Androstenediol Antagonizes Herpes Simplex Virus Type 1-Induced Encephalitis Through the Augmentation of Type I IFN Production

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Dehydroepiandrosterone and androstenediol (AED) have previously been found to protect mice from viral-induced encephalitis resulting in an increased survival rate of the animals. These hormones have been shown to antagonize corticosteroids, which have immunosuppressive effects in vivo and in vitro, suggesting the antiviral effect of DHEA and AED may be linked to the anticoagulicosteroid action. The present study was undertaken to address the immune response to herpes simplex virus type 1 (HSV-1) during the acute ocular infection with and without AED treatment focusing on the early immune events in the eye and trigeminal ganglion. AED treatment was found to significantly improve the survival of HSV-1-infected mice in a dose-dependent fashion. While AED did not antagonize the elevated serum corticosterone levels following acute infection, AED enhanced the expression of IFN-α mRNA and decreased the expression of HSV-1-infected cell polypeptide 27 mRNA in the trigeminal ganglion during the acute (day 6 postinfection) infection of mice, as determined by reverse transcription-PCR. However, there was no change in the viral load from the eye or trigeminal ganglion when comparing the AED-treated with the vehicle-treated mice. Neutralization Abs to IFN-α, -β, or -β/β, but not control Ab, blocked the protective effect following AED exposure, confirming the involvement of type I IFN in the enhancement of survival in AED-treated mice. Collectively, these results identify innate immunity as a key component in augmenting the survival of HSV-1-infected mice following AED treatment. The Journal of Immunology, 1998, 160: 3060–3066.

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3 Abbreviations used in this paper: DHEA, dehydroepiandrosterone; AED, androstenediol (5-androstene-3β, 17β-diol); AET, androstenediol (5-androstene-3β, 17β-diol); HSV-1, herpes simplex virus type 1; TG, trigeminal ganglion; p.i., postinfection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICP27, infected cell polypeptide 27; LAT, latency-associated transcript; MCP-1, monocyte chemotactic protein-1; pfu, plaque forming unit.

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immunoregulatory properties of biologic relevance in addition to the antiglucocorticoid action (26).

The present study was undertaken to assess the protective effects of AED on herpes simplex virus type 1 (HSV-1)-induced mortality. The immune response to ocular HSV-1 infection is well defined, involving innate (27, 28), cellular (29, 30), and humoral immunity (31, 32). Since the previous viral models characterizing the effects of DHEA and AED on survival employed viral pathogens that induce a rapid death, it was hypothesized that the protective effect mediated by AED would primarily modify some aspect of the innate, nonadaptive immune response. Within this first line of defense, neutrophils (33), macrophages (34, 35), NK cells (36), and cytokines (e.g., type I IFN (37, 38) are candidates previously shown to be involved in monitoring HSV-1 infection. Consequently, the present study focuses on the early immune (cytokine) events within the immediate vicinity of HSV-1 replication following ocular infection including the eye and trigeminal ganglion (TG).

Materials and Methods

Virus and cells

Vero and CV-1 African monkey kidney cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in complete media (RPMI 1640; Mediatech, Washington, DC) containing 5% FBS (Life Technologies, Gaithersburg, MD) and an antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, MO). Cells were incubated at 37°C, 5% CO₂, 95% humidity. HSV-1 was grown up and harvested as previously described (39).

Infection of mice

Female ICR mice (25–34 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized by i.p. administration of 0.1 ml of PBS containing xylazine (6.6 mg/kg) and ketamine (100 mg/kg). Following scarification, tear film was blotted from the eyes and the mice were inoculated with 210 plaque forming units (pfu) of HSV-1 (McKrae strain) in each eye in a volume of 3 μl. Infection was verified by swabbing the eyes 2 to 3 days postinfection (p.i.), placing the swabs in CV-1 monolayer cultures, and observing the cultures for cytopathic effects. Animals were assessed for survival following inoculation with virus or killed by CO₂ asphyxiation at 3 days, 6, and 30 days p.i. Blood was collected via the vena cava, and the eyes and TG were removed. TGs and eyes were processed for RNA isolation and serum was obtained from the clotted blood. Animals were handled and maintained in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals (40).

Treatment of mice

AED 3-sulfate (henceforth referred to as AED) (Sigma Chemical Co.) was reconstituted in a 1:1 DMSO:ethanol ratio that was then added to water for a final concentration of 10, 50, or 100 μg/ml of AED in 0.05% DMSO:ethanol. Following the inoculation of mice with HSV-1, the drinking water of the mice was replaced with either water containing the AED or vehicle (0.05% DMSO:ethanol). Due to the precipitation of AED within 96 h following reconstitution, the treated water was replaced every 72 h. In indicated experiments, mice received rabbit anti-mouse IFN-α/β (Access Biomedical, San Diego, CA; 1,000 neutralizing U), IFN-α (Hycult Biotechnology, Uden, The Netherlands; 1,000 neutralizing U), IFN-β (Access Biomedical; 1,000 neutralizing U), or normal rabbit Ig at the time of infection and 3 and 6 days p.i.

Reverse transcription (RT)-PCR

RT-PCR of TG was performed as described (39). Briefly, TG RNA was extracted in Ultraspec 228 RNA isolation reagent (Biotecx Inc., Houston, TX). First strand cDNA was synthesized using AMV reverse transcriptase (Promega Corp., Madison, WI). PCR was performed in a thermal cycler (Ericomp Δ cycler I; Ericomp, San Diego, CA) with 35 cycles of 94°C (for 1.25 min) → 57 to 60°C (for 1.25 min) → 72°C (for 0.5 min). PCR primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), infected cell polypeptide 27 (ICP27), IFN-γ, TNF-α, latency-associated transcript RNAs (LAT), IL-10, and RANTES were as previously described (39). IFN-α (consensus sequence for IFN-α1, -2, and -7) and CD8 primer sequences were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Primers for IL-6 were 5'-TTCATCCAGTTGCCTTCTTG-3' (sense) and 5'-TTTCATGTACTCCAGGTAG-3' (antisense), yielding a 359-bp product. Primers for IFN-induced protein 10 kDa and JE/monocyte chemoattractant protein-1 (MCP-1) yielding 431- and 582-bp products, respectively, and the settings for the amplification of the specific products were as described (41). Following electrophoresis of the amplified product, ethidium bromide-stained PCR products were visualized with a Bio-Rad 1000 gel documentation system (Bio-Rad, Hercules, CA). Densitometric analysis of gel images was performed using molecular analysis 3.3 software (Bio-Rad).

Measurement of HSV-1 titers in the tissues

TG, eyes, and cerebella were removed 5 to 6 days p.i. and homogenized in 0.8 ml of RPMI 1640 containing 5% FBS in 2.0-ml microfuge tubes. Homogenates were clarified by centrifugation for 1 min at 13,000 × g. HSV-1 titer in clarified supernatants was determined by plaque assay. Viral load was calculated as pfu × sample volume/tissue weight and expressed in pfu/ml.

Corticosterone determination

Sera from killed animals were assayed for corticosterone levels by radio immunosassay (RIA; ICN Biomedicals, Costa Mesa, CA). All samples were assayed in duplicate and collected at 10:00 AM. The corticosterone levels were extrapolated from the standard curve (R² ≥ 0.9900).

Statistics

One-way analysis of variance and Scheffe multiple comparison test were used to determine significant (p < 0.05) differences between the indicated groups using the GBSTAT program (Dynamic Microsystems, Inc., Silver Springs, MD).

Results

AED enhances the survival of mice infected with HSV-1

AED was assessed for its effects on the survival of mice ocularly infected with HSV-1. The results show that greater than 80% of vehicle-treated animals succumbed to the infection compared with 38 and 30% of mice treated with 10 and 50 μg/ml of AED, respectively (Fig. 1). Similar to vehicle, mice treated with 100 μg/ml were not able to manage the viral infection, with only 25% of the

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animals surviving although there was a modest delay in mortality compared with vehicle-treated animals (Fig. 1). There were no apparent differences in the consumption of water containing the AED between the HSV-1-infected groups of mice, which might explain the difference in the survival curve.

AED does not decrease the viral load during acute infection

To determine whether AED reduced the replication of virus during the acute stage of infection, HSV-1-infected mice treated with or without AED were screened for infectious virus. Although there was a tendency for a decrease in infectious virus in the eye and more in the TG of AED-treated mice, there were no significant differences in the viral titers in the eye, TG, or cerebellum (Fig. 2). However, only 45% (5 of 11) of the AED-treated mice had detectable virus in the cerebellum compared with 73% (8 of 11) of vehicle-treated mice. These results suggest that AED treatment antagonizes the spread of HSV-1 from the site of inoculation to the central nervous system.

AED does not alter corticosterone levels during the acute stage of HSV-1 infection

Since a previous study has shown that AED administered s.c. could antagonize the increase in corticosterone following an intra-nasal influenza infection (25), corticosterone levels were determined in mice treated with AED (50 μg/ml) or vehicle. The results show no differences in the serum corticosterone levels 3, 6, or 30 days p.i. when comparing the vehicle-treated with the AED-treated animals, suggesting that the antiglucocorticoid properties reported for AED are not applicable in this model system (Fig. 3).

AED augments MCP-1 and IFN-α mRNA in the eye and TG, respectively, during acute HSV-1 infection

To determine whether AED modifies the expression of cytokine genes during the course of HSV-1 infection, RT-PCR was performed to monitor viral and cytokine transcript expression using eye and/or TG samples obtained 3, 6, and 30 days p.i. Three days p.i., MCP-1 gene expression was elevated in the eye of AED-treated mice (Fig. 4 and Table I). When the PCR was conducted through a range of cycles from 20 to 35, MCP-1 mRNA expression was significantly elevated in the AED-treated animals, confirming the qualitative RT-PCR (data not shown). Similar to MCP-1, the immediate early gene ICP27 of HSV-1 was elevated in the AED-treated mice compared with vehicles (Fig. 4 and Table I). No other transcripts analyzed in the eye were found to be significantly different when comparing the two groups of animals (Table I). Samples obtained from the TG of vehicle- and AED-treated mice 3
days p.i. showed no significant differences in any of the transcripts tested (Fig. 5 and Table II). However, both CD8 and RANTES mRNA levels were elevated in the AED-treated group. Likewise, ICP27 gene expression in the TG was detected in two of eight AED-treated mice compared with zero of eight vehicle-treated controls.

TG samples surveyed for viral and cytokine gene expression 6 days p.i. showed a significant increase in IFN-α and decrease in ICP27 mRNA levels in AED-treated mice compared with vehicle controls (Fig. 6, Table II). Incremental increases in the cycling during the PCR for the detection of IFN-α mRNA ranging from 20 to 35 cycles confirmed the qualitative differences between the AED- and vehicle-treated groups (data not shown). No other transcripts assessed were found to be different between the two groups of treated animals.

Unlike days 3 and 6 p.i., there were no differences in the levels of transcripts tested (including LAT, IL-6, IFN-α, IFN-γ, CD8, and RANTES) in the TG comparing AED-treated with vehicle-treated mice 30 days p.i. (data not shown).

**Type I IFNs are involved in the protective effect in HSV-1-infected mice following AED administration**

Since AED-treated mice were found to have elevated IFN-α and lower ICP27 mRNA levels in the TG during the early, acute (day 6 p.i.) stages of the infection, the involvement of type I IFNs in the protective effect of the AED-treated animals was investigated. Similar to previous results, 33% of the vehicle-treated HSV-1-infected mice survived acute infection (Fig. 7). However, mice exposed to AED in the drinking water and treated with control Ab showed a significant improvement in survival (74%) of acute infection. When AED-treated mice were administered neutralizing Abs to IFN-α/β or IFN-α, the survival was reduced to nearly the same level as the vehicle-treated, HSV-1-infected animals, supporting the RT-PCR data showing involvement of IFN-α in AED-mediated protection (Fig. 7). All the AED-treated animals administered neutralizing Ab to IFN-β succumbed to acute infection, suggesting a key, protective role for IFN-β during the acute ocular infection with HSV-1.

**Discussion**

In the present study, the characterization of AED-elicted protection from HSV-1-induced mortality was undertaken. Based on previous studies showing the anticorticosteroid action of AED as well as the enhancement of IFN-γ and decrease in IL-10 production by Ag-stimulated lymphocytes in virally infected mice administered AED (22–25), it could be concluded that AED acted primarily to reduce circulating corticosteroid levels during infection and
thereby blocked the immunosuppressive effects mediated by this hormone. Similar to previous studies, ocular HSV-1 infection resulted in a significant rise in corticosterone levels during the acute stages. However, AED treatment had no effect on the steroid levels during this time period. The data suggest the anticorticosteroid action of AED is not a consideration in the current study. The discrepancy between the present and previous studies may reside in the route of administration of the compound. Previous studies have focused on the administration of AED s.c. in a single bolus (22–25). In the present study, AED was administered through the drinking water throughout the time course of the infection. Consequently, the metabolism of the compound via these two routes of treatment may greatly influence the action of the drug on physiologic processes, including immunocompetence. However, consistent with previous studies showing the antiviral nature of AED in vivo, the current investigation shows AED was found to significantly enhance the survival of HSV-1-infected mice.

Following ocular inoculation with HSV-1, the virus replicates in the eye, spreads to the sensory nerve endings, and by retrograde transport travels to the neuronal cell bodies within the TG (42). The result of the penetration and replication of the virus in the sensory ganglia is an intense inflammatory response (43). The control of viral replication during this period is crucial in eliminating the spread of the virus into the central nervous system, resulting in fulminating encephalitis. Accordingly, the immune response within the first 3 to 7 days p.i. will ultimately dictate the outcome of the infection. Inspection of cytokine transcripts in the TG during this time period showed a significant increase in IFN-α expression in the AED-treated mice. This increase coincided with a decrease in the viral transcripts ICP27 and LAT. Furthermore, the administration of neutralizing Abs to IFN-α or IFN-α/β but not control Ab blocked the protective effect observed in the AED-treated mice, confirming the observation assessing IFN-α gene expression within the TG of the AED-treated mice. Collectively, these results suggest that type I IFN is the primary mechanism by which AED treatment affords protection from death in the ocular HSV-1-infected mice. However, differences were observed when comparing Ab to IFN-α and IFN-β. AED-treated mice administered anti-IFN-α or IFN-α/β Ab succumbed to the infection at a level similar to the vehicle-treated mice, whereas all mice treated with anti-IFN-β Ab died. Since IFN-β is linked to a single gene whereas IFN-α is part of a multigene family (44), it is difficult to determine the nature of the neutralizing capacity of the anti-IFN-α Ab relative to the Ab directed against IFN-β. However, the PCR data using oligonucleotide primers that are specific for IFN-α1, -2, and -7 and showing increases in the mRNA expression following AED treatment suggest that one or more of these subtypes of IFN-α are likely candidates in the protective effect following AED treatment. Therefore, it is imperative to identify which of the IFN-α subtypes...
is involved in the protective effect mediated through AED against HSV-1 infection before a comparison in the efficacy of the subtype of IFN-α to IFN-β can be determined. Nevertheless, the results of the present study are consistent with previous findings illustrating the central role of the type I IFNs in controlling HSV-1 replication during acute infection in vitro (37, 38) and in vivo (27, 28). Type I IFNs are also thought to promote a Th1 response (45) and the generation and maintenance of memory CD8+ T cells (46), both of which are important attributes for the host during a viral infection.

The present study found that low to moderate concentrations (10–50 μg/ml) of AED in the drinking water antagonized viral-induced mortality while a higher concentration (100 μg/ml) had no protective effect. Although monitoring of individual mice for the consumption of AED was not determined, there were no obvious differences in the volume of vehicle- or AED-containing water consumed between groups of mice, which might explain the differences in the protective effect. It is possible that other neuroendocrine processes are involved upon reaching a threshold level of AED in the circulation that might influence the outcome of the viral infection in the central nervous system.

During the early events of HSV-1 replication in the eye, AED-treated mice displayed elevated levels of both MCP-1 and ICP27 gene expression compared with the vehicle-treated controls. The increased replication of HSV-1 in the eye of AED-treated mice as indicated by the expression of ICP27 may result in a greater degree of tissue pathology associated with an increase in MCP-1 in response to the infection. The increase in MCP-1 synthesized from immune and nonimmune sources within the eye would predictably enhance the extravasation of monocytes into the inflamed site (47, 48), providing an increase in Ag processing, presentation, and type I IFN induction compared with the normal course of the infection. An association of MCP-1 but not RANTES gene expression with herpes stromal keratitis has previously been found (41) and is consistent with the present results supporting the role of MCP-1 in tissue inflammation in the eye.

Since infectious HSV-1 was found in 45% of the cerebella of AED-treated mice and 73% of vehicle-treated controls, AED does not prevent death of the animal by preventing the spread of virus to the central nervous system. Therefore, AED may function in an additional capacity within the brain to reduce the sequela of HSV-1 pathogenesis (i.e., encephalitis) unrelated to the enhancement of type I IFN by AED. However, the enhancement of IFN-α is required to protect the animal from undergoing encephalitis. Similar to another study (22), the present investigation found that AED had no direct effect on viral replication (data not shown), implying that another extrinsic property (e.g., neuroendocrine hormone modulation) besides the induction of IFN might play a role in antagonizing the pathogenesis of HSV-1 within the central nervous system. The fact that AED did not significantly reduce the viral load in the tissues examined but reduced the mortality of the animals is similar to a recent finding investigating HSV-1-induced pneumonia (49). In that study, inhibition of inducible nitric oxide synthase was found to suppress viral pneumonia even though higher viral titers in the lung were found. The authors concluded that the inflammatory response rather than the cytopathic effect of the virus resulted in pneumonia. However, in the present study, both AED- and vehicle-treated mice presented with intense pericentral inflammation. Moreover, by RT-PCR the mice exhibited insignificant differences in the levels of cytokine and CD8 transcripts within the TG with the exception of IFN-α, suggesting that both groups of animals are undergoing a similar inflammatory response. Consequently, future studies will be required to further characterize the immunologic and nonimmunologic parameters that antagonize HSV-1 replication in both peripheral and central nervous systems in the context of the AED treatment regimen employing both oral and s.c. routes of administration.

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