Retinal Function and Morphology in Monkeys with Ethambutol-Induced Optic Neuropathy

Junzo Kinoshita, Noriaki Iwata, Takanori Maejima, Tomofumi Kimotsuki, and Mitsuya Yasuda

**Purpose.** Ethambutol-induced optic neuropathy is a well recognized adverse ocular event. However, abnormalities of the retina in this optic neuropathy are not fully understood. Therefore, the purpose of the present study was to investigate both functional and morphological alterations of the retina induced by ethambutol in monkeys.

**Methods.** Ethambutol was orally administered to three cynomolgus monkeys, initially at 400 mg/kg/day followed by 800 mg/kg/day, for a maximum of 39 weeks. Full-field electroretinograms (ERGs) were recorded at intervals of approximately one month. The protocol included standard ERG responses to white flashes obtained under dark-adapted conditions (rod, combined rod-cone, oscillatory potentials) or with a white background (single-flash cone response [R/B]). In addition, we measured the ERG elicited with red flashes under blue background light (single-flash cone response [R/B]). All the ethambutol-treated monkeys were euthanized, and the retinae and various other nervous system tissues were examined histopathologically.

**Results.** No obvious changes were observed in the standard full-field ERGs. On the other hand, selective attenuation of the photopic negative response (PhNR) of the single-flash cone response (R/B) was observed in two out of three ethambutol-treated monkeys at week 22 or 28. Histopathology of these two monkeys revealed single cell necrosis of the retinal ganglion cells (RGCs), decreased RGCs in the parafovea and increased microglial cells in the nerve fiber layer in the retina, in addition to demyelination and glial reaction in the optic nerve, chiasm and tracts.

**Conclusions.** The attenuated PhNR and histopathology of the retina indicated that RGCs were markedly damaged, both functionally and morphologically in monkeys with ethambutol-induced optic neuropathy. These results imply that RGCs are predominantly affected in the retina of patients with ethambutol-induced optic neuropathy. (Invest Ophthalmol Vis Sci. 2012;53:7052–7062) DOI:10.1167/iovs.12-10308

Ethambutol is widely used as an antimycobacterial drug for the treatment of tuberculosis. It is well known that long term medication with ethambutol produces dose-related alterations in the visual system of the patients, such as reduced visual acuity, dyschromatopsia, and central or cecocentral scotoma. Abnormal visual evoked potentials (VEPs) with normal electroretinograms (ERGs), indicating involvement of the optic nerve and/or the more proximal visual pathway, has been reported in ethambutol-treated patients. In animal experiments, ethambutol-induced histopathologic lesions, mainly consisting of demyelination in the visual pathway (extending from the optic nerve to the optic tract), have been reported in many species, suggesting that similar morphological changes occur in patients. Therefore, the aforementioned visual disturbances in ethambutol-treated patients are generally associated with retrobulbar optic neuritis (i.e., optic neuropathy).

Furthermore, in the retina of patients taking ethambutol, abnormal electro-oculogram (EOG) and decreased amplitude or delayed implicit time in multifocal ERG have been reported. The authors of these reports suggested that ethambutol functionally affects not only the optic nerve but probably the retina. Few studies, however, have been conducted focusing on the detailed function and morphology of the retina in such patients. Also, in animal experiments, there are very few reports that describe ethambutol-provoked abnormality in histopathology of the retina; though Heng et al. reported the selective loss of cells from the retinal ganglion cell (RGC) layer in rats treated repeatedly with ethambutol. Furthermore, as far as we know, no studies have examined retinal function in animals with histopathologically confirmed ethambutol-induced optic nerve lesions; though effects of ethambutol on ERG have been investigated in rats and dogs. Thus, abnormalities of the retina in ethambutol-induced optic neuropathy are not fully understood.

One of the ERG components, the photopic negative response (PhNR), a negative-going wave of the photopic ERG that appears immediately after the b-wave, is known to be an indicator of retinal function. Several lines of experimental evidence indicate that the PhNR originates from the spiking activity of the RGCs and their axons in several species including humans and monkeys. Consistent with the origin of the PhNR in humans, significantly reduced PhNR has been reported in patients with several pathological conditions in which RGCs were affected.

Therefore, the purpose of this study was to investigate retinal alterations in ethambutol-induced optic neuropathy, by means of both functional and morphological assessments of the retinas in animals with ethambutol-induced damage of the optic nerve. For this purpose, we repeatedly administered ethambutol to monkeys, a species in which the physiology and anatomical structure of the retina is widely known to be similar to that in humans. We serially recorded the ERG including the PhNR in these monkeys. Finally, tissues of the visual pathway including the retina were examined histopathologically in monkeys with attenuated PhNR.
Table 1. Dosage Regimen and Clinical Course

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No.</th>
<th>Dosage Regimen, mg/kg/d×wk, po</th>
<th>Clinical Course</th>
<th>Week (Day) of Necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>01M01</td>
<td>0×39</td>
<td>-</td>
<td>NE</td>
</tr>
<tr>
<td></td>
<td>01F01</td>
<td>0×39</td>
<td>-</td>
<td>Week 39 (day 274)</td>
</tr>
<tr>
<td></td>
<td>01F02</td>
<td>0×39</td>
<td>-</td>
<td>NE</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>02M01</td>
<td>400×13 → 800×26</td>
<td>Anorexia on Days 159 and 160; incomplete eyelid opening and prone position on Day 160*</td>
<td>Week 39 (day 274)</td>
</tr>
<tr>
<td></td>
<td>02M02</td>
<td>400×13 → 800×10</td>
<td>-</td>
<td>Week 23 (day 160)</td>
</tr>
<tr>
<td></td>
<td>02F01</td>
<td>400×13 → 800×15</td>
<td>-</td>
<td>Week 28 (day 196)</td>
</tr>
</tbody>
</table>

-, no noteworthy findings; NE, not examined pathologically; po, per os.
* Recovered mostly within 1 hour.

**METHODS**

**Animals**

A total of nine cynomolgus monkeys (Macaca fascicularis) between 3 and 8 years of age were used in this study. The animals were housed individually in stainless steel cages (W 60 cm × D 68 cm × H 75 cm) in an animal study room where the environmental condition was set as follows: room temperature, 24°C; relative humidity, 60%; illumination, 12 hour lighting (7:00 AM to 7:00 PM) at 150 to 300 luxes. The animals were fed 100 g/animal/day of pellet food for monkeys (PS; Oriental Yeast Co., Ltd., Tokyo, Japan). Tap water from a feed-water nozzle was supplied ad libitum to the animals. All experimental procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care Committee of Daiichi Sankyo Co. Ltd.

**Drug Administration**

Ethambutol was administered repeatedly to three monkeys. The first day of the repeated ethambutol dosing was referred to as day 1, and the period from days 1 to 7 was referred to as week 1; subsequent days or weeks were counted relative to day 1 or week 1. The starting dose level of ethambutol was 400 mg/kg/day. The dose level was increased to 800 mg/kg/day in week 14 (day 97), based on the report by Schmidt et al.22

**Animal Preparation for ERG Recording.** The animals were anesthetized with intramuscular injection of ketamine hydrochloride (Ketalar Intramuscular 500 mg; Daiichi Sankyo Co., Ltd.) (10 mg/kg initial dose, 5–10 mg/kg/h maintenance dose) and 0.6 mg/kg xylazine hydrochloride (Celactal; Bayer Medical Ltd.). The pupils were dilated with topical 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydri-P ophthalmic solution; Santen Pharmaceutical Co., Ltd., Osaka, Japan); the corneas were anesthetized with topical 0.4% oxybuproca cine hydrochloride (Benoxil ophthalmic solution 0.4%; Santen Pharmaceutical Co., Ltd.) and protected with topical hydroxyethylcellulose (Scopisol solution for eye; Takeda Chemical Industries, Ltd., Osaka, Japan).

**Visual Stimulation.** Full-field stimulation was produced with a Ganzfeld stimulator (BigShot Ganzfeld; LKC Technologies, Inc., Gaithersburg, MD) that was positioned just in front of animal’s face. The light flashes and steady background illumination were generated with the following light-emitting diodes (LEDs) housed in the stimulator: red (\(\lambda_{max} = 627 \text{ nm}\)), green (\(\lambda_{max} = 530 \text{ nm}\)), and blue (\(\lambda_{max} = 470 \text{ nm}\)). White flashes were produced by combining the output from these three LEDs. Duration of all stimuli was less than 5 minutes. The maximum intensity of white flash generated with the LEDs in the stimulator was 27.3 phot cd/s/m², as measured by a calibrated photometer (IL1700; International Light Technologies, Inc., Peabody, MA) and a calibrated optical detector (SED053/Y/R; International Light Technologies, Inc.). The stimulus intensity and the background luminance were altered by varying the LED pulse duration using software (Ganzfeld control panel; LKC Technologies, Inc.) installed in a personal computer.

**ERG Recording and Analysis.** The standard full-field ERGs were evaluated according to the guidelines25 of the International Society for Clinical Electrophysiology of Vision (ISCEV). In addition, the photopic ERG elicited with red flashes under blue background (i.e., single-flash cone response [R/B]) was evaluated. A bipolar contact lens electrode (H6515NFC; Mayo Corporation) was placed on the corneal surface of the left eye. A ground electrode (TN208-016; Unique Medical Co., Ltd., Tokyo, Japan) was attached to the parietal region of the scalp. Following 40 minutes or more of dark adaptation, the rod-driven response (the rod response) and the rod- and cone-driven response (the combined rod-cone response [standard flash]) were elicited by white light flashes at an intensity of 0.009 and 2.8 phot cd/s/m², respectively. Another rod-and cone-driven response (the combined rod-cone response [bright flash]) and the oscillatory potentials were simultaneously elicited by white light flashes at an intensity of 17.7 phot cd/s/m². Subsequently, after 10 minutes of light adaptation with a blue background light at 6.9 phot cd/m², the single-flash cone response (R/B) was elicited by red light flashes at an intensity of 2.9 phot cd/s/m² under blue background light. Finally, the photopic ERG elicited with white flashes under white background (i.e., single-flash cone response [W/W]) and the 50 Hz flicker...
response were elicited by white light flashes at an intensity of 2.8 phot cd/s/m² under white background light at 29.0 phot cd/m². Each ERG mentioned above was obtained during week –1, week 1 (days 2 and 3), and weeks 4, 9, 14, 18, 22, 26, 28, 30, 35, and 39. ERGs were recorded prior to dosing on all days except on day 2, when the ERGs were recorded 53 to 81 minutes after the oral dosing of ethambutol. For TTX-treated animals, a bipolar contact lens electrode was placed on each eye and the single-flash cone response (R/B) from each eye was recorded simultaneously before and 2 hours after intravitreal injection.

Responses were amplified at 10,000 times and were filtered with a band pass from 0.5 to 1000 Hz except for the oscillatory potentials, which were filtered with a band pass from 100 to 1000 Hz. The amplified signals were stored in the evoked potential test equipment (MEB-9104; Nihon Kohden Corporation, Tokyo, Japan). A limited number of waveforms (3–10) for each response were averaged to reduce variability and background noise. For the waveform analysis, (1) the amplitude of the a-wave, which mainly reflects photoreceptor function, was measured from baseline to the a-wave trough for the combined rod-cone response and the single-flash cone response, (2) the amplitude of the b-wave, which reflects function of the inner nuclear layer, was measured from the a-wave trough to the b-wave peak for all the responses, and (3) the amplitude of the PhNR, which mainly reflects RGC function, was measured from baseline to the PhNR trough for the single-flash cone response. In addition, the PhNR/b-wave amplitude ratio, which has been reported to be of smaller variability for the single-flash cone response. (2) combines the amplitude of the a-wave, which mainly reflects photoreceptor function, and OP2, respectively) were measured from baseline to each respective peak.

Ophthalmoscopy

After recording the ERGs, the fundi of both eyes were inspected with a binocular indirect ophthalmoscope (HEINE OMEGA 500; HEINE Optotechnik GmbH & Co. KG, Herrsching, Germany) by the same examiner throughout the study period.

Toxicokinetics

Immediately after recording the ERGs, a volume of approximately 0.3 mL of blood was collected from the femoral vein to measure the plasma ethambutol concentration. The blood was collected immediately after recording the ERGs on day 2, corresponding to 77 to 85 minutes after dosing, and on days 3, 28, 63, 94, 150, 178, 192, 206, 241, and 269, corresponding to 22 hours 52 minutes to 27 hours 43 minutes after dosing. Blood was also collected 1, 2, 4, 7, and 24 hours after dosing on day 1, prior to dosing and 1, 2, 4, 7, and 24 hours after dosing on days 23, 97, and 125 in the same manner. The plasma was prepared from the blood samples by centrifugation at 10,000 rpm for 5 minutes at 4°C. The plasma was then stored at –80°C until measurement. The plasma concentration of ethambutol was determined by liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS) (HPLC; Waters Alliance 2795 Separations Module; Waters Corp., Milford, MA; MS/MS, Quattro Premier XE; Waters Corp.). As toxicokinetic (TK) parameters, area under the plasma concentration time curve up to 24 hours after dosing (AUC_C0–24h), maximum plasma concentration (Cmax), and time to maximum plasma concentration (tmax) were calculated.

Pathology

Three ethambutol-treated animals, numbers 02M02, 02F01, and 02M01, were necropsied in weeks 23, 28, and 39, respectively. One out of three vehicle-treated animals (No. 01F01) was also necropsied in week 39. The animals were euthanized under general anesthesia and examined macroscopically. The eyes, optic nerves, optic chiasm, optic tracts, brain, spinal cord (cervical, thoracic, and lumbar parts), and sciatic nerves were collected. The eyes were fixed with glutaraldehyde-formalin acid or Bouin’s fluid and other tissues collected were fixed with 10% volume neutral buffered formalin. The fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) or Luxol fast blue-H&E (LFB-H&E). In addition, immunohistochemistry was performed by the labeled polymer method (EnVision kits; DAKO Japan, Tokyo, Japan) with 3,3′-diaminobenzidine (DAB) as a chromogen. Polyclonal rabbit antibodies against glial fibrillary acidic protein (GFAP; DAKO Japan); ionized calcium-binding adapter molecule 1 (Iba1; Wako Pure Chemical Industries, Ltd., Osaka, Japan); or oligodendrocyte lineage transcription factor 2 (Olig2; Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) were used. All slides prepared were examined under a light microscope.

Statistics

For statistical analysis of the ERG parameters, the paired t-test and the Student’s t-test were used to assess the difference between the values before and after repeated dosing and between vehicle-treated and ethambutol-treated groups, respectively. The observation time defined as “after repeated dosing” for each animal was as follows: week 22 for one ethambutol-treated animal (No. 02M02); week 28 for another ethambutol-treated animal (No. 02F01); and week 39 for the other one ethambutol-treated animal (No. 02M01), and all vehicle-treated animals (Nos. 01M01, 01F01, and 01F02). For the statistical analysis of the single-flash cone response (R/B) in the TTX-treated animals, the paired t-test and the Student’s t-test were used to assess the difference between the values before and after treatment and between vehicle-treated and TTX-treated eyes, respectively. The differences were considered to be significant when P was less than 0.05.

Results

Clinical Observation

The clinical course of ethambutol-treated monkeys is shown in Table 1.

One animal (No. 02M02) receiving ethambutol had anorexia in week 23 (day 159). The clinical condition of the animal deteriorated on the next day (day 160), and additional signs such as incomplete eyelid opening and prone position were observed. Based on the occurrence of these clinical signs within a couple of days, the prognosis of the animal was judged to be poor and it was euthanized on Day 160. No marked changes in clinical signs were observed in either of the other ethambutol-treated animals (Nos. 02M01 and 02F01).

Electoretinogram

Standard Full-Field ERGs. Typical waveforms of the standard full-field ERGs at baseline and those recorded after repeated dosing in ethambutol- and vehicle-treated monkeys are shown in Figure 1. The amplitude of the ERG components is summarized in Table 2. No obvious changes in waveform of the standard full-field ERGs were observed in any animals receiving ethambutol.

Single-Flash Cone Response (R/B). Typical waveforms of the single-flash cone response (R/B) after single intravitreal injection with TTX and after repeated oral dosing with ethambutol are shown in Figures 2A and 3A, respectively. Time course of the PhNR/b-wave amplitude ratio during repeated dosing with ethambutol in each animal is shown in Figure 4A.
In the animals given intravitreal injection of TTX, marked and selective attenuation in the PhNR was observed in all three animals after the treatment. The decrease in the PhNR amplitude after TTX was significant in comparison with both predosing value ($P < 0.05$) and vehicle-control eye ($P < 0.01$) (Fig. 2B).

In ethambutol-treated animals, no obvious changes in waveform of the single-flash cone response (R/B) were observed after dosing on day 2. Thereafter, selective attenuation in the PhNR was found in one animal (No. 02M02) receiving ethambutol in week 22 (day 150). Attenuation in the PhNR was also observed in another ethambutol-treated animal (No. 02F01) in week 28 (day 192). The decreases in the PhNR amplitude and the PhNR/b-wave amplitude ratio after repeated dosing of ethambutol were significant ($P < 0.05$) in comparison with vehicle-control values (Fig. 3B and Table 2).

Ophthalmoscopy
No apparent changes in fundus oculi, including optic disc or macula, were observed in any animals treated with ethambutol.

Toxicokinetics
Plasma concentrations and TK parameters of ethambutol after oral dosing are shown in Table 3.

The AUC$_{0-24h}$ of ethambutol in week 1 (day 1; the first day of dosing at 400 mg/kg/day), week 4 (day 23; 23rd day of dosing at 400 mg/kg/day), week 14 (day 97; the first day of dosing at 800 mg/kg/day) and week 18 (day 125) were $235 \pm 44.0, 221 \pm 57.0, 458 \pm 128$, and $441 \pm 145$ μg·h/mL, respectively. The $C_{\text{max}}$ of ethambutol in week 1 (day 1), week 4 (day 23), week 14 (day 97) and week 18 (day 125) were $33.0 \pm 10.2, 33.7 \pm 9.32, 66.4 \pm 11.1$, and $48.5 \pm 7.46$ μg/mL, respectively.

The plasma concentration of ethambutol at the time of ERG recording in week 1 (day 2; second day of dosing at 400 mg/kg/day) was $25.0 \pm 13.1$ μg/mL. The plasma concentrations of ethambutol in each animal at the time of ERG recording remained at a steady level during the each period at the doses of 400 mg/kg/day and 800 mg/kg/day.

Pathology
Histopathologic findings in the tissues of nervous system in ethambutol-treated monkeys are shown in Table 4.

No abnormalities were observed macroscopically in any animals treated with ethambutol. In both of the ethambutol-treated animals (Nos. 02M02 and 02F01) that had decreased PhNR in the ERG, single cell necrosis of the RGCs and decreased number of the RGCs were observed in the retina (Fig. 5). The decrease in RGCs was apparent in the parafovea, where there are normally more RGCs than in other retinal areas. Moreover, the decrease in RGCs in the parafovea was relatively more pronounced in the nasal area compared with that in the temporal area. An increase in microglial cells in the nerve fiber layer was also noted in the retina of these two animals. No apparent changes were observed in any retinal layers other than RGC and nerve fiber layers including the macula in these two monkeys. In the optic nerves of the same two animals, demyelination, single cell necrosis of the glial cells, increased microglial cells, and swelling of the oligodendrocytes were observed (Fig. 6). The optic chiasm of these two animals contained lesions similar to those found in the optic chiasm of ethambutol-treated monkeys.

**Figure 1.** Typical waveforms of the standard full-field ERGs at baseline and those recorded after repeated dosing in ethambutol- and vehicle-treated monkeys. Ethambutol or vehicle was orally administered to monkeys for a maximum of 39 weeks, and the standard full-field ERGs were serially obtained as described in the text. The ERGs after repeated dosing were recorded within weeks 22 to 39. Arrowheads indicate onset of the light flashes. The responses at baseline (gray trace) are superimposed on those obtained after repeated dosing (black trace). Each trace represents an average of 3 to 10 responses.
Table 2. Effects of Ethambutol on the Amplitude of ERGs in Monkeys

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Treated Group</th>
<th>Ethambutol-Treated Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>After Repeated Dosing†</td>
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<tr>
<td>Rod response</td>
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<tr>
<td>b-wave, µV</td>
<td>86.9 ± 10.01</td>
<td>68.5 ± 15.28</td>
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<tr>
<td>Combined rod-cone response, standard flash</td>
<td></td>
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<tr>
<td>a-wave, µV</td>
<td>69.4 ± 15.24</td>
<td>64.4 ± 15.10</td>
</tr>
<tr>
<td>b-wave, µV</td>
<td>170.9 ± 53.78</td>
<td>147.3 ± 49.13</td>
</tr>
<tr>
<td>Combined rod-cone response, bright flash</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-wave, µV</td>
<td>111.3 ± 25.43</td>
<td>108.4 ± 28.03</td>
</tr>
<tr>
<td>b-wave, µV</td>
<td>186.1 ± 52.62</td>
<td>167.4 ± 66.27</td>
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<tr>
<td>Oscillatory potentials</td>
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</tr>
<tr>
<td>OP1</td>
<td>22.1 ± 2.11</td>
<td>21.9 ± 5.49</td>
</tr>
<tr>
<td>OP2</td>
<td>18.9 ± 0.92</td>
<td>18.1 ± 4.11</td>
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<td>Single-flash cone response, R/B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a-wave, µV</td>
<td>16.1 ± 4.24</td>
<td>14.5 ± 2.54</td>
</tr>
<tr>
<td>b-wave, µV</td>
<td>49.8 ± 7.18</td>
<td>43.9 ± 4.74</td>
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<tr>
<td>PhNR, µV</td>
<td>18.9 ± 5.30</td>
<td>19.8 ± 5.39</td>
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<tr>
<td>PhNR/b-wave amplitude ratio</td>
<td>0.39 ± 0.121</td>
<td>0.46 ± 0.161</td>
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<tr>
<td>Single-flash cone response, W/W</td>
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<tr>
<td>a-wave, µV</td>
<td>16.7 ± 5.73</td>
<td>14.6 ± 3.00</td>
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<tr>
<td>b-wave, µV</td>
<td>68.6 ± 24.88</td>
<td>63.3 ± 22.41</td>
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<tr>
<td>PhNR, µV</td>
<td>20.5 ± 5.15</td>
<td>16.6 ± 1.51</td>
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<tr>
<td>PhNR/b-wave amplitude ratio</td>
<td>0.32 ± 0.110</td>
<td>0.29 ± 0.112</td>
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<tr>
<td>30 Hz flicker</td>
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<tr>
<td>b-wave, µV</td>
<td>74.9 ± 19.30</td>
<td>59.9 ± 8.76</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of three animals.

∗ P < 0.05, ** P < 0.01; significantly different in comparison with the vehicle-treated group by the Student’s t test.
† Week 39 (day 269) for all animal.
‡ Week 22 (day 150), week 28 (day 192), or week 39 (day 269) in one animal each.

Additionally, the optic chiasm of one of the two animals (No. 02M02) had an astrocyte reaction (increase in gemistocytic astrocytes) in its central portion. An increase in microglial cells was also observed in the optic tracts of these two ethambutol-treated animals, and the optic tracts of one of them (No. 02M02) showed single cell necrosis of the glial cells. Neither the other ethambutol-treated animal (No. 02M01) nor in one vehicle-treated animal (No. 01F01) showed apparent abnormality in histopathology of the visual pathway.

Increased microglial cells were observed in ventral horn of the cervical spinal cord in one ethambutol-treated animal (No. 02M02), in which the clinical condition had deteriorated on the day of necropsy. The spinal cord of this animal also contained axonal degeneration in lateral funiculus in its lumbar part.

**DISCUSSION**

**Comparison of Ethambutol Exposures in Monkeys and in Humans**

The clinical dosage of ethambutol is usually at 15 to 25 mg/kg/day. In a randomized crossover study, in which the pharmacokinetics of ethambutol in serum were studied with healthy human subjects, the mean AUC₀–∞ and Cmax of ethambutol after single oral dosing of 25 mg/kg under fed condition were 29.6 µg·h/mL and 3.85 µg/mL, respectively. In the monkeys in this study, the AUC₀–24h and Cmax at 400 mg/kg/day (week 4) were 221 ± 57.0 µg·h/mL and 35.7 ± 9.32 µg/mL, and those at 800 mg/kg/day (week 18) were 441 ± 145 µg·h/mL and 48.5 ± 7.46 µg/mL, respectively. In addition, there were no apparent changes in the TK parameters of ethambutol that were attributable to repeated dosing in the present study. Therefore, it is considered that compared with humans treated with ethambutol at a dose of 25 mg/kg/day, the AUC and Cmax of ethambutol during the first 14 weeks in this study, at the dose of 400 mg/kg/day, were approximately 8 and 9 times higher, respectively, and those after week 14 in this study, at the dose of 800 mg/kg/day, were approximately 15 and 13 times higher, respectively.

**Neuropathology**

The literature describes a dose-related incidence of the ethambutol-induced ocular side effects. The incidence of the ocular side effects was reported to be 50% in patients taking ethambutol at doses of 60 to 100 mg/kg/day. It was also reported that the average duration of ethambutol therapy before development of optic neuropathy was 235 days in a recent post-marketing surveillance. In animal studies, histopathologic lesions including demyelination in the visual pathway tissues (from the optic nerve to optic tract) have been reported in many species. These results imply that patients with ethambutol-induced optic neuropathy develop lesions similar to those of the animals; although morphological alteration of the visual pathway tissues in patients receiving ethambutol medication is not fully understood. In the present study, two out of three ethambutol-treated monkeys developed...
Figure 2. (A) Typical waveforms of the single-flash cone response (R/B) from TTX- and vehicle-treated eyes in a monkey. TTX and vehicle were injected into the right and left eyes, respectively, and the single-flash cone responses (R/B), elicited with red light flashes under blue background light, were recorded. The responses obtained before injection (gray trace) are superimposed on those obtained after injection (black trace) in the right column. Each trace represents an average of 6 to 10 responses. (B) Amplitudes of the a- and b-waves and PhNR recorded from TTX- and vehicle-treated eyes in monkeys. Data are expressed as mean ± SD of eyes from three monkeys. Significant differences in the PhNR amplitude were detected by the paired t-test (*P < 0.05) and the Student's t-test (†P < 0.01).

Figure 3. (A) Typical waveforms of the single-flash cone response (R/B) at baseline and those recorded after repeated dosing in ethambutol- and vehicle-treated monkeys. Ethambutol or vehicle was orally administered to monkeys for a maximum of 39 weeks, and the single-flash cone responses (R/B), elicited with red light flashes under blue background light, were serially recorded in the monkeys. The responses after repeated dosing were recorded within weeks 22 to 39. The responses at baseline (gray trace) are superimposed on those obtained after repeated dosing (black trace) in the right column. Each trace represents an average of 6 to 10 responses. (B) Amplitudes of the a- and b-waves and PhNR recorded in the ethambutol- and vehicle-treated monkeys. Data are expressed as mean ± SD of three animals. Significant difference in the PhNR amplitude was detected by the Student’s t-test (†P < 0.05).
demyelination and glial reaction in the optic nerve, chiasm and tract. These histopathologic lesions in monkeys were induced under an approximately 10-fold higher exposure to ethambutol compared with that in humans for approximately 6 months; one of these two monkeys was completely free from any neurological symptoms. Therefore, the two monkeys developing the histopathologic lesions in the visual pathway in the present study were considered to be in a pathologic condition corresponding to that in patients with ethambutol-induced optic neuropathy.

Heng et al.\(^{13}\) reported the selective loss of cells from the RGC layer in the retina of rats treated repeatedly with ethambutol. No ethambutol-induced histopathologic change in the retina, however, has been reported in any species other than rats as far as we know. In the present study, histopathology of the retina in two monkeys developing ethambutol-induced optic neuropathy revealed obvious damage of the RGCs (i.e., single cell necrosis of the RGCs, decreased RGCs in the parafovea, and increased microglial cells in the nerve fiber layer); the decrease in RGCs in the parafovea was more pronounced in the nasal area compared with that in the temporal area. The preferential decrease in RGCs in the nasal parafovea in monkeys in the present study was considered to correspond to the predominant loss of thickness in the retinal nerve fiber layer (RNFL) of the temporal quadrant detected with optical coherence tomography (OCT) in patients with ethambutol-induced optic neuropathy.\(^{26-27}\) These results imply that the papillo-macular bundle is vulnerable in ethambutol-induced optic neuropathy, as in the previous report.\(^{28}\)

Meanwhile in the retinal components other than those mentioned above, all components appeared to be within normal limits including the macula in these two animals. To our knowledge, the present report represents the first description of ethambutol-induced histopathologic lesions of the retina in monkeys, a species in which the anatomical structure of the retina is known to be similar to that in humans. As for histopathologic lesions of the nervous system other than those in the visual pathway mentioned above, increased microglial cells in the ventral horn of the cervical spinal cord and axonal degeneration in the lateral funiculus in the lumbar spinal cord were noted in one ethambutol-treated animal, which had shown incomplete eyelid opening and prone position on the day of necropsy. These changes in clinical signs including abnormal body position were considered probably attributable to the histopathologic lesions in the spinal cord, suggesting motor neuron involvement. No histopathologic abnormalities associated with neuronal damage were observed in any other tissues of the nervous system evaluated in this study. This finding indicates that among the nervous system tissues the visual pathway is vulnerable to ethambutol in cynomolgus monkeys as well as in humans.

### Retinal Function

No apparent changes were found in any ERGs recorded near the \(C_{\text{max}}\) of plasma ethambutol in any animals treated with ethambutol on day 2. Thus, it was considered that there was no acute ethambutol-induced dysfunction in the retina that was related to the plasma concentration. Thereafter, to investigate

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**Table 3. Plasma Concentrations and TK Parameters of Ethambutol after Oral Dosing in Monkeys**

<table>
<thead>
<tr>
<th>Dose, mg/kg/d</th>
<th>Week (d)</th>
<th>Prec</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>7 h</th>
<th>24 h</th>
<th>(\text{AUC}_{0-24h}), (\mu\text{g}\cdot\text{h/mL})</th>
<th>(C_{\text{max}}, \mu\text{g/mL})</th>
<th>(t_{\text{max}}, \text{h})</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>1 (1)</td>
<td></td>
<td>23.5 ± 6.43</td>
<td>26.9 ± 5.94</td>
<td>30.9 ± 11.3</td>
<td>8.49 ± 2.72</td>
<td>0.974 ± 0.127</td>
<td>235 ± 44.0</td>
<td>53.0 ± 10.2</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>4 (25)</td>
<td></td>
<td>1.07 ± 0.342</td>
<td>33.3 ± 10.1</td>
<td>26.9 ± 9.74</td>
<td>20.3 ± 8.49</td>
<td>8.50 ± 2.36</td>
<td>1.29 ± 0.207</td>
<td>221 ± 57.0</td>
<td>35.7 ± 9.32</td>
</tr>
<tr>
<td>800</td>
<td>14 (97)</td>
<td></td>
<td>1.89 ± 0.121</td>
<td>57.7 ± 4.12</td>
<td>51.5 ± 12.4</td>
<td>44.8 ± 29.8</td>
<td>18.3 ± 4.71</td>
<td>3.19 ± 0.748</td>
<td>458 ± 128</td>
<td>66.4 ± 11.1</td>
</tr>
<tr>
<td></td>
<td>18 (125)</td>
<td></td>
<td>5.10 ± 0.454</td>
<td>40.1 ± 16.5</td>
<td>47.3 ± 8.73</td>
<td>42.2 ± 11.7</td>
<td>19.1 ± 9.14</td>
<td>5.77 ± 0.596</td>
<td>441 ± 145</td>
<td>48.5 ± 7.46</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of three animals. Animals were treated with ethambutol hydrochloride at dose levels of 400 mg/kg/day on days 1 to 96 and 800 mg/kg/day on days 97 to 273. Lower limit of quantitation is 0.5 \(\mu\text{g/mL}\). NE, not examined.

* The first day of dosing at dose level of 400 mg/kg/day.
† The 29th day of dosing at dose level of 800 mg/kg/day.
chronic changes in retinal function, the ERGs were repeatedly recorded near the trough of ethambutol concentration in plasma at intervals of approximately one month.

As a result of the serial evaluations of ERGs, markedly and selectively attenuated PhNR of the single-flash cone response (R/B) was observed in week 22 or 28 in two animals receiving ethambutol, in which histopathologic abnormalities of the RGCs and their axons were later identified. A quite similar change in waveform of the single-flash cone response (R/B) was also observed in all monkeys treated with single intravitreal injection of TTX, which blocks Na⁺-dependent action potentials that occur in all RGCs and a subset of amacrine cells.20,21 Thus, these findings indicated that the function of RGCs were severely impaired in the two monkeys with ethambutol-induced optic neuropathy.

Meanwhile, no marked changes were noted in the a- or b-wave of the single-flash cone response (R/B) in ethambutol-treated animals. In addition, no obvious changes were observed in the standard full-field ERGs elicited under photopic conditions (i.e., the rod response, the combined rod-cone response [standard flash] and the combined rod-cone response [bright flash], respectively) in the ethambutol-treated animals. The origins of the waveform components in the scotopic ERG from cynomolgus monkeys have been shown to be as follows: the leading edge of the a-wave contains the response from the rod and cone photoreceptors and the postreceptoral components, and the contribution from the rods is much greater than the others 31; and activity in ON pathway cells (i.e., ON-bipolar cells in both rod and cone pathways) contributes to generation of the b-wave.32 Hence, the results of the scotopic ERGs in this study indicated that the function of photoreceptors and bipolar cells in the rod pathway was mostly preserved in the monkeys with optic neuropathy.

Furthermore, no obvious changes were observed in the standard full-field ERGs elicited by flashes of weak, moderate and strong intensity under scotopic condition (i.e., the rod response, the combined rod-cone response [standard flash] and the combined rod-cone response [bright flash], respectively) in the ethambutol-treated animals. The origins of the waveform components in the scotopic ERG from cynomolgus monkeys have been shown to be as follows: the leading edge of the a-wave contains the response from the rod and cone photoreceptors and the postreceptoral components, and the contribution from the rods is much greater than the others 31; and activity in ON pathway cells (i.e., ON-bipolar cells in both rod and cone pathways) contributes to generation of the b-wave.32 Hence, the results of the scotopic ERGs in this study indicated that the function of photoreceptors and bipolar cells in the rod pathway was mostly preserved in the monkeys with optic neuropathy.

In addition, no apparent changes in the oscillatory potentials (OPs) were detected in any of the ethambutol-treated animals. The most probable generators of the OPs would appear to be amacrine cells, interplexiform cells, and bipolar cells, although the exact mechanism of OPs generation is still unclear.33 Thus, these findings imply that there was no marked impaired function in any of these probable generators of the OPs in the ethambutol-treated monkeys.

Putting all the results of the standard full-field ERGs in the ethambutol-treated monkeys together, it is concluded that the functions of photoreceptors and bipolar cells in both the rod and cone pathways, amacrine cells, and interplexiform cells...
were mostly preserved in the monkeys with optic neuropathy.

On the other hand, several lines of electrophysiologic evidence imply that the retinal components are dysfunctional in patients with ethambutol toxicity. It has been reported that the EOG is abnormal, which implies dysfunction of the retinal pigment epithelium (RPE). Also, it has been reported that the amplitude of the multifocal ERG was decreased, especially in the macular area, which implies that the photoreceptors and bipolar cells in the macular area are impaired. In ERGs from monkeys in the present study, no evidence to suggest functional changes in these retinal components was detected, although the RPE function cannot be directly assessed by the ERGs recorded. One possible explanation for this difference is that, in the full-field ERGs from monkeys in the present study, the possibility of a decreased response from the photoreceptors and/or bipolar cells in the macular area may have been masked by the normal response from the other areas of the retina. An alternate explanation is that, as a result of the serial ERG recording, we detected a relatively early change in the retina, and the functional changes in the macular area had not occurred yet.

**Evaluation of the RGC Function by the PhNR**

VEP and OCT have been reported to be objective tools to detect optic neuropathy induced by ethambutol. By means of serial recordings of the PhNR in the present study, we have found that the function of RGCs was gradually and severely affected in two out of three monkeys receiving repeated doses of ethambutol. Meanwhile, no obvious change in the PhNR was detected in one other ethambutol-treated monkey that had no histopathologic abnormalities in the RGCs or their axons. These results in this study suggest that the PhNR is useful to detect abnormalities in the RGCs and their axons, as well as VEP and OCT. The PhNR

**Figure 5.** Photomicrographs of the retina in (A) vehicle- and (B) ethambutol-treated monkeys. Single cell necrosis of the RGCs (arrowhead) and decreased RGCs in the parafovea (arrow) were observed in the retinas of ethambutol-treated animals. Note that the decrease in RGCs in the parafovea was more pronounced in the nasal area compared with that in the temporal area. Stain, H&E. Animal Numbers: (A) 01F01; (B) 02F01.

**Figure 6.** Photomicrographs of the optic nerve in (A) vehicle- and (B) ethambutol-treated monkeys. Demyelination, swelling of the oligodendrocytes (arrow), and increased microglial cells (arrowhead) were observed in the optic nerves of ethambutol-treated animals. Stain, LFB-H&E. Animal Numbers: (A) 01F01; (B) 02F01.
has been reported to be an objective tool to detect dysfunction of RGCs in patients in several different pathological conditions: open-angle glaucoma caused by elevated IOP17,18; anterior ischemic optic neuropathy caused by ischemic injury19, or compressive optic neuropathy caused by a tumor.20 To our knowledge, the present report represents the first description of the use of PhNR to detect drug-induced damage to RGCs (i.e., toxic optic neuropathy).

It was not assessed in this study whether the reduced PhNR was reversible or not. However, changes of the VEP27,34 and OCT36 in ethambutol-treated patients have been reported be reversible. These results imply that the attenuated PhNR in monkeys in the present study would have recovered.

The PhNR, which was markedly attenuated in ethambutol-treated monkeys in the present study, was elicited with red light flashes under blue background light (i.e., the single-flash cone response [R/B]) (Figs. 3, 4A). Meanwhile, no apparent change was observed in the PhNR that was elicited with white light flashes under white background light (i.e., the single-flash cone response [W/W]) in the same monkeys (Figs. 1, 4B). This difference in the result of the PhNR depending on which color combination of stimuli and backgrounds was utilized is consistent with results previously reported by Rangaswamy et al.,29 in which the PhNR was elicited with a variety of color combinations of stimuli and backgrounds in cynomolgus monkeys. They concluded that the best stimulus for maximizing PhNR amplitude is one that primarily stimulates one cone type (i.e., at least a monochromatic or narrow-band stimulus). They also considered that preferential stimulation of the long wavelength sensitive cones (L-cones) is likely to be more successful than that of the short wavelength sensitive cones (S-cones) or the middle wavelength sensitive cones (M-cones). Thus, red stimuli in combination with blue background light are considered to be a suitable recording condition for the PhNR to evaluate the RGC function.

Our study has some limitations. First, both of the monkeys with diminished PhNR were euthanized and their retinas of them were examined histopathologically at the point of detecting marked PhNR changes. Thus, reversibility of the PhNR alteration could not be assessed in the present study. Second, a minimum number of animals to investigate both the ERG and retinal histopathology were used in this study. Further studies are needed to show the sensitivity of the PhNR to detect histopathological lesions of the RGCs. However, these limitations should not affect our conclusions, because the attenuated PhNR and histopathological changes in the RGCs observed in two ethambutol-treated animals were obvious.

CONCLUSIONS
The attenuated PhNR and histopathology of the retina indicated that RGCs were markedly damaged both functionally and morphologically in monkeys with ethambutol-induced optic neuropathy. These results implied that RGCs are predominantly affected in the retina of patients with ethambutol-induced optic neuropathy.

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References


