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Differential Expression of the Immediate-Early and Early Antigens in Neuronal and Glial Cells of Developing Mouse Brains Infected with Murine Cytomegalovirus

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Brain disorders induced by congenital cytomegalovirus (CMV) infection may appear at a later time after birth as a consequence of persistent infection and/or the activation of a latent infection of the neural cells. We have analyzed the infection dynamics of the neural cells in the neonatal mouse brains infected with murine CMV (MCMV) in the late stage of gestation. First we prepared a rat monoclonal antibody to the major immediate-early (IE)-89K antigen and then used the antibody for comparison of the expression of early and late viral genes in the developing mouse brains. The cells expressing the IE-89K antigen were mostly localized in the ventricular and subventricular zones and were preferentially double stained with anti-glial fibrillary acidic protein and anti-nestin antibodies. In contrast, the cells expressing the early nuclear antigen, detected by the monoclonal antibody D5, were diffusely distributed in the cortex and the hippocampus and were mostly double labeled with anti-neuron-specific enolase antibody. In neonatal mouse brains infected congenitally with recombinant MCMV, which expressed lacZ as a late gene, the number of the early nuclear antigen-positive cells was much higher than that of the β-galactosidase-expressing cells, the number of which was almost the same as that of the IE-89K antigen-positive cells. In addition, the distribution of viral DNA-rich cells detected by DNA-DNA hybridization was similar to that of the IE-89K antigen-positive cells. These results suggest that CMV may persistently infect neuronal cells, whereas lytic infection may preferentially occur in the glial cells in the developing brain. *(Am J Pathol 1997, 151:1331–1340)*

Cytomegalovirus (CMV) is the most significant infectious cause of congenital anomalies of the central nervous system (CNS) caused by intrauterine infection in humans,[1] with an average incidence of approximately 1% of all live births.[2,3] It is estimated that approximately 5 to 10% of infected infants have generalized inclusion disease at birth with symptoms such as microcephaly, periventricular calcification, and microphthalmia.[2,4,5] Another nearly 10% of infected infants have subclinical congenital infections and will subsequently have brain disorders including mental retardation, sensorineural hearing loss, visual disorder, seizures, and epilepsy.[6,7] The exact pathogenesis of these abnormalities has not been fully elucidated.

We have previously reported brain abnormalities induced in mouse embryos by injecting murine CMV (MCMV) into the conceptus at midgestation[8,9] and into the embryos at the late stage of gestation.[10,11] We also showed that MCMV infection disturbed neuronal migration and induced neuronal cell loss in the developing mouse brain,[12] and that a prolonged time of infection was observed in some of the neurons.[13] These neurotropic features of MCMV infection were observed only by using the monoclonal antibody specific to the early nuclear antigen of MCMV. In the present study, we prepared a monoclonal antibody (MAb) to the major immediate-early (IE) antigen of MCMV and found that the infectious dynamics of neurons were different from those of glia cells by the immunohistochemical double staining using these viral antibodies and antibodies to cell-specific markers.

Materials and Methods

Virus and Cell Cultures

The Smith strain of MCMV, which had been passaged in the mouse embryonic fibroblast (MEF) cells, was provided by Dr. Y. Minamishima, Miyazaki Medical College, Miyazaki, Japan.[14] The MEF cells were prepared from 11-day-old embryos of ICR mice as reported previously[15]

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and were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing penicillin (100 U), streptomycin (50 μg/ml), and 10% fetal calf serum (FCS). The titer of the virus stock was 1×10^8 plaque-forming units (PFU)/ml. Recombinant MCMV (RM461), in which the γ-0.85 gene was inserted with the *Escherichia coli* lacZ gene and which was constructed to express β-galactosidase (β-gal) under the control of the human CMV ie1/ie2 promoter/enhancer was provided by Dr. E. S. Mocarski (Stanford University, Stanford, CA).[16]

Preparation of MAb to the Immediate Early Antigen of MCMV

Six-week-old Wistar rats were immunized with the cell lysate of MEF cells infected with MCMV at a multiplicity of infection (MOI) of 3 PFU/cell for 24 hours. Spleen cells from the immunized animals were fused with mouse myeloma cells (Sp2/0-Ag14).[17] Hybridoma cells were obtained by growing cells in HAT medium in the presence of 5% BriClone (BioResearch, Dublin, Ireland). Hybridoma culture fluids were screened by immunofluorescence using MCMV-infected or uninfected MEF cells that were in the IE condition; namely, MEF cells were pretreated with cycloheximide (CH; 50 μg/ml) for 1 hour. CH was also added to the medium during and after the infection for 4 hours. The cells were then released from the CH-induced block in the presence of actinomycin D (10 μg/ml) for 2 hours.[18]. The selected hybridomas were cloned twice by the limited dilution method.

Immunoprecipitation

MEF cells plated in a 25-cm^2 flask were infected with MCMV in the IE condition or 24 hours after infection and labeled with [^{35}S]methionine (50 μCi/ml; Amersham, Buckinghamshire, England) in methionine-free DMEM for 2 hours. Radiolabeled cultures were immunoprecipitated as described previously.[19] The hybridomas were cultured in serum-free medium with the addition of insulin, transferrin, and bovine serum albumin (ITS+, Becton Dickinson, San Jose, CA), and the culture fluids were concentrated by Centricon-100 (Amicon, Beverly, MA; approximately 1:50) for immunoprecipitation. The immunoprecipitated samples were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After the electrophoresis, the gels were fluorographed as described previously.[18]

Virus Infection of Mouse Embryos

ICR mice were obtained from Japan Slc Co. (Hamamatsu, Japan). Pregnancies were dated as day 0 when copulation plugs were found after overnight mating. Embryos on day 15.5 of gestation (E15.5) were injected with 1 μl of MCMV or minimal essential medium (MEM) in the cerebral ventricles through the uterine walls using a microsyringe (Hamilton, Reno, NV) as described previously.[11,12] The embryos were allowed to develop and to be delivered and were then fed by the mothers during the postnatal days. The offspring were killed under anesthesia with ether, and the brains were removed and fixed with 4% paraformaldehyde (PFA).

Immunohistochemical Staining

After fixation and embedding in paraffin, the coronal serial sections of the MCMV-infected or uninfected brains were stained with hematoxylin and eosin (H&E). Adjacent sections were subjected to immunohistochemical staining using the mouse MAb D5, specific to both the early nuclear antigen and late nuclear inclusions of MCMV as described previously,[11,13,15] or using MAb N2, specific to the major IE antigen prepared in this study. After incubation with MAb D5 or N2, the sections were then sequentially incubated with goat anti-mouse or rat IgG biotinylated secondary antibody and with horseradish peroxidase (HRP)-conjugated streptavidin and then colored with 3,3′-diaminobenzidine as described previously.[15]

Detection of MCMV DNA in in Situ *Hybridization*

In situ DNA-DNA hybridization was performed according to the methods of Pomeroy et al.[20] A 30-bp oligonucleotide probe homologous to the sequences in exon 4 of the major IE MCMV gene 1 was labeled with digoxigenin (DIG) according to the manufacturer's specifications (Boehringer-Mannheim, Mannheim, Germany). After hybridization, the sections were incubated with anti-DIG Fab fragments conjugated with alkaline phosphatase and colored with 5-bromo-4-chloro-3-indoxyl phosphate (BCIP)/nitroblue tetrazolium.

Immunohistochemical Double Staining

Immunohistochemical double staining was performed according to the method of van Rooijen et al[21] with minor modifications. Deparaffinized sections were pretreated with 0.6% hydrogen peroxide in methanol to inactivate endogenous peroxidase, washed in phosphate-buffered saline (PBS), and incubated with goat serum blocking solution. The sections were first reacted with the rat MAb N2 or mouse MAb D5 for 20 minutes at room temperature, then sequentially incubated with a goat anti-rat or anti-mouse IgG biotinylated secondary antibody and alkaline-phosphatase-conjugated streptavidin, and then colored with Fast blue BB salt (Sigma Chemical Co., St. Louis, MO) in the presence of naphthol AS-MX phosphate (Sigma), resulting in a blue precipitate in the nuclei of MCMV-infected cells. Next, the sections were incubated with a sheep serum blocking solution and reacted with a rabbit anti-γ-neuron-specific enolase (NSE) antibody (Dr. K. Kato, Aichi Prefectural Colony, Aichi, Japan), a rabbit anti-glial fibrillary acidic protein (GFAP; Dako Corp., Carpinteria, CA) or a rabbit anti-nestin antibody specific to the neuronal precursor cells (Dr. H. Kitani, National Institute of Animal Health, Tsukuba, Japan).[22] The sections were then sequentially incubated with anti-rabbit

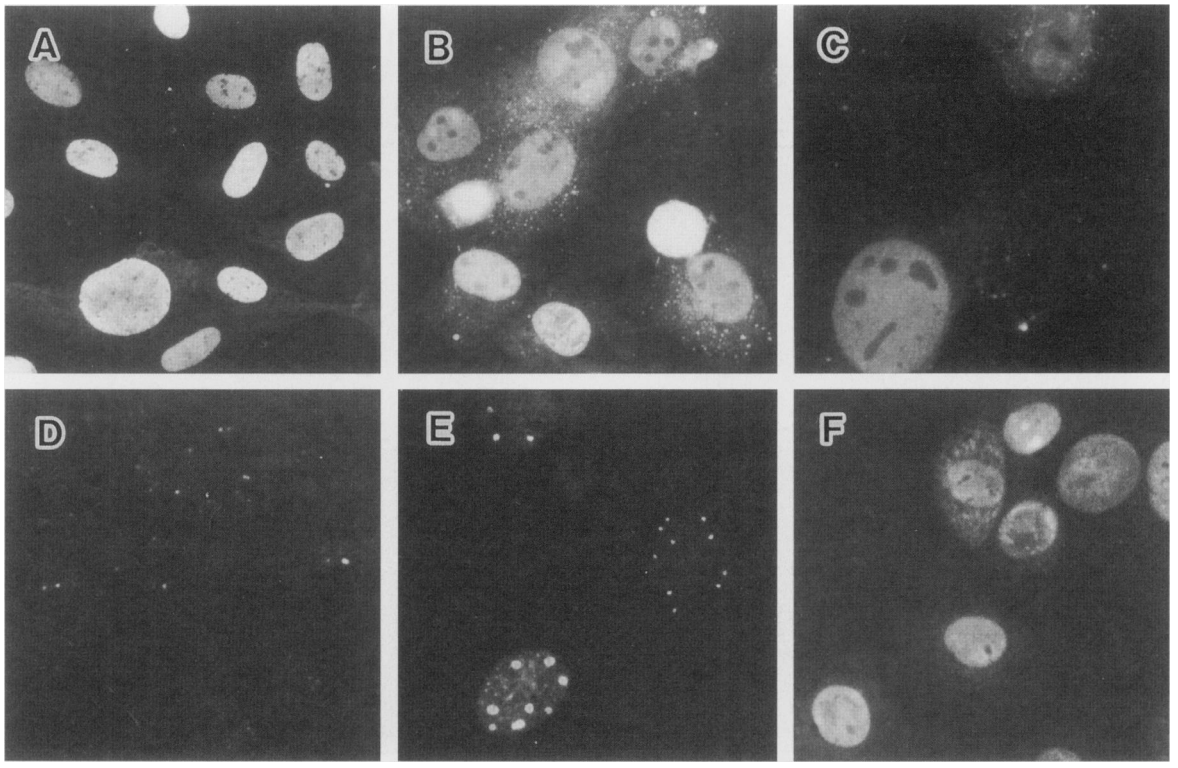


Figure 1. Indirect immunofluorescence photographs of MEF cells infected with MCMV using the MAbs N2 (**A** to **D**) and D5 (**E** and **F**). MEF cells infected (**A**) or mock-infected (**D**) with MCMV in the IE condition (see Materials and Methods) and infected for 24 hours (**B**) or 72 hours (**C**) without specific reagent. MEF cells were also infected for 8 hours (**E**) without reagent or infected for 24 hours in the presence of PAA (**F**) and were stained with the MAb D5. Magnification, ×720.

IgG DIG-conjugated Fab fragment (Boehringer-Mannheim) and anti-DIG peroxidase-conjugated Fab fragments (Boehringer-Mannheim) and then colored with 3-amino-9-ethylcarbazole (Dako), resulting in the formation of a red precipitate in the cytoplasm of these neural cells.

Combination of β-Gal Staining and Immunohistochemical Staining of the Viral or Neural Antigens

The histochemical staining of β-gal activity was performed by the method described by Reynolds et al[23] using the substrate 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) and then immunohistochemical staining with MAb N2 or D5 as described above but colored with Fast Red TR (Sigma), resulting in the formation of a red precipitate in the nuclei of infected cells. The mutant MCMV-infected brains fixed with 4% PFA were also subjected to immunohistochemical staining using rabbit anti-β-gal antibody (Organon Teknika Corp., Westchester, PA) as described in the staining for NSE, GFAP, and nestin.

Results

Isolation of Rat MAb Specific to Major IE Antigen 89K

We have tried many times to develop a MAb to the IE antigen of MCMV by immunizing mice with the MCMV-infected cells but had not been successful in all of the trials. By immunizing rats instead of mice, we obtained only one hybridoma producing the antibody specific to the IE antigen among more than 300 colonies after fusions with mouse myeloma SP2 cells. The hybridoma cell line, named N2, which produced antibody reacting with MCMV-infected MEF cells in the IE condition but not with uninfected cells, was found to be IgG2b with a serotype kit (Serotec, Oxford, UK).

An immunofluorescence study using the N2 antibody was performed on MEF cells uninfected or infected with MCMV. Diffuse and strong nuclear fluorescence was observed in more than 90% of the MCMV-infected MEF cells in the IE condition (Figure 1A). The nuclear fluorescence was also observed in the infected cells treated with phosphonoacetic acid (PAA), which blocks viral DNA replication (not shown). No fluorescence was observed in the uninfected cells (Figure 1D). At 24 hours after infection, granular cytoplasmic fluorescence was also observed in the cells in addition to nuclear fluorescence (Figure 1B). At 72 hours after infection, megalocytic cells with nuclear and cytoplasmic inclusions showed diminished fluorescence (Figure 1C), suggesting that the expression of the IE antigen decreased during the late phase of the MCMV infection. The immunoprecipitation analysis of the N2-reactive antigen using protein G Sepharose showed a single band defined as 89 kd in the MCMV-infected cells in the IE-conditioned cells and the cells 24 hours after infection (Figure 2A, lanes 2 and 4). A faint band was also observed in the infected cells treated with PAA (Figure 2A, lane 3).

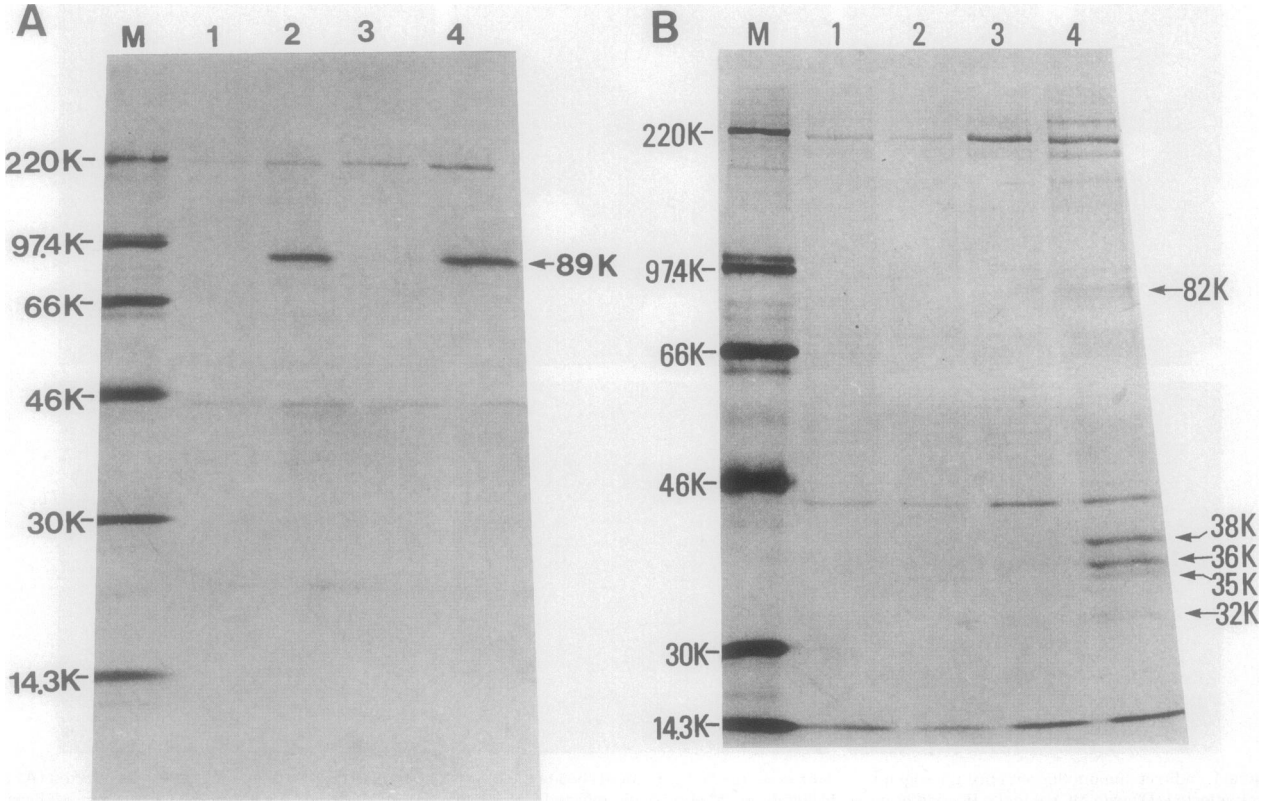


Figure 2. Immunoprecipitation of polypeptides of MCMV-infected cells with the MAb N2 (**A**) and MAb D5 (**B**). MEF cells were infected (**lane 2**) or mock-infected (**lane 1**) with MCMV in the presence of cycloheximide (50 μg/ml) for 5 hours and released from the block and incubated in the presence of actinomycin D and 50 μCi/ml of [^{32}S]methionine in methionine-free MEM for 2 hours. MEF cells were also infected for 24 hours in the presence (**lane 3**) or absence of phosphonoacetic acid (PAA; 100 μg/ml; **lane 4**) and labeled with the isotope for the last 2 hours. M, molecular markers.

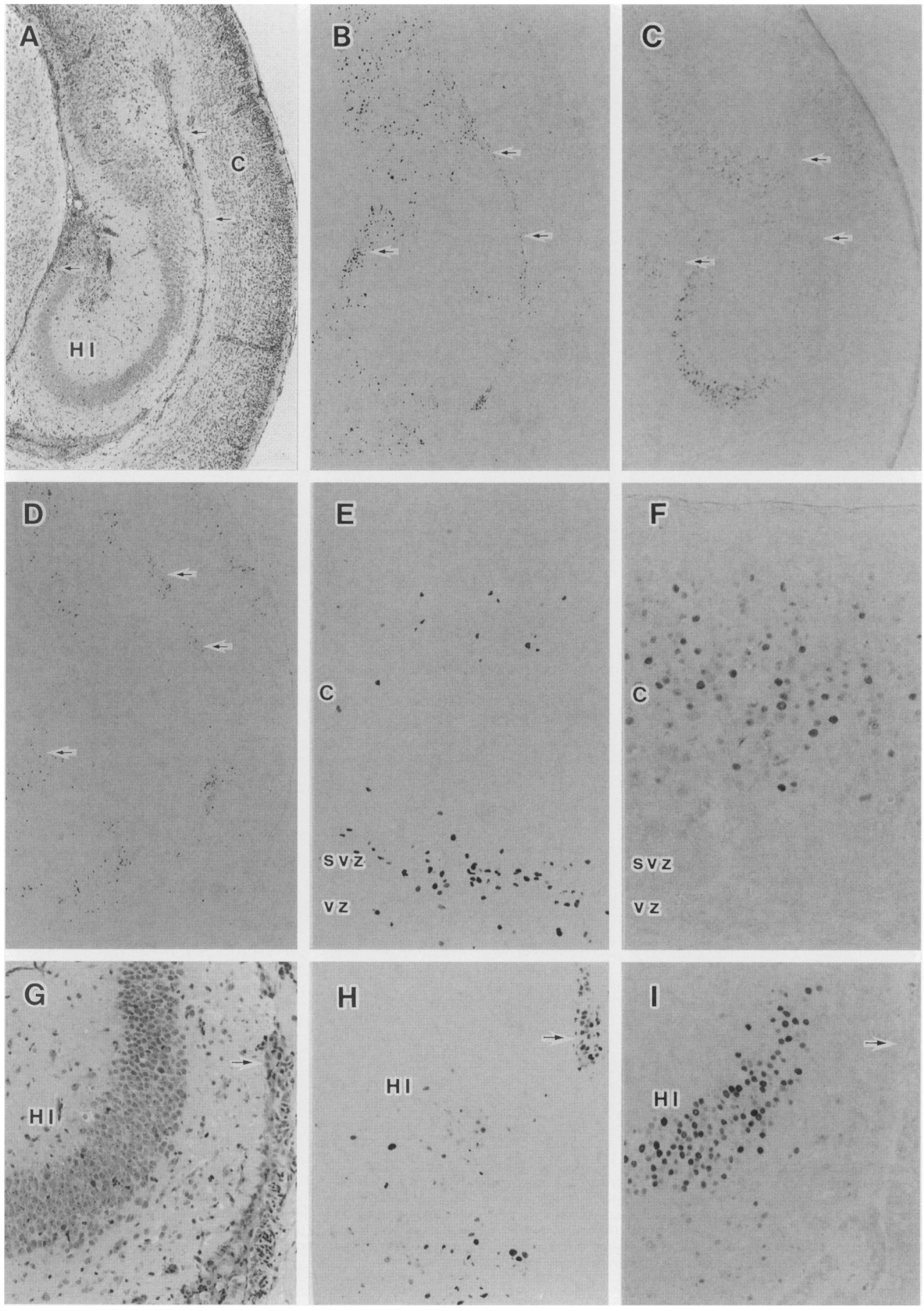
Using MAb D5, at least four bands at approximately 32 to 38 kd were detected by immunoprecipitation (Figure 2B), and immunofluorescence showed dotted nuclear fluorescence in the early phase of infection (Figure 1E), diffuse nuclear fluorescence in the cells treated with PAA for 24 hours (Figure 1F), and fluorescence with nuclear inclusion in the late phase of infection (not shown) as reported previously.[15]

Differences in the Distribution of the Brain Cells Expressing the 89K IE Antigen and the D5 Early Antigen

Mouse embryos on E15.5 were injected with MCMV (1×10^3 PFU) into the cerebral ventricles, allowed to be delivered, and then fed until 7 postnatal days (P7). The MCMV-infected mice had significantly lower body weights and brain weights than those of the uninfected group, as shown previously.[12] Sequential coronal sections of the MCMV-infected brains were subjected to immunohistochemical staining using the N2 antibody specific to major IE 89K antigen or the D5 antibody specific to the early nuclear antigen of MCMV. The PFA-fixed and deparaffinized sections were shown to be immunohistochemically stainable by the MAb N2, although these sections were also stainable by the MAb D5, as reported previously.[11]

We found that the distribution of the cells expressing the N2 antigen was different from that of the cells expressing the D5 antigen when the same part of the MCMV-infected P7 brains were compared in adjacent sections (Figures 3 and 5A). The N2-antigen-positive cells were mainly located in the ventricular zone and subventricular zone (Figures 3, B and E, and 5A), whereas the D5-antigen-positive cells were diffusely distributed in the cerebral cortex (Figures 3, C and F, and 5A). Small numbers of the N2-antigen-positive cells were also observed in the cortex (Figure 3E). In the hippocampus, the N2-antigen-positive cells were observed along the ventricular walls (Figure 3H), and the D5-antigen-positive cells were arranged in the regions of the pyramidal neuronal cells (Figure 3I). No staining was ob-

Figure 3. Immunohistochemical comparison between the IE antigen-positive cells detected by the MAb N2 and nuclear antigen-positive cells detected by the MAb D5 in the cerebral cortex and the hippocampus in the 7-day-old offspring infected with MCMV on E15.5. The serial coronal sections of the brain were stained with H&E (**A**), MAb N2 (**B**), or MAb D5 (**C**) and subjected to *in situ* DNA-DNA hybridization for the detection of viral DNA (**D**). The adjacent sections of the parietal cortex were stained with MAb N2 (**E**) and with MAb D5 (**F**). The adjacent sections of the hippocampus were stained with H&E (**G**), with MAb N2 (**H**), and with MAb D5 (I). **Arrows** show the ventricular zone (VZ). SVZ, subventricular zone; C, cortex; HI, hippocampus. Magnification, ×32 (**A** to **D**) and ×120 (**E** to I).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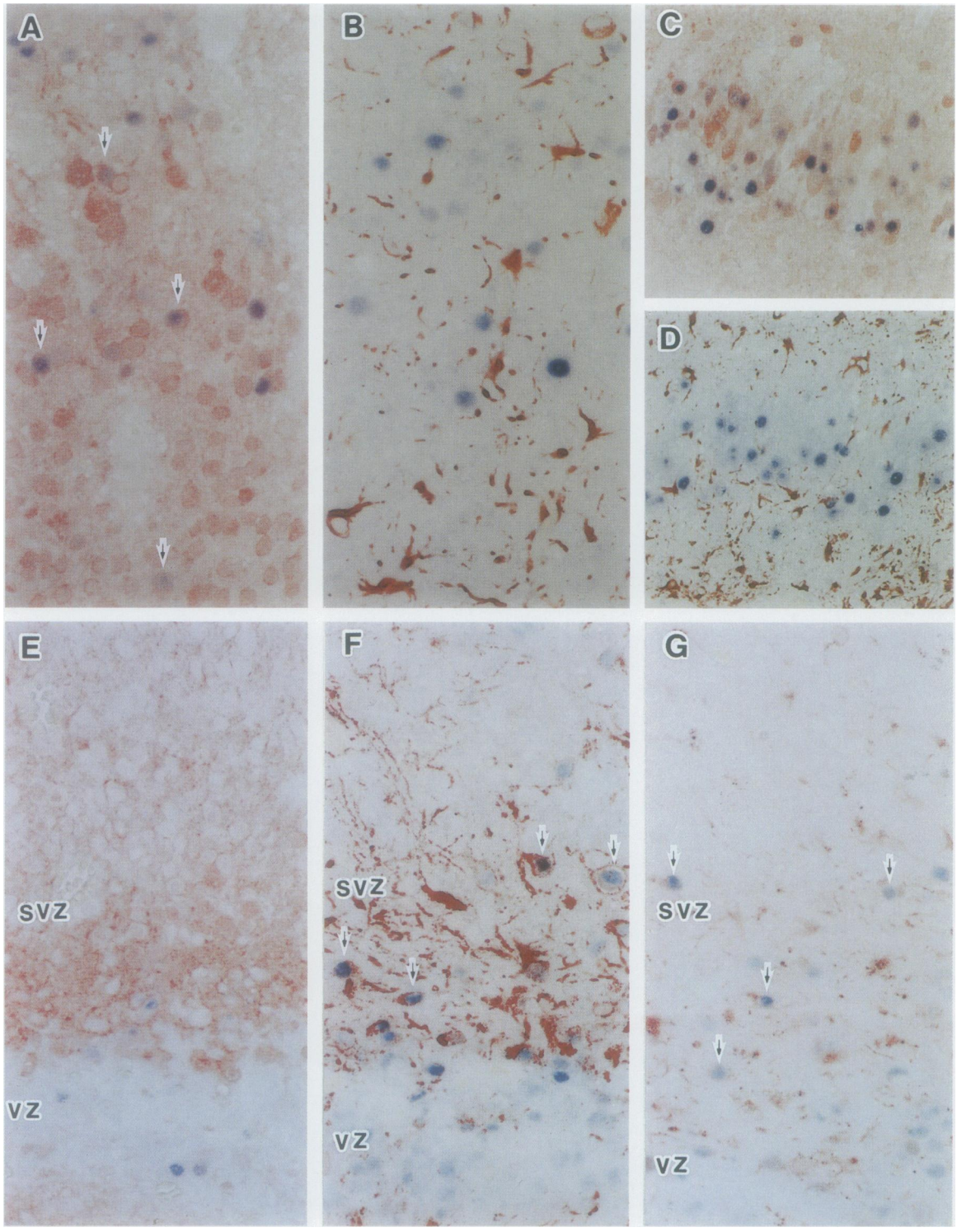

Figure 4. Immunohistochemical double staining of coronal adjacent sections of the cortex (**A** and **B**), hippocampus (**C** and **D**), and the ventricular and subventricular zones (VZ and SVZ; **E** to **G**) of brains of the offspring infected with MCMV on E15.5. Sections were first reacted with the MAb D5 (**A** to **D**) or the MAb N2 (**E** to **G**) followed by reactions with goat anti-mouse or anti-rat IgG-conjugated biotin, with alkaline-phosphatase-conjugated streptavidin, and with Fast blue BB salt (colored blue). The sections were then reacted with anti-NSE (**A**, **C**, and **E**) or with anti-GFAP (**B**, **D**, and **F**) or with anti-nestin antibody (**G**) followed by reactions with DIG-conjugated Fab fragments of anti-rabbit IgG and with aminoethylcarbazole (colored red). **Arrows** show some of the double-stained cells. VZ, SVZ, ventricular and subventricular zones, respectively. Magnification, ×180.

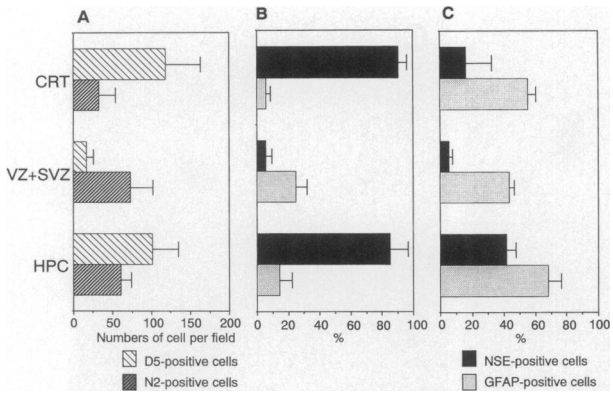

Figure 5. Histograms show the representative quantitations of the viral antigen-positive cells and their percentages of the cell-specific markers. **A:** Viral antigen-positive cells stained either with the MAb D5 or N2 were counted under a microscope with a 20× objective lens in the cortex (CRT), the ventricular and subventricular zones (VZ + SVZ), and the hippocampus (HI). Values are the averages of five fields. **B** and **C:** In the double-stained sections, the percentages of NSE- or GFAP-positive cells in the D5-positive cells (**B**) and the N2-positive cells (**C**) were calculated in each area. The NSE- and GFAP-positive cells in the viral antigen-positive cells were counted independently in the same areas. Values are the average percentages of five fields.

served in the uninfected brains either by the N2 or D5 antibodies (not shown).

The distribution of viral DNA-positive cells detected by the *in situ* DNA-DNA hybridization (Figure 3D) was almost the same as that of the IE antigen-positive cells detected by the N2 antibody (Figure 3B) in the same coronal adjacent section. This suggests that the cells in which active viral DNA replication occurs may have the abundant IE antigen.

Neuronal Cells Preferentially Expressed the Viral Early Nuclear Antigen Detected by the D5 Antibody

In a previous study, we showed by immunohistochemical double staining that some of the viral antigen-positive cells detected by the D5 antibody were also stained at the same time with antibody specific to NSE but rarely stained with the antibody to GFAP.[11] In the present study, we clarified the previous results by using an improved double-staining method. In the cortex of the postnatal offspring infected with MCMV on E15.5, approximately 90% of the viral nuclear antigen-positive cells detected by the D5 antibody were also stained with the anti-NSE antibody, although the staining of NSE decreased in the upper layers of the cortex (Figures 4A and 5B). In contrast, the viral antigen-positive cells detected by the D5 antibody were rarely stained with the anti-GFAP antibody (Figures 4B and 5B). It was also shown that the D5-antigen-positive cells in the hippocampus were pyramidal neuronal cells (Figure 4C) and that the D5-positive cells were not stained with anti-GFAP antibody (Figure 4D).

Glial Cells and Their Precursors Preferentially Expressed the Major IE 89K Antigen

By the immunohistochemical double staining method, more than 40% of the major IE 89K antigen-positive cells detected by the N2 antibody were double stained with the anti-GFAP antibody (Figures 4F and 5C), but fewer than 10% of the IE 89K antigen-positive cells were stained with the anti-NSE antibody (Figures 4E and 5C). Those N2-antigen-positive cells were also preferentially double stained with the anti-nestin antibody in the ventricular and subventricular zones (Figure 4G). Nestin was reported to be a marker of neural precursor cells.[22,24]

Correlation of the Major IE-89K Antigen or Early Nuclear Antigen-Expressing Cells to Late Viral Antigen-Expressing Cells

Mouse embryos on E15.5 were infected with the recombinant MCMV (RM461), in which the lacZ gene was inserted in late γ-0.85 gene[16] and their brains at the postnatal period were analyzed. The brains were fixed in 4% PFA, sliced, and stained with X-gal, and then embedded in paraffin and sectioned. Small numbers of X-gal-positive cells were scattered in the cerebral cortex (Figure 6, A–C). With the combined staining, more than 50% of the β-gal-positive cells were double stained with anti-GFAP antibody (Figure 6C), whereas only small numbers of β-gal-positive cells were double stained with anti-NSE antibodies (not shown). Most of the β-gal-positive cells were double stained with the N2 antibody specific to the IE-89K antigen, although small numbers of cells were only positive for the IE antigen (Figure 6A). In contrast, high numbers of nuclear antigen-positive cells detected by the D5 antibody appeared in the cortex in addition to a few β-gal-positive cells, some of which were also stained with the D5 antibody (Figure 6B). As the immersion of X-gal substrate might have been limited at the reaction in the brain slices, the recombinant virus-infected brains were fixed with 4% PFA and subjected to immunohistochemical staining using the anti-β-gal antibody. The β-gal-positive cells were mainly located in the ventricular and subventricular zones; there were a few in the cortex (Figure 6F). This distribution of the β-gal-positive cells was almost the same as that of the N2-positive cells (Figure 6D) but was different from that of the D5-positive cells in the adjacent sections, where the positive cells were scattered in the cortex and the hippocampus (Figure 6E), as shown in Figure 3.

Discussion

There has been no report of an immunohistochemical analysis of the expression of the IE antigen in MCMV-infected developing brains, although it was reported by Keil et al[25] that the MAb reacted to the IE-89K and IE-76K antigens. In the present study, we succeeded in preparing a MAb specific to the major IE-89K antigen by immunizing MCMV-infected cells to rats and fusing the sensi-

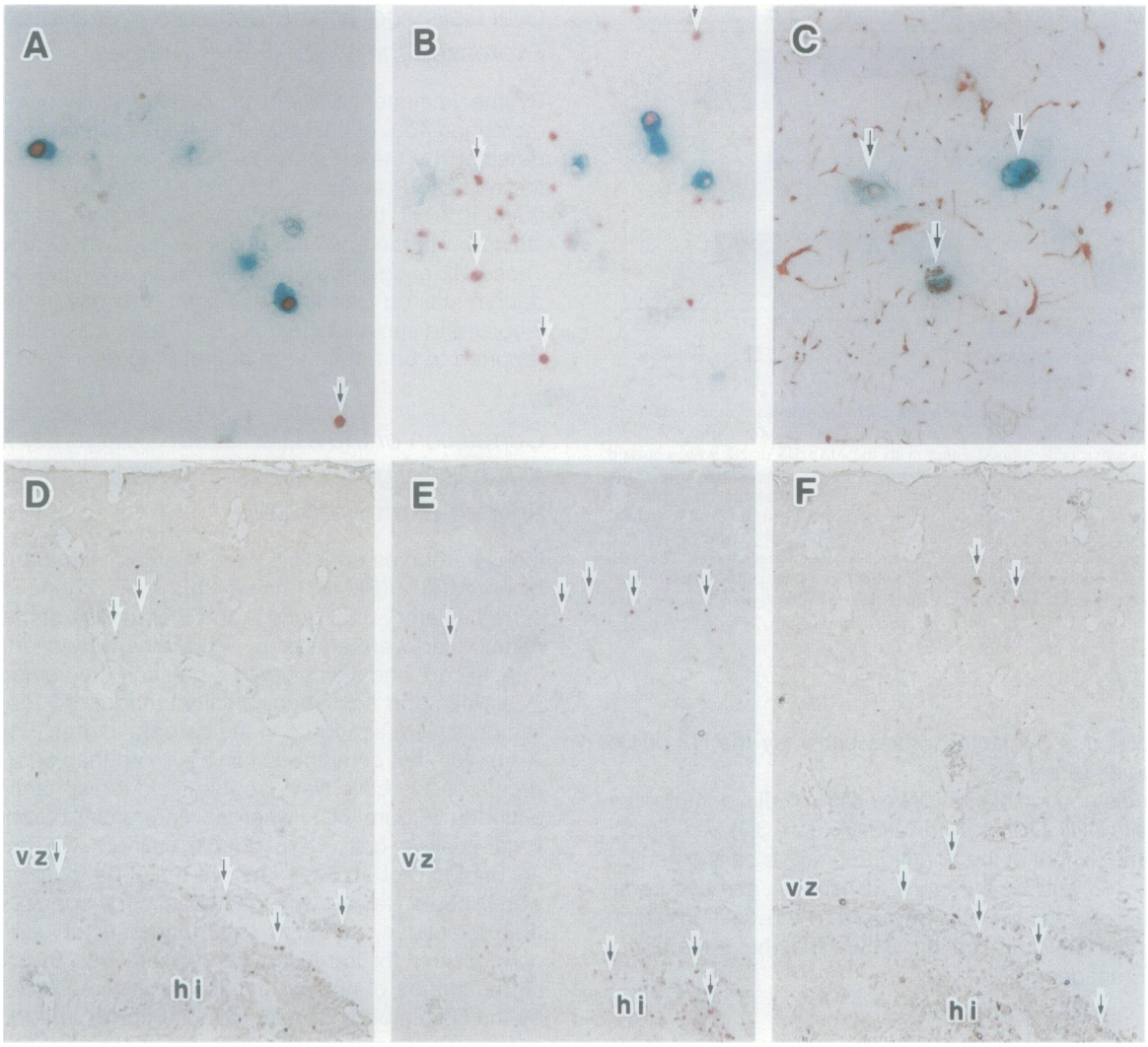

Figure 6. Combination of β-gal staining and immunohistochemical staining or single immunohistochemical staining using anti-β-gal antibody. The MCMV-infected neonatal brains were reacted with X-gal in slices and then embedded in paraffin. The sections were stained using MAb N2 (**A**), MAb D5 (**B**), and anti-GFAP (**C**). Alternatively, single immunohistochemical staining was performed using the MAb N2 (**D**), MAb D5 (**E**), and anti-β-gal antibody (**F**) in the serial adjacent sections. **Arrows** indicate single-stained cells with MAb N2 (**A**) or MAb D5 (**B**), double-stained cells (**C**), some viral antigen-positive cells (**D** and **E**), and β-gal-positive cells (**F**). VZ, ventricular zone; HI, hippocampus. Magnification, ×180 (**A** to **C**) and ×64 (**D** to **F**).

tized spleen cells to the mouse myeloma cells. We found that the antibody is able to be available to the histological samples that are fixed in 4% PFA and embedded in paraffin for immunohistochemical analysis.

It has been reported that the expression of genes from the CMV genome is temporally controlled and regulated in a cascade fashion; the IE (α) phase, the early (β) phase, and the late (γ) phase.[26,27] As reported in human CMV,[28] expression of the major IE antigen of HCMV may not be constant during the infection cycle; its expression was diminished in the late phase of infection during the lytic infection of MEF cells in culture (Figure 1C). This suggests that the expression of the IE antigen is dependent on the infection cycle, type of infection (such as permissive, abortive, or persistent), and the cell type of the infected cells.[29,30]

In the present study, it is notable that the expression of IE-89K antigen was markedly suppressed in the neuronal cells in the cortex and hippocampus compared with their constant expression of the early antigen detected by the D5 antibody. As it is unlikely that the IE-89K antigen was not expressed in the neuronal cells at all, its expression is thought to be below the sensitivity of the immunohistochemical method. It was confirmed by the improved immunohistochemical double-staining method that the early nuclear antigen detected by the D5 antibody was preferentially expressed in neuronal cells and rarely in glial cells.[11] In contrast, most of the cells preferentially expressing the IE-89K antigen detected by the N2 antibody had cell-specific markers of glial and neural precursor cells and mainly were located in the ventricular and subventricular zones. This marked contrast in expressing the viral antigens in neuronal and glial cells may reflect a difference in cellular factors necessary for their expression between the two cell lineages in the developing brain.

Neuronal cells differentiated from the neuronal precursor cells in the ventricular zone become postmitotic and migrate to the cortical plate or cerebral cortex.[31] We have previously shown that the infection of the developing

brain with MCMV disturbed neuronal migration to some degree and reduced the number of neuronal cells during migration.[12] Furthermore, viral antigen-positive neurons were retained for a prolonged time after birth when mouse embryos were infected with a low titer of MCMV.[13] These findings suggest that MCMV has prominent neurotropic features and is preferentially transferred to a persistent infection in neuronal cells, as shown in other viruses.[32–34] Joly et al[34,35] reported that neuronal cells are deficient in major histocompatibility class I molecules for the presentation of viral antigen to cytotoxic T lymphocytes and suggested that viral infections would be persistent in these cells.

The activation of the major IE enhancer/promoter of the CMV may be a critical step in determining CMV tissue-specific expression.[36] It has been reported in *in vitro* studies that the HCMV enhancer appears to be universally functional in multiple cell types.[30] However, Shering et al[37] recently reported that the HCMV major IE promoter/enhancer within a replication-deficient recombinant adenovirus vector was shown to produce cell-specific expression in rat nervous cell culture. Furthermore, expression of the major IE enhancer/promoter was highly regulated in transgenic mice.[38–40] These findings suggest that expression of the IE genes in the neurons *in vivo* may be markedly suppressed and highly regulated.

Concerning MCMV-infected glial cells, Poland et al[41] reported that less differentiated glial cell lines were partly or nonpermissive, whereas some astroglial cell lines were fully permissive. However, in the present *in vivo* study, the IE-89K protein-positive cells, located mainly in the ventricular and subventricular zones, had characteristics of glial or neural precursor cells detected by anti-GFAP or the anti-nestin antibodies. In human congenital CMV infection, CMV inclusion-bearing cells have been reported to be found often in these regions.[5] The MCMV-infected cells in these regions are thought to be a permissive infection, because the incidence of the IE-89K antigen-positive cells was almost the same as that of the viral late gene expressing-cells detected by lacZ expression produced by infection with recombinant MCMV (RM461).[16] In the present study, the distribution of the IE antigen-positive cells detected by the N2 antibody was almost the same as that of the viral DNA-positive cells detected by *in situ* DNA-DNA hybridization. This finding suggests that viral DNA is much higher in the IE antigen-expressing glial cells, in which active viral DNA replication may occur, than in the neuronal cells expressing the early nuclear antigen, but it does not show that viral DNA is not present in the infected neuronal cells, because of the methodological limitation of the sensitivity. Furthermore, there is no possibility that the D5 antibody widely cross-reacts with the damaged neuronal cells other than by MCMV infection, because we confirmed that the D5 antibody did not react with neuronal cells of the brain infected with herpes simplex virus type 1 (not shown).

Taking these findings into consideration, the glial cells expressing the IE-89K antigen extensively appeared to be lytic infected. The lytic-infected cells seem to be restricted mainly to the ventricular and subventricular zones, although small numbers of the IE-89K antigen-positive cells and late antigen-positive cells were observed in the cortex. This restriction of the extension of the lytic infection of the glial cells suggests that some of the infected glial cells may become a latent infection. Fritschy et al[42] showed that the HCMV IE promoter was normally silent in most astrocytes, but the activity was strongly induced in reactive astrocytes in response to a neocortical stab lesion in transgenic mice using a HCMV IE promoter connected with the lacZ reporter gene.

The difference in the expression of viral antigens between neuronal and glial cells in the developing brain may be important to clarification of the morphological and functional brain disorders caused by congenital CMV infections in humans.

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