# Detection of Severe Acute Respiratory Syndrome Coronavirus in the Brain: Potential Role of the Chemokine Mig in Pathogenesis

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**Background.** Previous studies have shown that common human coronavirus might be neurotropic, although it was first isolated as a pathogen of the respiratory tract. We noticed that a few patients with severe acute respiratory syndrome (SARS) experienced central nervous symptoms during the course of illness. In the present study, we isolated a SARS coronavirus strain from a brain tissue specimen obtained from a patient with SARS with significant central nervous symptoms.

Methods. Using transmission electronic microscopy and nested reverse transcription–polymerase chain reaction, the causative pathogen was identified in cultures of a brain tissue specimen obtained from the patient with SARS. Histopathologic examination of the brain tissue was performed using the methods of immunohistochemistry analysis and double immunofluorescence staining. Fifteen cytokines and chemokines were detected in the blood of the patient with SARS by means of a bead-based multiassay system.

**Results.** A fragment specific for SARS human coronavirus was amplified from cultures of the brain suspension, and transmission electronic microscopy revealed the presence of an enveloped virus morphologically compatible with a coronavirus isolated in the cultures. Pathologic examination of the brain tissue revealed necrosis of neuron cells and broad hyperplasia of gliocytes. Immunostaining demonstrated that monokine induced by interferon-γ (Mig) was expressed in gliocytes with the infiltration of CD68+ monocytes/macrophages and CD3+ T lymphocytes in the brain mesenchyme. Cytokine/chemokine assay revealed that levels of interferon-γ–inducible protein 10 and Mig in the blood were highly elevated, although the levels of other cytokines and chemokines were close to normal.

**Conclusions.** This study provides direct evidence that SARS human coronavirus is capable of infecting the central nervous system, and that Mig might be involved in the brain immunopathology of SARS.

The causative pathogen of severe acute respiratory syndrome (SARS) has been identified as a new member of the coronavirus family that exhibits a broad range of hosts, infecting many mammalian and avian species and causing upper respiratory, gastrointestinal, hepatic, and CNS diseases [1, 2]. Two known human coronaviruses, 229E and OC43, which cause up to one-third of all cases of common cold, were also found to infect the

CNS [3]. Our recent study of the SARS epidemic found that the spike protein, a surface antigen determining the tropism of coronavirus, had the strongest response to positive selection pressure [4]. We noticed that a few patients with SARS in hospitals in Guangzhou City, China, exhibited central nervous symptoms during the course of their illnesses. Lau and colleagues [5] reported that a CSF sample obtained from a 32-year-old woman with SARS tested positive for SARS coronavirus (SARS-CoV) by RT-PCR, indicating that SARS-CoV might cause an infection in the CNS of patients with SARS. In the present study, we isolated a SARS-CoV strain from a brain tissue specimen obtained during autopsy from a patient with SARS who became severely sick and showed significant central nervous symptoms during the course of his illness. Furthermore, we investigated the immunopathological mechanism of brain

damage in the patient with SARS. In this study, we detected a high level of monokine induced by IFN-γ (Mig), a member of CXC family [6, 7], in the patient's brain and found that gliocytes were a major source for Mig production in the brain. All results suggested that Mig was involved in the immunopathology of the brain on invasion of SARS-CoV.

## CASE REPORT

A 39-year-old doctor who was in charge of treatment of patients with SARS in the respiratory intensive care unit of the Chest Hospital (Guangzhou, China) developed a sudden onset of fever, chills, malaise, headache, dizziness, and myalgia and was hospitalized on 2 April 2003. Physical examination showed a temperature of 38.5°C, clear lungs, and no obvious focus of infection. His fever resolved with treatment with ribavirin and methylprednisolone (dosage, 120 mg per day), and his condition improved during the first week of treatment. A chest radiograph obtained on day 11 after the onset of symptoms revealed left lower lobe infiltrates. A complete blood count demonstrated an elevated WBC count of $16.4 \times 10^9$ cells/L, an increased level of neutrophils ($15.5 \times 10^9$ cells/L), and lymphopenia (lymphocyte count, $0.4 \times 10^9$ cells/L). This suggests that the condition of the patient with SARS was complicated by a secondary bacterial infection. Treatment with methylprednisolone was switched to 160 mg per 12 h intravenously, coincident with the initiation of antibiotic therapy. After initiation of the treatment mentioned above, his condition gradually improved during the fourth week after onset, at which time a series of chest radiographs showed obvious resolution of the left lower lobe consolidation. His total WBC count returned to a normal level, but lymphopenia still persisted. Treatment with methylprednisolone was then reduced to 40 mg per 12 h. For the patient's complaint of obscured monocular vision, eye-ground examination was performed and revealed an exudation around the visual yellow zone on day 26 after onset of illness (27 April). Two days later, the patient experienced progressive central nervous symptoms, including dysphoria, vomiting, and deliria, and was found to have progression of left lower lobe consolidation, for which he was treated with noninvasive ventilation and methylprednisolone at a dosage of 80 mg per 12 h, coincident with initiation of treatment with ceftazidime (Fortum; Glaxo Wellcome) and immunoglobulin. Despite the management described above, his condition deteriorated, and a series of chest radiographs obtained during the fifth week after onset showed progressive bibasilar infiltration with severe leucopenia (WBC count, $0.86 \times 10^9$/L), lymphopenia (lymphocyte count, $0.11 \times 10^9$ cells/L), and depressed marrow.

After receiving intravenous sedative, the patient developed coma on day 33 of illness, after which he was transferred into the respiratory intensive care unit of the Guangzhou Institute of Respiratory Diseases (Guangzhou, China). A CT scan of the brain revealed broad encephalic pathological changes of probably ischemia and necrosis and brain edema (figure 1). By day 2 after admission to the Guangzhou Institute of Respiratory Diseases, the patient developed breathness with slowing heartthrob; thus, he underwent intubation and received mechanical ventilation. His condition rapidly deteriorated; he experienced multiorgan dysfunction syndrome, and brain herniation occurred. He died on day 3 after his admission to the Guangzhou Institute of Respiratory Diseases (35 days after the onset of illness). Serum specimens obtained from the patient at early and later phases of the illness were forwarded to the Centers for Disease Control and Prevention (Guangzhou, China), where the diagnosis of SARS was confirmed by ELISA and indirect fluorescence assay [8].

## METHODS

***Autopsy materials.*** Full autopsy was performed on the patients with SARS. A detailed pathologic examination was performed on the brain tissue obtained during autopsy. A paraffin-embedded block of the brain tissue was sectioned at a thickness of 5 $\mu$m and dewaxed according to standard procedures.

***Immunohistochemistry and immunofluorescence staining.*** Histopathologic evaluation was performed on the specimen of brain tissue obtained during autopsy. Immunostaining was performed with monoclonal antibodies to N protein of SARS-CoV (supplied as a gift from Dr. Xiaoyan Che, Zhujiang Hospital, Southern Medical University, China), INF-γ–inducible protein 10 (IP-10; Abcam), CD3 and CD68 (DAKO), and a polyclonal antibody to Mig (Abcam). To identify the cell type with expression of Mig, double immunofluoresence staining was performed with a primary polyclonal antibody to Mig plus a monoclonal antibody to glial fibrillary acidic protein (Chemicon International) and secondary goat antirabbit antibody conjugated with tetramethylrhodamine isothiocyanate and goat antimouse antibody conjugated with fluorescein isothiocyanate (Sigma-Aldrich).

***Isolation and identification of SARS-CoV.*** We inoculated the suspension of the brain tissue onto 2 cell lines: Vero-e6 (C1008; ATCC) and human embryo lung fibroblast. The initial cytopathic effect (CPE) was observed between days 3 and 5. The CPE was a refractive appearance of cell rounding followed by cell detachment and quickly spread to the entire cell monolayer within 24–48 h after the initiation of CPE. Specimens were prepared for electronic microscopy by fixing a washed cell pellet with 2.5% glutaraldehyde and embedding it with epoxy resin.

***RT-PCR.*** Nucleic acids were purified from the supernatants of cell cultures with CPE using a nucleic acid extraction kit (Amplimedical SpA-Bioline Division). The cDNA synthesis from the extracted RNA was performed using a reverse-tran-
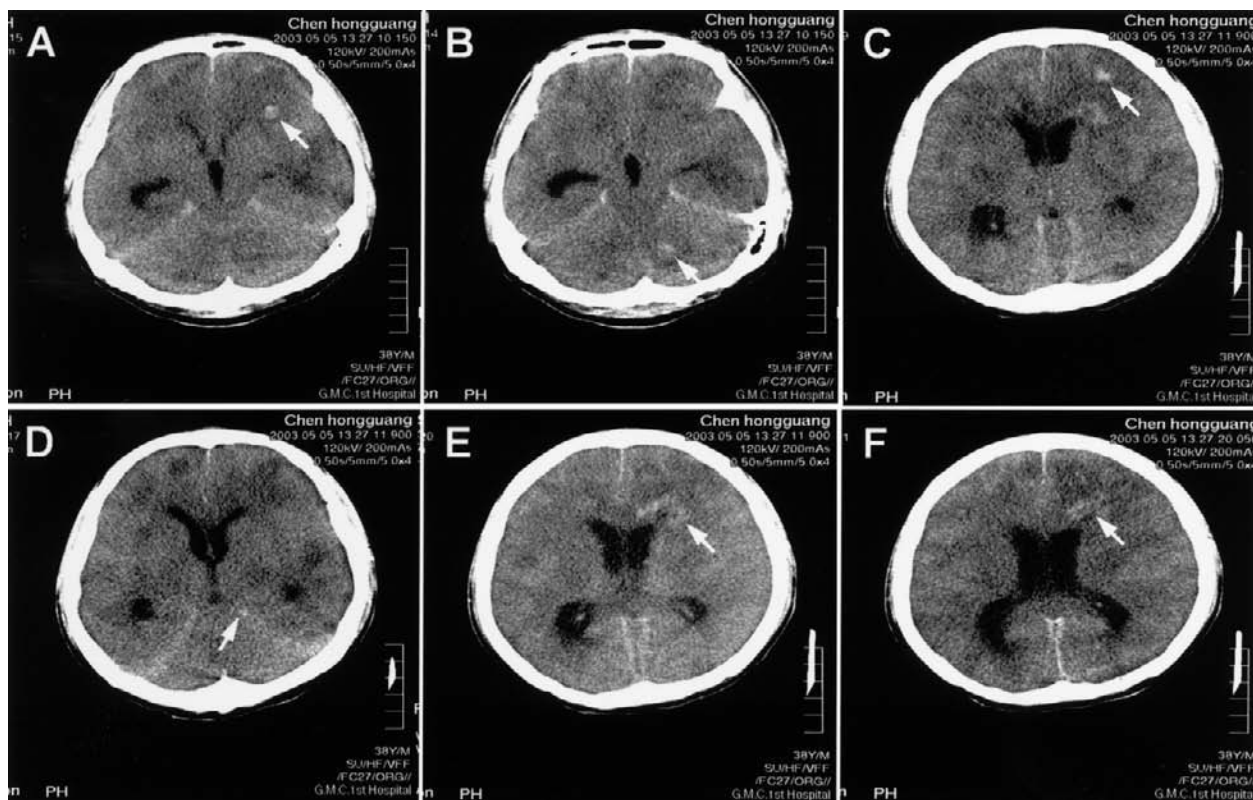
**Figure 1.** A CT scan of different brain sections shows a diffuse brain edema with multiple high-density lesions *(A–F)*. *Arrows,* high-density lesions.

scription kit (Amplimedical SpA-Bioline Division). The procedure is based on the reverse-transcription reaction utilizing the random primer technique. A nested RT-PCR was performed in accordance with the protocol developed by Dr. Christian Drosten with outer primers 5′-ATGAATTACCAAGTCAATGG-TTAC-3′ and 5′-CATAACCAGTCGGTACAGCTA-3′ and innerprimers 5′-GAAGCTATTCGTCACGTTCG-3′ and 5′-CTG-TAGAAAATCCTAGCTGG AG-3′ [8].

*Cytokine/chemokine assay.* Quantification of multiple cytokines/chemokines was performed using a LiquiChip work-station (Qiagen GMBH), which is a bead-based system for immunoassays that allows simultaneous assays of multiple analytes from a single sample [9]. A blood sample obtained from the patients with SARS on the day before death was used for the cytokine/chemokine assay. The 15 cytokines/chemokines assayed in this study included the following: IL-1$\beta$, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-$\alpha$, IFN-$\gamma$, granulocyte macrophage–colony-stimulating factor, Mig, IP-10, monocyte chemoattractant protein 1, macrophage inflammatory protein 1$\alpha$, and regulated on activation, normal T cell expressed and se-

**Table 1.** Results of general laboratory examination of the patient with severe acute respiratory syndrome (SARS), by number of days after onset of illness.

| Laboratory measurement | Day after onset of SARS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 11 | 21 | 29 | 31 | 33 | 34 | 35 |
| WBC count, ×10⁹ cells/L | 4.72 | 16.40 | 5.87 | 21.01 | 7.01 | 3.41 | 1.51 | 0.86 |
| Lymphocyte count, ×10⁹ cells/L | 0.72 | 0.40 | 0.48 | 0.32 | 0.32 | 0.31 | 0.24 | 0.11 |
| Neutrophil count, ×10⁹ cells/L | 3.46 | 15.50 | 4.95 | 20.22 | 6.24 | 2.80 | 1.07 | 0.66 |
| Platelet count, ×10⁹ cells/L | 212 | 160 | 45 | 37 | 37 | 25 | 26 | 28 |
| LDH level, U/L | 151 | 326 | 208 | 458 | 464 | 465 | 521 | 1233 |
| AST level, U/L | 26 | 35 | 37 | 24 | 20 | 12 | 13 | 14 |
| ALT level, U/L | 32 | 103 | 86 | 60 | 43 | 30 | 29 | 28 |
| CRP level mg/L | 2.9 | 124.1 | 24.9 | 298.6 | 278.0 | 306.7 | 418.0 | 417.8 |

**NOTE.** ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; LDH, lactate dehydrogenase.
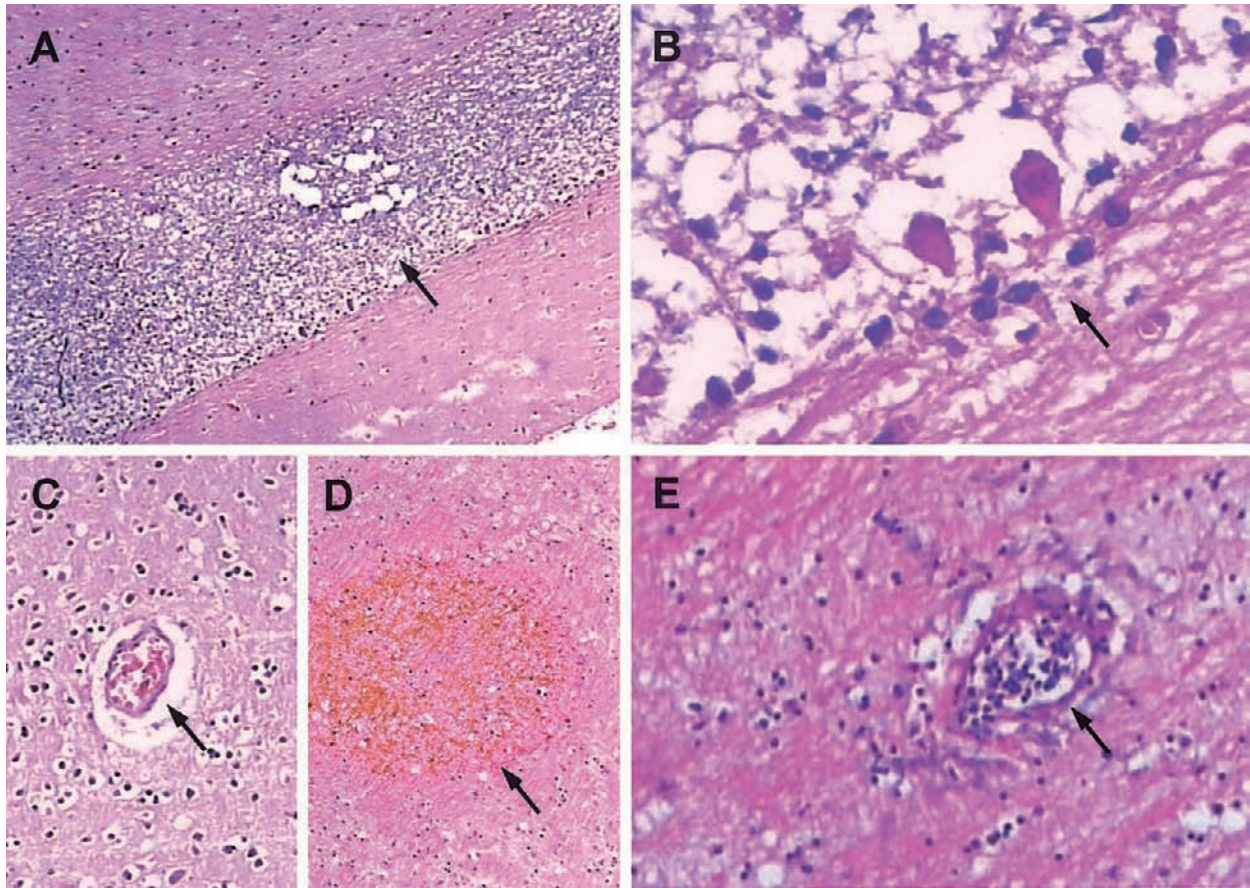
**Figure 2.** Histochemistry stains showing neuron denaturation and necrosis with striated encephalomalacia *(A;* original magnification, ×100) *(B;* original magnification, ×400), vasocongestion with widening peripheral space the around vessel *(C;* original magnification, ×200), and focal hemorrhage *(D;* original magnification, ×100) and gliosome formation in the brain tissue *(E;* original magnification, ×200). *Arrows,* pathological alterations described above.

creted (RANTES). Serum samples were obtained from 10 healthy control subjects aged 22–51 years. Samples were analyzed on the LiquiChip system (Qiagen GMBH), according to the manufacturer's instruction. Approval of the local ethics committees and informed consent were obtained.
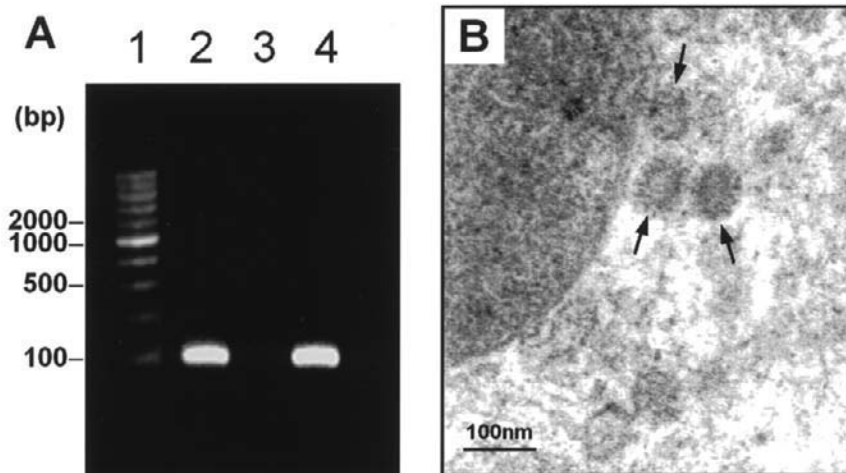
## RESULTS

***General laboratory examination.*** General laboratory examination revealed a peak C-reactive protein level of 418.0 mg/L and an increase in the lactate dehydrogenase level to 1233.0 U/L between days 1 and 35 after the onset of illness. Detailed information is shown in table 1.

***Histopathologic examination.*** Histopathologic examination revealed invasive *Aspergillus* pneumonia with multiple abscess, constitutional diffuse mycohemia, and fungal multiabscess in multiple organs. Pathologic examination of the brain tissue specimen under microscope revealed neuron denaturation and necrosis, broad gliocytes hyperplasia with gliosome formation, and encephalic edema (figure 2).

***Identification of SARS-CoV in the brain.*** The result of the nested RT-PCR showed that a predicted cDNA fragment (BNI-1) of ∼108 bp specific for SARS-CoV [8] was amplified from Vero-E6 cell culture inoculated with the brain suspension, but not from the culture that included medium only (figure 3*A*). Examination by transmission electronic microscopy revealed the presence of enveloped virus particles with a diameter of ∼80–90 nm and a surface morphology compatible with a coronavirus (figure 3*B*) in the culture showing CPE [8].

***Immunohistochemistry and double immunofluoresence staining.*** Immunohistochemistry staining of gliocytes and neurocytes obtained from the brain of the patient with SARS revealed them to be positive for N proteins (figure 4*A*), but those obtained from a patient who died in a traffic accident were not (figure 4*B*). We found that Mig, but not IP-10, expressed with infiltration of CD68^{+} monocytes/macrophages and CD3^{+} T lymphocytes in the brain mesenchyme of the patient. Double immunofluoresence staining revealed that Mig was mainly expressed in gliocytes (figure 5).

**Figure 3.** Identification of severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) in the brain tissue of the patient with SARS. *A,* Results of nested RT-PCR for BNI-1 fragment (108 base pairs) of SARS-CoV in the supernatants obtained from cultured Vero-E6 cells with cytopathic effect. *Lane 1,* DNA standard; *lane 2,* Vero-E6 cell culture inoculated with the lung suspension; *lane 3,* Vero-E6 cell culture used as negative control; *lane 4,* Vero-E6 cell culture inoculated with the brain suspension. *B,* Transmission electronic microscopy reveals the presence of enveloped virus particles with morphology compatible with coronavirus. Extracellular particles were found clustering and adhering to the surface of the plasma membrane.

***Cytokine/chemokine profile in the blood.*** As shown in figure 6, the levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, granulocyte macrophage–colony-stimulating factor, RANTES, macrophage inflammatory protein 1α, and monocyte chemoattractant protein 1 were close to those of the control subjects, but the levels of IP-10 and Mig in the blood of the patient with SARS were highly elevated.

## DISCUSSION

On review of the patient's vital record, we found that his general condition was nearly normal, and a chest radiograph showed resolving left lower pulmonary infiltration when he developed initial central nervous symptoms with an exudation around the visual yellow zone, indicating that the pathologic change in the brain was independent of pulmonary superinfection in the patient. In addition to findings of CT scanning indicating brain damage, pathologic examination revealed gliocytes hyperplasia, neuron denaturation, and necrosis combined with a striated encephalomalacia, suggesting that a chronic progressive viral cerebritis was present in the patient during the course of illness. Neuroinvasion by SARS-CoV was also directly evidenced by the viral morphology observed under electronic microscope, genetic identification, and the viral antigen (N protein) found in the brain.

Previous studies found that coronavirus might be neurotropic, neuroinvasive, and neurovirulent, although it was first isolated as a pathogen of the respiratory tract [1, 3]. At least 3 routes, including olfactory nerve, a hematogenous route, and lymphatic systems could be used by coronavirus to gain access to the CNS [10–12]. The cell lines of astrocytoma, neuroblastoma, neuroglioma, and oligodendrocyte were all susceptible to the infection of human coronaviruses [13–16]. Mouse hepatitis virus, a murine coronavirus, has been found to induce demyelinating disease of the CNS [17, 18]. Moreover, Glass and colleagues [19] recently reported a model of SARS-CoV
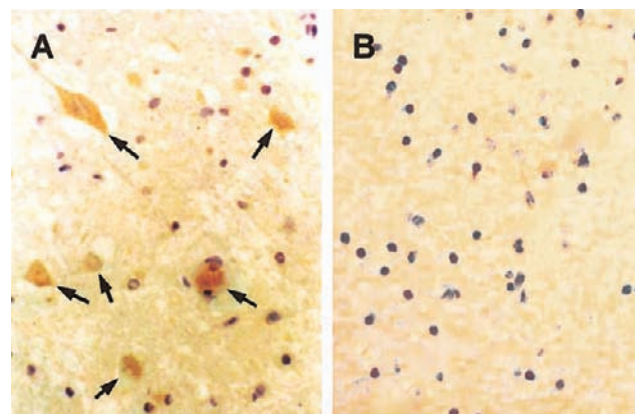


**Figure 4.** Immunohistochemistry stains for N protein of severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) in a specimen of brain tissue obtained from the patient with SARS during autopsy. A monoclonal antibody against N protein of SARS-CoV and the secondary antibody of horseradish peroxidase (HRP) conjugated goat antimouse were used for the immunohistochemical staining of brain tissue specimens obtained from the patient with SARS *(A; original magnification, ×200)* and a patient who died in a traffic accident *(B; original magnification, ×200).* Staining was performed with diaminobenzidine (brown). *Arrows,* positive staining cells.
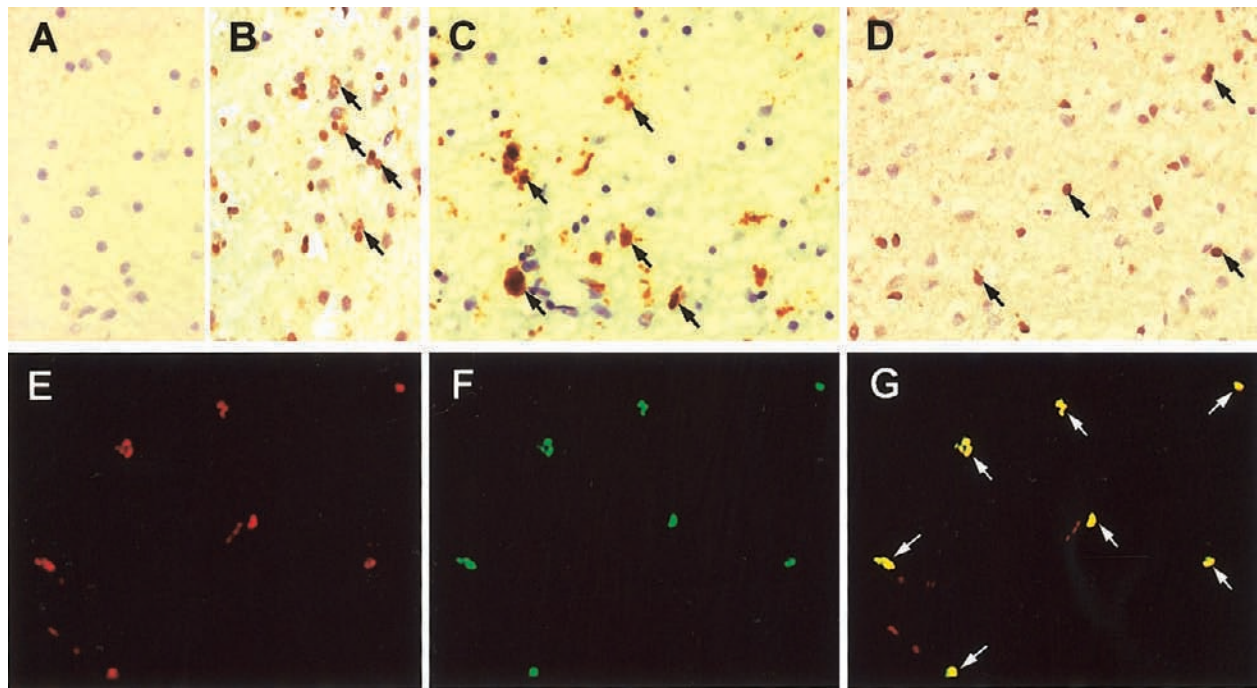
**Figure 5.** Immunohistochemistry and double immunofluoresence staining of specimens of brain tissue sections obtained from the patient with severe acute respiratory syndrome during autopsy. Antibodies against IFN-γ–inducible protein 10 (IP-10) *(A;* original magnification, ×200), monokine induced by IFN-γ (Mig) *(B;* original magnification, ×200), CD68 *(C;* original magnification, ×200), and CD3 *(D;* original magnification, ×200) were used in the immunohistochemical staining. Antibodies against Mig *(E;* original magnification, ×400) and glial fibrillary acidic protein *(F;* original magnification, ×400) were used in double immunofluoresence staining. *A,* Brain tissues without evidence of IP-10 from staining. *B,* Mig-positive staining cells detected in the brain tissue. *C,* Infiltration of CD68+ monocyte/macrophage cells. *D,* A few CD3+ T lymphocytes. *E–G,* A double immunofluoresence stain showing that glial fibrillary acidic protein positive cells *(F)* were expressing Mig *(E)* and displaying yellow immunofluoresence *(G;* original magnification, ×400) when the 2 images were merged. *Arrows,* positive staining cells. *A–D* were stained by diaminobenzidine. *E–G* were stained by both tetramethylrhodamine isothiocyanate (red) and FITC (green), respectively.

infection in C57BL/6 mice that demonstrated that SARS-CoV was able to infect the brain. The finding that SARS-CoV infected the brain of the patient with SARS was consistent with findings of previous reports on neuroinvasion of the coronavirus family [13–19].

Genomic comparison of strains of SARS-CoV showed that immunological pressures influenced the evolution of SARS-CoV in the human population by a change of the spike protein [4]. However, DNA sequence analysis showed that there was no variation with significant substitution of amino acids on the spike protein of the SARS-CoV isolated from this patient with the primary isolates of SARS-CoV [4]. It is possible that SARS-CoV is characterized by behavioral abnormalities similar to those in human coronaviruses OC43 and 229E, which have the capacity to infect the CNS but do not necessarily yield severe pathological symptoms [13, 16], because local immune surveillance limits viral replication on a large scale [20]. On the basis of the consideration that a progressive lymphopenia and leukopenia developed [21, 22] during the disease course of this patient, we speculate that local immune surveillance in his brain was removed by severe immunodepression [21–23], leading to the amplification of infectious viruses.

With regard to the superinfection with invasive *Aspergillus* in the brain and other organs of the patient, we think that severe immunodepression resulting from the damage to the immune system induced by SARS-CoV infection, combined with high-dosage treatment with a corticosteroid, provided access for conditional pathogens, causing a superinfection with invasive *Aspergillus* in multiple organs [24]. In this case, *Aspergillus* infection might have synergized or deteriorated the harmful effects of the SARS-CoV infection in the brain [24], which brought about a more complicated clinical process.

Our previous study showed that the IP-10 level, one of the non–ELR CXC chemokines, was augmented markedly in the blood of patients in the early stage of SARS and remained at a high level during the course of illness [25]. Consistent with that finding, this study showed that IP-10 in the blood of the patient with SARS was also highly increased. Furthermore, we found that the level of Mig, another CXCR3 chemokine, was highly elevated in the blood of the patient with SARS. In contrast to our finding, Wong et al. [26] reported that Mig could not be detected with significant increase in the blood of patients with SARS. The difference between the findings of Wong and colleagues and our results provides evidence for the hypothesis
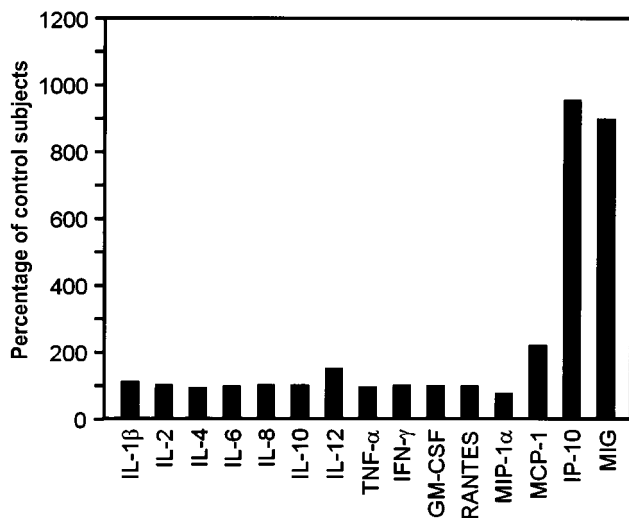
**Figure 6.** The profile of cytokines and chemokines in the blood of the patient with severe acute respiratory syndrome (SARS). Each cytokine/chemokine is expressed as a ratio of that of the patient with SARS to the average among control subjects. Of all the detected cytokines/chemokines, only IFN-γ–inducible protein 10 (IP-10) and monokine induced by IFN-γ (Mig) showed highly increased production.

that the high level of Mig in the blood of this patient with SARS was associated with the invasion of SARS-CoV in the brain tissues. It has been reported that both Mig and IP-10 were involved in host defense and immune damage by attracting activated T cells, NK cells, and monocytes that express CXCR3 [27]. We could not detect a significant elevation in the IP-10 level, but we did detect a high expression of Mig in the brain. This result is different from findings of our previous study, which revealed a high level of IP-10 in the lungs of patients with SARS. The results of histopathologic examination and immunostaining indicated that the invasion of SARS-CoV of brain tissues brought about the induction of Mig in gliocytes, which in turn attracted CD68+ monocytes/macrophages and CD3+ T lymphocytes to the sites of virus infection [27, 28]. Infiltrated immune cells should be helpful to limit SARS-CoV infection of the brain. However, because the virus could not be eliminated effectively while the patient was immunodepressed, a high number of cytokines/chemokines released in response to SARS-CoV infection would contribute to the tissue damage [29, 30]. The brain damage caused by SARS-CoV infection might take the same immunopathological process that happened in the lung tissues of patients with SARS [25], although different chemoattractant factors were involved.

In conclusion, to our knowledge, our findings are the first direct evidence that SARS-CoV has the ability to infect the CNS and that the infection causes immunopathological damage leading to a more serious and complicated clinical syndrome. Mig, but not IP-10, may be involved in the brain immunopathology by attracting immune effector cells to the site of virus infection.

However, the specific mechanism for Mig as a key mediator in the brain damage induced by SARS-CoV infection needs further investigation.

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