West Nile Virus Infection Induces Interferon Signalling in Human Retinal Pigment Epithelial Cells

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PURPOSE. In addition to neuroinvasive disease, West Nile virus (WNV) infection is frequently associated with self-limiting chorioretinitis and vitritis. However, the mechanisms of ophthalmic WNV infection are rarely investigated, in part because of the lack of reliable in vitro models. The authors therefore established the first model of ocular WNV infection and investigated interaction of WNV with IFN signal-transduction mechanisms.

METHODS. Human retinal pigment epithelial (RPE) cells were infected with WNV strain NY385-99 at a multiplicity of infection of 5. Virus replication was evaluated by virus titers at different times after infection. The susceptibility of RPE cells to WNV infection was confirmed by transmission electron microscopy. IFN-β expression was assessed by quantitative real-time PCR and by measurements of antiviral activity in cell culture supernatants. IFN signaling was evaluated by phosphorylation of transducer and activator of transcription 1 and 2 (STAT1/2) proteins, with immunoblot analysis.

RESULTS. RPE cells appeared to be highly sensitive to WNV infection. Maximum viral titers were found 24 hours after infection, followed by a continuous decline during the course of infection. WNV infection of RPE cells was followed by increased IFN-β expression associated with IFN signaling and subsequent inhibition of WNV replication.

CONCLUSIONS. In this study, the first cell culture model of ophthalmic WNV infection was developed and characterized in RPE cells, and the molecular mechanisms of WNV infection were studied. The data suggest that WNV induces a general antiviral state in RPE cells. This general antiviral state correlates with WNV-induced IFN signalling in retinal cells. (Invest Ophthalmol Vis Sci. 2006;47:645–651) DOI:10.1167/iovs.05-1022

West Nile virus (WNV), a member of the Japanese encephalitis virus serocomplex, is a single-stranded RNA flavivirus. Initially isolated in 1937, it is now realized as one of the most widely distributed flaviviruses, endemic in Africa, the Middle East, and parts of Asia and Europe.1,2 Since 1999, the virus has been recognized in North America as causing an epizootic among birds and horses and an epidemic of meningitis and encephalitis in humans.3 WNV causes a high frequency of latent infection in humans but may also cause fatal encephalitis in the elderly and children.4,5 Approximately 20% of infected persons become symptomatic with a febrile illness, and 1 in 150 infections result in meningitis or encephalitis. Several retrospective case reports and a recent prospective study with serologically confirmed WNV-infected patients described common, but frequently asymptomatic and self-limiting ophthalmic involvement in association with WNV infection.5,6,7 Chorioretinitis associated with vitritis occurs in >80% of hospitalized patients. Other reported findings include uveitis without chorioretinitis, retinal vasculitis, optic neuritis, and congenital chorioretinal scarring. Transient or permanent visual loss may occur due to anterior chamber or vitreous inflammatory reaction, central chorioretinal lesion, late choroidal neovascularization, optic neuritis, or occlusive retinal vasculitis.5,9,10

Viral infection of mammalian cells leads to an innate immune response characterized by α and β interferon (IFN) secretion and subsequent transcriptional upregulation of IFN-stimulated genes (ISGs).12 ISGs mediate an antiviral state within the infected cells and in neighboring tissues. In the early phase of virus infection, the constituatively produced transcription factor IFN regulatory factor (IRF)-5 is activated and forms dimers that are retained in the nucleus and interact with the cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 coactivator. Activated IRF3 along with several other transcription factors, including IFRF1, NF-κB, and ATF-2/c-Jun, weakly activates the IFN-β promoter. Secreted IFN-β binds to the IFN receptor on the cell membrane, initiating the Janus kinase (JAK)/signal transducer and activator of transcription (STAT)/IRF9 IFN signal-transduction cascade, resulting in the induction of IFN regulatory factor 7 (IRF7). In the late phase, IRF3 and IRF7 collectively amplify IFN-α/β gene induction, leading to full ISG stimulation through the IFN signaling cascade.12,13

Viruses have developed different strategies to counteract IFN responses, to ensure their survival in an infected host. In initial studies Darnell et al.14 discovered that WNV exhibits a marked resistance to the innate immune response elicited by IFN-α. Moreover, IFN-α did not inhibit WNV replication after the establishment of an infection.15,16 In several studies, the nature of the resistance of WNV to the antiviral activity of IFNs was investigated in human-transformed cell lines. WNV overcomes the host antiviral response despite demonstrated IFN-β transcription through the IRF3 activation pathway.17–19 WNV nonstructural proteins may be involved in preventing the activation of IFN signal-transduction pathways by inhibition of STAT1 and -2 phosphorylation.18

An in vitro model of viral infection is an important tool for understanding key mechanisms of virus-induced diseases and for the development of antiviral treatments. However, no in vitro models of ophthalmic WNV infection have been described. In the present study, we established the first model for ocular WNV infection in RPE cells and investigated the inter-

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action of WNV with IFN expression and its signal-transduction mechanisms.

**Materials and Methods**

**Cell Culture**

Human RPE cells were isolated from two globes from different donors freshly enucleated for corneal transplantation (tenets of the Declaration of Helsinki were followed). RPE isolation and culture were performed as described previously. The cells were grown in Iscove’s modified Dulbecco’s medium (IMDM), supplemented with 20% fetal bovine serum (FBS). Homogeneity of cultured RPE cells was confirmed by positive immunostaining with monoclonal antibodies (mAbs) to cytokeratins (pan) and to cellular retinaldehyde binding protein. mAbs were kindly donated by John Saari (Department of Ophthalmology, School of Medicine, University of Washington, Seattle, WA). The cell cultures used were designated RPE-I and -II. The cells were routinely monitored for mycoplasma and were not used in the experiments later than passage 5.

Monkey kidney cell line Vero was obtained from the American Type Culture Collection (Manassas, VA). Human foreskin fibroblasts (HFFs) were described as described previously. Both Vero and HFF cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS.

**Virus Preparation**

The WNV strain NY385-99 was kindly provided by Jan ter Meulen (Institut für Virologie, Philipps-Universität, Marburg, Germany). Virus stocks were prepared in Vero cells grown in MEM with 4% FBS. The respective infectious virus titers in stock solutions were determined by titration on a Vero cell monolayer in 96-well plates and determined as 50% tissue culture infective dose (TCID50).

In accordance with World Health Organization’s (WHO) recommendations, all work involving infectious WNV was performed under biosafety level (BSL)-3 conditions in a BSL-3 facility.

Human cytomegalovirus (HCMV) strain Hi91 was isolated from the urine of an AIDS patient with HCMV retinitis. Virus stocks were prepared in HFF grown in MEM with 4% fetal bovine serum (FBS). The respective titers were determined by plaque titration in HFF cells, as described previously.

**Preparation of Virus-Free Supernatants**

Virus-free supernatants were prepared by filtering of culture medium collected from WNV-infected cultures at different time points after infection. For this purpose a microcentrifuge with a cutoff of 300,000 molecular weight (Microsep; Filtron Technology Corp., Northborough, MA) was filled with cell culture supernatants and centrifuged at 3,000g for 1 hour at 4°C. The filtrate samples were free of infectious virus, as demonstrated by titration on a Vero cell monolayer. Aliquots of virus-free supernatants were stored at −80°C before use in the experiments.

**Testing of IFN Activity**

To test IFN activity produced by RPE cells infected with WNV, noninfected RPE cells were incubated with different dilutions of virus-free supernatants for 24 hours before infection with WNV at multiplicity of infection (MOI) 5. In some experiments antiviral activity of recombinant IFN-β was tested. RPE cells were either incubated with IFN-β 24 hours before infection, or IFN-β was added 1 hour after virus inoculation. The antiviral effects were evaluated 24 hours after infection (PI) with immune staining for WNV NS1 protein. For this purpose, cells were fixed with 60/40 methanol acetone for 15 minutes. Immune staining was performed with a mouse mAb directed against WNV NS1 protein (Chemicon, Hohenheim, Germany) and biotin-conjugated secondary goat anti-mouse mAb. The proteins were visualized with streptavidin-peroxidase complex with 3-aminio-9-ethylcarbazole (AEC) as a substate. The extent of staining was estimated by two independent investigators and expressed as a percentage of nontreated virus control (100% WNV antigen expression).

**Immunoblot Analysis**

Cells were lysed in radioimmunoprecipitation assay buffer (10 mM Tris, 150 mM NaCl, 0.02% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS) containing protease inhibitors (Sigma-Aldrich, Diesenhofen, Germany) and 1 mM okadaic acid. Proteins (20 mg) were resolved on 10% to 12.5% polyacrylamide gels containing SDS. After electrophoresis, proteins were transferred to a nitrocellulose transfer membrane, and blots were blocked overnight at 4°C. The following antibodies (all obtained from Biornol, Hamburg, Germany) were used to probe the blots: rabbit anti-STAT1-α, anti-phosphorytrosine 701 STAT1, anti-STAT2, anti-phosphorytrosine 689 STAT2, and peroxidase-conjugated donkey anti-rabbit antibody. Protein bands were visualized by using chemiluminescence detection reagents (ECL-Plus Immunoblotting; GE Healthcare, Freiburg, Germany) followed by exposure of the blot to film.

**WNV and HCMV Coinfection**

RPE monolayers were infected with WNV at MOI 5. After 24 hours, the cells were washed with PBS and infected with HCMV at MOI 10. HCMV infection was examined by immunostaining for HCMV-specific antigens, as described previously. Briefly, HCMV antigens were detected after fixation of the RPE monolayer 24 and 120 hours PI with HCMV, using mAbs directed against 72 kDa immediate early antigen (IEA; DuPont, Bad Homburg, Germany) and 67-kDa late antigen (LA), respectively. The number of antigen-positive cells was quantified microscopically by the examination of 500 cells.

**Electron Microscopy**

RPE cells infected with WNV at MOI 5 were processed for ultrastructural analysis, as described previously. Briefly, cells were centrifuged, and the pellets were fixed with 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol, and embedded in resin (Durcupan-Epon; Fluka Chemie GmbH, Buchs, Germany). Thin sections were contrasted with uranyl acetate and lead citrate and viewed with a microscope (model JEM 2000 CX; JEOL, Arishima, Japan).

**Quantitative Real-Time PCR**

Total RNA was isolated (RNaseasy Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer’s protocol with an additional DNase digestion step using the RNeasy free DNase set (Qiagen). Reverse transcription was performed with reagents from Applied Biosystems, Inc. (ABI), Foster City, CA, according to the manufacturer’s instructions. cDNA was quantified by quantitative real-time PCR (QRT-PCR; Prism 7900HT; ABI). All reactions were performed with primer–probe assays according to a standard thermal profile: denaturation at 95°C for 15 seconds and annealing-extension at 60°C for 60 seconds, with 40 repeats. Sequences were selected by using the NCBI database (nucleotide database; National Center for Biotechnology Information, Bethesda, MD [http://www.ncbi.nlm.nih.gov]; and Primer Express Software v2.0; Table 1). All the samples were performed at least in duplicate. Threshold levels and baseline were optimized depending on the probes used. Relative quantification was determined with the software (SDS2.1; ABI) provided with the PCR system (Prism 7900HT; ABI). Normalization was obtained by using β-actin or 18s rRNA as endogenous control and the uninfected cell culture as calibration sample in comparison to treated cell culture. The results are presented as x-fold increase.

**Results**

**Sensitivity of RPE Cells to WNV Infection**

Confluent layers of RPE cells obtained from two different donors (RPE-I and -II) were infected at MOI 5 with WNV strain...
NY385-99. Maximum infectious virus titers were achieved 24 hours PI (4.9 \times 10^7 H500 for RPE-I and 1.9 \times 10^8 H500 for RPE-II). The virus titers decreased at later times after infection. Four days after infection a 10^3- to 10^4-fold reduction of infectious titers was observed compared with titers at 24 hours PI (Fig. 1A). No cytopathic effect (CPE) was observed in RPE cultures 24 hours PI (Fig. 1B). Four days after infection, phase contrast microscopy revealed CPE characterized by enlarged cells with increased granularity (Fig. 1B).

The susceptibility of RPE cells to WNV infection was confirmed by electron microscopy. Mock-infected cells are shown in Figure 2A. Incubation with WNV resulted in approximately 50% infected cells as indicated by convolute membranes/paracrystalline arrays characteristic of flavivirus infection in the cytoplasm 24 hours PI (Fig 2B) as well by enveloped viral particles found in the intercellular space (Fig. 2C). Twenty-four hours after infection only a few infected cells were destroyed, as characterized by formation of enveloped vesicles with numerous viral particles (Fig. 2D). Most of the infected cells (Figs. 2B, 2C) showed ultrastructural features similar to those of mock-infected cells (Fig. 2A).

**Effect of WNV Infection on IFN-β Expression in RPE Cells**

Because virus-induced transcription of the IFN-β gene and IFN-β production initiate the antiviral response in other cell types, we investigated whether the decrease in WNV titers after reaching its maximum at 24 hours PI could be associated with the antiviral response. IFN-β and α mRNA levels in WNV-infected RPE-I cells (MOI 5) were determined by QRT-PCR at different times PI. As shown in Figure 3, WNV infection significantly increased IFN-β mRNA levels as early as 12 hour (6-fold) and 24 hours (44-fold) PI compared with mock-infected RPE cultures and reached a maximum at 4 days PI (265-fold). In contrast, IFN-α mRNA expression was not induced at time points before 24 hours PI. Four days after infection 3.9-fold
increased levels of IFN-α mRNA were found in WNV-infected cultures (Fig. 3).

To confirm the functionality of the secreted IFN-β by WNV-infected RPE cells, we preincubated native RPE cells with virus-free supernatants from WNV-infected RPE cultures and subsequently inoculated the cells with infectious WNV. Conditioned medium from WNV-infected RPE cells harvested after 24 hours PI inhibited virus replication concentration dependently (Fig. 4). The antiviral activity was higher when conditioned medium was collected at later times after infection. Supernatants collected from mock-infected cultures had no effect on WNV infection.

Effect of WNV Infection on IFN Signal-Transduction Pathways in RPE Cells

Because of the finding that WNV induces antiviral IFN-β expression in RPE cells from early times after infection, we investigated the levels of IFN-β-related tyrosine phosphorylation of STAT1 and -2 in WNV-infected RPE cells. Indeed, WNV infection increased accumulation of total STAT1 and -2 proteins and induced tyrosine phosphorylation of both STAT1 and -2 24 hours PI (Fig. 5A). Experiments with virus-free supernatants from WNV-infected cultures (collected 24 hours PI) but not from mock-infected cultures revealed phosphorylation of both STAT1 and -2 protein in native RPE cells after 1 hour of incubation (Fig. 5B). Similar to WNV supernatants, IFN-β stimulated both STAT1 and -2 phosphorylation, whereas IFN-γ activated STAT1, but not STAT2.

Effect of WNV Infection on the Expression of ISGs in RPE Cells

To investigate transcriptional activity resulting from IFN-β signaling, we studied by QRT-PCR whether WNV infection of RPE cells induces expression of ISGs including MX1, MX2, STAT1, IRF1, and IRF9, which are responsive to IFN-α/β but not to other stimuli, such as IRF3. As shown in Table 2 WNV infection upregulated the expression of ISGs in WNV-infected RPE cells relative to mock-infected cells in a manner similar to IFN-β.

Effect of Treatment with IFN-β on WNV Replication in RPE Cells

To examine the sensitivity of WNV replication in RPE cells to IFN-β treatment, we added IFN-β before or after virus inoculation. IFN-β 20 IU/mL added before or after virus inoculation completely suppressed the expression of WNV antigen (Fig. 6). In addition, pretreatment with IFN-β resulted in only moderately increased anti-WNV activity compared with IFN-β addition after infection.

WNV-Induced Antiviral Response and HCMV Replication

To investigate whether the WNV-induced antiviral response specifically inhibits the WNV replication or expresses nonspe-
cific inhibitory effects against other viruses, we investigated the dynamic of HCMV replication in WNV-infected RPE cells. RPE cells infected for 24 hours with WNV were superinfected with HCMV strain H91. The expression of HCMV IE and L antigens was assessed 1 and 5 days PI, respectively (Fig. 7). HCMV IE antigen expression, which occurs independent of HCMV replication, was not inhibited by WNV infection or by IFN-β. In contrast, HCMV L antigen expression, which is indicative of HCMV replication, was completely suppressed by WNV-infection and by IFN-β.

**DISCUSSION**

Ophthalmic manifestations of WNV infection include self-limiting optic neuritis, chorioretinitis, retinal hemorrhages, and vitreous inflammation; however, no mechanisms of ocular diseases have been investigated. In the present study, we showed that RPE cells are highly sensitive to WNV infection, as demonstrated by measurements of virus titers and electron microscopy. Thus, we developed and characterized the first cell culture model of ocular WNV infection. We also studied molecular mechanisms of host cell antiviral response to WNV infection.

Similar to most other viruses, WNV developed mechanisms to escape the innate immune response. In our model, we found that WNV-infected RPE cells expressed high levels of IFN-β and that WNV replication was highly sensitive to inhibition by IFN-β. WNV inhibited IFN signaling through blockage of STAT1 and -2 activation by several WNV nonstructural proteins in different transformed human cell lines. Because of this blockage of IFN signaling, IFN treatment failed to inhibit WNV replication when added after infection. In contrast to these previous findings, our data demonstrate that IFN signaling can be detected early after WNV infection of RPE cells (i.e., during the period of maximum virus replication and thus in the presence of maximum amounts of WNV proteins). This finding suggests that RPE cells possess mechanisms that counteract the inhibitory effects of WNV proteins on IFN-β signaling. Superinfection of WNV-infected RPE cells with HCMV completely prevented replication of the latter. Our data show that WNV induced a general antiviral state in RPE cells, not restricted to WNV.

The antiviral response elicited by viruses is initiated by IRF3 activation, which acts along with several other transcription factors as activator of the IFN-β promoter. Although IRF3 response to WNV infection in previous studies did not block viral replication, it was found to constrain WNV infection and limit cell-to-cell virus spread. Secreted IFN-β stimulates expression of numerous ISGs, some of which may also be directly induced by transcriptional activity of IRF3. The MX1 gene, which is rapidly induced on infection with a variety of viruses, strictly depends on IFN-α/β activity and is nonresponsive to IRF3. Therefore, MX1 is a marker for endogenous IFN activity. In addition to MX1, WNV infection stimulated the expression of several other ISGs that depend on IFN-β activity including MX2, STAT1, IRF1, and IRF9. In concordance, WNV-infected RPE cells responded rapidly to secreted and exogenously added IFN-β. Therefore, our results demonstrate that WNV induced secretion of IFN-β by host cells. Secreted IFN-β bound to the IFN-α/β receptor, subsequently activating the JAK/STAT pathway at early times after infection in RPE cells.

The retina, similar to other ocular compartments, has an immune-privileged status and thus differs from other tissues in their means of eliminating pathogens or inducing immune responses to infections. The retinal pigment epithelium

### Table 2. Induction of ISGs by WNV Infection or IFN-β Treatment in RPE Cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>WNV*</th>
<th>IFN-β*</th>
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<tbody>
<tr>
<td>MX1</td>
<td>6408.05 ± 941.1</td>
<td>5120.75 ± 596.2</td>
</tr>
<tr>
<td>MX2</td>
<td>79.65 ± 9.3</td>
<td>35.55 ± 5.9</td>
</tr>
<tr>
<td>STAT1</td>
<td>17.05 ± 0.4</td>
<td>16.45 ± 0.9</td>
</tr>
<tr>
<td>IRF1</td>
<td>20.0 ± 1.7</td>
<td>11.85 ± 0.8</td>
</tr>
<tr>
<td>IRF9</td>
<td>3.4 ± 0.4</td>
<td>3.05 ± 0.6</td>
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Data are expressed as the x-fold increase and are the mean ± SD of results in three independent experiments.

* x-Fold increase in WNV-infected or IFN-β-treated cultures relative to the mock infected cells determined by QRT-PCR.

![Figure 5](image-url) **Figure 5.** Western blot analysis of STAT1 and -2 expression and phosphorylation in mock-infected (mock) or WNV-infected (WNV) RPE cells 24 hours PI (A) and in noninfected RPE cells incubated with supernatants collected from mock-infected (mock) or from WNV-infected (WNV-sup.) cultures or RPE cells incubated with IFN-β (1000 IU/mL) or IFN-γ (1000 IU/mL) (B). Cell culture supernatants were collected 24 hours PI. The incubation with cell culture supernatants and IFNs was performed for 1 hour.

![Figure 6](image-url) **Figure 6.** Inhibition of WNV replication in RPE cells treated with recombinant IFN-β. The incubation with IFN-β was initiated 24 hours before infection or 1 hour PI. Virus replication was evaluated 24 hours PI by immune staining for WNV NS1 protein and microscopic investigation.
can be regarded as a front-line defense against invading organisms. It is strategically located between the neural retina and the blood-rich choroids and forms the outer blood–retina barrier.\(^\text{34-35}\) Therefore, RPE cells may develop mechanisms that enable more efficient activation of innate immune responses to WNV infection when compared with other cell types that are not involved in the front line of defense against pathogenic organisms. For example, RPE cells constitutively express TLR3, not involved in the front line of defense against pathogenic WNV infection when compared with other cell types that are involved.\(^\text{34-35}\) Immunostaining showed IE antigens in the nucleus (arrows) and L antigens in the cytoplasm (arrowheads) of infected cells. *Mean (± SD) of results of three independent experiments.

**Figure 7.** WNV infection of RPE cells induced an antiviral response that prevented HCMV replication. RPE cells were infected with WNV (MOI 5) or treated with IFN-β (1000 IU/mL) 24 hours before infection with HCMV (MOI 10). The number of HCMV IE and L antigen-expressing cells was assessed by immunostaining 24 and 120 hours PI, respectively. Immunostaining showed IE antigens in the nucleus (arrows) and L antigens in the cytoplasm (arrowheads) of infected cells. *Mean (± SD) of results of three independent experiments.

**References**


