Digoxin Inhibits Induction of Experimental Autoimmune Uveitis in Mice, but Causes Severe Retinal Degeneration

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Purpose. Digoxin, a major medication for heart disease, was recently reported to have immunosuppressive capacity. Here, we determined the immunosuppressive capacity of digoxin on the development of experimental autoimmune uveitis (EAU) and on related immune responses.

Methods. The B10.A mice were immunized with interphotoreceptor retinoid-binding protein (IRBP) and were treated daily with digoxin or vehicle control. On postimmunization day 14, the mouse eyes were examined histologically, while spleen cells were tested for cytokine production in response to IRBP and purified protein derivative. The immunosuppressive activity of digoxin was also tested in vitro, by its capacity to inhibit development of Th1 or Th17 cells. To investigate the degenerative effect of digoxin on the retina, naïve (FVB/N × B10.BR)F1 mice were similarly treated with digoxin and tested histologically and by ERG.

Results. Treatment with digoxin inhibited the development of EAU, as well as the cellular response to IRBP. Unexpectedly, treatment with digoxin suppressed the production of interferon-γ to a larger extent than the production of interleukin 17. Importantly, digoxin treatment induced severe retinal degeneration, determined by histologic analysis with thinning across all layers of the retina. Digoxin treatment also induced dose-dependent vision loss monitored by ERG on naïve mice without induction of EAU.

Conclusions. Treatment of mice with digoxin inhibited the development of EAU and cellular immune response to IRBP. However, the treatment induced severe damage to the retina. Thus, the use of digoxin in humans should be avoided due to its toxicity to the retina.

Keywords: experimental autoimmune uveitis, digoxin, retinal degeneration

Digoxin has been used to treat heart disease for many decades, due to its positive inotropic effect on the heart via the plasmalemmal Na^+/K^+ ATPase.[1,2] However, digoxin is a broad acting drug, and, when administered orally, acts systemically with toxic effects.[3–6] Side effects in humans have been reported to include instances of disturbed vision and photoreceptor dysfunction.[7,8] A recent *Nature* paper reported that digoxin is an efficient inhibitor of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, and suggested that digoxin and the family of derived compounds could be used for treatment of autoimmune conditions.[9]

Noninfectious uveitis, an umbrella term for various intraocular inflammatory diseases, is one of the leading causes of vision loss in developed countries.[10–12] Treatments for these conditions are still lacking, consisting mostly of broad immunosuppressants.[13] It is commonly assumed that autoimmunity plays a major role in many of these eye conditions,[14,15] and the search for more targeted medications is carried out mostly in experimental animals in which an inflammatory eye disease, experimental autoimmune uveitis (EAU), is induced.[15–17] Experimental autoimmune uveitis in mice is induced by immunization with the retinal interphotoreceptor retinoid-binding protein (IRBP),[15,18] or peptides from its sequence.[19] Recent studies have shown that EAU is mediated by both Th1 and Th17 cells,[20,21] with Th17 cells reported to be responsible for sustained intraocular inflammation.[22,23] The study of Huh et al.,[9] mentioned above, reported that digoxin is able to bind to the ligand binding domain of retinoic acid receptor (RAR)-related orphan receptor gamma (RORγ)t, the major transcription factor responsible for the generation of Th17 cell lineages,[24] by acting as an inverse agonist to reduce the level of transcription of RORγt.[25,26] Huh et al.[9] suggested, therefore, that in their EAE model, the major target of digoxin are the immunopathogenic Th17 cells.

Digoxin treatment of mice developing EAU was found in the present study to inhibit the ocular inflammatory process and the cellular response to IRBP. In addition, however, digoxin caused severe thinning of the retina, primarily affecting the photoreceptor cell layer. The extent of the retinal damage was also analyzed by ERG.

MATERIALS AND METHODS

Mice

For the EAU model, female B10.A mice were purchased from Charles River Laboratories, Inc. (Frederick, MD, USA), while the

studies on digoxin toxicity were performed on (FVB/N × B10.BR)F1 mice, bred at the National Eye Institute (NEI) animal facility. (These hybrid mice were generated as "byproducts" of breeding carried out for other studies.[27,28]) All mice were housed in a pathogen-free facility and all manipulations were performed in compliance with the National Institutes of Health Resolution on the Use of Animals in Research and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental procedures used in this study were approved by the NEI Animal Care and Use Committee, under NEI Animal Study Protocols NEI-555 and NEI-624.

Induction of EAU

We induced EAU in female B10.A mice, aged 6 to 10 weeks, by immunization with IRBP as described elsewhere,[29,30] with minor modifications. The mice were immunized with 40 μg bovine IRBP emulsified with complete Freund's adjuvant (CFA) and injected subcutaneously into the base of the tail and both thighs. In addition, the mice were injected intraperitoneally with 0.2 μg pertussis toxin (List Laboratories, Campbell, CA, USA). On postimmunization (pi) day 14, mice were euthanized and eyes were collected for histopathologic examination. Spleens were collected for assessment of the specific cellular immune response.

Treatment With Digoxin

Digoxin (Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in DMSO and diluted in PBS to 1% DMSO for injection. Mice were treated with digoxin daily (1 or 2 mg/kg.), administered intraperitoneally, on pi days 1 through 13 and euthanized on pi day 14. Control mice were similarly treated with 1% DMSO.

Histologic Analysis

Eyes were fixed in 4% glutaraldehyde for 30 minutes before being transferred to 10% formalin until processing. Eye tissues were embedded in methacrylate, and stained with hematoxylin and eosin. Severity of ocular inflammation in the IRBP-immunized mice was evaluated, on a scale of 0 to 4, as described elsewhere.[29,30]

Cytokine Production

Cytokine production by splenocytes from the immunized mice was measured as detailed elsewhere.[29,30] Briefly, cells were cultured in 24-well plates at 5×10^6 cells per well in 1 mL medium, with the indicated stimulants, IRBP and purified protein derivative (PPD [tuberculin]; Parke-Davis, Morris Plains, NJ, USA). Culture supernatants were collected after a 48-hour incubation, and the levels of interleukin (IL)-17 and interferon (IFN)-γ were measured by ELISA kits, (R&D Systems, Minneapolis, MN, USA).

Test for Digoxin Effects on Lymphocytes In Vitro

Naïve mouse CD4 spleen cells, purified by T-cell columns (R&D Systems) followed by MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), were stimulated by incubation in 24-well culture plates coated with anti-CD3 and anti-CD28 antibodies (BD Bioscience, San Jose, CA, USA). The coating antibodies were added to the wells at 1 mg/mL and the free antibodies were removed before the cells suspension was added. The cells were cultured at 5×10^6 per well, in a total volume of 1 mL of RPMI 1640 medium supplemented with HL-1 (Lonza, Walkersville, MD, USA).

Digoxin, dissolved in DMSO, was added to the cell cultures at the indicated concentrations and DMSO at the corresponding dilutions was used as controls. The supernatants were collected following incubation of 48 hours. Levels of IL-17 and IFN-γ in the supernatants were measured by ELISA, as described above.

Assessment of Retinal Damage in Digoxin-Treated Naïve Mice: Histology

Naïve (FVB/N × B10.BR)F1 mice were treated with digoxin as described above. Eyes of the mice were collected for histopathologic examination, as detailed above. The effect of digoxin treatment on the retina was assessed by measuring the thickness of six layers (i.e., outer segment layer [OSL], outer nuclear layer [ONL], outer plexiform layer [OPL], inner nuclear layer [INL], inner plexiform layer [IPL] and ganglion cell layer [GCL]). Thickness measurements were performed on eye sections of vehicle-treated controls and of treated mice, collected on days 6 and 14 of digoxin treatment. Measurements were performed using eye sections of 2 mice of each group, at different points, 0.5 to 1.0 mm from the optic nerve head.

Assessment of Retinal Damage in Digoxin-Treated Naïve Mice: ERG

Retinal function was evaluated by ERG, using commercial equipment (Espion E2 system with ColorDome and Rodent table; Diagnosys LLC, Lowell, MA, USA). Electroretinography data were recorded on days 7 and 14 of the treatment. Following overnight dark adaptation, animals were sedated with intraperitoneal (IP) injection of a ketamine and xylazine mixture, 76.8 mg/kg and 4.6 mg/kg, respectively. Details of ERG recording procedures were described in Reference 31.

Statistical Analysis

To allow comparison of ELISA data across multiple experiments with lymphocytes from treated mice, the results were normalized 0 to 1 with feature scaling,[32] where:

$$X' = \frac{x_i - x_{\min}}{x_{\max} - x_{\min}}.$$

Statistical significance was determined using unpaired t-tests with $P \leq 0.05$, as determined by the Holm-Sidak method. Where nonnormal distributions were compared, the Mann-Whitney U test was used. For the comparison among groups of mice tested for ERG responses, a multiple t-test was used.

RESULTS

Digoxin Treatment Inhibits EAU Induction

To determine the ability of digoxin to inhibit the induction of EAU, we immunized groups of B10.A mice with IRBP and treated them daily with digoxin or vehicle control for 13 days. The mouse eyes, collected on day 14, were analyzed histologically and the accumulated data are summarized in Figure 1A. Histologic scoring shows a significant difference ($P < 0.0001$) between the groups treated with digoxin and their controls; eyes from control mice exhibit more inflammation than the digoxin-treated mice. Figure 1B shows typical histologic eye sections of a digoxin-treated mouse and a control mouse. Inflammatory changes are seen in the control eye, whereas essentially no inflammation is seen in the eye of the treated mouse.
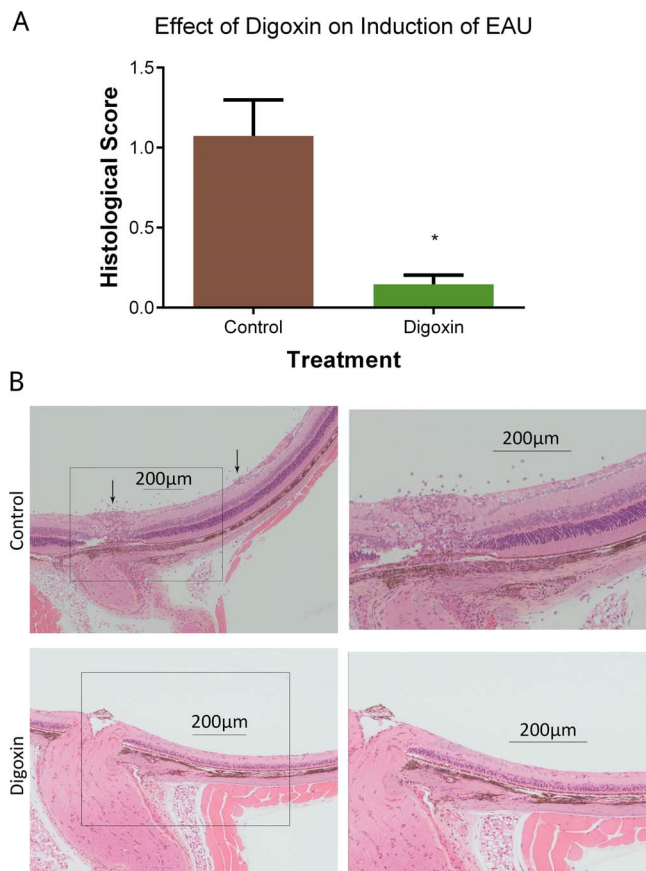
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FIGURE 1. Induction of EAU is inhibited by treatment with digoxin. The B10.A mice immunized with IRBP were treated daily with digoxin at 2 mg/kg and their eyes were collected on pi day 14 and analyzed for histologic changes. (**A**) Mean histopathologic changes in mice treated with digoxin and their vehicle-treated controls ($n = 20$ in each group). *$P \leq 0.0001$. (**B**) Representative sections of a treated and a control mouse eyes. The changes in the control eye include inflammatory cells infiltrating through the optic nerve head and retinal blood vessels, as well as lymphoid cells in the vitreous. In contrast, essentially no inflammatory changes are seen in the digoxin-treated mouse eye, but the retina of this mouse exhibits severe thinning.

Digoxin Treatment Inhibits Cytokine Production by Spleen Cells

Experimental autoimmune uveitis is initiated by helper T (Th) cells, specifically of the Th17 and Th1 subtypes, which act in large part by releasing their signature cytokines, IL-17, and IFN-γ, respectively.[20,21] To assess the immunomodulatory effects of digoxin on the Th17 and Th1 populations in vivo, we cultured spleen cells of immunized mice with IRBP, the uveitogenic protein, and PPD, a component of CFA, and measured in the culture supernatants the levels of IL-17 and IFN-γ. Data of three individual experiments are summarized in Figure 2, which shows the mean cytokine levels ± SEM in the different cultures. Significantly lower levels of IFN-γ were measured in cultures of spleen cells from digoxin-treated mice compared with their controls. The differences between the two groups in their IL-17 production, however, were insignificant.

Digoxin Selectively Inhibits Th17 Cell Development in an In Vitro System

Digoxin inhibits immune responses by selectively blocking the activity of RORγt,[9] the key transcription factor for the
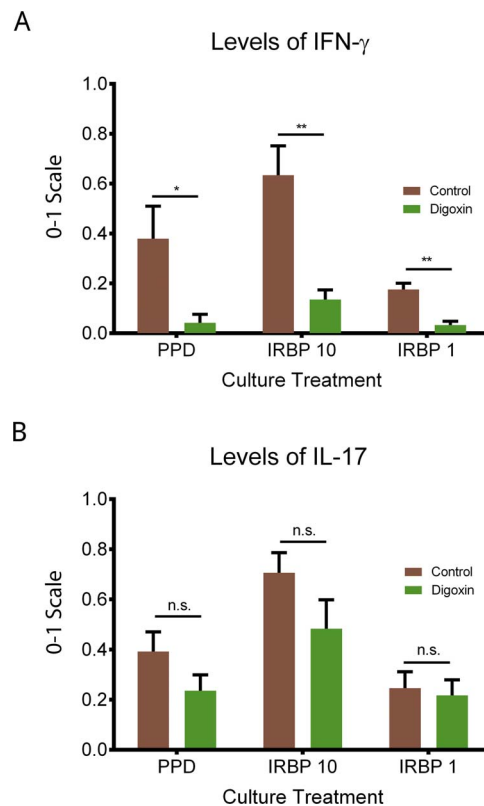

FIGURE 2. Reduced cytokine production by lymphocytes of digoxin-treated mice. Spleen cells from B10.A mice immunized with IRBP and treated with digoxin or vehicle control were cultured with PPD and IRBP. Their release of IFN-γ and IL-17 was measured by ELISA. The data of three repeated experiments were normalized and averaged across these experiments. The secretion of both cytokines was lower in digoxin-treated splenocyte cultures than in their controls, but the differences reached significance only for IFN-γ secretion. *$P \leq 0.05$. **$P \leq 0.001$.

differentiation of Th17 lineages.[24] The finding that splenocytes from mice treated with digoxin exhibited significant inhibition of IFN-γ expression, but just moderately reduced IL-17 production was, therefore, unexpected. To further analyze this observation, we examined the effect of digoxin in an in vitro system in which naïve CD4 cells are activated by anti-CD3/CD28 antibodies and concurrently acquire polarity toward either the Th1 or Th17 phenotypes. Data of a representative experiment are shown in Figure 3 and demonstrate that digoxin selectively inhibited the production of IL-17, but had no suppressive effect on the production of IFN-γ. This observation thus verifies the selective inhibitory effect of digoxin against the development of Th17, but not Th1. The unexpected observation in our study, of inhibitory effect of digoxin on IFN-γ production in vivo is further dealt with in the Discussion section, below.

Digoxin Treatment Causes Retinal Degeneration

Histologic analysis of eyes from the digoxin-treated mice revealed that, in addition to reduction in inflammatory changes in eyes developing EAU, treatment with digoxin also caused remarkable thinning of the retina (Fig. 1). To further analyze this effect of digoxin, we examined histologically eyes of controls and digoxin-treated mice without induction of EAU. Eye sections of representative mice, collected on days 6 and 14 of treatment with digoxin at 2 mg/kg, are shown in Figure 4A.
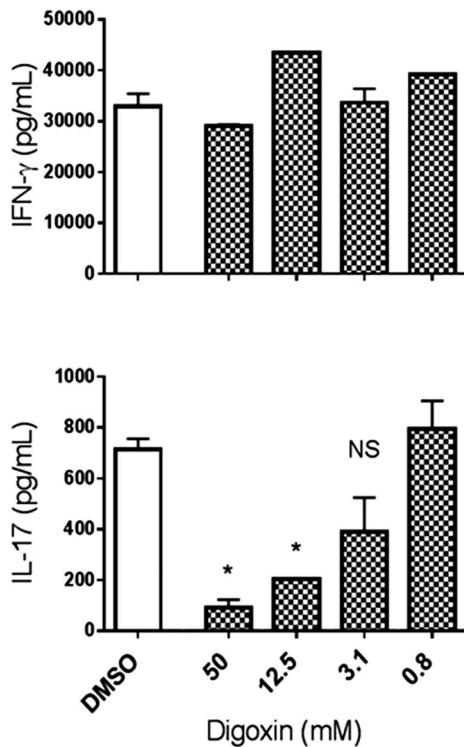
FIGURE 3. Digoxin selectively suppresses IL-17 production in cultures of naïve CD4 cells stimulated by anti-CD3/CD28 antibodies. Digoxin was added at the indicated concentrations to cultures of naïve CD4 spleen cells stimulated with the antibodies as detailed in the "Materials and Methods" section. Supernatants were collected following 48-hour incubation and the levels of IFN-γ and IL-17 were determined by ELISA. Data shown were collected in a representative experiment; similar data were collected in two additional experiments. *$P < 0.005$.

Remarkable levels of tissue damage are seen in eyes of the treated mice, indicated by the substantial thinning of retinal tissue in the treated mice, compared with the control eye sections. Substantial thinning is seen on day 6 and further severe damage is apparent on day 14 of treatment. In order to quantify the thinning process, we measured the thickness of individual six retinal layers, as presented in Figure 4B. The most affected layers were those of the photoreceptor cells, with the OSL showing the damage earlier than the ONL. The originally thin OPL could not be defined on day 14, whereas lower degrees of thinning were noted in the INL and the IPL. The ganglion cell layer was the least affected layer, with thinning measured only on day 14 of treatment.

Digoxin Causes Loss of Visual Function

To analyze the effect of digoxin treatment on the visual function of the mice, we conducted ERG analysis on days 7 and 14 of daily treatment. A summary of ERG results (Fig. 5A) shows a dose-dependent loss of visual function. Reductions of ERG responses with digoxin treatments were observed under both dark- and light-adapted conditions. For b-wave amplitudes of dark- and light-adapted ERG, mice treated with 2 mg/kg digoxin showed lower peak potential than mice treated with 1 mg/kg digoxin, than did mice treated with vehicle control (1% DMSO). For a-wave amplitudes of dark-adapted ERG, mice treated with both 1 mg/kg and 2 mg/kg digoxin had similar peak amplitudes which were much lower than those from control mice.
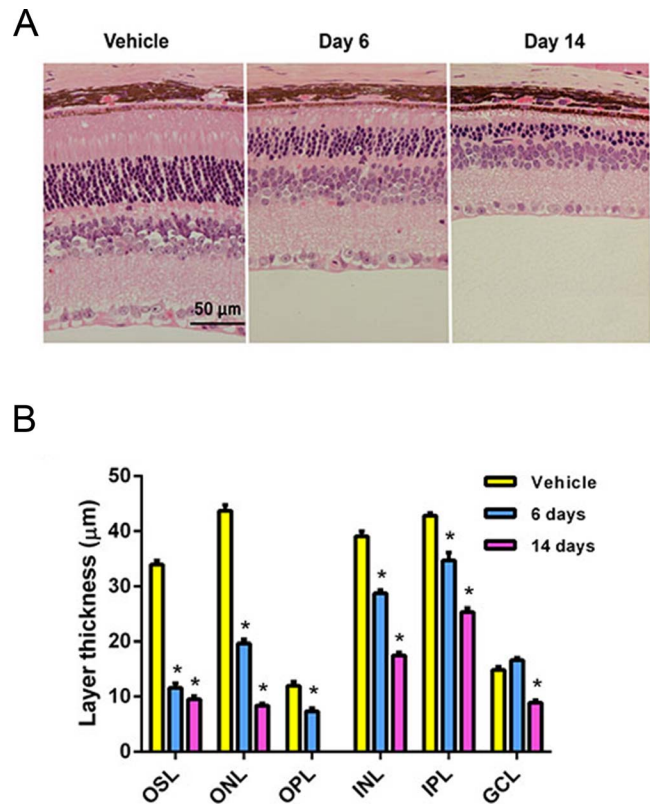

FIGURE 4. Treatment with digoxin reduces retinal thickness. Naïve (FVB/N × B10.BR)F1 mice were treated daily with digoxin at 2 mg/kg, or with the vehicle, and were euthanized on days 6 or 14 of treatment. (A) Histologic sections of eyes of representative control and treated mice, as indicated. Gradual thinning of the retinas in the digoxin-treated mice, with the photoreceptor layers showing the most severe damage. (B) Quantification of the damage in the digoxin-treated eyes, determined by thinning of the six retinal layers. The outer segment layer and ONL, as well as the OPL are affected more than the INL and IPL, while the GCL is the least affected. *$P < 0.001$.

Electroretinography waveforms of a representative experiment are shown in Figure 5B. Although there were significant reductions in the amplitudes of the responses, waveforms were similar among those recorded from control and treated animals under both dark- and light-adapted conditions. Figure 5C compares mean amplitudes of dark-adapted ERG a- and b-wave and light-adapted b-wave amplitudes recorded at day 7 and day 14 of treatment, and demonstrates that, interestingly, the degree of visual function did not change significantly between these time points, despite the continued daily treatment with digoxin.

DISCUSSION

Results reported here show that treatment with digoxin inhibits the development of EAU in mice immunized with IRBP. It is of note that inflammation in control mouse eyes averaged only 1.07 ± 0.22, levels that are lower than those in a previous study with B10.A mice.[29] The unusually low levels of inflammatory changes in mice of the present study could be attributed at least in part to the microbiome of these mice. The effect of the microbiome on the immune response is well established (see reviews in Refs. 33 and 34) and it is assumed that the microbiome of the mice in the present study was different from that of the mice in the cited study,[29] since the mice of the two studies were housed in different animal
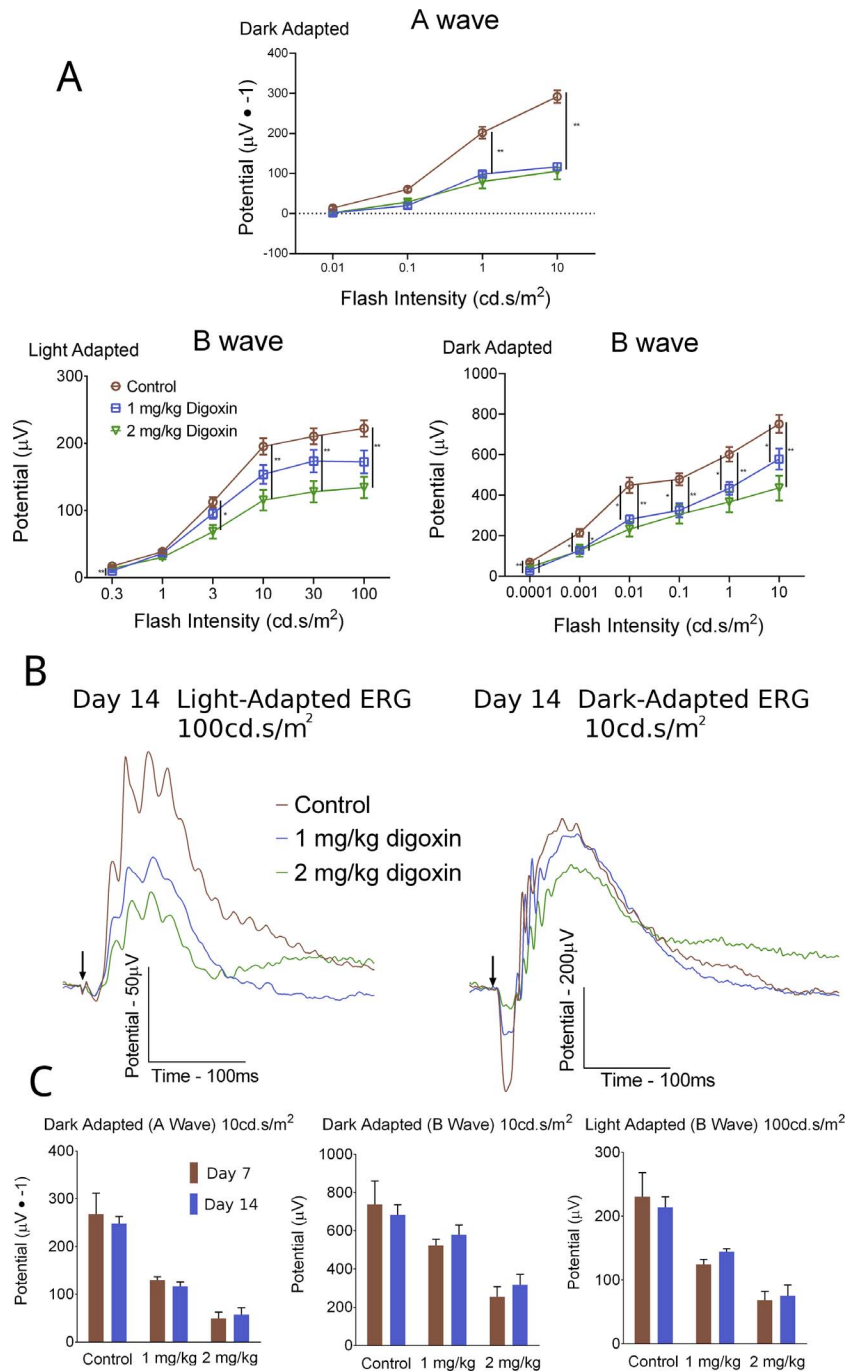
FIGURE 5. Treatment with digoxin affects the ERG responses in mouse eyes. (FVB/N × B10.BR)F1 hybrid mice were treated daily for 14 days with digoxin at 1 or 2 mg/kg or with vehicle control. (**A**) Mean a- and b-wave peak intensities of electroretinography waveforms, taken on day 14 of treatment, dark- and light-adapted responses from all experiments. *$P \leq 0.05$, **$P \leq 0.001$. Controls: $n = 16$; 1 mg/kg, $n = 6$; 2 mg/kg, $n = 20$. (**B**) Representative ERG waveforms elicited by the highest intensity light flashes in each sequence (dark- or light-adapted) from mice that received 1 mg/kg, 2 mg/kg digoxin, or vehicle control. (**C**) Comparison of mean a- and b-wave amplitudes recorded on days 7 and 14 of digoxin treatment, dark- and light-adapted responses from a representative experiment. $n = 8$ for each group.

facilities. Indeed, we have shown in another study that mice of the same line differ in their EAU development when housed in different facilities[35] and microbial products were found to facilitate ocular autoimmune processes.[36]

The immunosuppressive mechanism of digoxin in the EAU system was analyzed by comparing spleen cells of mice treated with the compound or with the vehicle for their production of IFN-γ and IL-17, the signature cytokines for Th1 and Th17, respectively.[22,23] Unexpectedly, the production of IFN-γ·by

spleen cells from the digoxin-treated mice was remarkably inhibited, whereas no significant differences were noted between the two groups in their IL-17 production. This observation contradicts the known mode of action of digoxin (i.e., selective inhibition of Th17 development).[9] The selectivity of digoxin's inhibitory effect was verified; however, in our mouse system by showing that the compound inhibits the generation of Th17 cells but not of Th1 cells, when added to cultures in which both subpopulations are concurrently

generated from naïve CD4 cells (Fig. 3). The unexpected observation with spleen cells from immunized mice treated with digoxin could be explained by the plasticity of Th17 cells; these cells readily acquire Th1 phenotype in immunized animals, as reported by our group and others.[37–39] We suggest that the subpopulation of Th17 cells that switched phenotype in the immunized mice in our study contribute substantially to the total IFN-γ in the splenocyte cultures and that these cells are highly susceptible to the digoxin cytotoxic effect, so that their elimination brings about the remarkable reduction in IFN-γ levels in these cultures. This notion is in line with the finding of Xiao et al.,[40] that the double phenotypic Th cells that increased substantially in their proportion in mice immunized for EAE induction, are highly susceptible to the immunosuppressive effects of TMP778, another RORγt inhibitor.

Due to digoxin's cytotoxic properties, namely, its ability to inhibit Na^+/K^+ ATPase, its effects on the retina are drastic. It is of note that the dose of digoxin used in our study, as well as in the mentioned study of Huh, et al.[9] (2 mg/kg) is higher by more than 2 orders of magnitude than doses used to treat heart failure in humans [280–1120 times higher].[6] The reason that such a relatively high dose should be applied in mice is due to the amino acid polymorphisms in murine Na^+/K^+ ATPase, which makes mice profoundly more resistant to digoxin's effects than its human counterpart ATPase.[41,42] Therefore, the daily dose used in mice in the present study and in the one by Huh et al.[9] was accordingly calibrated to be equivalent in its toxic capacity to the median range of doses given to humans.

Huh et al.[9] did not notice any toxic effects of digoxin in the treated mice, but the eye was found in the present study to be highly susceptible to the toxicity of this compound when given at the same dosage (2 mg/kg). The toxicity of digoxin was particularly damaging to the retina, with essentially all layers losing cells and volume and becoming dramatically thinner, as demonstrated in Figure 4. Consequently, digoxin treatment affected the animal vision, as indicated by ERG analysis, indicating considerable levels of vision loss in a dose-dependent manner: mice treated with only 1 mg/kg digoxin retained greater ERG b-wave than those mice treated with 2 mg/kg (Fig. 5). It is also of interest that the damage in retinal function remained constant between days 7 and 14 for mice treated with digoxin, despite the continued daily treatment with the compound (Fig. 5C). This suggests that the damage inflicted upon the retina by the dose of digoxin used here may not increase past a certain level, indicating that dose-dependence is of greater importance than time-dependence for digoxin toxicity.

Digoxin is not the only compound to affect the ROR family of molecules. As reviewed by Solt and Burris,[25] many compounds act nonspecifically on the ROR ligand binding domain. Synthetic analogs of digoxin such as SR1001, SR2211, and TMP778, now being evaluated for use in humans, are able to bind to RORγt specifically, but do not possess the cytotoxic properties of digoxin, namely, inhibition of the plasmalemmal Na^+/K^+ ATPase.

In summary, our study shows that digoxin may reduce uveal inflammation, but its cytotoxic properties also cause retinal degeneration, leading to loss of visual function. Therefore, not only is digoxin just an improper therapeutic choice for uveitis, but treatment with this compound should be avoided in humans with heart disease, as to avoid causing retinal degeneration in an otherwise healthy eye.

Acknowledgments

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