Ultrastructural Localization of Viral Antigens in the CNS of Mice Persistently Infected With Lymphocytic Choriomeningitis Virus (LCMV)

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Lymphocytic choriomeningitis virus (LCMV) produces a persistent infection of the nervous system in susceptible mice. To map the localization of viral antigens in the central nervous system (CNS), the authors studied, by means of ultrastructural immune peroxidase techniques, 4–6-month-old mice persistently infected with LCMV following an intracerebral inoculation at birth. The greatest number of infected neurons was observed in the cortex, particularly of the limbic system, and certain nuclei of the hypothalamus. In the cerebellum, Purkinje cells selectively expressed viral antigens. Moderate numbers of infected neurons were found in the anterior horns of the spinal cord, basal ganglia, and thalamus. The immunoperoxidase technique using monoclonal antibodies showed that persistently infected neurons primarily expressed the nucleocapsid protein antigens of LCMV. Glycopeptide antigens were minimally expressed. Electron-microscopic examination of selected individual infected neurons showed viral antigens exclusively associated with ribosomes. No staining was seen on cell surfaces. Glutaraldehyde-fixed CNS tissue studied by electron microscopy did not reveal morphologic abnormalities or mature viral particles. This study demonstrates that LCMV persistently infects specific neuronal populations. Infected neurons express viral antigens in association with host ribosomes and show no significant morphologic alterations. (Am J Pathol 1983, 110:95–100)

MICE infected at birth with lymphocyte choriomeningitis virus (LCMV) provide an excellent animal model for the study of persistent infection in a natural host. These LCMV "carrier" mice develop a lifelong infection with persistent viremia and virus growth in most major organs.[1,2] Persistently infected mice may have an almost normal life span but usually develop glomerulonephritis, focal hepatic necrosis, or arteritis.[3,4] In contrast, infection in immunocompetent adult mice can result in an acute leptomeningitis and death within 6–8 days of infection.

Previous investigation of mice persistently infected with LCMV using immunofluorescence and histopathologic light-microscopic techniques indicated both expression of viral antigens in central nervous system (CNS) neurons and absence of injury to neurons.[5] We undertook the present study to map CNS sites of neurons persistently infected with LCMV and to use high resolution electron microscopy to study infected neurons and localize intracellular loci of viral antigen expression.

Materials and Methods

Virus

The Armstrong (CA 1371) strain of LCMV was propagated in BHK-21 cell culture and quantitated by plaque assay in Vero cells as described.[6]

Antibodies (Polyclonal and Monoclonal)

Polyclonal antibodies were prepared by inoculation of adult Hartley strain guinea pigs with Armstrong strain LCMV as described elsewhere.[7] Monoclonal antibodies to LCMV were prepared and characterized

Supported by NIH Grants NS 09053, NS 07078, AI 16102, AI 09484, and NS 12428. Dr. Rodriguez was the recipient of the 1982 Weil Award given by the American Association of Neuropathologists for the best paper presented on experimental neuropathology at the annual meeting in Philadelphia, Pennsylvania. This work was done during the tenure of an Established Investigatorship granted to Dr. Buchmeier by the American Heart Association.

Accepted for publication August 12, 1982.

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0002-9440/83/0107-0095$01.10

as reacting against viral nucleocapsid protein (NP), glycoprotein 1 (GP-1), or glycoprotein 2 (GP-2).[8] For the purposes of this study, two separate mixtures of 3 antibodies to viral nucleocapsids (anti-NP) and three antibodies directed against viral GP-1 and GP-2 (anti-GP) were used for immunoperoxidase staining studies. We found it necessary to use such mixtures because insufficient signal was obtained with the use of individual antibodies alone. Specificity of staining was insured by staining of infected but not uninfected tissue culture cells *in vitro* as described[8] and by staining of brain sections prepared from mice acutely infected with LCMV, but not of uninfected mice. Further, the ability of antiserums and monoclonal antibodies to stain LCMV-infected cells could be adsorbed out by mixing antibody with purified viral antigen, and the specificity of monoclonal and polyclonal antibodies was determined by precipitating specific viral polypeptides.[8]

Tissue Preparation and Immunoperoxidase Techniques

Fifteen C_3H/ST, 10 BALB/C WEHI, and 8 SWR/J mice were inoculated intracerebrally at birth with 10^3 plaque-forming units of the Armstrong (CA 1371) strain of LCMV. At 4–6 months of age, following intracardiac puncture, these mice were perfused with either 3% glutaraldehyde in PBS (5 mice) or a mixture of 4% paraformaldehyde and 0.5% glutaraldehyde (28 mice). Brains and spinal cords of animals perfused with the paraformaldehyde–glutaraldehyde mixture were carefully removed and 30-μ sections were made with the use of a vibratome (Ted Pella, Inc., Tustin, Calif). Twenty to 40 sections from each mouse were processed with immunoperoxidase techniques described by Sternberger.[9] Coronal sections of each brain were also embedded in paraffin and stained with hematoxylin and eosin.

Viral Antigen Localization With Polyclonal Antibody (PAP Technique)

Sections were treated with 10% egg albumin and 3% normal rabbit serum in phosphate-buffered saline (PBS) for 30 minutes and then sequentially incubated with guinea pig antibody to LCMV—1:400 to 1:800 dilution for 45 minutes, rabbit antibody to guinea pig IgG 1:10 dilution for 30 minutes (Cappel Laboratories, Cochranville, Pa) and then in guinea pig peroxidase–antiperoxidase (PAP) complex diluted 1:100 in PBS (Cappel Laboratories, Cochranville, Pa) for 30 minutes. Sections were then reacted with Hanker–Yates reagent (Polysciences, Inc., Warrington, Pa) in the presence of hydrogen peroxide for 15 minutes.

Viral Antigen Localization With Monoclonal Antibodies (Protein-A–Peroxidase Technique)

The sections were incubated with 10% egg albumin for 30 minutes and then treated with mouse monoclonal antibodies against either the nucleocapsid protein or glycopeptide in 1:100 dilution for 30 minutes. The sections were then sequentially incubated with rabbit antimouse IgG (Cappel Laboratories, Cochranville, Pa) diluted 1:10 for 30 minutes, which had been previously adsorbed with acetone powder prepared from mouse brain (Sigma, Saint Louis, Mo). Sections were then treated with staphylococcal protein-A–peroxidase (EY Laboratories, San Mateo, Calif) diluted 1/200 for 20 minutes and reacted with Hanker–Yates reagent in the presence of hydrogen peroxide for 15 minutes.

After staining, the sections were thoroughly washed in PBS, dehydrated in alcohol, and osmicated. The tissues were infiltrated with araldite resin and placed between 2 coverslips, and pictures were taken with Kodak Plus X black and white film. Selected sections were then placed on araldite blocks and polymerized. Areas of interest were trimmed, and ultrathin sections were viewed with a Zeiss 10 electron microscope.

Brains of mice that had been perfused with 3% glutaraldehyde were cut into 1-mm cubes and processed routinely for electron microscopy. The tissues were dehydrated, osmicated, and embedded in araldite. These ultrathin sections were counterstained with uranyl acetate and lead citrate.

Sections of brain from 5 age matched C_3H/ST mice were used as negative controls for immunoperoxidase studies and electron microscopy. Additional immunoperoxidase controls included substitution of PBS or normal rabbit serum for the primary antibody against LCMV.

Results

Morphology

Paraffin sections stained with hematoxylin and eosin (H&E) showed an absence of morphologic abnormalities in the CNS of persistently infected LCMV mice. The meninges and parenchyma showed no inflammatory infiltrates. There was no neuronophagia or astrocytic proliferation. Electron-microscopic examination failed to reveal any significant abnormalities. Cortical neurons showed prominent granular endoplasmic reticulum and Golgi complex with prominent polyribosomes. Lipofucsin granules were seen occasionally, but their numbers did not appear different from control animals. The neuropil was normal, without vacuolation. No mature viral particles were observed.

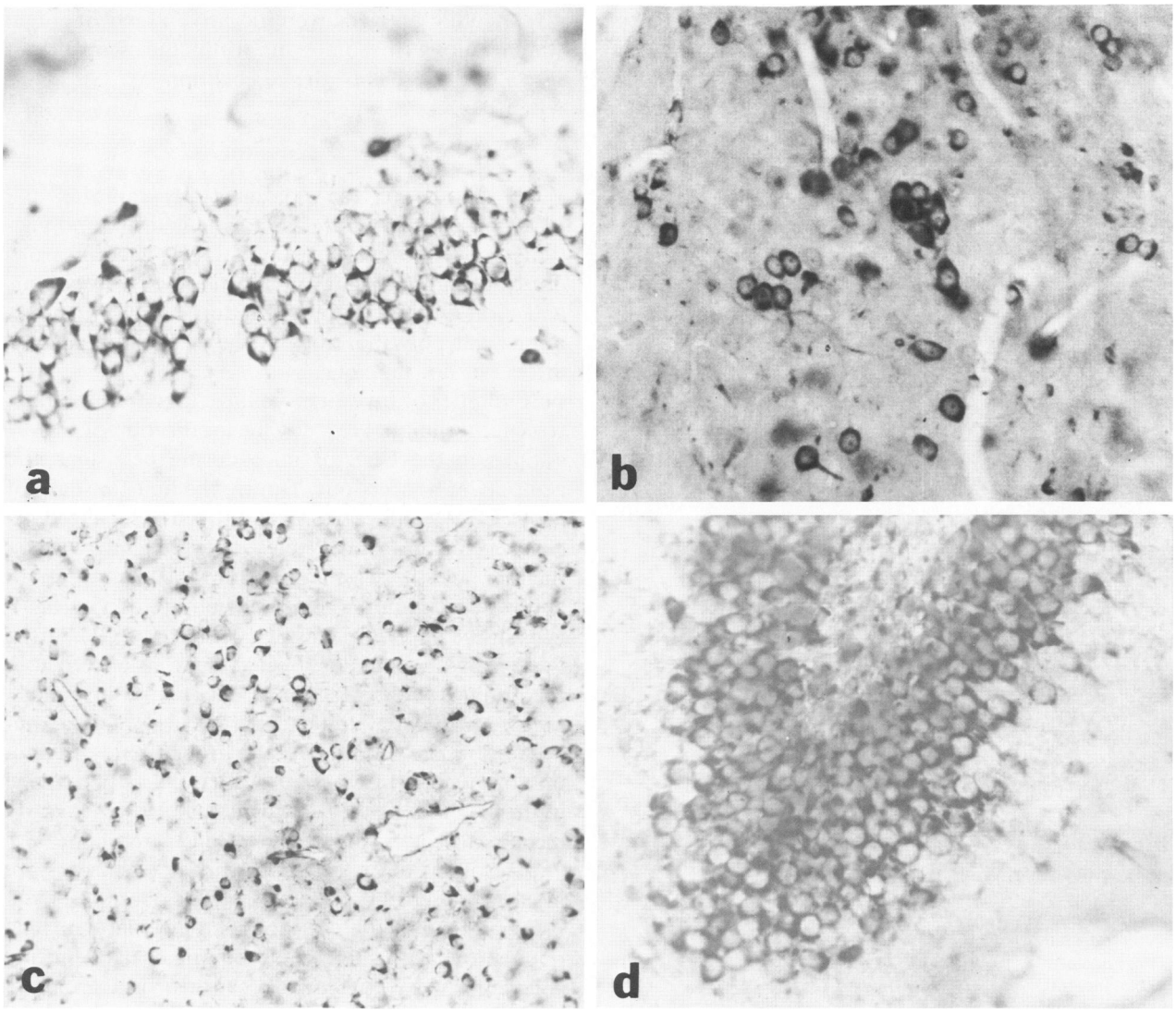

Figure 1a—Localization of LCMV antigens in hippocampal neurons. Immunoperoxidase staining of neurons from a vibratome section using the polyclonal guinea pig antibody against LCMV. (×750) **b**—Localization of LCMV antigens in parietal cortical neurons. Immunoperoxidase staining of neurons from a vibratome section using the polyclonal guinea pig antibody. (×750) **c**—Viral antigen localization of the nucleocapsid protein in frontal cortical neuron using the monoclonal hybridoma antibodies. (×500) **d**—Viral antigen localization of the nucleocapsid protein in hippocampal neurons. Immunoperoxidase staining using monoclonal antibodies against the nucleocapsid protein. (×750)

Immunoperoxidase Staining

Neurons of mice persistently infected with LCMV showed intense viral-antigen-specific staining with both the polyclonal guinea pig antibody against LCMV (Figure 1a and b) and the mouse monoclonal antibody against the nucleocapsid protein of the virus (Figure 1c and d). The staining was limited to the cytoplasm and dendrites of neurons. Astrocytes, oligodendrocytes, and microglial cells did not stain. Immunoperoxidase staining with the monoclonal antibody to LCMV glycopeptides was negative except for occasional very light staining in rare Purkinje cells of the cerebellum.

The topography of neurons persistently infected with LCMV is shown in Table 1. Neurons of the cerebral cortex, particularly of the limbic system, and certain nuclei of the hypothalamus consistently contained viral antigen. In the cerebellum, the Purkinje cells stained selectively; essentially no staining was present in the granular or molecular layers. Only moderate numbers of neurons stained in the anterior horn cells of the spinal cord, basal ganglia, or thalamus. Ependymal cells of the lateral ventricle occasionally showed weak staining.

Electron-microscopic examination of selected staining neurons from the cortex, hippocampus, and cerebellum showed intense staining primarily associ-

Table 1—Topography of Persistent LCMV-Infected Neurons

Cerebral cortex	
Frontal	+3
Temporal	+3
Parietal	+3
Occipital	+3
Cerebellum	
Purkinje	+3
Granular	0
Molecular	0
Dentate	+2
Basal ganglia	
Putamen	+1
Caudate	+1
Globus pallidus	+1
Thalamus	
Anterior nuclei	0
Ventral nuclei	+1
Posterior nuclei	+2
Brainstem and spinal cord	
Pontine nuclei	+2
Anterior horns (cervical)	+2
Anterior horns (thoracic)	+2
Limbic system	
Pyriform cortex	+3
Clingulate gyrus	+2
Hippocampus	+3
Dentate gyrus	+3
Mamillary bodies	+2
Hypothalamus	
Paraventricular	+3
Suprachiasmatic	+1
Supraoptic	0
Dorsomedial	+2
Ventromedial	+3
Post nuclei	+3
Zone incerta	+3
Lateral septal	+2
Preoptic	0

0, no neurons expressing LCMV antigens.
+1, few neurons staining.
+2, moderate number of neurons staining.
+3, many neurons staining.

ated with polyribosomes (Figures 2 and 3). Most identifiable ribosomes of a sectioned neuron stained with the immunoperoxidase product. No staining was seen in association with cell surfaces. Similar staining along ribosomes was seen with the use of both the polyclonal guinea pig antibody (Figure 2) and the monoclonal mouse antibody against the nucleocapsid protein of LCMV (Figure 3). Prominent staining was also occasionally seen within the Golgi complex of infected neurons. Dendrites were frequently identified with viral antigen associated with ribosomes. Axons, which do not ordinarily contain ribosomes, were consistently negative. Experiments using the monoclonal antibodies directed against the glycopeptide antigens were negative except for light staining along ribosomes and within the cytoplasm of rare Purkinje cells of the cerebellum.

Discussion

The results of the present study indicate that LCMV persistently infects specific neuronal populations in the mouse. Virus infection in these neurons does not cause significant morphologic alterations, and viral antigen is found predominantly in association with polyribosomes in the cell body and dendrites but not in axons.

A number of investigators have used immunofluorescence techniques to study the localization of LCMV antigen in the CNS of mice during persistent and acute LCMV infections. During the first few days of acute LCMV infection, viral antigen is localized exclusively in the choroid plexus, the ependymal epithelium, and the arachnoid cells of the meninges.[10] In contrast, immunofluorescence staining of viral antigens in the CNS of persistently infected LCMV mice have shown scattered infected neurons throughout the brain,[11] while infection is uncommon in the meninges, ependyma, and choroid plexus. Using immunofluorescence techniques, the topographic distribution of infected LCMV neurons has not been mapped in detail, but studies have suggested greatest involvement in the cortex, hippocampus, and cerebellum.[3,12-14] Our results using immunoperoxidase techniques are in close agreement with these earlier observations.

Of particular interest is our finding that neuronal populations with specific axosomatic or axodendritic connections do not equally express viral antigens. For example, some selective nuclei of the thalamus, (ie, the anterior nucleus) rarely stain, while their corresponding projecting cortical neuronal population (cingulate gyrus) frequently shows immunoperoxidase product. This suggests that the virus probably does not spread by axonal transport. This contention is substantiated by electron-microscopic examination. Axons consistently show an absence of viral antigens, while positive immunoperoxidase staining is associated with ribosomes in the perikaryon and dendrites.

The finding of LCM viral antigen associated with host cell ribosomes is of interest. LCM virions contain host-derived ribosomes,[15] and Abelson and coworkers[16] have described a similar association of viral antigens with ribosomal aggregates in infected 3T3 cell cultures. The predominant expression of viral nucleocapsid protein with polyribosomes suggests several possibilities. First, this may reflect active synthesis of NP on the polyribosomes. Alternatively, this protein may have an unusual affinity for ribo-

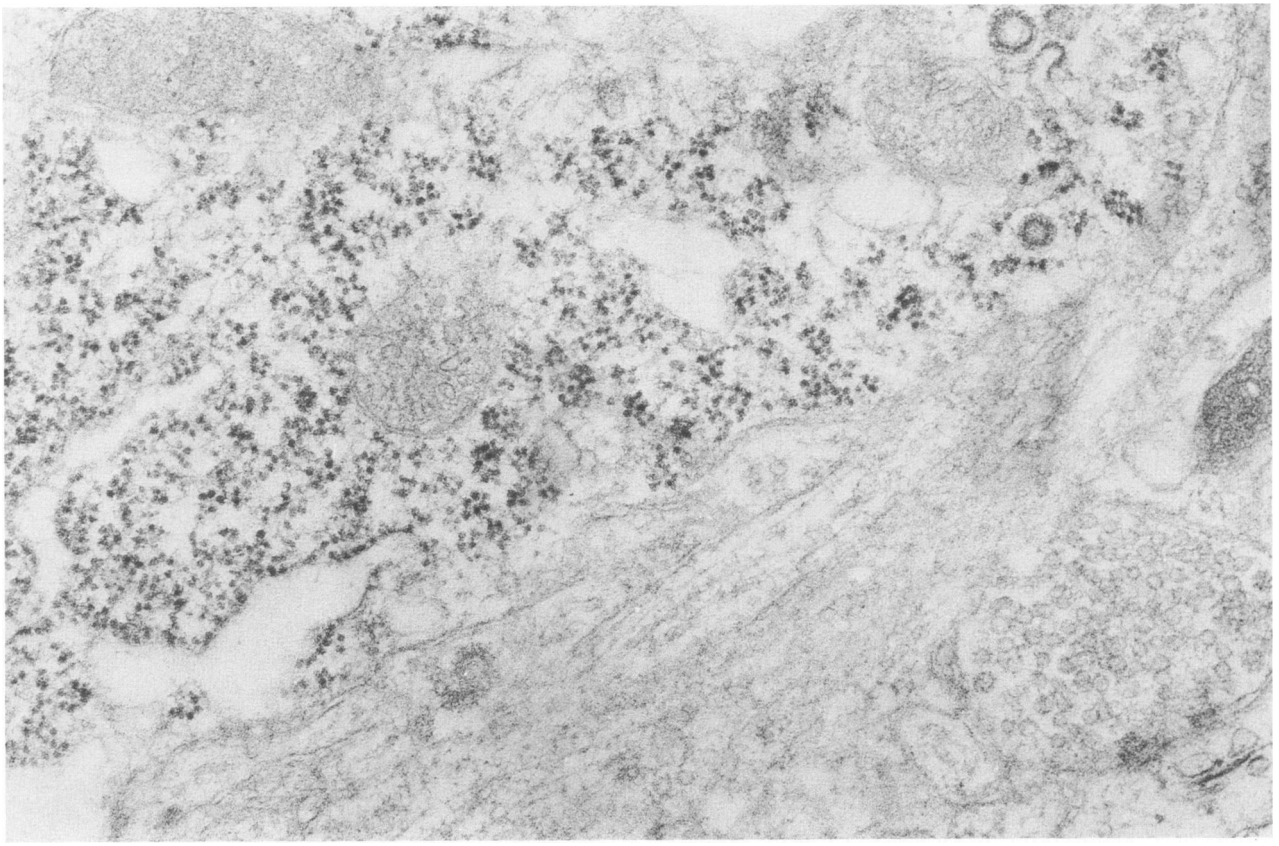

Figure 2—Electron micrograph of a positively staining neuron taken from the vibratome section shown in Figure 1c stained with the guinea pig polyclonal antibody against LCMV. Note that the immunoperoxidase product is associated selectively with host cell ribosomes. Synaptic vesicles, mitochondria, and the adjacent neuropil are not staining.

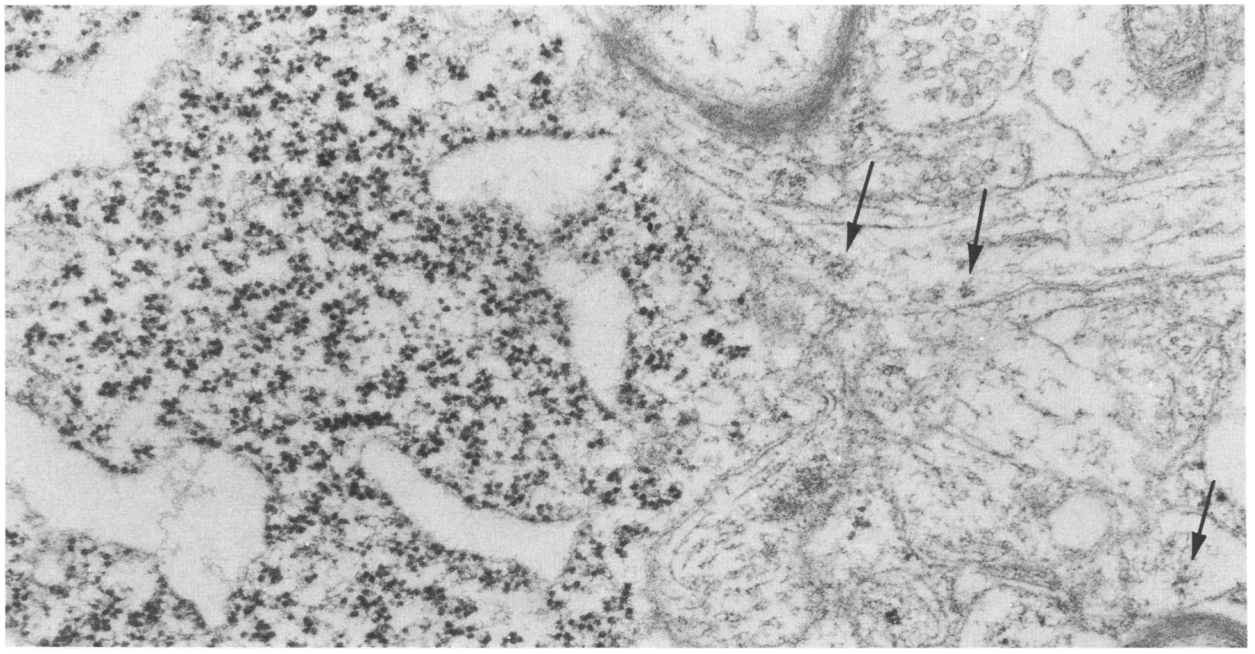

Figure 3—Ultrastructural localization of the nucleocapsid protein of LCMV in a cortical neuron using the monoclonal hybridoma mouse antibodies. Note similar immunoperoxidase staining along host polyribosomes. *Arrows* indicate unstained ribosomes in the adjacent uninfected neuropil. (×75,000)

somes, thus causing aggregation. Finally, the presence of abundant NP but apparent lack of GP suggests either a dissociated synthesis of these components, NP being the dominant species synthesized; or, alternatively, the half-life for degradation of NP in the cell may be greater than that of GP. The fact that viral proteins are not expressed at the plasma membrane suggests a mechanism by which these cells may be refractory to immune attack. Differential synthesis and expression of LCMV polypeptides has been observed *in vitro* in persistently infected mouse and hamster cells.[6,17] The present observations suggest that this mechanism operates *in vivo* as well.

There is evidence indicating that LCMV may interfere with the function of neuronal populations without causing morphologic alterations. Using persistently infected neuroblastoma cells with LCMV, Oldstone et al[18] have shown in tissue culture a decrease in the cell's ability to make choline acetyltransferase and acetylcholine esterase. Persistent LCMV infection does not alter cellular morphology nor affect RNA, DNA, or total protein synthesis. This may provide a mechanism whereby a virus can cause cell dysfunction without cell destruction. In addition, some evidence exists suggesting that mice persistently infected with LCMV may show behavioral alterations.[13,14] Neonatally inoculated LCMV carriers tested between 2 and 6 months after infection are more reluctant to explore a novel environment and are more sensitive to low-current electric shock than control mice. These behavioral findings correlate well with the localization of viral antigens in the cortex, hippocampus, and limbic system.

The present *in vivo* studies using LCMV suggest that a virus may selectively infect specific neuronal populations and possibly induce neurochemical and functional alterations without causing morphologic injury. In addition, virus may persist in neurons as a result of the differential expression of virus-associated protein antigens. This mechanism of viral persistence may play a role in the pathogenesis of some degenerative diseases of the CNS that are caused by a progressive loss of specific neuronal populations.

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Acknowledgments

We appreciate the excellent technical help provided by Bob Garrett, Hanna Lewicki, Alison Barbee, and Anne Lampert and the secretarial assistance of Cathy Doria.