Effect of Aminoguanidine, a Nitric Oxide Synthase Inhibitor, on Ocular Infection with Herpes Simplex Virus in Balb/c Mice

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PURPOSE. To study the effect of aminoguanidine (AMG), an inhibitor of nitric oxide production, on the ocular infection of Balb/c mice with herpes simplex virus (HSV) type 1 strain F and HSV-2 strain G.

METHODS. Animals were treated with different amounts of AMG (0.5, 0.1, and 0.05 mg/mouse) by topical application in the eye from postinfection (PI) days 2 through +5, considering 0 the day of infection. At different PI days, development of herpetic keratitis was evaluated in treated and control mice.

RESULTS. Treated animals showed a dose-dependent increase in ocular disease after viral infection, compared with control animals. Viral titers in ocular washings were higher in AMG-treated mice (PI day 2: HSV-1: AMG 0.5 mg, 1.3 × 10³ plaque-forming units (PFU/ml; control, 0.22 × 10³ PFU/ml, P < 0.025). At PI day 3, control corneas had only scattered inflammatory cells, whereas those from treated animals showed a conspicuous infiltrate consisting primarily of neutrophils. Viral titers were also higher in brains of treated mice. These animals died earlier and in a greater proportion than control animals (percentage of mortality, PI day 12, HSV-1: AMG 0.5 mg, 40% ± 4%; control, 18% ± 3%, P < 0.05).

CONCLUSIONS. These data indicate an inhibitory effect of nitric oxide on HSV ocular infection. (Invest Ophthalmol Vis Sci. 2001;42:1277–1284)

Herpes simplex virus (HSV) is a pathogen that infects the mucosal surfaces of the eye, mouth, and genitalia, causing ulcerative lesions. In a primary infection, the virus quickly replicates in peripheral tissues, enters nerve endings, and travels to sensory nerve ganglia, where it remains in a latent state. HSV transcription during latency is confined to the repeat regions of the viral genome. Once latent in the ganglia, the HSV replicates in peripheral tissues, enters nerve endings, and travels to sensory nerve ganglia, where it remains in a latent state. HSV transcription during latency is confined to the repeat regions of the viral genome. Once latent in the ganglia, the virus appears to avoid detection by the cells of the immune system.1–2 The timing of appearance and incidence of isolation of virus from the trigeminal ganglia suggests that is the most likely source of reactivated virus to produce recurrent ocular disease and shedding in the tear film. The most probable route of spread of virus reactivated in the trigeminal neurons is within the axons of ocular nerves.3 Under conditions such as stress or exposure to UV light, the virus may be reactivated and cause recurrent lesions at mucosal surfaces or on the skin.

Such recurrence is particularly harmful in the case of ocular infection, in which damage to the eye can lead to blindness. In some cases, the virus may spread through the nerves to surrounding areas of dermatome, resulting in zosteriform lesions, or, more rarely, to the central nervous system, leading to encephalitis and death. Topical corneal infection with HSV results in a chronic inflammatory response of the corneal stroma called herpetic stromal keratitis (HSK) mediated by CD4+ T-helper 1 (Th1) cells. Replicating virus as well as viral mRNA is absent from the cornea at the time when lesions are clinically apparent, but it has been demonstrated that an initial period of viral replication is necessary to induce HSK.

The chronic inflammatory response that typifies HSK occurs at a location where and time when viral antigens cannot be demonstrated. Such observations could indicate that the T cells present in the cornea may be activated to orchestrate the inflammatory event, not by reacting with viral peptides, but perhaps in response to host-derived determinants unmasked as a result of the virus infection.4 In this way, Zhao et al.5 identify one reoviral host antigen that shares some homology between sequences in the Cγ3 region of IgG2a (amino acids 292-308) and the HSV-1 encoded UL6 peptide that contains identical or similar amino acids at seven of eight sequential positions that contribute to T-cell recognition. HSK could be induced in animals genetically incapable of generating HSV antigen-specific CD4+ T cells, indicating that clinical HSK may not require viral antigen recognition by CD4+ T cells and that T cells of irrelevant specificity can be recruited, activated, and driven into effector function in the HSV-infected cornea.

This is suggested to represent a bystander activation effect caused by the presence of proinflammatory mediators that result from HSV replication.6–8 Histologically, HSK is characterized by neovascularization, corneal swelling, and an intense infiltrate that is pronounced in the stroma by 2 weeks after infection. Neutrophils are the most prominent cell type seen, although monocytes and lymphocytes are also present. Recruitment of cells from the blood to the site of tissue injury is thought to depend on the synthesis of specific sets of inflammatory mediators and the establishment of chemoattractant gradients in the endothelium and in the extracellular matrix.7 The mechanisms involved in innate immunity may be crucial in controlling primary infection with HSV in ocular mucosa thus diminishing viral replication and consequent HSK.

Nitric oxide (NO) is a free radical gaseous molecule that is a mediator of vital physiological functions including host defense.8–10 Many cell types are able to produce NO through the enzymatic conversion of l-arginine to l-citrulline by nitric oxide synthase (NOS). Macrophages are the best characterized sources of an inducible form of NOS (iNOS) that is activated in response to microbial infections. Throughout the past decade, NO has been shown to play an important role as a first-line
defense against various pathogens. More recently, different investigators have reported antiviral activity of NO has against several viruses such as HSV-1, ectromelia virus, vaccinia virus, encephalomyocarditis virus, vesicular stomatitis virus, and Japanese encephalitis virus.\(^{11-24}\) The antiviral activity of NO, at least in vitro, involves a blockade at the stage of DNA replication but has no effect on early protein synthesis.\(^{4}\)

In this work we studied the effect of aminoguanidine (AMG), an iNOS inhibitor of ocular infection with HSV-1 and -2 in Balb/c mice.

**MATERIALS AND METHODS**

**Chemicals**

The iNOS inhibitor, aminoguanidine hemisulfate (Sigma, St. Louis, MO), was directly dissolved in sterile phosphate-buffered saline (PBS) at the indicated concentrations for each experiment. Oligonucleotides primers used in this study were synthesized by Gibco (Grand Island, NY).

**Animals**

Male Balb/c mice, 4 to 10 weeks old, were used for all experiments. Mice were housed five per cage with sterile wood-chip bedding and were provided with Chow pellets and tap water ad libitum. The animals quarters were maintained at 21°C to 24°C, and 40% to 60% humidity with a 12-hour light–dark cycle. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Virus Preparation**

HSV-1 strain F and HSV-2 strain G were maintained at a low passage in our laboratory. Pools of virus stocks were prepared in Vero cells and stored at \(-70°C.\) Plaque-forming unit (PFU) assays were performed on Vero cells grown in 16-mm tissue culture plates containing 24 wells. Virus dilutions (0.1 ml/well) were allowed to adsorb for 1 hour at 37°C, and then each well was overlaid with 1 ml of minimum essential medium (MEM; Gibco) containing 1% methylcellulose (4000 cp). After 3 days of incubation at 37°C in 5% CO\(_2\), the plates were fixed with 10% formalin.

**FIGURE 1.** iNOS mRNA expression in the eyes after HSV-1 infection. Balb/c mouse corneas were scarified and infected with \(10^5\) PFU HSV-1 strain F. Eyes were excised at the indicated times, after which 4 \(\mu\)g of the extracted total RNA was subjected to RT. PCR was then performed for iNOS and \(\beta\)-actin. The experiment was repeated three times. Lane 1: mock infected; lane 2: PI day 2; lane 3: PI day 4; lane 4: PI day 6.

**FIGURE 2.** Effect of AMG treatment on ocular illness induced by HSV infection in Balb/c mice. Eyes in groups of 10 male Balb/c mice (4 – 6 weeks of age) were infected with 5 \(\mu\)l of a viral suspension containing \(10^5\) PFU HSV-1 strain F (A) or HSV-2 strain G (B). The animals received daily application of 0.5, 0.1, or 0.05 mg AMG in PBS, topically applied to the eye for 7 days, beginning 2 days before infection. Control mice received an equal volume of PBS. Criteria for keratitis included stromal opacity due to edema and cellular infiltration, mydriasis, corneal neovascularization, and corneal ulceration. Signs of illness were recorded daily for 2 weeks. Data are expressed as mean ± SD of three independent experiments (*\(P < 0.05\)).
Inoculation of Balb/c Mice

Animals were anesthetized by intraperitoneal injection of 2 mg ketamine hydrochloride (Ketalar; Parke Davis, Morris Plains, NJ) and 0.04 mg xylazine (Rompun; Mosby, Inc., St. Louis, MO) in 0.1 ml of PBS.

TABLE 1. Effect of AMG Treatment on the Clinical Course of HSK in Balb/c Mice Inoculated with HSV-1

<table>
<thead>
<tr>
<th>AMG Dose</th>
<th>Symptoms</th>
<th>Control</th>
<th>0.5 mg</th>
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<td>0</td>
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<td>80 ± 7</td>
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<td>52 ± 6</td>
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Data are expressed as percentage of infected animals ± SD.

They were inoculated by scarification of the left cornea with a 26-gauge needle through a 5-μl drop of medium containing 10^5 PFU of HSV. Control mice were inoculated similarly with a preparation of uninfected Vero cells prepared in the same way as the viral inoculum (mock inoculum). Animals with eyes accidentally perforated at the time of corneal inoculation were not included in clinical or histopathologic studies.

AMG Treatment of Mice

Mice were separated in four experimental groups. Between days −2 through +5 each mouse was anesthetized as previously described and topically treated three times per day with AMG: 0.5 mg (group 1), 0.1 mg (group 2), 0.05 mg (group 3), and PBS (control). Mice in an additional control group received 0.5 mg AMG but were mock infected. All dilutions were prepared in PBS.

Clinical Observations of Corneal Disease Progression

Mice were clinically evaluated daily for 2 weeks after corneal inoculation and examined using a slit lamp biomicroscope. Cornea, iris, and lids of animals were examined for signs of disease. Criteria for keratitis included stromal opacity due to edema and cellular infiltration, corneal neovascularization, and corneal ulceration. Edema was considered positive when stromal opacity was severe (iris not visible). Corneal neovascularization was considered positive when eyes showed unequivocal signs of hyperemia. These signs did not appear simultaneously but developed gradually after infection. Animals showing at least two of these signs were considered positive for illness. Infected animals also show blepharitis, but this symptom was not considered for determining whether an animal was positive for keratitis. Clinical evaluations.
were performed in a masked fashion. The experiments were repeated three times to test the reproducibility of the results.

**Evaluation of Histopathologic Lesions**

Representative eyes were removed and placed in 10% buffered neutral formalin, embedded in paraffin, stained with hematoxylin and eosin, mounted (Permount; Fisher Scientific, Fairlawn, NJ), and covered with a coverslip for microscopic examination. Five representative sections of each eye were examined to evaluate histopathology and inflammatory infiltrate characteristics.

**Isolation of Virus from Eye Washings, Eyes, and Brains of Infected Mice**

To evaluate viral replication in ocular mucosa, eyes were washed with 20 μl of medium at 12, 24, 48, and 72 hours after infection, and virus was titrated on Vero cells. For determining virus in eye tissues, groups of five mice were killed on postinfection (PI) days 1 through 7, and their left eyes removed. Each eye was ground in 0.5 ml of medium and then frozen and thawed three times to disrupt the cells. The resultant cell-free suspensions were titrated on Vero cells by the PFU assay.

Groups of five mice were killed on PI days 3 through 15 to obtain tissue samples of brains. Tissues were excised and collected into 1 ml MEM, minced with scissors, and homogenized using an electric blender. After sonication in an ice-cold water bath for 1 minute and centrifugation at 3000 rpm for 10 minutes, virus in supernatants was evaluated by PFU assay.

**PCR Analysis of Viral DNA in Trigeminal Ganglia**

Groups of five mice were killed on PI days 1 through 7, trigeminal ganglia were dissected, rinsed in PBS, and blotted on tissue paper to remove traces of blood. Individual trigeminal ganglia were homogenized, and the cell pellet was used to detect viral DNA by polymerase chain reaction (PCR) analysis. The pellet was washed twice with PBS, and the trigeminal ganglia pellets were suspended in 100 μl of Tris-EDTA containing 0.1% sodium dodecyl sulfate (SDS) and 100 μg proteinase K per milliliter. The mixture was incubated at 55°C for 16 hours. The DNA was extracted and PCR performed using two primers (sense: 5'-TTTCTCCAGTGCTAGCTGAAGG-3', antisense: 5'-TCAACTCGCAGACACGACTCG-3') that generated a 283-bp product corresponding to the ICP27 viral gene.

Trigeminal ganglia DNA (3.5 μl) was combined with 1× Taq buffer, 0.25-μM concentrations of each PCR primer, 100-μM concentrations of each deoxyribonucleoside triphosphate, and 2.5 U of Taq polymerase (Promega, Madison, WI) in a 50-μl reaction volume and overlaid with mineral oil. PCR was performed in a thermocycler (Eppendorf, Freemont, CA). The first cycle of PCR was at 95°C for 5 minutes, 52°C for 1 minute, and 72°C for 1 minute, followed by 35 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute. PCR products were resolved in 1.5% agarose gels and were visualized by ethidium bromide staining.

**RT-PCR Analysis of iNOS in Corneal Samples**

To evaluate iNOS expression, groups of five mice were killed on PI days 0, 2, 4, and 6; left corneas were collected; and RNA was extracted with a commercial system (RNAgents Total Isolation System; Promega) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 4 μg of total corneal RNA using an oligo-dT15 primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (RT; Promega) in a 40-μl reaction volume. PCR on iNOS cDNA was performed using two primers (sense: 5'-CTTCGGAGAGACAGACTCG-3', antisense: 5'-GAGCCTGCTGCTTGGTGGCTCTC-3')
that generated a 487-bp product corresponding to the iNOS gene.26 Expression of \( \beta \)-actin mRNA in the corneal tissue sample was used as a control for RNA isolation. The first cycle of PCR was at 95°C for 5 minutes, 52°C for 1 minute, and 72°C for 1 minute followed by 45 (iNOS) or 32 (\( \beta \)-actin) cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute. PCR products were resolved in 1.5% agarose gels and were visualized by ethidium bromide staining.

**Statistical Analysis**

Statistical analyses were performed by computer using analysis of variance (ANOVA; Statgraphics Plus for Windows, ver. 3.0; Manugistics, Rockville, MD).

**RESULTS**

To determine whether HSV ocular infection induces iNOS expression, RT-PCR analyses of infected corneas were performed on different PI days. As shown in Figure 1, HSV-1 infection induced iNOS mRNA expression in corneas of infected animals from PI day 2. Similar results were obtained with HSV-2 infection (data not shown). To study the role of NO in controlling HSV ocular infection, we topically treated animals with different amounts of AMG. This compound significantly altered the development of stromal keratitis in the higher concentrations used. As shown in Figure 2A, nearly 60% of mice infected with HSV-1 and treated with 0.5 mg AMG showed signs of illness by PI day 4, whereas only 18% of the control animals did so. Similar results were observed in HSV-2-infected mice (Fig. 2B). In this case, data obtained with 0.5 and 0.1 mg AMG where very similar, and therefore only data obtained with 0.1 mg of AMG are shown.

The progression of disease was exacerbated by AMG treatment in a dose-dependent manner. Tables 1 and 2 show that the different pathologic signs of ulcerative keratitis were more frequent and appeared earlier in treated animals. These results were further confirmed by microscopic examination of hematoxylin and eosin-stained histopathologic eye sections obtained by standard procedures. As shown in Figure 3, by day 3 PI, corneas from treated (0.5 mg) or control groups showed slight extracellular edema and congestive blood vessels. Control corneas (Fig. 3A) had only scattered inflammatory cells, whereas those from treated animals (Fig. 3B) showed a conspicuous infiltrate consisting primarily of neutrophils. Two days later, the inflammation was established in corneas from nontreated and treated animals (Figs. 3C, 3D), although it was more pronounced in the last group. Edema and numerous dilated blood vessels were present in the stroma, and a few plasmacytes (<5% of total inflammatory cells) were seen in the inner side of the stroma. By PI day 7, there were no differences in corneas from both groups, and first signs of ulceration were observed (Figs. 3E, 3F).

Viral titers in eye washings obtained 24 hours after infection were significantly higher in animals treated with 0.1 and 0.5 mg AMG (Fig. 4A, 4B). This augmentation of virus titers in treated animals was maintained during the following 2 days. In the same way, by PI day 1 larger viral titers were recovered from eye tissues of animals treated with the higher AMG concentration (Fig. 5). In this experiment, diminution in viral titers from PI days 3 through 7 was less pronounced in treated animals. In contrast, significantly more virus was recovered at PI day 8 in brains of treated animals. HSV-1 levels (in PFU per milliliter) were as follows: AMG 0.5 mg, 1.46 \( \pm \) 0.1 \( \times \) 10^3 (\( P < \)
deaths were recorded in this group. but mock infected did not show any sign of morbidity. No group (**HSV-2**). By PI day 12, 30% of the animals infected with HSV-1 and treated with 0.5 mg AMG had died, compared with 14% (**HSV-2**). Levels were as follows: 0.1 mg, 2.3 ± 0.6 × 10^2; 0.05 mg, 2.4 ± 0.5 × 10^2; nonsignificant (NS); control, 1.3 ± 0.6 × 10^2. HSV-2 levels were as follows: 0.1 mg, 2.3 ± 0.6 × 10^3 (P < 0.05); 0.05 mg, 2.0 ± 0.6 × 10^2 (NS); control, 2.3 ± 0.8 × 10^2.

In addition, PCR analysis of viral DNA in trigeminal ganglia revealed that from PI day 3, 100% of the animals treated with the higher AMG dose and infected with **HSV-1** were positive for **ICP27** DNA, whereas at PI day 3, 40% of the control animals showed the presence of **HSV DNA** (Fig. 6). No samples were positive by PI day 1, whereas no differences were observed between treated and control mice on PI days 5 and 7. Infected mice that died on PI day 7 showed signs of encephalitis (ataxia, ruffled fur, and paralysis). The higher doses of **AMG** significantly augmented lethality of **HSV infection**. As shown in Fig. 7, 40% of the animals infected with **HSV-1** and treated with 0.5 mg died by PI day 9, whereas values close to 10% were recorded in control animals. Similar results were obtained with **HSV-2**. By PI day 12, 30% ± 4% of the animals treated with 0.1 mg **AMG** had died, compared with 14% ± 2% in the control group (P < 0.05). Finally, animals receiving only 0.5 mg **AMG** but mock infected did not show any sign of morbidity. No deaths were recorded in this group.

**DISCUSSION**

In humans, infection of the cornea with **HSV** results in a recurrent immune-mediated inflammatory response, **HSK**, which is one of the most common infectious cause of blindness in many countries. There seems to be general agreement that **HSK** largely represents an immunopathologic disease, and clinicians usually treat the lesions with anti-inflammatory drugs along with or even without anti-herpesvirus drugs. The mechanistic nature of **HSK** remains uncertain, but from studies in experimental animals, evidence for a variety of mechanisms has been forthcoming. These include toxic immune complexes, delayed-type hypersensitivity, and lymphocyte cytotoxicity.**1**.27** Productive infection with **HSV** is crucial for **HSK** manifestation. NO production by different cellular types in response to viral infection or cytokines may be one of the mechanisms of innate immunity involved in limiting viral replication after ocular infection with **HSV**.

In this work, we investigated the effect of **AMG**, an **iNOS** inhibitor, on the ocular infection of Balb/c mice that had been infected with **HSV-1** or **HSV-2**. We observed an increase in viral titers in ocular washings, eye tissue, and brains of treated animals. Viral DNA also was detected earlier in trigeminal ganglia of treated mice. This correlates with the observed augmentation in mortality and neuropathologic signs in the treated groups. All these data agree with previous reports from other investigators showing antiviral activity in vivo of NO against different viruses.28,29** The PMNs cause progressive destruction of the corneal tissue, which appears to be responsible for the blinding complications of **HSV** corneal infections in humans. The **Th2** cytokines IL-4 and IL-10 are not detected in cells that infiltrate the **HSV**-infected corneas. IL-10 injection in the infected cornea was shown to inhibit corneal inflammation.**30** A replication-induced proinflammatory milieu in the cornea may be crucial for the subsequent progression of **HSK**, possibly because of enhance-
ment of the expression of corneal agonists that drive HSV manifestation.\textsuperscript{30} Productive infection with HSV resulted in rapid upregulation and sustained expression of chemokines such as N51/KC, macrophage inflammatory protein (MIP)-1\textalpha, MIP-2, monocyte chemotactic protein (MCP)-1, or such cytokines as IL-1, IL-6, IL-8, IL-12, and TNF-\textalpha.\textsuperscript{31–35} In particular, IL-8 has been pointed out as an important chemokine during HSK.\textsuperscript{34}

In this work, we observed that AMG-treated animals showed increased PMN infiltration in corneas at PI days 3 and 5 compared with control animals. Recently, it has been reported that peroxynitrite, formed by the reaction between NO and superoxide, regulates cytokine function during inflammation. Peroxynitrite attenuates neutrophil and monocyte chemotaxis induced by MIP-1\textalpha and IL-8 in a dose-dependent manner, possibly by inhibition of chemokine binding to neutrophils and monocytes.\textsuperscript{35,36} Thus, we hypothesize that the observed increase in the influx of PMNs to corneas of treated mice could be due to inhibition of NO production by AMG treatment during HSV infection, thus suppressing the NO-inhibitory effect on PMN chemotaxis. In the same way, preliminary data from our laboratory obtained by the RT-PCR technique indicate an earlier TNF-\alpha response in corneas of AMG-treated animals, although no differences were observed in IFN-\gamma induction between treated and control animals (data not shown). These data could be consistent with previous reports indicating an important chemokine (MIP-2, IL-8)-inducing activity of TNF-\alpha.\textsuperscript{37–39} and enhanced Th1 responses as a consequence of NO impairment.\textsuperscript{40,41} We are currently investigating this issue.

Last, although we observed that inhibition of iNOS during the first days of infection increased HSV ocular infection, according to Fuji et al.,\textsuperscript{42} intraperitoneal treatment of HSV-1 rats infected intranasally with N-nomonomethyl-L-arginine (L-NMMA) from PI days 3 through 7 decreased neurologic symptoms and increased survival of treated animals. They demonstrated that NO production is related to histopathologic changes in the brain during infection. Thus, although NO production can be beneficial as an antiviral effector against HSV and other viruses, it also may be detrimental by contributing to disease during immune responses, as previously reported.\textsuperscript{45,44} Thus, to unravel the role of NO in the natural resistance to HSV infections, it may be crucial to determine the stage of viral disease in which this molecule exerts its major effect.

**Acknowledgments**

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**References**


40. Mac Lean A, Wei XQ, Huang FP, Al-Alem UAH, Chan WL, Liew FY. Mice lacking inducible nitric-oxide synthase are more susceptible to herpes simplex virus infection despite enhanced Th1 cell response. J Gen Virol. 1998;79:825–830.


