Analysis of the mechanism of spongiform degeneration induced by neuropathogenic mouse hepatitis virus
神経病原性マウス肝炎ウイルスによる海綿状脳症成立機構の解析

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要旨

マウス肝炎ウイルス MHV-JHM cl-2 株は神経病原性が強く、MHV のレセプター(MHVR)を発現していない神経細胞にも感染が広がる。一方、cl-2 株由来可溶性レセプター抵抗性ウイルス変異株 srr7 は cl-2 に比べ病原性が低く、MHVR 依存的に感染を広げ神経細胞への感染は認められない。感染後初期の両者の脳内でのウイルス増殖とウイルス抗原分布はほぼ同じである。しかし、感染後 48 時間になると病変およびウイルス抗原分布は cl-2 では灰白質に認められる一方で srr7 では白質に認められることが確認されている。我々は感染後初期には全ての種類の免疫担当細胞にウイルス抗原が認められ、両者における感染細胞の種類に違いがないことを確認した。さらに srr7 感染マウスでは、感染後 48 時間という短時間で脳実質に空胞変性が認められ、主として小脳や橋に海綿状脳症という病変が生じることを明らかにした。感染後 48 時間以降には脳室壁の上衣細胞やタイプ B 細胞へ感染が広がることを確かめたが、空胞変性の近傍にはウイルス抗原が検出されなかった。感染部位と初期の空胞変性の形成部位が離れていたことから変性はウイルスの直接傷害の結果ではないと考えられたため、間接的な要因としてサイトカインが関与している可能性を検討した。感染後初期のサイトカイン産生細胞は脳の広範囲に分布しており、ウイルス抗原が検出されない場所にも認められた。脳内のサイトカイン産生量は cl-2 感染マウスの方が srr7 感染マウスに比べ非常に高いことが確かめられた。さらに初期の感染の広がりは同じであるにもかかわらず cl-2 と srr7 の病変分布が灰白質と白質で異なることから、サイトカインによる脳内の情報伝達機構の違いが両者の病変の違いに関与している可能性が考えられた。リンパ組織では情報伝達機構を担う繊維構造が見つかっており、サイトカインや抗原物質を運ぶ役割をしている。感染が最初に髄膜に認められ次に脳室へ広がること、ウイルス抗原が繊維状に認められるなどの特徴からリンパ組織の繊維構造に相当するものが脳内にも存在することが示唆されたため、この構造を脳内で検索した。感染後 48 時間の脳内にリンパ組織で見つかった構造と類似した特徴をもつ繊維構造が広範囲に分布していた。その近傍には多数のサイトカイン産生細胞が分布しており、感染マウスの脳内では何らかの情報伝達が行われている可能性が明らかになった。以上の結果から、ウイルスによる病変発症機序にはサイトカインによる傍観者効果（Bystander effect）が関与し、cl-2 と srr7 の病変の違いに影響していることが示唆された。

Keywords：mouse hepatitis virus, spongiosis, cytokines, FRN, immune communication

1. Introduction

Mouse hepatitis virus (MHV), a member of the coronavirus family, is an enveloped virus with single-stranded, positive-sense genomic RNA that is about 30 kilobases long. The principal receptor for MHV is murine carcinoembryonic antigen-related cell adhesion molecule 1 (MHVR). MHVR is abundantly expressed in various tissues and cells. However, only microglial cells were found to express MHVR in the central nervous system (CNS).

cl-2 is a highly neurovirulent MHV-JHMV that infects various brain cells, including neurons, to induce encephalomyelitis. Infection of neurons is unique, because they do not express MHVR to which the MHV spike protein binds. In the case of cl-2 infection, the rapid viral spread leads to high mortality rates within short incubation periods, usually within 3 days post-inoculation (pi) (Takatsuki et al. 2010). A soluble receptor-resistant mutant (srr7) cloned from cl-2 also exerts neurovirulence, but with a lower virulence than cl-2. Previous studies have revealed that srr7 fails to infect neurons. A different distribution and intensity of cl-2- and srr7-induced lesions became apparent at 48 hours pi, that is, rapid and widespread destructive changes in the grey matter of the brain infected with cl-2, whereas srr7 infection induced lesions after a longer incubation period than cl-2, with predilection to the white matter. Therefore, the difference in neuropathogenesis between cl-2 and srr7 has been considered to be due to ability to infect neurons.

However, there is no difference in the viral growth rate during the initial phase of infection between these two strains as determined by a titration assay. It might be possible that the ability to infect neurons is not a sole reason of different neuropathogenesis induced by infection with cl-2 and srr7. These two viruses exhibit similarities in the initial infection before 24 hours pi, when the viral antigens are not yet

observable in the infected brain parenchyma including injection sites, *i.e.,* at 12 hours pi; both of the viruses were already found to have infected the monocyte lineage infiltrating into the meningeal regions, inducing viral and F4/80-antigen positive syncytial giant cells (Takatsuki et al. 2010). Another intriguing feature of viral antigen localization after infection with cl-2 or srr7 is that the viral antigens were found in fibrous structures, which appeared to be extracellular matrix (ECM).

However, such colocalization of viral antigen or viral particles with ECM has been reported to occur in the lymphoid organs. For example, after infection with lymphocytic choriomeningitis virus or Ebola virus, viral antigens are found colocalized with the components of reticular fibers, These viruses infect fibroblastic reticular cells (FRCs) in the lymphoid organs, followed by immune dysfunction, allowing viral persistence (Mueller et al. 2007) or tissue destruction (Steel et al. 2009). FRCs are considered to maintain reticular fibers, which comprise a fibroblastic reticular network (FRN) in the lymph nodes and spleen. Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7) has been used to define FRCs (Van Vliet et al. 1986) although the antigen of ER-TR7 has yet to be determined.

The FRN has been reported to function as a conduit system for immunocompetent cells and inflammation-associated molecules such as cytokines and ligands or foreign antigens to reach appropriate sites and cause an immune reaction in the lymph nodes and spleen (Nolte et al. 2003), or to guide the homing of cells. Furthermore, it functions as immune communication network in lymphoid organs.

However, the function of FRN in the brain remained unclear. Previous report described the presence of an analogous reticular system at inflammatory sites within the brain; this system was not present in the normal brain tissues examined, but was associated with areas of parasite replication and local inflammation (Wilson et al. 2009). The manner of viral spread after infection and area of lesions with cl-2 or srr7 mentioned above indicates that immune communication in the brain parenchyma might contribute to the different neuropathogeneses. In this study, we investigated the detail neuropathologic feature of infection with srr7 which has not been studied. We also characterized the expression of FRN in the brain, a structure that could function as immune communication.

2. Materials and methods.

2-1. Animals and viruses

Fut9$^{-/-}$ mice with the BALB/c background (Kudo et al. 2007) and specific-pathogen-free inbred BALB/c mice purchased from Charles River (Tokyo, Japan) were housed in a specific pathogen-free animal facility, and were kept according to the guidelines set by the committee of our university. For infection, mice were transferred to the P3-level laboratory. Each mouse was injected with $1x10^2$ of the srr7 or cl-2 virus into the right frontal lobe under deep anesthesia.

2-2. Immunostaining and neuropathology

After exsanguination of the infected animals under deep anesthesia, removed parts of the organs were frozen for viral titration and for frozen section, and the remaining portions were fixed in 4% paraformaldehyde buffered with 0.12 M phosphate to obtain paraffin-embedded sections for histological staining with hematoxylin and eosin (HE) or for immuno-histochemistry. Details of the immunostaining procedure has been described elsewhere (Kashiwazaki et al. 2011a; Kashiwazaki et al. 2011b).

2-3. Cytospin Procedure

Single-cell suspensions were prepared by teasing the spleens in phosphate-buffered saline with DMEM supplemented with 1% FBS, and passing them through a stainless mesh. They were centrifuged and the resulting cell pellets were suspended in a 0.83% NH_4Cl-Tris buffer (pH 7.65) to lyse the red blood cells. The spleen cells were suspended in PBS (Nissui, Japan) containing 0.02 % Ethylenediaminetetraacetic acid tetrasodium salt dihydrate and 0.5% bovine serum albumin (both from Sigma, Japan) for the cytospin procedure. A total of 0.1 ml of the fluid sample containing $1x\ 10^6$ cells was added to the chamber fixed and secured on the glass by a metal clip. Filter cards, sample chambers, and metal clips were all obtained from SHANDON, Pittsburgh, PA, USA. After spinning at

2000 rpm for 4 minutes, slides were removed from the cytospin chamber, and were further fixed in 100% alcohol for 2 minutes and acetone for 5 minutes (Wako, Japan), and then stored at −35°C until use.

2-4. Protein preparation and analysis using multiplex bead assays.

To prepare protein for multiplex bead analysis, brains, spleens, and livers were harvested and flash frozen. Tissues then homogenized and the supernatants collected for protein analysis. Splenic cells were also cultured for 24 hours in 24-well plates with various stimuli. The supernatants were collected and frozen at −80°C until time of the cytokine assay. Samples were analyzed using a Luminex analyzer (Luminex, USA) and procarta immunoassay kit (Affimetrix, Santa Clara, Calif., USA) according to manufacturer's instructions.

3. Results

3-1. Neuropathologic feature of infection with srr7

A neuropathogenic JHM strain, srr7, was found to induce spongiform encephalopathy in the infected mice. The distinct vacuolar degeneration could be detectable at 48 hours pi in the area where no viral antigens were detected (Kashiwazaki et al. 2011a). After 4-10 days pi., large areas with extensive spongiotic changes appeared mainly in the brainstem and cerebellum of the infected mice.

The spongiosis exhibited several neuropathological characteristics. First, the lesion was fairly well-demarcated. Second, the lesions were accompanied by few or no inflammatory reactions. This phenomenon occurs rather rarely in viral infections with apparent destructive lesions. The third neuropathological characteristic of extensive spongiotic lesion is that neurons looking viable were found in the lesion, where vacuoles attached to the neurons directly.

3-2. Viral distribution

All kinds of cell population investigated with double-colored immuno-fluorescence for viral antigens and for specific cell marker proteins of immuno-competent cells, such as F4/80 for macrophages, TCR for T cells, or CD20 for B cells, at every time point between 12 and 48 hours p.i. were infected (Kashiwazaki et al. 2011b). We found many of the $B220^{+}Ly\text{-}6C^{+}$ and viral antigen-positive cells appeared as typical pDCs in shape with a shifted localization of the nucleus.

To study vacuolar formation during the early phase of infection, we investigated infected mice at 48 hours pi, because the viral antigens are distributed only in the restricted area at this time while vacuolar lesions become apparent. Viral antigen-bearing cells were observed in cell components of the ventricular wall, including ependymal cells and type B cells which locate in the subventricular zone (SVZ), before viral spread into the brain parenchymal cells. In addition, astroglial activations were most prominent in the area near the very restricted site of infection around the ventricle, and found extending deep into the brain parenchyma.

3-3. Cytokine production

The brain parenchymal cells near and distant to infected cells produced cytokines. In the pons, interferon (IFN)-β, interleukin (IL)-1β, IL-6, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α levels were strongly elevated after infection. There was significant difference in cytokine levels between these two strains. Higher levels of circulating IFN-β, IL-1β, IL-6, MCP-1, and TNF-α were detected in the brain of mice infected with cl-2 compared with those in srr7-infected mice at 48 hours pi. Furthermore, we compared cytokine levels between α1,3-fucosyltransferase 9 ($Fut9^{-/-}$) mice and wild-type mice. $Fut9^{-/-}$ mice are unable to synthesize the Lewis x (Le^{x}) structure (Kudo et al. 2004). No fundamental differences in virulence among the different strains of mice after infection were observed, although viral titers obtained from the brains of mutant mice were lower than those of wild-type mice. There were no clear differences in the levels of cytokines examined in the brains between $Fut9^{-/-}$ and wild-type mice except for IFN-β expression.

3-4. Induction of the components of FRN after infection

To visualize FRN, we used anti-ER-TR7 (Van Vliet et al. 1986). There was also an increase in the expression of ER-TR7 antigens at 48 hours pi with cl-2 compared to that found in mice without viral infection. We confirmed that the expression of ER-TR7-positive fibers play a role as a conduit system in the brain,

analogous to the roles reported in the lymphoid organs by use of fluorescence-labeled dextran. ER-TR7 antigen-producing cells, including neurons and astrocytes, as well as cytokine-producing cells, were found scattered over a wide area in the brain parenchyma, where covered with meshes of glial fibrillary acidic protein (GFAP)-positive fibers.

4. Discussion

The possible mechanisms of pathogenesis

srr7 as well as cl-2 infection could have induced a rapid viral spread in the infected CNS after effectively suppressing initial innate immunity through inducing cytopathic effects on the infected immuno-competent infiltrating cells and infecting pDCs during the initial phase of infection.

In the case of srr7 infection, the distribution of spongiotic lesions indicates that the viral spread into the brain starts from the junctional area between the arachnoid and choroid plexus where viral antigens are detected during the initial phase of infection. Type B cells have a long basal process that terminates on blood vessels and an apical ending at the ventricle surface, with long cilia projecting into the ventricle (Alvarez-Buylla and Lim, 2004), which can be a target of infection via infected monocytes that have infiltrated the ventricle during the initial phase of infection (Takatsuki et al. 2010). We did not consider these type B cells directly induce spongiosis because of the discrepancy in viral antigens and legions. Instead, these cells might have triggered a chain reaction of astrogliosis which spread from the ventricle reaching the deep white matter of cerebellum. Those activated astrocytes in a large area must have communicated with each other, leading to vacuole formation away from the infection sites.

Another reason of remote effect might be infection of type B cells. Type B cells have been considered as "nursing cells" capable of maintaining the extracellular homeostasis and providing metabolic support for the neuronal functioning. Infection could alter this important role of type B cells for neurons, inducing neuronal degeneration.

Contribution of cytokines to pathogenesis

The levels of several cytokines and ER-TR7 positive fibers were elevated at 48 hours pi. These cytokine-producing cells and ER-TR7-positive fibers were distributed in wide parenchymal area and connected to the main network by fine GFAP-positive fibers. It is reported that FRN in the lymphoid organs are able to transfer information through micro- and macromolecules including antigens and cytokines. An important question is how signals that reach the parenchymal cells are then transmitted further. Two possibilities might be cell-to-cell-mediated immune cross-talk through an immune synapse (Lee et al. 2002), as reported in the lymphoid cells, or the use of immune-related molecules and several neurotransmitters as a tool. In this way, it might trigger an immune reaction in the brain parenchymal cells. Astrocytes may play a role in cellular communication because a GFAP-positive fibrous network was found to connect ER-TR7 antigen-positive areas around the ventricle facing the dorsal pons and ventral meninges.

We suggested that the central nervous system employs a unique conduit system for immune communication. In this immne communication network, there might be differences between cl-2 and srr7, leading to different neuropathogenesis. Although further studies are required, this possibility is supported by the result of the analysis in Fut9$^{-/-}$ mice.

5. References

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