CD68, methyl green counterstain; **B**, ×200; **C**, ×200; **D**, ×400).

adjacent to one another. The CXC chemokines are chemotactic for neutrophils and lymphocytes. On the other hand, the CC chemokines are chemoattractive only for monocytes and lymphocytes, not neutrophils.[15-17] In fact, the profuse infiltrate of macrophages in the infected lung and the atrophic white pulp of the spleen considered together with the lack of lymphocytes in the damaged lung clearly indicate that SARS viral infection–specific product(s), which
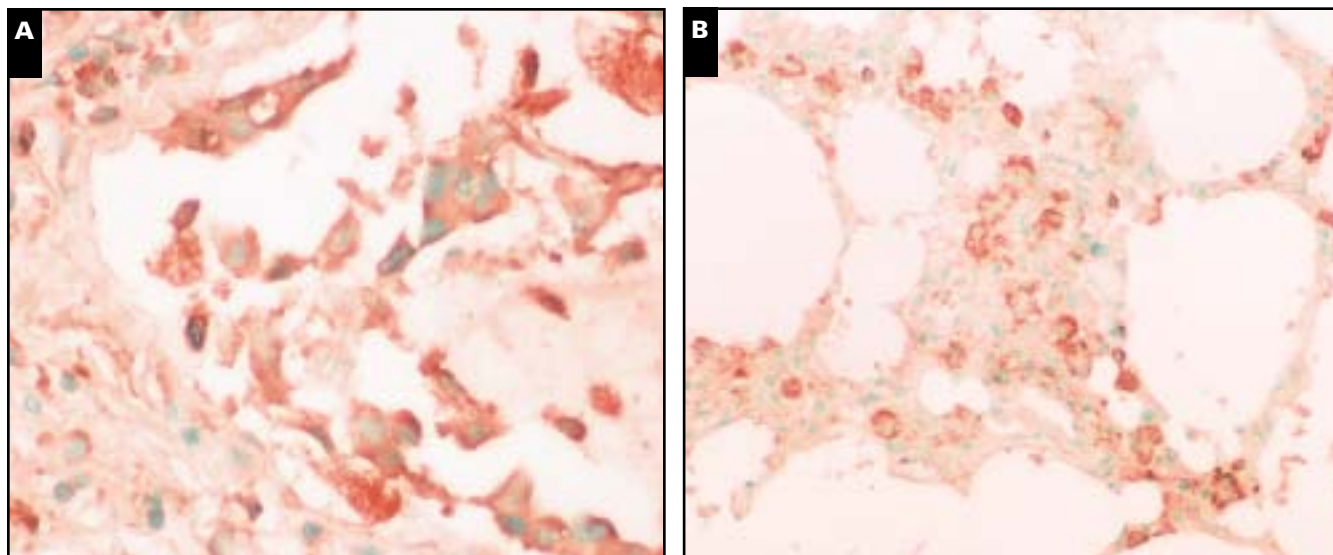
**Image 3** The severe acute respiratory syndrome (SARS) virus–infected alveolus and the neighboring macrophage-infiltrated region. **A**, In addition to the presence of various degrees of alveolar deformation and tissue fibrosis in the infected lung, transudate and exudate were found mainly around epithelial cells that were positive for the SARS viral signal (in situ hybridization, purple-blue cytoplasmic precipitates). In this area, only a small number of macrophages were identified (cells with crimson precipitates in the cytoplasm) (immunohistochemical analysis, CD68, ×400). **B**, No transudate or exudate was identified in the neighboring regions, which had abundant macrophages (cells with crimson precipitates in the cytoplasm) (immunohistochemical analysis, CD68, methyl green counterstain, original magnification, ×200).

**Table 3**

**Intensity of In Situ Hybridization Signal in Affected Alveolar Specimens From Patients With SARS Viral Infection**

| Case No. | F-S Interval (d)[†] | Intensity of In Situ Hybridization Signal[*] | | Contagious |
|---|---|---|---|---|
| | | REP Probes | N and M Probes | |
| 1 | 18 | – | +++ | No[‡] |
| 2 | 22 | – | +++ | No |
| 3 | 11 | ± | +++ | No |

SARS, severe acute respiratory syndrome; +, positive; +++, strong positive; –, negative; ±, intermediate.

[*] Antisense oligonucleotide probes specific, respectively, to the replicase-encoding region (REP) and the nucleic acid binding protein–encoding (N) and membrane protein–encoding (M) regions of the SARS viral genome were used to determine the viral signal by in situ hybridization. Intensity of the viral signal was measured by scanning of the specific cell types following in situ hybridization.

[†] The interval between the onset of fever and the sample collection.

[‡] Although case 1 was traced epidemiologically as the origin of outbreak in Taiwan, the subject was not contagious at the time when open-lung biopsy was performed. In the clinical behavior of SARS infection, the patient is most contagious when the fever persists; after fever subsides, the patient becomes less contagious or not contagious.[6]

**Table 4**

**Potential Chemokine-Like Motifs[*]**

| Motif | Amino Acid Sequence |
|---|---|
| IPCVCGRDATQYLVQQESSFVMMSAPPAEYKLQQGTFLCANEYTGNYQCGHYTH | 1762-1815 |
| ACCHLAKALNDFSNSGADVLYQPPQTSITSAVLQSGFRKMAFPSGKVEGCMVQVTCGT | 3207-3264 |
| LYCRCHIDHPNPKGFCDLKGKYVQIPTTCANDPVGFTLRNTV | 4305-4346 |
| NTCCNLSHRFYRLANECAQVLSEMVMCGG | 5012-5040 |
| RTCCLCDKRATCFSTSSDTYACWNHSVGF | 6107-6135 |

[*] The putative CXC and CC motifs are underlined.

are potentially monocytotactic and lymphotoxic, could be expressed in an atypical manner.[18]

It is worth noting that the amino acid sequence of SARS viral replicase also contains papain-like endopeptidase (NSP1) and 3C-like endopeptidase motifs.[1-4] With potential chemokine-like motifs and intrinsic enzyme activity, it is likely that SARS viral products would elicit an unconventional immune response that resembles the acute phase of histiocytic necrotizing lymphadenitis, with a dramatic increase of the CD68+ histiocyte population

locally.[9] In the chronic phase, the extent of CD68+ histiocytes decreased considerably, and foamy histiocytes became predominant. In this phase, the REP signal in enlarged and multinucleated cells became limited. Unlike histiocytic necrotizing lymphadenitis, though, no karyorrhectic bodies were detected within the cytoplasm of infected epithelial cells or histiocytes, and these pathologic appearances would lower the possibility of adenovirus or cytomegalovirus involvement, in which karyorrhexis, necrotizing exudates, and intranuclear inclusion bodies are abundant.[19] Moreover, these pathologic observations also corresponded well with the clinical behavior of the disease, in which patients were most contagious when the fever persisted.[6] On the other hand, the infected lesion limited to the lower respiratory tract, ie, the type II pneumocyte in the alveolus, further correlated with dry cough, and the symptoms would be less consistent with infection by the influenza virus, parainfluenza virus, hantavirus, or respiratory syncytial virus that could broadly infect the bronchiolar epithelium.[19]

Our results demonstrate that in the SARS virus–infected lung, the viral signal is defined mostly within epithelial cells, in particular, pneumocytes. The impact of SARS viral expression on mortality could be via the downregulation of surfactant production, which in turn leads to alveolar filling, alveolar collapse, tissue fibrosis, and pulmonary dysfunction. Expression of the SARS viral REP region, in which certain regions resemble a chemokine motif, would further result in an increased histiocyte population and, hence, deteriorated local inflammation. Undoubtedly, both hypotheses remain to be verified, and the effect of SARS viral infection on host gene regulation, in particular, the "cytokine dysregulation" that might be induced aberrantly in the macrophages and the surrounding tissues of the afflicted lung,[18] deserves more in-depth study. It is clear, however, that REP expression can be associated directly or indirectly with the severity of SARS disease and, possibly, the mechanism of tissue dysfunction, and this in and of itself should provide an interesting target for discovering and designing a novel therapeutic protocol for SARS viral infection, if the disease should persist and recur.[13]

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