Enhanced Atherogenesis Is Not an Obligatory Response to Systemic Herpesvirus Infection in the ApoE-Deficient Mouse
Comparison of Murine γ-Herpesvirus-68 and Herpes Simplex Virus-1

Dagmar G. Alber, Patrick Vallance,* Kenneth L. Powell*

Abstract—Viral and bacterial infectious agents have been implicated in the etiology of atherosclerosis. We have previously shown that a γ-herpesvirus can accelerate atherosclerosis in the apolipoprotein E−/− mouse. To address whether a virally induced systemic immune response is sufficient to trigger enhanced atheroma formation, we infected apoE−/− mice with murine γ-herpesvirus-68 (MHV-68) or herpes simplex virus-1 (HSV-1). In this study, we show that both viruses were able to induce a cell-mediated and humoral immune response in the apoE−/− mouse, which was sustained over a period of 24 weeks. Although intranasal or intraperitoneal infection with MHV-68 induced similar levels of virus-specific IgG1 and IgG2a antibodies in the serum of apoE−/− mice, those infected with HSV-1 showed higher anti–HSV-1 IgG2a compared with IgG1 antibody levels. In addition, viral message was not detected in the aortas of HSV-1–infected animals, whereas we have shown previously that MHV-68 mRNA can be detected in the aortas of infected mice as early as 5 days after infection. Compared with control mice, apoE−/− mice infected with MHV-68 showed accelerated atherosclerosis, whereas mice infected with HSV-1 did not. These data indicate that a systemic immune response to any particular infectious agent is insufficient to induce enhanced atherosclerosis in the apoE−/− mouse and point to specific infections or immune mechanisms that might be essential for virally enhanced atherogenesis.

Key Words: atherosclerosis • infection • herpesvirus • animal models

Accumulating evidence suggests that infectious agents may play a role in the pathogenesis of atherosclerosis.1–6 However, with the large number of infectious agents implicated in disease progression, it has been postulated that their effect is nonspecific and that any infectious agent could potentially accelerate atherogenesis, either through systemic or local immune response mechanisms.7

Herpesviruses have been implicated in the development of atherosclerosis, yet the mechanism is unclear. On the one hand, herpesviruses have been shown to be present in the vessel wall, where they might directly induce molecular and cellular changes that could be proatherogenic.8–13 On the other hand, systemic immunological effects produced by the virus might exert effects on an injured vessel wall without the need for the pathogen to infect or reside within the vessel locally. Recently, and consistent with the latter possibility, Zhou et al14 have reported that cytomegalovirus infection in rats increases the neointimal response to vascular injury without the replicating virus being present in the injured carotid arteries. Establishing whether viruses enhance atherogenesis through local virus-specific mechanisms or through general contribution to systemic inflammation would clearly be of importance.

In the present study, we used 2 herpesviruses, murine γ-herpesvirus-68 (MHV-68) and herpes simplex virus type-1 (HSV-1) to address this issue. MHV-68 naturally occurs in wild murid rodents.15 The virus infects laboratory mice, in which it causes an acute and persistent infection16 and accelerates atherosclerosis in the apoE-deficient (apoE−/−) mouse.17

HSV-1 infection has been associated with atherosclerosis in humans8,18 and is one of the most studied herpesvirus infections in mice, in which it causes an acute infection. The virus causes a strong inflammatory response characterized by the production of interferon-γ and various cytokines.19,20 Infected animals mount a cytotoxic and T helper (Th) cell response, and antibodies specific to the virus can be detected for several months.21–23

The aim of the present study was to test the hypothesis that acceleration of atherosclerosis in the apoE−/− mouse requires a specific type of infection or immune response and is not due to an obligatory response to a systemic immune response to any herpesvirus infection.
**Methods**

**Experimental Mice**

Apoe−/− mice (C57BL/6J-ApoE, Jackson Laboratory, Bar Harbor, Me) were bred at the Royal Veterinary College, London, UK, under specific pathogen-free conditions. Mice were screened regularly for the presence of common adventitious mouse pathogens, including murine cytomegalovirus and MHV-68. Experiments were carried out according to the guidelines of the Animals (Scientific Procedures) Act 1986. For all experiments, mice were age- and sex-matched. In addition, when possible, litters were divided between the experimental groups to account for potential variation in the amount of atherosclerosis between different groups of litters.

**Virus Propagation and Infection of Mice**

MHV-68 was propagated, purified, and titrated on BHK-21 cells as previously described. HSV-1 strain SC-16 was grown on Vero cells. Virus was purified by infecting subconfluent Vero cell layers with a multiplicity of infection of 0.5. The virus was separated from cell debris and media by velocity gradient sedimentation on a 10% to 40% sucrose gradient as previously described. Virus was purified by infecting subconfluent Vero cell layers with 1 × 10^5 pfu HSV-1 in 100 μL PBS either intranasally or intraperitoneally. Mice infected with HSV-1 received a second intraperitoneal inoculation (14 weeks later) with 1 × 10^4 pfu HSV-1 in 100 μL PBS. Control mice received an equivalent volume of PBS either intranasally or intraperitoneally. Mice were culled 20, 24, or 40 weeks after inoculation.

**Virus Isolation From Tissues of Infected Mice**

To isolate HSV-1, lungs and spleens were removed aseptically, homogenized in Glasgow minimum essential medium (GMEM, Sigma Chemical Co) containing 2% FBS, and centrifuged. The supernatants were titrated and added to a suspension of BHK-21 cells, which were shaken at 37°C for 30 minutes before the addition of 8 mL of maintenance medium (GMEM, 2% FBS, 2 mmol/L glutamine, 200 μM penicillin, 100 μg/mL streptomycin, and tryptose phosphate broth; Sigma) containing 0.8% carboxymethylcellulose. Cells were plated out and then fixed with formal saline and stained with 0.1% crystal violet after 4 days. For MHV-68 isolation, lungs and spleens were homogenized in DMEM (Life Technologies) containing 2% FBS and centrifuged. Serial dilutions of the supernatant were added to preformed NIH3T3 monolayers and allowed to absorb for 45 minutes before the addition of 2 mL of overlay (0.8% agarose [Life Technologies], DMEM, 2% FBS, 2 mmol/L glutamine, 200 μM penicillin, and 100 μg/mL streptomycin). Cells were fixed and stained after 5 or 6 days.

**Quantification of Atherosclerotic Lesions**

Arteries were dissected away from residual excised adventitia, opened up longitudinally from the cusps to the iliac bifurcation, and divided into two stripes as previously described. These were then stained with oil red O (Sigma) and mounted en face in glycerol-gelatin mountant (Sigma). Images were analyzed by using the computer program ImageStat 1.0, as previously described. The amount of atheroma was expressed as a percentage of the total area of the aorta.

**Serology**

Briefly, 96-well Maxisorp plates (Nunc) were coated with an optimum concentration of purified MHV-68 or HSV-1 in bicarbonate buffer (pH 9.6). Plates were washed and blocked with 1% powdered milk solution (Marvel) in PBS for 60 minutes at 37°C and incubated with mouse antisera (1:50 to 1:6400 dilution in PBS containing 0.5% Marvel and 0.05% Tween 20) at 37°C for 1 hour. Plates were washed and incubated with a 1:1000 dilution of a rabbit anti-mouse horseradish peroxidase conjugate (Dako) or individual horseradish peroxidase-conjugated isotype-specific antibodies (added at a dilution of 1:300 [Serotec]). After 1 hour of incubation at 37°C, the substrate o-phenylenediamine (Sigma) was added to the washed plates. The reaction was stopped with 3 mol/L sulfuric acid, and optical densities were assessed at 492 nm.

**Detection of Viral Message by RT-PCR**

Reverse transcription (RT) and PCR were performed as previously described. As previously reported, mice infected intranasally or intraperitoneally with MHV-68 showed no signs of illness (data not shown). Virus was recovered from the lungs and optical densities were assessed at 492 nm. To calculate the proportion of labeled cells, the substrate 3,3′-diaminobenzidine (Sigma) was used as a positive control. PCR amplification with primers against HSV-1 ribonucleotide reductase 1 (5′-AAC CTC TAC CAG CAC TTC GA-3′) and 5′-AGA GTG CTG TCG ATC ATG AT-3′) and glycoprotein B (5′-TCA TCG ACA AGA TCA CCG CC-3′) and 5′-TGT ACA CAA AGT CCG CAG CTC-3′), β-Actin primers (5′-GAC ATG GAG AAG ATC TCG CGC CA-3′ and 5′-GCT CGA AGT CTA GAG CAA CA-3′) were used as a positive control. PCR amplification with Taq DNA Polymerase (Life Technologies) was performed for 35 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, followed by a final extension at 72°C for 7 minutes. Samples were run on a 2% agarose gel containing ethidium bromide, and bands were made visible under a UV transilluminator.

**Results**

**Virus Isolation From Lungs and Spleens of ApoE−/− Mice Infected With MHV-68 or HSV-1**

As previously reported, mice infected intranasally or intraperitoneally with 5 × 10^5 pfu MHV-68 showed no signs of illness (data not shown). Virus was recovered from the lungs but not the spleen from mice infected intranasally with MHV-68, whereas intraperitoneal infection resulted in virus replication primarily in the spleen, with no virus being recovered from the lungs (Table). Because HSV-1 infection...
at a dose of $5 \times 10^5$ pfu would be lethal, mice were infected with $5 \times 10^4$ pfu virus. This dose was sufficient to cause infection, as characterized by replication of the virus in the lungs at 6 days after infection (Table). This dose caused death in a small number of mice (4 of 19), but in general, it was well tolerated in 8- to 10-week-old apoE / H11002 / H11002 mice.

Isotype-Specific Antibody Response of MHV-68–and HSV-1–Infected ApoE / H11002 / H11002 Mice

Total anti–MHV-68 IgG antibody titers were similar in serum samples of apoE / H11002 / H11002 mice infected intranasally or intraperitoneally with MHV-68 for 20, 24, and 40 weeks (end-point IgG ELISA titers were as follows: 1/165±1/97, 1/476±1/219, and 1/552±1/216, respectively, for intranasally infected mice and 1/358±1/24, 1/318±1/86, and 1/348±1/90, respectively, for intraperitoneally infected mice). Comparable levels of virus-specific IgG2a and IgG1 antibodies were detected at 20 (n=6 to 8), 24 (n=4), or 40 (n=4 to 5) weeks after infection (Figure 1a and 1b shows results at 24 weeks after infection) in mice that were either infected intranasally or intraperitoneally with MHV-68. Mice infected with HSV-1 showed similar total virus-specific IgG antibody levels compared with mice infected with MHV-68 (end-point IgG ELISA titers were as follows: 1/280±1/53 and 1/365±1/159 for mice inoculated once or twice with HSV-1, respectively). However, higher levels of anti–HSV-1 IgG2a antibodies than of anti–HSV-1 IgG1 antibodies were measured at 24 weeks after infection in serum samples of mice infected once (n=6, Figure 1c) or twice (n=4, Figure 1d) with HSV-1.

Virus-Specific Cell-Mediated Immunity in Infected Mice

To determine whether infected mice mounted a systemic cell-mediated immune response, splenocytes were isolated 12 to 20 weeks after infection. A virus-specific T-cell immune response was measured in the spleen of MHV-68– and HSV-1–infected mice (Figure 2), indicating that infected animals mounted a systemic immune response. The SI to HSV-1 was not significantly different from that measured in MHV-68–infected mice. Interestingly, as shown previously, mice infected intranasally or intraperitoneally with MHV-68 mounted a local T-cell response in the para-aortic lymph nodes throughout the course of infection (data not shown). This response was absent in mice infected with HSV-1 at SI 1.1 (n=4) at 2 to 3 months after infection, suggesting that HSV-1 may not reach or reside in the aorta or the surrounding tissue.

MHV-68 but Not HSV-1 RNA Is Present in Aortas of Infected Mice

To determine whether HSV-1 RNA was present in the aorta, an RT-PCR reaction was set up with the use of HSV-1–specific primers against the ribonucleotide reductase and glycoprotein B. Both genes are abundantly expressed during lytic infection. Primers against $beta$-actin were used as positive control. No viral message was detected in the aortas of HSV-1–infected mice (n=10) 3 to 13 days after infection.
Consistent with our previous studies, 17 MHV-68 RNA was detected as early as 5 days after infection in the aortas of mice infected with MHV-68 (data not shown). HSV-1 Infection Does Not Accelerate Atherosclerosis in the ApoE−/− Mouse

To determine whether a systemic immune response to a herpesvirus infection is sufficient to trigger accelerated atherosclerosis, aortas were dissected to remove any residual fat and stained with oil red O. Atherosclerotic lesions are stained red. Aortas of a control mouse inoculated with PBS (a), of an MHV-68–infected mouse (b), of an HSV-1–infected mouse (c), and of a mouse that received 2 inoculations of HSV-1 (d) are shown.

Figure 3. Atherosclerotic lesions in aortas of apoE−/− mice infected with MHV-68 or HSV-1. Mice aged 8 to 10 weeks were infected and culled 24 weeks later. Aortas were dissected to remove any residual fat and stained with oil red O. Atherosclerotic lesions are stained red. Aortas of a control mouse inoculated with PBS (a), of an MHV-68–infected mouse (b), of an HSV-1–infected mouse (c), and of a mouse that received 2 inoculations of HSV-1 (d) are shown.

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HSV-1 Infection Does Not Accelerate Atherosclerosis in the ApoE−/− Mouse

To determine whether a systemic immune response to a herpesvirus infection is sufficient to trigger accelerated atherosclerosis, aortas were dissected to remove any residual fat and stained with oil red O to assess atheroma formation macroscopically. As expected, control mice showed small lesion development in the cusps, in the great vessels, and at the branching points of side vessels (Figure 3a). Compared with control mice, MHV-68–infected mice showed a dramatic acceleration of atherosclerosis at 24 weeks after infection (Figure 3b). In contrast, mice inoculated with HSV-1 showed an amount of atheroma similar to that seen in control (uninfected) mice (Figure 3c). Even boosting the immune response with a second inoculation of HSV-1 had no effect on enhancing atherosclerosis in these mice (Figure 3d).

Quantification of lesion area by image analysis showed that in mice infected intranasally (n=10) or intraperitoneally (n=4) with MHV-68, atheroma increased by 2.3-fold (P=0.03 for infection versus control) and 2.0-fold (P=0.02 for infection versus control), respectively, compared with levels in control mice (15.5% atheroma [n=8] for intranasal inoculation and 10.4% atheroma [n=7] for intraperitoneal inoculation in control mice, Figure 4). There was no significant difference in the acceleration of atherosclerosis between mice infected intranasally or intraperitoneally with MHV-68 (P=0.51). The average amount of atheroma in mice inoculated once (n=7) or twice (n=4) with HSV-1 was 12.2% and 14.9% (ratios of 1 and 1.2 compared with amount in uninfected mice), respectively, which was not significantly different from that seen in control mice (12.1%; P=0.97 and P=0.55, respectively; Figure 4). The present data clearly show that whereas both viruses induced a systemic immune response, only MHV-68 enhanced atherogenesis.

Discussion

The present results confirm our previous finding that a single inoculation of MHV-68 enhances atherosclerosis in the apoE−/− mouse. 17 To test the hypothesis that it is a specific type of infection or immune response rather than a generalized inflammation against an infectious agent that triggers accelerated atherosclerosis, we infected mice with HSV-1. The results clearly show that whereas both viruses induce a systemic immune response, only MHV-68 enhances atherogenesis. The present study further indicates that infection of the aorta and the induction of a local immune response may be necessary to accelerate atheroma formation.

HSV-1 Infection in Humans and Mice

HSV-1 was chosen for the present study because the virus causes a persistent and latent infection in humans, with frequent periods of reactivation, and has been implicated as a potential causative cofactor in human atherosclerosis.1 Recently, it has been suggested that the presence of IgG antibodies to HSV-1 is associated with an increased risk of myocardial infarction and coronary heart disease death in older adults.26 HSV-1 is a human herpesvirus, but it is one of the most studied herpesviruses in the mouse, in which it...
causes a productive and latent infection. Even though the virus does not spontaneously reactivate in the mouse, it induces a humoral and cell-mediated immune response, which lasts several months, as shown in the present study. In addition, HSV-1 was selected as a representative of the \(\alpha\)-Herpesviridae to establish whether herpesviruses as a family are able to accelerate atheroma formation. It has been previously shown that murine cytomegalovirus, a \(\beta\)-herpesvirus, can contribute to the formation of early atherosclerotic lesions in Balb/c mice and accelerate lesion formation in apoE\(--/--\) mice. Furthermore, the present study and the studies of others have shown that \(\gamma\)-herpesviruses accelerate atheroma formation.

As expected, HSV-1 replicated in the lungs of infected mice and produced a systemic immune response, yet no acceleration in atheroma formation was seen. This indicates that not all herpesviruses are able to accelerate atheroma formation in the apoE\(--/--\) mouse and that enhanced atherosclerosis is not an obligatory response to systemic infection and immune response.

Possible Explanation for the Lack of Enhanced Atherogenesis After HSV-1 Infection

HSV-1 infection may not have enhanced atherogenesis for several reasons. First, whereas MHV-68 causes a natural infection in the mouse, HSV-1 is a human pathogen. However, this and numerous other studies have shown that HSV-1 readily infects mice, and in the present study, we reinoculated mice with HSV-1 to ensure an ongoing immune response. It is feasible to argue that the difference in virus distribution and the amount of virus recovered from the individual organ may account for the lack of enhanced atherogenesis in HSV-1-infected mice. In these mice, the virus was found to replicate in the lungs but not in the spleen after intraperitoneal infection. However, intraperitoneal infection of mice with MHV-68 resulted in replication of the virus in the spleen but not in the lungs, whereas after intranasal infection, MHV-68 was recovered from the lungs but not the spleen. The amount of virus recovered was also different. Nevertheless, both routes of infection with MHV-68 accelerated atheroma formation. Therefore, although we cannot conclude that these factors do not play a role in the proatherogenic effect of the virus, their roles are likely to be complex and, thus, require further detailed investigation.

Interestingly, the immune response generated by the 2 viruses was different, and this may provide an insight into the mechanisms determining whether atherogenesis is stimulated or not. Thus, HSV-1 induced a strong IgG2a response and low levels of virus-specific antibodies of the IgG1 subclass throughout the course of infection. This pattern of responses suggests T-cell help by proinflammatory Th1 cells. In contrast, virus-specific antibody levels of the IgG1 and IgG2a isotype were similar in MHV-68–infected mice. Induction of an IgG1 response in the mouse requires Th2 cell assistance. Thus, the Th2 component of the MHV-68 response may be important. Consistent with this hypothesis, a switch from a Th1 to a Th2 response has been implicated in the progression of atherosclerosis in the uninfected apoE\(--/--\) mouse. Furthermore, Frostegard et al have suggested that although a predominant Th1 proinflammatory cytokine response is present in mature human plaques, a balance between proinflammatory and anti-inflammatory cytokines might be crucial in the progression of plaque development. The current model of MHV-68 and HSV-1 infection in the apoE\(--/--\) mouse may provide a good model in which to test further the role of Th1/Th2 immune responses.

There was no significant difference between the T-cell proliferative response measured in either MHV-68– or HSV-1–infected mice. However, there was a rather large variation in the proliferative response of mice that received MHV-68. Inasmuch as all the mice infected with MHV-68 showed accelerated atherosclerosis compared with control mice, the degree of the T-cell proliferative response does not seem to influence the enhancement of atherogenesis.

An alternative explanation for the lack of enhanced atherosclerosis after HSV-1 infection may be that the site of viral infection is important. It is conceivable that the virus must infect the aorta, causing a local immune response, in order to have a proatherosclerotic effect. In fact, unlike MHV-68, HSV-1 mRNA was not detected in the vessel wall of infected mice, nor was a local T-cell immune response detected in the para-aortic lymph nodes. It should be noted that this is different from the human situation, in which HSV-1 has been found in the vessel wall; thus, in this respect, the mouse model should not be taken as a surrogate for the possible effect of HSV-1 in humans. Rather, it should be taken as a route to understanding atherogenic mechanisms in response to viral infections.

Finally, it is possible to reason that specific viral factors interacting with the immune system may explain the proatherosclerotic effect of MHV-68 but not HSV-1. Although the viruses share a large number of homologous genes, MHV-68, like other \(\gamma\)-herpesviruses, carries additional cellular gene homologues, which may play a role in the pathogenesis of atherosclerosis. Further studies with viruses in which these genes have been deleted may allow this hypothesis to be tested directly.

In summary, the present study shows that not all systemic herpesvirus infections enhance atherogenesis in the apoE\(--/--\) mouse. The finding that HSV-1 causes a significant and sustained immune response but does not accelerate disease progression, whereas MHV-68 does, paves the way toward identifying immune processes that deserve future consideration as having causative roles in the process of immune-mediated enhancement of atherosclerosis.

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References


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