Studies of Ocular Murine Cytomegalovirus Infection

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The pathogenesis of ocular cytomegalovirus (CMV) infection in mice was studied in detail as a model of ocular involvement of human CMV infection. Smith strain of mouse CMV (MCMV) was inoculated into the anterior chamber of the eye and viral antigen was located by immunofluorescence. When salivary gland passaged MCMV (SG-MCMV) was inoculated into the 12- to 18-day-old ICR/Sic mice, it elicited transient uveitis, retinitis, and scleritis during the early phase of infection followed by spread into the lacrimal glands, extraocular muscles and salivary glands. Mouse embryonic fibroblasts (MEF) passaged attenuated MCMV (CC-MCMV) caused mild uveitis with a short duration even inoculated into young mice, and the viral antigen was detected only in salivary glands thereafter. SG-MCMV titer in the eye taken from the young mice decreased from $10^{4.5}$ TCID$_{50}$/0.1 g at 2 days postinfection (PI) to $10^{1.0}$ TCID$_{50}$/0.1 g at 21 days PI, whereas in the salivary glands, it became detectable at 5 days PI with a titer of $10^{5.0}$ TCID$_{50}$/0.1 g, which increased up to $10^{8.7}$ TCID$_{50}$/0.1 g at 21 days PI. CC-MCMV was detectable only in the young mice salivary glands ranging from $10^{4.7}$ TCID$_{50}$/0.1 g at 9 days PI to $10^{3.3}$ TCID$_{50}$/0.1 g at 21 days PI. When the lens capsule had been damaged during the inoculation procedure, the mice developed cataracts with evidence of viral growth in the lens. When SG-MCMV was inoculated onto the cornea, viral antigen was not detected during the period of the present experiment. Balb/c (H-2d) mice, which had experienced latent ocular infection for 5-10 months, yielded MCMV after coculture of minced tissues such as retinochoroid, sclera, cornea, iris, lens, whole eye ball, optic nerve, trigeminal ganglion, hypophysis, lacrimal gland, and salivary gland with allogeneic mouse (C3H/HeJ, H-2k) embryonic fibroblasts. When mice intraperitoneally infected for 26 days to 14 months were examined for the content of MCMV using allogeneic coculture assays, MCMV was recovered from various organs including the trigeminal ganglion, eye ball, lacrimal gland, and salivary gland. The recovered virus was identified as MCMV by immunofluorescence. Invest Ophthalmol Vis Sci 26:486-493, 1985

Cytomegalovirus (CMV) is an ubiquitous agent causing one of the most common human infections, mostly in a latent form, as evidenced by seroepidemiology, particularly among newborns and lower socioeconomic groups.1-4

In Japan, over 60% of newborns are infected by this virus,5 and it is feared that congenital CMV infection may lead to serious birth defects, which include mental retardation, blindness and deafness.6-8 Although most of the infection is subclinical, the virus remains in the body as a potentially dangerous agent throughout the life of the individual.9

Ocular involvement of CMV such as uveitis and retinitis have been reported in the literature10-13 as a manifestation of systemic CMV infection. These were seen with a high frequency among recipients of renal, bone marrow, or cardiac allograft, blood transfusion, immunosuppressive therapy and also among patients of acquired immunodeficiency syndrome.14-21

In a previous study of experimental ophthalmitis, necrosis of the uveal tracts and retina was seen in Swiss CD-1 mice when mouse cytomegalovirus (MCMV) was inoculated into the anterior chamber, simulating the human cases.22 These features seemed to provide a useful model for understanding ocular CMV infections in humans. By using the murine model, therefore, more detailed experiments were designed to determine whether CMV infection could be established by intraocular inoculation of mice and, if successful, to locate viral antigens in the ocular and surrounding tissues. Further, recovery of latent virus from various tissues was attempted during the chronic period of infection by cocultivating with allogeneic mouse embryonic fibroblasts (MEF). Based on the results obtained, the relevance of this model to the human ocular infection will be discussed.
Materials and Methods

Mice

ICR/Sic mice 4 to 10 weeks old (male and female) in specific pathogen free condition were obtained from Shizuoka Experimental Animal Farm (Hama-matsu, Shizuoka, Japan). Twelve- to eighteen-day-old ICR mice were bred in our laboratory. Balb/c mice 2 to 8 weeks old were obtained from the Charles River Company, Japan. They were handled under adequate care and humane treatment according to the ARVO Resolution on the Use of Animals in Research.

Virus

Smith strain 23 of MCMV was kindly supplied by Dr. Y. Minamishima (Miyazaki Medical College, Miyazaki, Japan). Salivary gland passaged MCMV (SG-MCMV) was prepared by homogenating infected salivary glands in Eagle minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Submandibular glands were obtained from ICR/Sic mice which had been infected intraperitoneally with Smith strain of MCMV 14 days earlier; 10% homogenate were prepared and stocked at -80°C until use. Virus titer of the stock viruses ranged from 10^4.8 to 10^6.8 TCID_50/ml. A working stock of avirulent MCMV (CC3-MCMV) was prepared by consecutive three times passage of SG-MCMV in secondary mouse embryonic fibroblasts (MEF). Virus titer of CC3-MCMV ranged from 10^4.1 to 10^6.0 TCID_50/ml.

Immunofluorescence

Adult ICR/Sic mice were inoculated intraperitoneally with 0.1 ml of SG-MCMV 14 days earlier; 10% homogenate were prepared and stocked at -80°C until use. Virus titer of the stock viruses ranged from 10^4.8 to 10^6.8 TCID_50/ml. A working stock of avirulent MCMV (CC3-MCMV) was prepared by consecutive three times passage of SG-MCMV in secondary mouse embryonic fibroblasts (MEF). Virus titer of CC3-MCMV ranged from 10^4.1 to 10^6.0 TCID_50/ml. 5 min at room temperature. They were then flooded with the labeled antibody overnight at 4°C, washed with several changes of PBS and observed under the fluorescence microscope.

Inoculation of Virus

Intraocular inoculation of virus: Under ether anesthesia, a group of mice was inoculated with 0.012 ml to 0.025 ml of MCMV (10^5.0 TCID_50/ml) into the anterior chamber of the eye through corneal limbus using a 27-gauge needle under a stereoscopic microscope. Before the inoculation, the anterior chamber of the eye had been punctured to make a space for the inoculum. Another group of mice was inoculated with 0.012 ml of SG-MCMV (10^6.0 TCID_50/ml) onto the scarified cornea. The animals were observed daily for clinical signs and sacrificed at the scheduled times thereafter.

Intraperitoneal inoculation: Balb/c mice were inoculated with 0.25 ml (10^5.0 TCID_50/ml) of the virus into the peritoneal cavity using a 26 gauge needle.

Study of Acute Infection

Various organs including the eye balls, optic nerve, trigeminal ganglia, preauricular lymph nodes, lacrimal glands, Harder's glands, submandibular glands and hypophyses were removed aseptically from four animals at each timepoint and were frozen in cooled n-hexane for the immunofluorescence test. For determination of the virus titers, five animals were sacrificed at the scheduled times and paired organs or tissues from each animal were pooled and then were homogenized to make 10% emulsions in MEM supplemented with 10% calf serum. After centrifugation at 2500 rpm for 10 min, the supernatant fluid was removed, diluted in serial 10-fold steps with MEM, and then inoculated in 0.1 ml amounts to MEF grown on 96-well microtitration plates (Linbro Co.; Hamden, CT). The plates were incubated at 37°C in a humid atmosphere containing 5% CO_2 and observed for appearance of cytopathic effects (CPE).

Coculture Assay

Various organs were removed aseptically from the Balb/c mice in the chronic phase of infection. They were minced finely with scissors and cocultivated with C3H/He mouse embryonic fibroblasts for recovery of infective virus. Infected eye balls were separated into retina, choroid, sclera, cornea, iris and lens under the stereoscopic microscope. The cells were refed with fresh growth media at 5-day intervals. They were observed for CPE until the end of 2 mo of cocultivation.
vation. When CPE appeared, the virus was identified by immunofluorescence.

Results

Acute Phase of Infection

Different distribution of MCMV antigen depending on the animal age and passage history of the virus:

Distribution of MCMV antigen was studied by immunofluorescence. Differently aged mice (14 days old and 8-10 weeks old) were inoculated with either SG-MCMV or CC3-MCMV into the bilateral anterior chambers and examined for viral antigen by direct immunofluorescence. When 0.012 ml of CC3-MCMV was inoculated into 14- to 18-day-old mice, scattered viral antigen was first detected 3 days after the inoculation at the root of the iris. Subsequently at 7 days postinfection (PI), it was seen in the ocular choroid and the acinar epithelium of the submandibular glands. Fourteen days PI, antigen was occasionally seen only in the acinar cells of the submandibular glands.

When 8- to 10-week-old adult mice received CC3-MCMV, no viral antigen was detected in the eye, lacrimal gland, optic nerve, trigeminal ganglion, parotid gland, submandibular gland, hypophysis, liver or spleen at 3 and 7 days PI and thereafter.

On the second experiment, SG-MCMV was inoculated into the bilateral anterior chambers of young and adult mice. In young mice (12 to 18 days old), scattered viral antigen had been detected at 3 days PI at the root of the iris and it became widely distributed in the entire layers of the iris at 7 days PI (Fig. 1). Viral antigen was then extended to the sclera, choroid, retina, ocular muscles (Fig. 2), acinar cells of Harder’s and lacrimal glands, submandibular and parotid glands. Viral antigen in most of these tissues lasted for 2 weeks.

At three weeks PI, viral antigen was seen only in the submandibular gland (Fig. 3).

When adult mice (8-10 weeks old) had received SG-MCMV, viral antigen was first seen in the iris at 6 days PI. However, unlike the case of young mice, it was not extended to a wider area but confined to the iris until 10-12 days PI. Viral antigen was detected only in the submandibular glands 14 days PI and thereafter until 28 days PI. During the inoculation procedure, the lens capsules of some young mice had been damaged and they developed cataract with an intense positive fluorescence of viral antigen in the epithelium and lens fibers when examined at 3-5 days PI (Fig. 4).

By histopathologic examination, the infected areas which corresponded to the immunofluorescence positive sites showed focal necrosis with scattered small round cell infiltration and occasional intranuclear and cytoplasmic inclusion bodies.

Failure to detect infection in the corneal epithelium:

When 0.012 ml of SG-MCMV (10^6.0 TCID50/ml) was instilled into the conjunctival sac after corneal scratching, viral antigen as detected by immunofluorescence
Viral growth in the affected organs as assessed by titrating the infectivity of organ homogenates: The immunofluorescence study revealed that the virus, either SG-MCMV or CC3-MCMV, could invade in the ocular and surrounding tissues in young mice. These results were confirmed by titrating the infectivity of organ homogenates obtained from infected young mice (14–16 days old).

Twenty-four CC3-MCMV-infected mice (14 day old) were killed at appropriate times after bilateral intraocular infection. Various organs including the eye ball, optic nerve, trigeminal ganglia, submandibular glands, parotid glands, lacrimal glands, Harder's glands, and hypophyses were removed aseptically and homogenized into 10% (w/v) emulsions for infectivity titration in MEF. Results are shown in Figure 5. CC3-MCMV was positive only in submandibular glands at 9 days PI ($10^4.7$ TCID_{50}/0.1 g of the tissue).
and the infectivity increased up to $10^{7.3}$ TCID$_{50}$/0.1 g at 21 days PI. Infectivities in all the other organs were less than the detectable level.

Results of similar viral titration experiments using SG-MCMV are summarized in Figure 6. The ocular virus titer in young mice (14 to 16 day old) decreased gradually from $10^{4.5}$ TCID$_{50}$/0.1 g at 2 days PI to $10^{3.0}$ TCID$_{50}$/0.1 g at 21 days PI.

**Chronic Phase of Infection**

Virus harbored in various parts of the eye and surrounding tissues: In this experiment, Balb/c (H-2d) mice were used instead of ICR/Sic mice, since they had shown similar or a higher susceptibility to MCMV. Two- to three-week-old Balb/c mice were inoculated with 0.012 ml of SG-MCMV into the anterior chamber of the eye. Various ocular and surrounding tissues were taken in the chronic phase of infection and cocultured with allogeneic C3H MEF (H-2$k$).

Table 1 records the data obtained from these mice latently infected with the virus for varying time periods. As can be seen, infectious virus was recovered from the various parts of the eye and surrounding tissues.
tissues. All the cytopathic effects (CPE) observed were identified by direct immunofluorescence as being due to growth of MCMV.

The mean duration of cocultivation before the appearance of CPE was about a month, the shortest being 7 days with the iris of mice killed at 10 months PI, and the longest being 2 months with the whole eye ball, retina, choroid, optic nerve, trigeminal ganglion and salivary gland of mice killed at 6 months PI.

Presence of latent virus in ocular tissues after systemic infections: Table 2 summarizes results of attempts to recover virus by the allogeneic cocultivation with eye and surrounding tissues obtained from mice first infected intraperitoneally and bred for varying duration times. It is interesting that infectious virus was recovered from the eye, lacrimal gland and trigeminal ganglion even after the intraperitoneal inoculation. It was confirmed by immunofluorescence that most of the organs harboring latent virus coincided with the organs susceptible to MCMV during the acute phase of infection (data not shown). Infectious virus was recovered from over 15% of the salivary and lacrimal glands tested. The positive ratios recorded for others organs were less than 10%.

A part of these organs was homogenized and the supernatant was assayed for infectious virus using mouse embryonic fibroblasts. Most of the results was negative except for a few positive cases with the salivary glands of mice killed at 35 days PI.

Discussion

Although ocular involvement during systemic viral infections, notably those accompanying congenital infections, have been documented, detailed studies on the pathogenesis and sequelae of human cytomegalovirus infection have been hampered by the lack of susceptible laboratory animals. However, the immunofluorescence study of the ocular MCMV infection provided a sensitive and specific method to reveal detailed insights of the distribution of viral antigens in the affected parts of the ocular and surrounding tissues.

Intraocular viral growth and spreading were rather transient as was shown by both immunofluorescence and virus titration. After the acute phase of infection, viral antigen became detectable in glandular tissues such as the lacrimal and salivary glands.

Table 1. Recovery of murine cytomegalovirus by cocultivation from chronically infected mice

<table>
<thead>
<tr>
<th>Organ</th>
<th>Duration after intraocular infection (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Five</td>
</tr>
<tr>
<td>Whole eye ball*</td>
<td>2/2</td>
</tr>
<tr>
<td>Retina  chorioid</td>
<td>1/6</td>
</tr>
<tr>
<td>Sclera</td>
<td>0/6</td>
</tr>
<tr>
<td>Cornea</td>
<td>0/6</td>
</tr>
<tr>
<td>Iris</td>
<td>0/6</td>
</tr>
<tr>
<td>Lens</td>
<td></td>
</tr>
<tr>
<td>Optic nerve†</td>
<td>0/1</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>0/1</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>0/1</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0/1</td>
</tr>
<tr>
<td>Hypophysis</td>
<td>0/1</td>
</tr>
</tbody>
</table>

* Positive/number of eyes examined.
† Positive/number of mice examined.

Two- to three-week-old Balb/c mice were inoculated with 0.012 ml of salivary gland passaged MCMV (SG-MCMV) (10^6 TCID 50/ml) intraperitoneally. In the chronic phase of infection, the eye and surrounding tissues from each mouse were pooled and were allococultured for recovery of the virus. The number of positive isolation of the virus from individual mouse tissue is indicated in numerator and the total number of eyes or mouse tissues examined in denominator.

Table 2. Recovery of murine cytomegalovirus by cocultivation from chronically infected mice

<table>
<thead>
<tr>
<th>Organs</th>
<th>Duration after intraperitoneal infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>26</td>
</tr>
<tr>
<td>Trigeminal ganglion*</td>
<td>1/4</td>
</tr>
<tr>
<td>Hypophysis</td>
<td>0/2</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>0/2</td>
</tr>
<tr>
<td>Eye</td>
<td>0/2</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>(3/4)†</td>
</tr>
<tr>
<td>Peripheral blood lymphocytes</td>
<td>0/7</td>
</tr>
</tbody>
</table>

* Positive/number of mice examined.
† At 35 days postinfection, infectious virus was recovered by cocultivation and also detected in the supernatant of the salivary gland homogenate.

Two- to three-week-old Balb/c mice were inoculated with 0.25 ml of salivary gland passaged MCMV (SG-MCMV) (10^6 TCID 50/ml) intraperitoneally. In the acute phase of infection, the data of viral antigens became detectable in glandular tissues.
These features again ascertained the relevance of this experimental animal system as a model of the human CMV infection. It is interesting to note that, unlike the case of herpes simplex virus, the cornea is insusceptible to MCMV infection. Although there are few reported cases of corneal diseases accompanying systemic CMV infections, these were seen only in the terminal stage of the diseases or in the case of severe general conditions without evidence of CMV infection of the cornea itself. Whether MCMV can infect the cornea of immunocompromised or exhausted mice is yet to be studied.

The pathogenicity of MCMV is different depending upon the passage history of the virus. Salivary gland passaged virus is comparatively more virulent and spreads to various parts of the intraocular (iris, choroid, and retina) and surrounding (extraocular muscles, Harder's and lacrimal gland) tissues. This phenomenon has been reported also in systemic infection. Attenuated MCMV can be obtained after a few passages in MEF. In the present experiment, three times passaged virus was used throughout. It was shown that viral antigen was detected transiently in the iris and choroid only when it was inoculated into young mice (14-16 days old).

The susceptibility of ICR/Sic mice to MCMV is age-dependent. Virus can spread to various tissues in young mice (12-18 days old), while in adult mice (8-10 weeks old) it infects only limited parts of the iris. There might be many factors accounting for this age dependent susceptibility, but discussion on this point is out of the scope of this paper.

The latent viral infection differs from the acute or chronic infection in that infectious virus is absent despite the continued presence of viral genome or a nonproductive form of the virus in the cell. But none of these qualifications is absolute since detection of infectious as well as noninfectious virus may depend on the sensitivity and specificity of the test used.

In our experiments, infectious virus was no longer detectable when the supernatant of organ homogenates was added to susceptible mouse embryonic fibroblasts 60 days postintraocular inoculation and 35 days postintraperitoneal inoculation for all organs tested except the salivary glands.

Our experiments showed that this virus was rendered latent with a potential activity but could not tell precisely when the acute or chronic infection ceased. Criteria on which to distinguish between the chronic infection and the reactivation of truly latent virus might be necessary in further experiments. For in vitro reactivation of CMV, we cultivated host cells (tissues) with susceptible cells of an allogeneic origin (C3H/He H-2b) for a prolonged time, such as up to 2 months. These methods may have provided an increased sensitivity of the culture because the mean duration needed for recovery of the virus was about a month, and frequently, virus was recovered after 2 months of cocultivation.

The major findings of this paper was that in both intraocular and intraperitoneal infections, virus was reisolated from various organs that had experienced virus growth in the acute phase of infection. Thus, from the intraocularly infected group, virus was recovered from the retina, choroid, iris, sclera, lens, optic nerve, lacrimal gland, salivary gland, trigeminal ganglion and hypophysis. Some of the eye had become phthisical after the acute phase of infection and virus was recovered more frequently from these eyes. Whether these results are attributable to an initial extensive growth of virus or the incapability of the tissues to eliminate virus due to lowered metabolism or lowered defense mechanism in the phthisical eye is not known at present.

It is interesting to note that the virus was recovered from the lacrimal glands and the eye 45-70 days postintraperitoneal inoculation. These results might indicate the importance of possible virus recrudescence in ocular and surrounding tissues during systemic CMV infection.

Identifying those cells that harbor CMV during the latent or chronic infection is an important problem in both clinical and experimental researches.

In the previous reports, MCMV can establish latency in macrophages, T-and B-lymphocytes. MCMV also causes a prolonged period of chronic infection in the epithelial cells of the renal canaliculari, acinar and ductal cells of the salivary glands.

Whether inflammatory cells accumulating during the acute phase of infection persist and harbor virus or whether cells constituting those tissues are the base of the viral latency has not been elucidated and further experiments are now underway.

Key words: ocular cytomegalovirus infection, mouse cytomegalovirus, immunofluorescence, latent infection, allogeneic cocultivation

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