The apoptosis of pyramidal neurons in CA2 and CA3 subregions of the hippocampus is induced after infection with Mu-3 virus (Mu-3), a neuropathogenic strain of the JHM virus (JHMV), at 4–5 days post-inoculation (dpi). The viral antigens in the hippocampus are mainly found in the CD11b-positive cells distributed in the stratum oriens located outside the pyramidal layer, and only a few pyramidal neurons are infected. Furthermore, the apoptotic cells, indicated as showing caspase 3 (Cas3) activation, consist of a high number of uninfected cells. Therefore, it is considered that the apoptotic lesions occur through the indirect effects of infection, and not as a result of direct infection with Mu-3, similar to the reported neuronal apoptosis in the hippocampus after other types of infection. The apoptosis in the pyramidal neurons is accompanied by various types of proinflammatory cytokines depending on the causative agents. Thus, the local expression of proinflammatory cytokines was studied, revealing no correlation in the distribution of cytokine expression with the subregions showing apoptosis. However, the anti-inflammatory cytokine IL-10 was produced by pyramidal neurons of CA2 and CA3 at 3 dpi when there is no destructive change or viral invasion in the hippocampus.

Key words: apoptosis, hippocampus, IL-10, JHM, pyramidal neuron.

INTRODUCTION
The mutant virus Mu-3 has been isolated from the srr7 virus (srr7), which is a neuropathogenic strain of the mouse hepatitis virus (MHV), and is cloned as a soluble receptor-resistant mutant from the highly neuropathogenic JHM virus (JHMV) strain cl-2 virus (cl-2). Mu-3 infection causes a characteristic neuropathological picture in the hippocampus involving the apoptosis of pyramidal neurons in CA2 and CA3 subregions, which occurs in 100% of the mice infected at 4 to 5 days post-inoculation (dpi), mostly at 4 dpi, without showing neuropathological changes or viral invasion in the hippocampus at 3 dpi. Apoptosis of the pyramidal neurons in the hippocampus is observed in the ischemic brain, or after infection with various kinds of viruses, including human immunodeficiency virus (HIV) and Theiler’s murine encephalomyelitis virus (TMEV), as introduced in our previous report. The subregional distribution of the apoptotic lesions is variable depending upon the causative agents, and the mechanisms have yet to be clarified. It has been considered that apoptosis in the hippocampus occurs as a result of immune-triggered bystander effects during the course of infection, which has been indicated by our previous report showing that the viral antigens in the hippocampus are mainly found in the CD11b-positive cells distributed in the stratum oriens located outside the pyramidal layer, and only a few pyramidal neurons are infected. Furthermore, the apoptotic cells, indicated as showing caspase 3 (Cas3) activation, consist of a high number of uninfected cells. Such bystander effects causing pyramidal cell apoptosis are also observed in experimental autoimmune encephalomyelitis (EAE). The bystander effects are considered to be the result of elevated levels of proinflammatory cytokines detected in the course of diseases, such as TNF-α, IL-6, IL-1β and MCP-1. Therefore, we examined the local expression of several kinds of proinflammatory cytokines including those reportedly associated with hippocampal damage. However, the distribution of proinflammatory cytokines after infection with Mu-3 did not correspond with that of subregional lesions in CA2 and CA3 induced by Mu-3 infection.

Thereafter, in order to clarify the contribution of cytokines to the development of subregional lesions, we studied the local expression of anti-inflammatory cytokines, IL-10 and TGF-β, instead of examining many other kinds of
proinflammatory cytokines, because it has been reported that an immunosuppressive state is induced after infection with cl-2, its attenuated viral clone, srr7,10 and Mu-3,2,11 which cause encephalomyelitis with a wide area of brain damage without prominent inflammatory cell infiltration in the brain parenchyma. Intensive cell infiltration, leading to a dense population of monocytes/macrophages (Mo/Mas), is only observed in the meninges during the early phase of infection.1,10,12 Furthermore, it has been indicated that the immunosuppressive state after infection is mediated by expression of the Lewis X (Leα) carbohydrate structure13 using mutant mice that lack α1,3-β1-fucosyltransferase 9 (Fut9−/−) and are unable to synthesize the Leα structure.14 Our study comparing wild-type and Fut9−/− mice demonstrated that leukocytes derived from infected wild-type mice were unresponsive to lipopolysaccharide (LPS) challenge, whereas those from Fut9−/− mice responded to the challenge, although the stimulation of leukocytes derived from the two strains through Toll-like receptor (TLR) 9 similarly caused high-level responses.10,15 The immunosuppressive state observed in the wild-type mice after infection mimics the state in endotoxin tolerance (ET), or LPS tolerance, known as a classic example of a protective mechanism to prevent an excessive inflammatory response.16 The state of ET is mediated by suppressor cells, which are a Gr-1- and CD11b-positive (Gr-1− CD11b+) population of Mo/Mas.16

In this report, although we showed the IL-10 expression of the pyramidal neurons in CA2 and CA3 subregions, for the first time in the world, after infection with Mu-3, we could not demonstrate that Mu-3 is capable of inducing the production of IL-10 after infection using brain tissue culture. However, we revealed that Mu-3 infection triggers the production of IL-10 and TGF-β by Gr-1+ or CD11b+ peritoneal exudate Mo/Mas.17

**MATERIALS AND METHODS**

**Viruses and animals**

A neuropathogenic MHV strain, Mu-3, was used in this study. This virus was propagated and titrated in DBT cells maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma, Tokyo, Japan), as previously described.2 Specific pathogen-free inbred BALB/c mice purchased from Charles River (Tokyo, Japan) were housed in a specific pathogen-free animal facility, and maintained according to the guidelines set by the ethics committee of our university. For the experiment, mice were transferred to a biosafety level 3 (BSL-3) laboratory after obtaining permission from the committee of our university. Six 7-week-old mice were used for all experiments.

**Preparation of primary mixed neural cell culture**

Primary mixed neural cell cultures were established from the brains of neonatal mice as previously described,18 with minor modifications. Briefly, the brains obtained from newborn mice were dissociated in PBS (Nissui, Tokyo, Japan) after the removal of the meninges. The tissues were then centrifuged at 800 × g for 7 min and resuspended in DMEM (Gibco) supplemented with 1% FBS, containing dispase (Roche, Branchburg, NJ, USA) and collagenase (Invitrogen, Carlsbad, CA, USA). After incubation with DNase I (Roche), cells were suspended in DMEM supplemented with 10% heat-inactivated FBS (Gibco) containing B27 supplements (Invitrogen), and were plated at a density of 3 × 10^4 cells per well in eight-well plastic chamber slides (Nalge Nunc International, Rochester, NY, USA). Experiments were performed using 7- to 10-day-old cultures. For immunostaining, the cells adhering to chamber slides were fixed in cold ethanol for 1 min followed by fixation in cold acetone for 5 min and stored at −80°C until staining. Mice were inoculated with 1 × 10^7/50 μL of Mu-3 virus into the right frontal lobe under deep anesthesia using ethyl ether (SHOWA-ETHER, Tokyo, Japan). Sham infections were conducted using the medium for DBT cell culture.

**Tissue preparation for histology**

After the exsanguination of mice under deep anesthesia, tissues were isolated and embedded in OCT compound (Sakura, Tokyo, Japan). Tissue blocks were frozen on dry ice and 10-μm sections were cut using a cryostat (Sakura), air dried, fixed in acetone for 10 min and stored at −80°C until staining. A part of the brain was fixed in 4% paraformaldehyde buffered with 0.12 mol/L phosphate buffer at a density of 3 × 10^4 cells per well in eight-well plastic chamber slides (Nalge Nunc International, Rochester, NY, USA). Experiments were performed using 7- to 10-day-old cultures. For immunostaining, the cells adhering to chamber slides were fixed in cold ethanol for 1 min followed by fixation in cold acetone for 5 min and stored at −80°C until staining. Mice were inoculated with 1 × 10^7/50 μL of Mu-3 virus into the right frontal lobe under deep anesthesia using ethyl ether (SHOWA-ETHER, Tokyo, Japan). Sham infections were conducted using the medium for DBT cell culture.

Fixed cryosections and cells cultured on slide-glasses were treated with PBS containing 0.05% Tween 20 (Sigma, Tokyo, Japan), 1% bovine serum albumin (BSA) (Sigma), 0.1% sodium azide, chicken anti-mouse IgG (BioFX Laboratories, Inc., Owings Mills, MD, USA), and 5% horse serum for blocking. After incubation with the relevant primary antibodies (Table 1) for 1 h at room temperature, the slide-glasses were thoroughly washed with PBS containing 0.1% BSA and 0.05% Tween 20. The slide-glasses were then processed for incubation with a secondary or tertiary antibody, followed by nuclear counterstaining using Hoechst 33342 (Invitrogen). Stained sections and cell cultures were mounted with gold antifade reagent (Invitrogen) and examined using a confocal laser scanning microscope (Leica Microsystems, Knollhill, UK) or fluorescence microscope (KEYENCE, Osaka, Japan).
Tissue lysate preparation

The brains of mice with sham infection, viral infection, or without treatments were quickly removed, placed on an ice-cold surface, and the hippocampus was dissected. All tissues were snap-frozen and stored at −80°C until processing. The frozen tissues were powdered using an SK-100 mill (Tokken Inc., Chiba, Japan). Protein was purified from dissected tissues in radio-immunoprecipitation assay buffer (Wako, Osaka, Japan) and cell lysis buffer (Afmetrix, Santa Clara, CA, USA) supplemented with protease inhibitors (leupeptin, pepstatin and chymostatin; Sigma, Tokyo, Japan). After being centrifuged at 4°C and 10000 g for 30 min, the supernatant was collected and stored at −80°C.

Detection of cytokines

Cytokines in the tissue lysate and culture supernatant were assayed using the Luminex microbead-based multiplexed assay (Luminex Corp., Austin, TX, USA) and commercially available kits according to the manufacturer’s protocol (Affimetrix, Santa Clara, CA, USA). Cytokines analyzed by the array include IFN-α, IFN-β, IL-1β, IL6, IL-10, TNF-α, monocyte chemoattractant protein 1 (MCP-1) and TGF-β.

RESULTS

Proinflammatory cytokines

We did not observe increases in the amounts of proinflammatory cytokines in the hippocampus in specific association with viral infection compared with those in the sham-infected hippocampus (Fig. 1a). The injection of medium into the frontal lobe led to higher-level responses in the hippocampus than viral infection, which is typically observed as elevated levels of type I interferons (IFN-I) and TNF-α (Fig. 1a). In the pons, such dissociation was not seen, and relatively higher-level responses were observed on viral infection compared with sham infection (Fig. 1b), as has been reported on srr7 infection.13 These rather conflicting data obtained from the measurement of cytokines of total tissues, which has been the major method to show the contribution of cytokines to induce hippocampal damage, described in the Introduction, led us to study the local production of cytokines by immunofluorescence at 3 dpi. This time, no tissue damage in the hippocampus was found (Supplemental Fig. 1A), as previously reported,2 and no viral antigens were detected in the hippocampus (Supplemental Fig. 1B), as previously reported.2 Furthermore, most of the cytokine production after sham infection subsided (Fig. 1a). At 4 dpi, tissue destruction starts in the hippocampus,2 which would...
trigger the production of cytokines not involved in pyramidal cell apoptosis as causative cytokines. However, no proinflammatory cytokine examined showed expression localized in the CA2 and CA3 subregions at 3 dpi. IL-1β and TNF-α were detected in the CA1 and CA2 subregions. IL-6 and MCP-1 were expressed in none and all of the subregions of the hippocampus, respectively (Table 2). IFN-β was not detected in the hippocampus at 3 dpi (Table 2), although its expression was observed in the pons both in the areas adjacent to viral antigen-positive cells and at a distance from infected sites in astrocytes20 and in neurons (Supplemental Fig. 1C), as reported.21

**Table 2** Frozen sections were prepared from the hippocampus of Mu-3-infected mice at 3 days post-infection to detect the distributions of proinflammatory cytokines MCP-1, IL-1β, IL-6, TNF-α and IFN-β in the pyramidal layer.1 Only faint staining in a few cells was detected.

| Cytokines | Subregions in the hippocampus |
|-----------|-------------------------------|
|            | CA1  | CA2  | CA3 |
| MCP-1      | +    | +    | +   |
| IL-1β      | +    | +    | -   |
| IL-6       | -    | -    | -   |
| TNF-α      | -    | +1   | -   |
| IFN-β      | -    | -    | -   |

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**Anti-inflammatory cytokines in the hippocampus**

Similar to the study of proinflammatory cytokines, the measurement of total tissue cytokines did not indicate the involvement of the anti-inflammatory cytokines IL-10 and TGF-β in causing regional lesions (Fig. 1c). An immunofluorescent study revealed that TGF-β is expressed at a low level in the pyramidal layer of all three subregions, and most intensively in the area of the ventricular wall located below the CA3 pyramidal layer. IL-10 was expressed

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**Fig. 2** Frozen sections were prepared from the hippocampus of Mu-3-infected mice (/V) or sham-infected with medium (/Med) at 3 days post-infection (dpi) to show the distributions of anti-inflammatory cytokines TGF-β (A and C), IL-10 (B and D), and IFN-β (E). Nuclear counter-staining was performed using Hoechst 33342, and is shown by a blue color. IL-10 expression was observed in the pyramidal layers of CA2 and CA3 subregions, where apoptosis of pyramidal neurons occurs at 4–5 dpi, whereas weak immunofluorescence was distributed in all subregions based on TGF-β staining. Strong expression of TGF-β was observed in the ventricular wall, indicated by arrows. The dotted areas in A2 and C3 show double staining with nuclear counter-staining at higher magnifications of the same areas. D: IL-10 was not detected in the pyramidal layer of sham-infected mice (IL-10/Med). An IL-10-positive cell (arrow in D2) was occasionally found outside the pyramidal layer and is shown at a higher magnification in the dotted area of D2. White bars indicate 20 μm.
at 3 dpi in the pyramidal layers of CA2 and CA3 subregions (Fig. 2) where pyramidal cell apoptosis is observed 1 day later. Neither IL-10 nor TGF-β were expressed in the sham-infected hippocampus (Fig. 2). At 2 dpi, the immunofluorescence for these anti-inflammatory cytokines was too faint to clearly show their localizations (data not shown). In the pyramidal layers at 3 dpi, IL-10 was produced in the neurons, and was not detected in the CD11b- or GFAP-positive cells (Fig. 3).

**In vitro assays**

In order to examine whether Mu-3 infection is able to trigger the production of anti-inflammatory cytokines, the levels of cytokines in the supernatant of primary brain cultures were measured. However, the levels of proinflammatory cytokines did not change after infection except for TNF-α, and those of anti-inflammatory cytokines remained at background levels after infection (Fig. 4). The immunofluorescent study did not reveal clear differences in cytokine expression between mock and viral infection, and IL-10-producing neurons were not detected (data not shown). The neurons in the culture could be a different population from those in vivo. We did not find large neurons, like pyramidal neurons, in the culture. Another possibility is that the neurons that expressed IL-10 in vivo were triggered by a signal sent through a pathway or by cell-to-cell contact (see Discussion).

**DISCUSSION**

It is important to show that the expression of cytokines is limited to CA2 and CA3, because cl-2, the maternal viral clone of srr7, induces the apoptosis of neurons in CA1 and CA2 subregions, and not in CA3.2 In this report, we focused on the apoptosis induced by Mu-3 infection, because the apoptosis induced by cl-2 occurs at a low frequency, and tissue destruction and viral invasion in the hippocampus can already be observed at 2–3 dpi.2,19

Although many reports have indicated the contributions of cytokines to the pathogenesis of...
hippocampal lesions, on measuring the levels of cytokines in the homogenized tissues,\textsuperscript{4,7–9} we could not obtain persuasive data supporting the contribution using homogenized tissues. The failure to detect cytokines in the tissue extracts with the multiplex bead-based immunoassay, in spite of their local expression being detected by tissue immunostaining, is due to their more sensitive assessment by the latter compared with the former, shown in our previous study.\textsuperscript{17} The level of IFN-\(\beta\) in the supernatant of peritoneal exudate cell (PEC) culture was undetectable after Mu-3 infection when measured by the multiplex bead-based immunoassay, although, by immunostaining of cultured PECs after infection, IFN-\(\beta\)-producing cells were detected at a distance from infected cells. Nevertheless, the multiplex bead-based immunoassay revealed that the infection inhibited productions of proinflammatory cytokines in the hippocampus compared with the pons. The levels of cytokines measured in the pons might be a reflection of intensive and moderate inflammatory cell invasion in the meninges and fourth ventricle adjacent to the pons, respectively.\textsuperscript{10,12,22} The hippocampus can be more sensitive, hence more vulnerable, to external stress\textsuperscript{4,23} than other areas of the brain, leading to elevated levels of cytokines in the hippocampus of sham-infected mice at 2 dpi. The response observed on sham-infection, which did not induce anti-inflammatory cytokines in the hippocampus at a detectable level, might have been suppressed by viral infection. Viruses have evolved various strategies to circumvent the innate immune response,\textsuperscript{24} and coronaviruses are known to block type I IFN (IFN-I) signaling and counter the action of downstream effector molecules.\textsuperscript{25} Our findings indicate that the production of many proinflammatory cytokines other than IFN-I is also suppressed in the brain by Mu-3 infection.

However, until 4 dpi, viral antigens are undetectable in the hippocampus.\textsuperscript{2} How can the area sense viral invasion that has occurred distant from the hippocampus? The same question arose when we detected IL-10 expression in the hippocampus at 3 dpi. Furthermore, it was the pyramidal neurons that expressed IL-10 and not CD11b-positive Mo/Mas, which can migrate into the hippocampus. The failure of the production of anti-inflammatory cytokines by Mo/Mas or microglia after infection was also detected by the brain primary culture, where a large population of CD-11b-positive cells are found,\textsuperscript{20} although we have shown, for the first time, that coronaviral infection can trigger the production of anti-inflammatory cytokines by Mo/Mas in PECs within 8 h post-infection (hpi).\textsuperscript{17} The answer to the question of how information on infection reaches the hippocampus is provided by our previous reports.\textsuperscript{20,22} As early as 12 hpi with cl-2, a unique reticular network (RN) is formed in the meninges, ventricle, ventricular wall, and brain surface facing the meninges, which extends deep into the brain parenchyma with an association with astrocytic fibers within 48 hpi.\textsuperscript{22} The fibers of the RN (RNfib) are positive for antigens, such as collagen, laminin, and ER-TR7 antigen (ERag), the most important marker of the fibroblastic RN (FRN) in lymphoid organs. The fibers of FRN in the lymphoid organs contain collagen fibers as a core, which is wrapped by an ERag-positive extracellular

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Seven days after seeding a CNS cell suspension obtained from newborn mice on eight-well chamber slides, the culture cells were infected with Mu-3, or left uninfected (Mock). Production levels of cytokines in the culture supernatant were measured by the multiplex bead-based immunoassay at 12 and 24 h post-infection; pg/1 mL of the supernatant is shown. Vertical lines indicate SD.}
\end{figure}
matrix (ECM), and further by a basement membrane enriched with laminin. The FRN functions as a conduit system, transferring substances such as cytokines and antigens to facilitate immune responses, and guiding the homing of leukocytes. The RN in the brain, referred to as astrocytic RN (ARN), could have quickly transferred the information about the viral invasion to the cells deep in the brain parenchyma, like FRN does in the lymphoid organs. In addition, cell-to-cell-mediated cross-talk, like that occurring through an immune synapse formed between lymphoid cells, using either immune-related or non-immune-related molecules such as neurotransmitters, might contribute to transmit the signal from the infected site to neurons in the hippocampus in order to produce IL-10. Although a number of articles have reported that many kinds of cytokines are produced in the CNS, under either healthy or unhealthy states, by either invading cells such as inflammatory cells or domestic cells, including neurons and astrocytes, to our knowledge, the in vivo production of IL-10 by neurons has not been reported. Among domestic cells in the CNS, astrocytes that produce IL-10 are found in chronic active lesions of multiple sclerosis (MS). Using the cell line PC12, it was shown that neuronal cells are capable of producing IL-10 in vitro under a hypoxic state, which causes apoptosis of the cells in vitro as well as pyramidal neurons of the hippocampus in vivo. The blocking of the cascade to produce IL-10 reduces the apoptosis. It is an important issue whether IL-10 production by neurons is involved in neuronal apoptosis, because the anti-inflammatory function of IL-10 is considered as a therapeutic tool to handle an autoimmune state in the CNS.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Fig. S1. A: HE staining using a paraffin section of the hippocampal area at 3 dpi with Mu-3 shows no destructive changes. B: Immunofluorescence for viral antigens using frozen sections at 3 dpi with Mu-3 shows that there is no viral invasion in the hippocampal area (B1), while many viral antigen-positive cells are detected in and around the third ventricle near the hippocampus (B2). C: IFN-β-producing neurons, detected by anti-NeuN antibody, in the gigantocellular reticular nucleus of the pons at 3 dpi with Mu-3. Double and single bars indicate 100 and 20 μm, respectively.