Role of nitric oxide in murine cytomegalovirus (MCMV) infection

K. Tanaka and S. Noda
Department of Infectious Diseases, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa, Japan

Summary. Cytomegalovirus (CMV) is a typical pathogen of an opportunistic infection. In this review article, various roles of nitric oxide (NO) in murine CMV (MCMV) infections, including acute, persistent and latent infections, are discussed. In the acute phase of MCMV infection, NO plays a protective role against MCMV infection. In contrast, NO has been proven to act as a pathogenic factor in a model of MCMV pneumonitis. In MCMV persistent infection, when viral DNA was detected in the absence of any infectious particle, NO increased the amount of persistently-infected MCMV-DNA. As a result, NO was found to act as "a double edged sword" in the CMV-host relationship.

Key words: Cytomegalovirus, Nitric oxide, Inducible NO synthase (iNOS), iNOS—gene knock out mouse

Introduction

Furchgott, Ignarro and Moncada revealed that Endothelial cell relaxing factor (EDRF) was actually nitric oxide (NO). Thereafter, the development of various methods to measure NO and its derivatives, identification of NO synthases (NOS), syntheses of various NO inhibitors and the making of NOS gene-targeting mice has allowed us to identify numerous roles of NO in various fields of biology (MackMicking et al., 1997).

NO is a highly reactive radical formed from L-arginine, a semi-essential amino acid, by the action of NOS, which possesses 3 isoforms; neuronal NOS (nNOS, NOS-1), inducible (iNOS, NOS-2), and endothelial NOS (eNOS, NOS-3). The enzymatic activities of nNOS and eNOS require Ca2+, whereas iNOS acts in a Ca-independent way. The iNOS can be induced in various cells including macrophages, endothelial cells, epithelium cells and mesangium cells, by such cytokines as TNF-α and IFN-γ, and bacterial endotoxins. In general, iNOS can produce a higher amount of NO than other isoforms of NOS. NO spreads by diffusion, and then passes through the cell membrane without requiring any specific receptor on it, and finally reaches its target molecule. Although NO was originally found to be a physiologically indispensable material, it is now well recognized that NO, especially that derived from iNOS, can also damage normal tissues. As a result, NO is therefore now considered to be a double-edged sword.

Various reports from many laboratories have been published regarding the various roles of NO in viral infections. When the virus infects the host, several anti-viral cytokines are produced in the early stages of infection. Type 1 interferons (IFN α/β) are produced by the infected cells, and IFN-γ and TNF-α are then released from NK cells and/or T cells. Although the direct anti-viral effect of IFN-γ is weaker than that of Type 1 interferons, IFN-γ is a strong activator of iNOS. In addition, TNF-α demonstrates a synergistic effect with IFN-γ to induce iNOS. Sequentially, a large amount of NO is produced by iNOS. The purpose of this article is to demonstrate the various aspects of NO in viral infections. However, NO has been reported to have various roles in various virus infections (Reiss et al., 1998). We therefore focused our attention on one virus, cytomegalovirus (CMV).

Human CMV (HCMV) is a DNA virus, which belongs to β-Herpesvirinae of Herpesviridae. HCMV infection is endemic in almost all human populations. For example, around 90% of adults are seropositive in Japan. Primary HCMV infection, which mainly occurs
in childhood, is usually asymptomatic. Similar to other herpes viruses, the primary HCMV infection is followed by a persistent or latent infection. The persistently or latently infected HCMV seldom causes severe infectious disease in an immunocompetent host. However, in immunocompromised patients, especially in immunocompromised hosts such as developing fetuses, AIDS patients and organ transplant recipients, the primary infection or reactivation of the virus may cause various inflammatory diseases such as encephalitis, pneumonitis, retninitis, hepatitis, gastritis, and colitis. CMV is thus considered to be one of the most important microorganisms in opportunistic infections. In addition, the number of AIDS patients and transplant recipients has dramatically increased in developing and advanced countries, respectively. Such increases in immunocompromised patients raise severe concerns about CMV infection.

All of the results shown in this article were obtained in experiments using mouse CMV (MCMV). Although MCMV cannot infect to humans, it resembles its human counterpart, HCMV, in many ways with respect to the establishment of acute and chronic infection, viral persistency and latency, and host-virus interaction (Hudson, 1979). In this review article, we have demonstrated the roles of NO in protection during the early stage of primary MCMV infection, in the pathogenesis of CMV-mediated pneumonitis, and in MCMV latency (Fig. 1).

Role of NO in early stage of primary CMV infection

In HCMV primary infection, the virus usually spreads throughout a population by direct or indirect person-to-person contact (Britt et al., 1996). In such cases, the sources of the virus are the secretions from the infected patients such as saliva, urine, cervical and vaginal secretions, semen, breast milk, urine, tears, feces and blood. Following a primary HCMV infection, the virus enters the bloodstream. Though it has yet to be elucidated as to how the virus enters the bloodstream, HCMV-DNA can be detected in leukocytes such as monocytes, lymphocytes and neutrophils (Bruggeman, 1993). Using the leukocytes as vehicles, HCMV spreads to various organs including the salivary glands, kidney, liver, spleen, heart, lungs, intestines and so on (Sinzger et al., 1996).

In the mouse model of CMV infection, a mouse is usually infected with MCMV via the intraperitoneal (ip) route. It was previously reported that ip-inoculated MCMV replicated in macrophages and mesothelial cells in the peritoneal cavity (Stoddart et al., 1994). Thereafter, the virus disseminated to various organs such as the spleen, livers, and so on, using the mononuclear phagocytes as the vehicles. As a result, although the infection route is completely different from that in a clinical setting, MCMV by ip infection disseminates in a host in a way similar to that of HCMV. Therefore, the acute injection of MCMV by an ip injection of the virus is considered to be an appropriate acute infection model of HCMV.

The immune responses to acute infection can be classified into two categories: the innate response in the early phase and the specific response in the late phase (Ahmed et al., 1999). In innate immunity against MCMV infection, NK cells (Bukowski et al., 1984; Welsh et al., 1991), macrophages (Selgrade et al., 1979; Hamano et al., 1998), and cytokines collaboratively work together as the first line of anti-MCMV. In specific immunity, CD4+ T cells, CD8+ T cells, and antibody act to eliminate the virus, thus resulting in a protective effect on the virus-associated pathogenesis (Reddahase et al., 1987; Jonjic et al., 1989). Proinflammatory cytokines such as IFN-γ and TNF-α, are also involved in the elimination of MCMV both in the early and a late phase of infection (Lucin et al., 1994; Heise et al., 1995; Presti et al., 1998). Especially, IFN-γ has been shown to play an important role against MCMV (Lucin et al., 1992; Orange et al., 1995, 1996).

It has recently been demonstrated that not only the anti-viral cytokines but also macrophage-derived NO, which is mainly produced by an iNOS-dependent pathway, participates in anti-virus mechanism (Ahmed et al., 1999). We thus examined the role of the iNOS-dependent pathway in the early stage of MCMV primary infection by using an iNOS-gene knock-out mouse (iNOS−/−) together with its littermate ([C57BL/6x129/SvEv] F1, iNOS+/+) (Noda et al., 2001). When, 1x10⁶ plaque-forming unit (PFU), an indicator of a number of productive virus) of MCMV was ip infected, the iNOS−/− mice were more susceptible to a lethal infection with MCMV than the iNOS+/+ mice (Fig. 2). In addition, the iNOS−/− mice generated a much higher peak virus titer in the salivary gland, which was the main organ of MCMV replication after primary infection (Log10 PFU/or gan = 10.5 at 4wk post infection).

Various effects of Nitric oxide (NO) in murine cytomegalovirus (MCMV) infection

![Fig. 1. A schematic representation demonstrating the various effects of nitric oxide (NO) in murine cytomegalovirus (MCMV) infections including acute, persistent and latent infection.](image-url)
vs. 6.3 at 2wk post infection in iNOS+/+ mice). The titers of MCMV in other organs, such as the lung and the spleen increased slightly more in iNOS−/− mice than in iNOS+/+ mice. NK cell cytotoxicity and CTL response in NOS2−/− mice were comparable to those of NOS2+/+ mice. In an in vitro analysis, the peritoneal macrophages from NOS2−/− mice exhibited a lower antiviral activity than those from iNOS+/+ mice in spite of the presence of a similar phagocytic activity, thus resulting in an enhanced viral replication in these cells. The treatment of macrophages from NOS2−/− mice with a selective NOS2 inhibitor, (2-mercaptoethy1)-guanine (MEG), decreased the antiviral activity to a level below that obtained with iNOS−/− mice. The iNOS-mediated antiviral activity of macrophages via NO was thus considered to play a protective role against MCMV infection at an early stage of primary infection (Fig. 3).

Pathogenesis of CMV-mediated pneumonitis

CMV-associated pneumonitis is a serious concern because of its high frequency of occurrence and high mortality in immunocompromised patients. CMV can damage the tissues in organs, including the lung, liver, intestine, stomach, retina and the brain. The damage in various organs, except the lungs, has been believed to result from direct viral cytopathogenicity, although the mechanism for this is unclear. Anti-CMV agents, such as ganciclovir, which inhibits DNA replication of CMV, are effective for CMV infection in without any infectious particles. As a result, we observed that MCMV-DNA was detected up to 32 wk after the primary infection in the lungs of iNOS−/− mice, whereas the viral DNA could not be detected even 2 wk after infection in the lungs of NOS2+/+ mice (Noda et al., 2001). iNOS was thus considered to play a significant role not only in the initial clearance of the primary infection, but also in its latency following a primary infection. It is thus plausible that the viral burden load in the early stage of primary infection may regulate the viral load of latency.

The role of NO in anti-MCMV innate immunity

![Fig. 3. A schematic representation demonstrating the role of NO in anti-MCMV innate immunity. Regarding innate immunity, macrophage-derived NO played a protective role against MCMV. Such cytokines as IFN-α/β and IFN-γ also act as anti-MCMV factors. In addition, NK cells have been reported to be important cells in the protection against a primary infection of MCMV.](image-url)
Expression levels of mRNA in the spleen cells of MCMV-infected mice treated with anti-CD3

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![Fig. 4](image-url).

The expression of cytokine mRNA analyzed by RNA-PCR (agarose gel electrophoresis). The mice, which had been infected intraperitoneally with LD50 (50% lethal dose) of MCMV 4 wk earlier, were injected with 50ug of anti-CD3 monoclonal antibody (clone: 145-2C11). The lungs were removed at 0, 3, 6, and 12 hrs after the antibody injection to extract RNA. The expression of mRNA for the cytokines and the control (β-actin) were examined by RNA-PCR. Commercially available primers were used. The RNA-PCR products were stained with ethidium-bromide. FX174/HaeIII digest was used as a base pair marker.

Several models of MCMV pneumonitis have been demonstrated (Table 1). In the first model, unirradiated F1 hybrid mice infected with MCMV developed severe interstitial pneumonitis, when spleen cells were adoptively transferred from an uninfected parental mouse strain (Grundy et al., 1985). In this model, MCMV infection alone, or the induction of GVH (graft-vs-host) reaction alone could not lead to pneumonitis. In the second model, a single injection of cyclophosphamide 24 hr after the intranasal injection of MCMV provoked interstitial pneumonitis (Shanley et al., 1985). In this model of MCMV pneumonitis, interstitial pneumonitis still remained even after reductions in the MCMV titers after administration of acyclovir or anti-MCMV serum. In the third model, Reddehase et al. (1985) successfully induced MCMV pneumonia in γ-irradiated BALB/c mice. The fourth model of MCMV pneumonitis was reported by our group (Tanaka et al., 1994). Adult BALB/c mice were intraperitoneally infected with 0.2 LD50 (50% lethal dose) of murine cytomegalovirus (MCMV). At 4 wk after the infection, MCMV remained detectable only in the salivary glands, but not in the lungs or other organs. When the T cells of these mice were then activated in vivo by a single injection of anti-CD3 monoclonal antibody (mAb), interstitial pneumonitis was thus induced in lungs that were free of the virus with an excessive production of the cytokines. In the lungs of such mice persistently infected with MCMV, the mRNA of the cytokines such as IL-2, IL-6, TNF-α and IFN-γ were abundantly expressed 3 hr after the anti-CD3 injection, and the elevated levels continued thereafter (Fig. 4). A marked expression of iNOS was then noted in the lungs, thus suggesting that such cytokines as TNF-α and IFN-γ induced iNOS (Tanaka et al., 1997). To confirm whether MCMV-associated pneumonitis was attributed to iNOS-derived NO, iNOS−/− mice were utilized. As mentioned above, iNOS−/− were sensitive to MCMV. The MCMV genome thus still remained in some lungs even at 4 wk after the injection of 0.2LD50 of MCMV (Fig. 5A). In spite of such a high amount of MCMV-DNA, none of iNOS−/− died after anti-CD3 mAb injection (Fig. 5B).

Although, NO itself can moderately damage the tissues, peroxynitrite (ONOO−), an intermediate molecular species formed by the rapid reaction of NO with O₂⁻ or OCIO−, has been considered to be a major molecule in NO-mediated tissue injury (Crow et al., 1995). Evidence for the role of peroxynitrite in vivo is largely based on the detection of 3-nitrotyrosine in injured tissue (Kaur et al., 1994; Kooy et al., 1995; Saleh et al., 1997). An immunohistochemical analysis was done to examine the cells injured through NO-mediated cytotoxicity. As a result, nitrotyrosine was homogeneously detected in the bronchiolar epithelial cells (Tanaka et al., 2001). iNOS has been previously reported to be abundant in airway epithelial cells (Barnes et al., 1993; Asano et al., 1994; Robbins et al., 1994). Since the bronchiolar epithelial cells align in the respiratory tract, which is constantly exposed to an external environment, it is logical that both iNOS molecules and their activities would be abundant in respiratory epithelial cells in order to protect the host from invasive microorganisms. However, NO derived
from iNOS is also known to act in some cases as "a double-edged sword". In the case of MCMV infection, T-cells in infected mice are modified as to release supraphysiological amounts of cytokines. This modification thus allows the bronchiolar epithelial cells to induce high amounts of iNOS, which results in NO-mediated cytotoxicity in the epithelial cells and surrounding tissue (Fig. 6).

Which kind of modification occurs in the T cells of MCMV-infected mice? As shown in Fig. 4, anti-CD3 mAb induced a transient expression of mRNA of various cytokines 3 hr after the antibody injection in the spleen cells of mock-infected mice. Thereafter, no such expression levels of mRNA were detected, thus suggesting that some feedback signals were presumably induced in the T cells from uninfected mice to terminate the positive activation. In contrast, anti-CD3 mAb induced a high expression of mRNA of such cytokines as IL-2, IL-6, TNF-α and IFN-γ starting 3 hr after mAb injection, and such high levels thereafter continued until 12 hr in the cells from MCMV-infected mice (Fig. 4). These results suggested that the feedback signal pathways were impaired in the T cells of MCMV-infected hosts, and thus those activation states were prolonged in these cells.

Although the precise mechanism regarding how anti-CD3 mAb provokes MCMV-pneumonitis remains unclear, it is interesting to note that neither a permissive virus nor even viral DNA could be detected in T cells or the lung tissue of our mouse model of MCMV-pneumonitis. These results suggested that a modification of T cells in MCMV-infected mice was induced not by MCMV but by the host’s immune responses against MCMV. We previously observed such an effect of MCMV in other cell populations including the thymocytes and hematopoietic progenitor cells. In the
mice which had been infected with MCMV 4 wk., before, a single injection of 20 μg of anti-CD3 mAb dramatically eliminated the thymocytes by apoptosis, while the same dose of anti-CD3 mAb was unable to induce apoptosis in the thymocytes in uninfected mice (Koga et al., 1994). Interestingly, in the post-infection period, MCMV-DNA was undetectable in the thymocytes. Similarly, Fas-mediated apoptosis was enhanced in the hematopoietic progenitor cells of MCMV-infected mice, in which MCMV-DNA were undetectable (Mori et al., 1997). These results suggested that various types of immune cells including T cells, thymocytes and hematopoietic progenitor cells, were supposed to be functionally altered through host responses to an MCMV infection.

Role of NO in latent infection and reactivation of MCMV

Latency and reactivation are important characteristics of viruses in the herpes virus family. A latent infection is defined as a state in which the viral genome is present without producing any infectious particles. Reactivation means the state in which the latent virus becomes when producing the infectious particles upon some external stimuli. In mice, various organs such as the salivary glands, brain, heat, lung, liver, spleen, kidney and blood have been reported as sites of MCMV latency. Regarding cell types, endothelial cells and blood cells, which include polymorphonuclear cells, macrophages and bone marrow cells have been reported to be the site of MCMV latency. In various organs, the lungs, which are rich in macrophages, are reportedly the major sites of CMV latency (Balthesen et al., 1993; Kurtz et al., 1997).

NO has been suggested to be a major substance governing GVHR (graft-versus-host reaction) (Hoffman et al., 1996, 1997; Kichian et al., 1996; Krenger et al., 1996). We therefore used the mouse model of GVHR to examine the role of NO in MCMV latency and reactivation in vivo. At 5 wk after the intraperitoneal injection of MCMV in adult (C3H/He x BALB/c) F1 (CBF1) mice, MCMV-DNA was mainly noted in the salivary glands (SG, Fig. 7A) by the assay of PCR (Detection limit: 1 pg of pAMB25, a plasmid integrating whole immediate early gene segment of MCMV). The detection limit corresponded to about 6.1x10^5 copies of MCMV virus. When parental BALB/c spleen cells were then intravenously transferred to these mice to induce GVHR, a high copy number of MCMV DNA was thus observed in the lungs as well as the hearts and livers at 4 to 6 wk after cell transfer, whereas the copy numbers in SG after the cell transfer were comparable to those before the transfer (Fig. 7A). Similar results were obtained in PCR using primers to enhance the late gene segment of MCMV (Fig. 7B), thus suggesting that GVHR increased the amount of the whole genome of latently infected MCMV from an undetectable level up to a detectable level (>6.1x10^5 copies). Since a marked expression of mRNA of iNOS was noted in the lungs and the hearts during the course of GVHR (Okada et al., 1999), the role of NO in the increase of MCMV-DNA after the cell transfer was examined. To confirm that the increase in the MCMV genome was attributed to NO, L-arginine, a substrate for iNOS, and Phenyl-N-tert-butyl nitrite (PBN), which inhibited the induction of iNOS (Miyajima et al., 1995) were intraperitoneally injected to these mice suffering from GVHR. The administration of L-arginine increased the amount of MCMV DNA in the lungs and the hearts after the induction of GVHR (Fig. 8A). In contrast, PBN inhibited the emergence of MCMV DNA (Fig. 8B). Therefore, the NO generated

![Fig. 7. The detection of MCMV-DNA in the IE (A) and gH (B) regions in the organs of CBF1 mice, which had been inoculated 5 wk earlier with 0.2LD50 of MCMV, after the transfer of spleen cells from uninfected BALB/c mice. The organs were harvested at 0, 2, 4, 6 wk after the transfer, and thereafter DNA was extracted. One μg of the DNA was enhanced by PCR. The PCR product was gel electrophoresed, and then Southern blotting was performed using the probe labeled with the ECL system. A. The primer pair amplified a 700bp segment for the IE gene sequence of MCMV. 10-0.1 pg of pAMB25 was used for the positive controls. B. The oligonucleotide primer pairs, which were selected from the gH gene (late gene) of MCMV-DNA, amplified the 506bp segment of the gH sequence of MCMV. The DNA extracted from the salivary glands of BALB/c mice, which had been infected intraperitoneally with 0.2LD50 of MCMV 2 wk previously, was used as a positive control. Representative results are shown.](image-url)
during the course of GVHR increased the amount of the viral DNA in the lungs and the hearts of the mice latently infected with MCMV. However, no transcripts of MCMV DNA could be detected in these mice (Okada et al., 1999). If the increase in the amount of viral genome is the initial step of viral reactivation from the latent state, NO could thus be concluded to trigger the initial step of MCMV reactivation. Taken together with the fact that the lungs are the major organs to produce NO after systemic stimulation, this hypothesis may explain why the lungs are the major sites of CMV latency and why GVHR triggers CMV reactivation.

**Concluding remarks**

As shown in this article, NO plays various roles in MCMV infection. Although its protective role in the acute infection of MCMV and the pathogenical effect are comprehensive, its role in the viral latency and viral reactivation remains unclear. The reason for this is due to the fact that the mechanism of the viral latency has yet to be elucidated in the field of virology. We hope that NO provide us with a clue to solve the mechanism of viral latency.

**References**


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