

SHORT COMMUNICATION

Virus RNA Persists within the Retina in Coronavirus-Induced Retinopathy

YUSUKE KOMURASAKI, CHANDRASEKHARAM N. NAGINENI, YUN WANG, and JOHN J. HOOKS¹

Immunology and Virology Section, Laboratory of Immunology, National Eye Institute, National Institutes of Health, Bethesda, Maryland

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The murine coronavirus, mouse hepatitis virus (MHV), JHM strain, induces a biphasic retinal disease in adult BALB/c mice. In the early phase, Day 1 to Day 7, a retinal vasculitis is noted which is associated with the presence of viral proteins and infectious virus. In the late phase, Day 10 to Day 140, a retinal degeneration is associated with the absence of viral proteins, infectious virus, and inflammatory cells. The purpose of this study was to determine if viral RNA persists within the retina during the retinal degenerative phase of the disease. BALB/c mice were inoculated by the intravitreal route with $10^{4.0}$ TCID₅₀/5 μ l of virus. The presence of viral RNA was detected by *in situ* hybridization with a viral cDNA probe and viral proteins were identified by immunocytochemical staining. During the acute phase of the infection, viral RNA was found in the retina, RPE, ciliary body epithelium, and the iris epithelium. During the late phase of the infection, viral RNA was almost exclusively found within the retina and RPE and not in the anterior segment of the eye. Within the retina, viral RNA was detected in the ganglion cell layer, the inner retina, the outer retina, and the RPE cell. Immunocytochemical staining identified viral protein within the retina only from Day 1 to Day 8. This ocular disease was also associated with a persistent systemic infection. Both viral RNA and viral proteins were identified within the liver during the first 8 days. However, only viral RNA was detected in the liver from Day 8 to Day 60. These studies demonstrate that MHV established an acute infection (Day 1–8) where infectious virus and viral proteins were identified. This was followed by a persistent infection within the retina and liver where only viral RNA were detected by *in situ* hybridization. © 1996 Academic Press, Inc.

Retinal degenerative disorders consist of a diverse group of diseases frequently associated with a genetic predisposition; however, many cases are of unknown cause. Viruses may trigger some of these pathologic processes. In fact, viral infections often precede acute multifocal placoid pigment epitheliopathy and acute macular neuroretinopathy, and therefore are under suspicion as causative agents (1–2). In addition, herpes simplex virus and varicella zoster virus have been implicated in acute retinal necrosis (3, 4) and cytomegalovirus frequently induces a retinitis in immunosuppressed individuals (5). Retinal degenerative changes were also seen in Creutzfeldt-Jacob disease and in subacute sclerosing panencephalitis (6, 7). In order to evaluate the varied ways by which viruses may trigger retinal degenerative processes, we have developed a model of virus-induced retinopathy.

The murine coronavirus, mouse hepatitis virus (MHV), induces an acute and chronic ocular disease in BALB/c mice (8–10). In the early, acute phase, 1 to 7 days after inoculation, a mild retinal vasculitis was observed. The second stage was seen by Day 10 and progresses for several months. This stage was characterized by a retinal

degeneration in the absence of vasculitis or inflammation. This degenerative process was associated with a reduction of the photoreceptor layer, loss of interphotoreceptor retinoid binding protein, abnormalities in the retinal pigment epithelium (RPE), and retinal detachments. The development of the degenerative phase of the disease was controlled by a genetic predisposition of the host and was associated with the development of anti-retinal and anti-RPE cell autoantibodies (11).

One of the intriguing aspects of this disease process is the nature of viral clearance. The acute phase of the disease was associated with the presence of viral proteins and the detection of infectious virus within the retina (8, 9). However, after Day 8, infectious virus and viral proteins were not detected. At this time, anti-virus neutralizing antibody were readily detected within the sera of infected animals. In the absence of infectious virus, retinal degenerative changes became apparent at Day 10 and continued for months. The purpose of this study was to determine if the virus persisted within the retina and other tissues during the course of this disease process. *In situ* hybridization was selected as a way to demonstrate virus persistence and to identify the cellular location of the virus.

The JHM strain of mouse hepatitis virus (MHV-JHM) was obtained from American Type Culture Collection (ATCC, Rockville, MD) and passaged five to seven times

¹ To whom correspondence and reprint requests should be addressed. Fax: (301) 402-0485.

in mouse L2 cells. Viral infectivity titrations were performed on L2 cells propagated in 96-well microtiter plates. The infectivity of stock virus was $10^{5.6}$ TCID₅₀/0.1 ml. Clone 2-2, which contained cDNA representing MHV-A59 genes 4–6 cloned into *Pst*I site of pGEM-1 (Promega, Madison, WI), was provided by Dr. Susan R. Weiss (University of Pennsylvania, Philadelphia, PA) (12). The insert was excised from the vector and purified by gel electrophoresis. cDNA labeling with digoxigenin-11-dUTP was carried out by the random primed method using a commercially available kit (Boehringer Mannheim, Indianapolis, IN).

Intravitreal inoculation of 12-week-old male BALB/c mice was performed as previously described (8, 9). Mice were inoculated in the right eye with 5 μ l of MHV-JHM in culture medium. The contralateral eye was not treated. On selected days after inoculation mice were sacrificed, eyes and livers were removed, fixed in 10% buffered formalin, and then embedded in paraffin wax. Before hybridization, the slides were deparaffinized, rehydrated, digested with proteinase K, and air-dried. Twenty-five microliters of prehybridization solution (50% deionized formamide, 1 \times Denhardt's solution, 4 \times SSC (1 \times SSC is 150 mM NaCl plus 15 mM sodium citrate), 10% dextran sulfate, and 100 μ g/ml denatured salmon sperm DNA) was added to each slide, followed by incubation for 1 hr at room temperature. Five microliters of the labeled probe was added to the slides (final concentration of labeled cDNA was 100 ng/ml). Coverslips were placed on slides and the probe DNA was denatured on a hot plate at 90° for 10 min. The slides were cooled on ice and hybridization was carried out for 16 hr at 37° in a humidified box. After removal of coverslips the slides were washed in 2 \times SSC twice for 10 min at room temperature, 1 \times SSC for 30 min at room temperature with mild agitation, and 0.1 \times SSC twice for 10 min at 42°. Negative controls, RNase A (Boehringer Mannheim) treated tissue sections, digoxigenin-labeled linearized pBR328 (Boehringer Mannheim) as nonhomologous probe, and *in situ* hybridization without probe were processed similarly.

The slides were washed twice for 5 min in buffer 1 (100 mM Tris–HCl, pH 7.5, 150 mM NaCl), then they were immersed in buffer 2 (0.5% blocking reagent solution, Boehringer Mannheim) for 60 min. After brief dipping in buffer 2, they were incubated with anti-digoxigenin antibody (1 in 500 dilution with buffer 2) conjugated to alkaline phosphatase for 1 hr. This was followed by two 15-min washes in buffer 1 and immersion in buffer 3 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl) for 3 min. Visualization was performed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate solution in a dark sealed box for up to 16 hr and the reaction was stopped by immersion in buffer 4 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) before mounting in Aqua-Mount (Lerner Laboratories, Pittsburgh, PA). Paraffin sections were also assayed for viral proteins by an immuno-

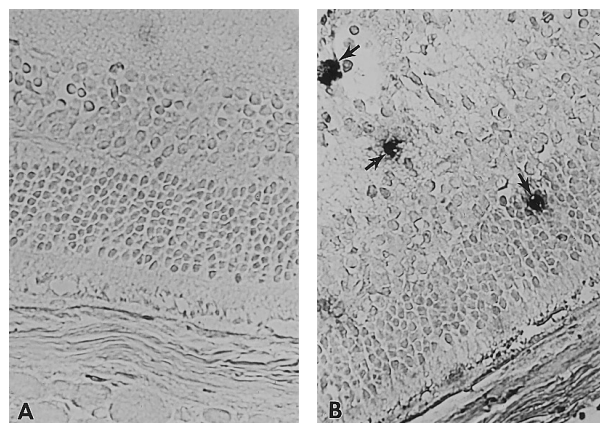


FIG. 1. Representative photomicrographs of *in situ* hybridization with viral cDNA probes in normal (A) and virus-infected (B) BALB/c mouse eyes (Day 3). (A) No hybridization was detected in the retina of the uninfected eye. (B) Positive hybridization was seen within the retina. Arrows indicate reactivity from top to bottom: inner plexiform layer, inner nuclear layer, and outer nuclear layer segments. Magnification 200 \times .

peroxidase procedure, as described previously (8, 9). Rabbit polyclonal antibody to MHV (F88, provided by Dr. Katherine Holmes, Uniformed Services Univ Health Sci, Bethesda, MD) or normal rabbit sera were used.

In order to evaluate the detection of viral RNA by *in situ* hybridization, we first studied JHM virus replication in L2 cells. The virus cDNA probe hybridized to virus RNA in the infected cultures. In contrast, no reactivity was noted in the untreated L2 cells. Moreover, RNase treatment of infected cultures inhibited reactivity and incubation of the infected cultures with plasmid cDNA resulted in no reactivity (data not shown). Analysis of *in situ* hybridization for viral RNA in JHM virus infected BALB/c mouse eyes is shown in Fig. 1. When uninfected BALB/c mouse eye sections were incubated with the viral cDNA probe, no reactivity was observed (Fig. 1A). In contrast, when JHM virus-infected BALB/c mouse eye sections were incubated with the viral cDNA probe, a positive signal was noted within the retina (Fig. 1B). If the virus-infected eye sections were pretreated with RNAase, the reactivity with the viral probe was inhibited. Likewise, when JHM virus-infected BALB/c mouse eyes were incubated with plasmid cDNA, no reactivity was noted.

This assay system was then used to evaluate mouse eyes harvested from 1 to 60 days after JHM virus inoculation. By *in situ* hybridization, the viral RNA was detected within the retina from Day 1 to Day 60. The mean number of positive signals of reactivity with the viral cDNA probe are summarized in Fig. 2. Multiple areas of viral RNA were detected within the eye throughout the 60-day period, while the maximal number of positive signals were noted between Day 5 and Day 10. We next evaluated the cellular distribution of viral RNA within the eye. As is seen in Table 1, the majority of viral RNA was detected in the retina. Low levels of viral RNA were detected in

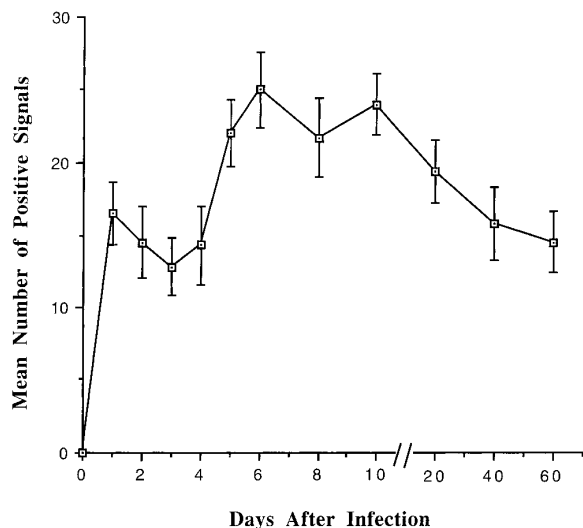


FIG. 2. Reactivities of the viral cDNA probe in JHM virus-infected BALB/c mice eyes. Eyes were harvested on selected days postinoculation (Days 0–60) and hybridized with the viral cDNA probe. The mean numbers of positive hybridization signals in the eyes are shown. Five whole eye sections from each mouse were evaluated in a masked fashion. Vertical bars indicate standard errors of the mean.

the ciliary body epithelium and the iris epithelium in eye sections obtained from animals from Day 1 to Day 6 postinoculation. Samples were further analyzed to localize the areas within the retina which hybridized with the viral cDNA probe (Table 2). The retina was classified into four areas: ganglion cell layer, inner retina, outer retina, and RPE. The inner retina contains the inner plexiform layer, the inner nuclear layer, and Muller cells. The outer retina consists of the outer plexiform layer, outer nuclear layer, photoreceptor inner and outer segments, and Muller cells. The Muller cell which is known to allow JHM virus replication actually transverses the retina and is therefore a part of the inner and outer retina area.

TABLE 1

Distribution of JHM Virus RNA within the Eyes of BALB/c Mice

Day	Number of mice examined	Mean number of reactions with viral cDNA probe		
		Retina	Ciliary body	Iris
0	2	0	0	0
1–2	4	13	3	1.5
3–4	7	13	0.8	0.2
5–6	8	23	0.2	0.2
8–10	7	23	0	0
20	5	19	0	0
40	5	15	0.8	0
60	4	15	0	0

Note. Viral RNA was hybridized in paraffin sections by digoxigenin-labeled cDNA probe. Five whole eye sections from each mouse were evaluated in a masked fashion.

TABLE 2

Distribution of JHM Virus RNA within the Retina of BALB/c Mice

Day	Number of mice examined	Mean number of reactions with viral cDNA probe			
		GCL	Inner retina	Outer retina	RPE
0	4	0	0	0	0
1–2	4	3	4	5	0.5
3–4	7	3	3	7	0.4
5–6	8	6	9	9	0.3
8–10	7	4	9	10	1
20	5	2	7	9	1
40	5	3	7	4	1
60	4	2	5	7	1

Note. GCL, ganglion cell layer; Inner retina contains inner plexiform layer (IPL), inner nuclear layer (INL); Outer retina contains outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor inner segments, photoreceptor outer segments; RPE, retinal pigment epithelial layer. Muller cells exist from GCL to ONL.

Reactivity was noted throughout the various layers of the retina. The maximal number of positive signals seen within the retina occurred between Day 5 and Day 10 postinoculation. It should be noted that at this time, the retina is infiltrated with macrophages.

The data presented indicates that the viral RNA remains within the retina throughout the 60-day period of analysis. Since our earlier studies showed that infectious virus and viral antigen was not detected within the retina after Day 7, we wanted to compare the two assay systems on the same samples (8, 9). Both viral RNA and viral protein were detected in 100% of the eyes removed during Day 1 to Day 6 postinoculation. Between Day 8 and Day 60, viral RNA was detected in 100% of the retinas. However, viral antigen was detected in only one of four eyes removed 8 to 10 days postinoculation and was not observed in any of the eyes harvested at 20 to 60 days postinoculation. These studies showed that although the viral RNA was identified within the retina for 60 days, viral antigen was detected only during the first 8 days of the infection.

Since JHM virus RNA persisted within the retina during the 60-day period of analysis, we wanted to determine if the virus was disseminated and persisted in other organs. Livers were removed from the same animals which have been evaluated for the ocular studies reported. Viral RNA could be detected in all of the liver samples tested from Day 1 to Day 60 postinoculation. The maximum number of positive signals were seen between Day 3 and Day 8 postinoculation. In contrast, viral RNA was not detected in liver samples from uninfected mice. The production of viral protein was evaluated on the adjacent liver sections by immunoperoxidase staining. As was seen in the retina, viral protein could be detected within

the liver at Day 1 through Day 8 postinoculation. However, after Day 8, viral protein was not detected by immunocytochemical staining even though viral RNA was detected by *in situ* hybridization (data not shown).

In this study we demonstrated that JHM virus established a persistent infection within the retina of BALB/c mice. Throughout the 60-day period, viral RNA was detected by *in situ* hybridization within the retina. Moreover, evaluation of the liver revealed a similar pattern of viral persistence, indicating that the virus-induced retinopathy was associated with a systemic persistent virus infection. The technique of *in situ* hybridization allowed us to identify some of the cell types within the retina which contained the viral RNA. During the acute phase of the infection, viral RNA was found in the retina, RPE, ciliary body epithelium, and the iris epithelium. During the late phase of the infection, viral RNA was almost exclusively found within the retina and RPE and not in the anterior segment of the eye. Within the retina, viral RNA was detected in the ganglion cell layer, the inner retina, and the outer retina. The Muller cell is considered the most prominent glial element in the retina. These cells transverse the layers of the retina in a radial direction and are therefore found in each of the layers of the retina. Earlier studies showed that the virus infects the Muller cell (13). The presence of viral RNA transversing the layers of the retina probably reflects the presence of virus within the Muller cells.

The RPE cell is a potent regulatory cell, maintaining physiologic and structural balance within the retina. We have also found that this cell appears to play a critical immunologic role. The cells may be activated to express MHC molecules enabling them to process and present both foreign and retinal proteins to helper T cells (14–17). The continued presence of viral RNA within retinal cells may contribute to the maintenance of persistence and to the development of autoimmune responses.

A number of studies indicate that MHV can cause acute and persistent infections in the central nervous system (CNS) and liver (18). For example MHV infection in mice resulted in an acute necrotizing encephalitis followed by a chronic demyelinating CNS disease (19, 20). In fact, in C57BL/6 mice, viral RNA was detected in oligodendrocytes for 10 months after it was possible to recover infectious virus or detect viral antigens (21). Perlman and coworkers also identified MHV, JHM strain, viral RNA in C57BL/6 mice with demyelinating encephalomyelitis (22, 23). These investigators suggested that persistence may be maintained due to viral spread from cell to cell thereby escaping immune elimination (24, 25). In a separate study, the hepatotropic MHV strain, MHV-3, was evaluated in C3H mice. Viral RNA was detected in the liver during the first 7 days after inoculation. However, after Day 21 viral RNA and proteins were not detected in the liver. In contrast, in the brain, viral RNA was de-

tected in meningeal cells up to 77 days after inoculation. Thus the persistent infection was associated with the CNS and not the liver in this model system (26). In addition to these studies which identify persistent infection in mice, MHV persistent infections have also been detected in the rat and monkey CNS (27, 28).

The data presented here identified that virus RNA was detected within the retina for 8 weeks after infectious virus and viral proteins were detected. The loss of infectious virus and viral proteins was correlated with the activation of several components of the immune system. Anti-virus neutralizing antibodies and anti-retinal autoantibodies were observed in the sera 6 to 7 days after infection (8, 11, 29). This may be a critical period for the virus to avoid the immune surveillance (30, 31). In this model system of murine coronavirus-induced retinopathy, the continuous presence of viral RNA and the possible production of low levels of viral proteins may contribute to the multifaceted pathogenic processes within the retina.

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