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

Fabian Benencia, María C. Courreges, Gisela Gamba, Hernán Cavalieri, and Ernesto J. Massoub

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 × 10^6 plaque-forming units (PFU)/ml; control, 0.22 × 10^3 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 virus appears to avoid detection by the cells of the immune system, and HSV-1 encoded UL-6 peptide that contains identical or irrelevant specificity can be recruited, activated, and driven into effector function in the HSV-infected cornea.

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.

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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 $-70\degree$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%

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**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 μg of the extracted total RNA was subjected to RT. PCR was then performed for iNOS and β-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 μl 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 (*$P < 0.05$).
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>
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<th>0.5 mg</th>
<th>0.1 mg</th>
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<tbody>
<tr>
<td></td>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td></td>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Day 9</td>
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<td>100</td>
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</tr>
<tr>
<td></td>
<td>Edema</td>
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<td>100</td>
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</tr>
<tr>
<td></td>
<td>Ulceration</td>
<td>54 ± 8</td>
<td>80 ± 7</td>
<td>63 ± 6</td>
<td>52 ± 6</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of infected animals ± SD.

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.

They were inoculated by scarification of the left cornea with a 26-gauge needle through a 5-μl drop of medium containing 10⁵ 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

TABLE 2. Effect of AMG Treatment on the Clinical Course of HSK in Balb/c Mice Inoculated with HSV-2

<table>
<thead>
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<tr>
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<td>Day 7</td>
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<td>52 ± 3</td>
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<td>88 ± 2</td>
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<td>Ulceration</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Day 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blepharitis</td>
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<td>70 ± 8</td>
</tr>
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<td>63 ± 4</td>
<td>85 ± 4</td>
<td>58 ± 6</td>
</tr>
</tbody>
</table>

Data are expressed as percentage of infected animals ± SD.
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 PBS 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 method.

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. 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 50-µl reaction volume.

**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 50-µl reaction volume. PCR on iNOS cDNA was performed using two primers (sense: 5' TTTCTCCAGTGCTAGCTGAAGG-3', antisense: 5'-TCAACTGCGAGACAGCTCG-3') that generated a 283-bp product corresponding to the ICP27 viral gene. Trigeminal ganglia DNA (3.5 µl) was combined with 1 X 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.

**FIGURE 4.** Effect of AMG treatment on viral replication in ocular washings of HSV-infected mice. For 5 days after infection, ocular washings with 20 µl of PBS were performed in Balb/c mice infected with HSV-1 (A) or HSV-2 (B) and treated with AMG. Obtained suspensions were centrifuged to eliminate cellular debris and were titrated in Vero cell monolayers by the PFU method. Data are expressed as mean ± SD of three independent experiments (*P < 0.05).
that generated a 487-bp product corresponding to the iNOS gene. Expression of β-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 (β-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 7 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 ± 0.1 × 10³ (P <
1.3 deaths were recorded in this group. but mock infected did not show any sign of morbidity. No record in control animals. Similar results were obtained with 0.5 mg died by PI day 9, whereas values close to 10% were significantly augmented lethality of HSV infection. As shown in Fig- 6, between treated and control mice on PI days 5 and 7. Infected 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- 102 (NS); control, 0.05 mg, 2.0 \pm 0.6 \times 10^2 (NS); control, 2.3 \pm 0.8 \times 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- 102 (NS); control, 0.05 mg, 2.0 \pm 0.6 \times 10^2 (NS); control, 2.3 \pm 0.8 \times 10^2.

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 mecha- nistic 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.5,27 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,8,9,15,17,20 thus indicating a role of NO in the natural resistance against ocular infection with HSV. This was supported by RT-PCR experiments showing iNOS mRNA induction in corneas of infected mice. In treated animals, we also observed an earlier appearance together with an aggravation of the symptoms associated with HSK, such as blepharitis, edema, neovascularization, and ulceration.

HSK in mice involves a chronic immune-mediated inflammatory response that leads to total destruction of the corneal architecture and to corneal perforation. The corneal inflammam- tion characterized predominantly by polymorphonuclear neutrophil (PMN) infiltration and T cells belonging to the CD4+ population is regulated by the Th1 cytokine interleukin (IL)-2, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ.28 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.29 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 HSK manifestation.\textsuperscript{30} Productive infection with HSV resulted in rapid upregulation and sustained expression of chemokines such as N51/KC, macrophage-inflammatory protein (MIP)-\textit{a}, MIP-2, monocyte chemotactic protein (MCP)\textit{-1}, or such cytokines as IL-1\textit{-}, IL-6, IL-8, IL-12, and TNF-\textit{a}.\textsuperscript{31–33} 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-\textit{a} 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-\textit{a} 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-\textit{a} and\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-monomethyl-L-arginine (\textit{N}-NMA) from PI days 3 through 7 decrease 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{43,44} 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

The authors thank Juan Flo for valuable help in molecular biology.

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