Ethambutol Alters Spinule-type Synaptic Connections and Induces Morphologic Alterations in the Cone Pedicles of the Fish Retina

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Purpose. Ethambutol can cause optic neuropathy and deficiencies in color-opponent visual processing in patients treated for tuberculosis. In fish, Ethambutol induces color vision deficiencies similar to those observed in humans and affects color coding in retinal ganglion cells. Color opponency in fish is mainly mediated by a horizontal cell feedback onto cones thought to be provided by spinules. The authors examined whether Ethambutol affects spinules and is, therefore, able to alter color processing at a distal stage, that is, at the first synaptic connection within the retina.

Methods. Ethambutol was injected into the vitreous of either dark- or light-adapted fish. After drug application, fish were held under different illumination conditions. Thereafter, the retinas were dissected and prepared for electron microscopy. Ultrathin tangential sections of retinas were examined at the level of the outer plexiform layer.

Results. In already light-adapted retinas, a high dose of Ethambutol (10 mM) reduced the number of spinules by 30%. Ethambutol application in the dark with subsequent light adaptation resulted in severe dose-related inhibition of light-induced spinule formation. In these experiments, low doses (0.1 mM) of Ethambutol caused 40% inhibition, and high doses (10 mM) caused 70% inhibition. Besides affecting spinules, Ethambutol occasionally induced a degeneration of cone pedicles. This neurotoxicity only occurred in cones exposed to light.

Conclusions. Results show that Ethambutol alters synaptic connections between horizontal cells and cones in a dose-related fashion; Ethambutol treatment can be toxic for cone pedicles and can cause their degeneration; and the rod pathway is not affected by the drug. This indicates that Ethambutol influences the color-coding process already at the level of the cone-horizontal cell synapse. Invest Ophthalmol Vis Sci. 1995;36:1046-1055.

Ethambutol (Eb), a commonly used tuberculostatic drug, causes deficiencies in visual processing of approximately 1.5% to 3% of treated patients. These patients develop optic neuropathy, central scotomas, and, as a first sign of intoxication, changes in red-green color vision. The acquired deficiency in red-green color vision can lead to an absolute red-green color blindness. Usually, all these failures recover completely after the drug is suspended. In a combined electrophysiological and psychophysical study in humans, Zrenner and Krüger pointed out that, besides the aforementioned effects, Eb did not alter the electroretinographic results and that cone mechanisms remained unaffected by the drug up to the visual cortex. They concluded that Eb-induced color blindness is caused by abnormal functioning at the level of the color-opponent neurons in the retina, which are horizontal cells, ganglion cells, or both.

Van Dijk and Spekreijse demonstrated that Eb induces changes in the color coding of ganglion cells of tetrachromatic carp without changing the sensitivity of the underlying receptor processes, thus verifying the suggestion of the clinical observation in an animal model. Moreover, in goldfish, Eb was able to induce color blindness similar to that observed in humans. In the fish retina, the first color coding steps occur at the level of the horizontal cells. The chromaticity-types
of horizontal cells generate color-opponent responses. Besides testing color discrimination and ERG, Spekreijse and coworkers also recorded intracellularly from chromaticity horizontal cells in the fish retina. The horizontal cell (HC) recordings showed a clear reduction of the color-opponent components after application of Eb.

In lower vertebrates, color-opponent responses of HCs are generated by a negative feedback interaction between HCs and cones.7 In the fish retina, the appearance of color opponency in horizontal and ganglion cells is correlated with the appearance of newly formed neurites at the synaptic endings of horizontal cell dendrites.9-11 located laterally at cone synaptic ribbons. During light adaptation, these finger-like neurites, called spinules,12 protrude from the dendritic endings further into the cytoplasm of the bell-shaped cone pedicle. During dark adaptation, the spinules are retracted. This remarkable synaptic plasticity is predominantly triggered by the ambient conditions and, to a lesser extent, by endogenous factors.13,14 Because the course of spinule formation and degradation closely correlates with the color response behavior of HCs, spinules are considered the site of the feedback synapse.10,11 Therefore, it seems that a drug such as Eb, which in electrophysiological experiments affects color opponency in horizontal cells and ganglion cells,15,16 may do so by affecting the putative color-coding horizontal cell synapse.

To examine this possibility, we looked for the influence of Eb on spinule formation in the carp retina. Our results show that spinule outgrowth during light adaptation is vigorously reduced by Eb and that spinules already expressed can be affected by the drug at higher concentrations. Moreover, Eb can be toxic to cone pedicles and is capable of causing their necrosis and degeneration.

MATERIAL AND METHODS

Carps (Cyprinus carpio) with body lengths of 14 to 18 cm were used for all experiments. All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Fish were kept in aerated tanks at 19° to 21° C under natural light conditions. Experiments were performed between noon and 4 PM (light phase) and between 11 PM and 3 AM (dark phase). At the relevant times, fish were light- or dark-adapted for at least 4 hours. Fish were then lightly anesthetized with MS 222 (Sigma, Munich, Germany) and received an intraocular injection of 1.0, 0.1, 0.01, or 0.001 mg Ethambutol dihydrochloride (Eb, Sigma) dissolved in 10 ml saline (pH corrected to 7.2 with NaOH) into the right eye. With respect to the size of the eyeball, this resulted in a calculated concentration at the retinal surface ranging from 10 mM to 0.01 mM. Data will be presented using the calculated concentrations. In some cases, a commercially available aqueous solution of Eb (Myambutol; Lederle, Wolfratshausen, Germany), which is used in tuberculostatic therapy, was injected. The results for the saline and aqueous solutions were identical and were not treated separately. The left eye was used as a control in all experiments and was injected with the vehicle alone.

In an additional series of experiments, to exclude any indirect influence by the release of endogenous neurotransmitters, cells within the retina were isolated synthetically. For this purpose, CoCl2 dissolved in saline was injected into the vitreous 10 minutes before Eb application. The CoCl2 concentration was chosen in accordance with the size of the eyeball to give an estimated concentration of 8.5 mM at the surface of the retina. The control eye was injected with the CoCl2 solution alone.

The injections were performed during light phases under room light and during dark phases under dim red light. After the injections, fish were held under the initial illumination conditions for 10 additional minutes to allow dissociation of the drug within the vitreous. During this dissociation phase (approximately 5 minutes), the animals recovered from anesthesia. They were kept for as long as 45 minutes under the same illumination conditions, or the illumination conditions were changed—light-adapted fish were dark-adapted and vice versa.

Thereafter, the animals were decapitated, and the brains were pithed, the eyes enucleated, the cornea and lens removed, and the retinas separated from the retinal pigment epithelia by dissection. All surgery was performed under appropriate illumination. Retinas were immediately immersed in ice-cold fixative (1% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M phosphate buffer with 3% sucrose, pH 7.2) and fixed for 1 hour at room temperature under the same illumination conditions used during the experiments and at 4°C overnight. After fixation, retinas were washed, postfixed in 1% osmium, stained en bloc in 2% uranyl acetate, dehydrated in graded acetone, and embedded in Taab-resin (Taab Laboratories, Reading, UK). Pieces from the central retina dorsal to the optic nerve were collected. Sections measuring 80 to 90 nm were cut tangentially to the retinal surface and prepared for electron microscopy by conventional protocols.

Spinule formation was observed in cross-sections of cone pedicles. The spinule-to-ribbon ratio was determined in a minimum of 30 pedicles per retina. Three to five animals were used for each series of experiments. To quantify spinule formation in a retina, spinules and ribbons were counted, and the spinule-to-ribbon ratio was calculated.17 For the histo-
grams, the calculated spinule-to-ribbon values were normalized to the values of fully (4 hours) light-adapted controls, and the standard deviations were included. To determine if differences between the means were statistically significant, analysis of variance followed by the Student-Newman-Keul test was used.

RESULTS
Spinule-type Synapses
In tangential sections through light- and dark-adapted cone pedicles, synaptic ribbons appear as electron-dense strips within the cytoplasm of the pedicle, capped along their central ridge by an arciform structure. They are flanked by a pair of electron-lucent horizontal cell dendrites (Figs. 1A, 1B). Spinules with well-developed membrane densities protrude from the dendritic terminals in light-adapted retinas (Fig. 1A), as described by Wagner, whereas the terminals appear round and smooth in dark-adapted retinas (Fig. 1B). Spinule formation is quantified and summarized in the histogram in Figure 1C for both naturally occurring adaptation conditions.

When a high dose of Eb (calculated concentration at the retinal surface, 10 mM) was injected into the vitreous of a light-adapted animal, the spinules already formed declined from 2.2 ± 0.3 spinules per ribbon (spr) in the control eye to 1.6 ± 0.2 spr in the Eb-treated eye (Fig. 2A, and 4A). This reduction by approximately 30% was highly significant (P < 0.001). A concentration of 1 mM of the drug did not reduce spinules significantly, and spr-values were identical to those of the controls at 0.1 mM retinal surface concentration (Fig. 4B).

In these light-adapted and Eb-treated retinas, spinules were occasionally bent away from their lateral position at the synaptic ribbon (Figs. 2A, 2B, arrows). They then ran within the bell-shaped cavity of the pedicle along the border between the pedicle’s membrane and the central bundle of invaginating dendrites. Bent-back spinules sometimes even overlapped one another (Fig. 2B, arrowheads) as if the spinules were repelled by the cone pedicle and were no longer able to penetrate it (Figs. 2A, 2B). Small electron-dense strips of incorporated plasmalemma were found within the cytoplasm of the cone pedicles. These plasmalemma strips were located in the pedicle cytoplasm opposite to the spinule tips (Fig. 2A) and adjacent to horizontal cell terminals with membrane densities but without finger-like protrusions (Figs. 2A, 3). Spinule reflection and plasmalemma incorporation were observed in retinas exposed to 10 mM and 1 mM Eb.

In dark-adapted retinas, Eb did not show any effect on the dendritic terminals of the horizontal cells (Fig. 2B).

Intravitreal injection with an Eb concentration at the retinal surface of 10 mM in the dark, 10 minutes before light adaptation, resulted in severe inhibition of light-induced spinule formation (Fig. 4). During the 45 minutes of light adaptation, the spr-values of the control eyes attained a clearly light-adapted level (spr 1.9 ± 0.3), whereas the Eb-injected eyes retained spr-values that were still within the spr-range of dark-
FIGURE 2. Electron micrographs showing Eb effects in light- and dark-adapted retinas. (A1, A2) In a light-adapted retina, a high concentration of Eb (10 mM) reduces the number of spinules already formed by approximately 30%. Occasionally, spinules are bent away from the synaptic ribbon (arrows), and sometimes they even overlap one another (arrowheads). In the cone cytoplasm, small electron-dense stripes of incorporated plasmalemma are visible (open arrows). (B) In dark-adapted retinas, Eb does not show any effect. Calibration bars = 500 nm.
adapted retinas (spr 0.5 ± 0.15; paired difference test, \( P < 0.001 \)). However, in some HC terminals, bulges were formed bearing the typical submembranous densities normally seen in spinules (Figs. 1A and 3 for comparison). In addition, plasmalemma stripes were found in the cone pedicles (Fig. 3).

No Eb effect on the retraction of spinules during dark adaptation was detectable.

The histograms in Figure 4A summarize the normalized data obtained with 10 mM Eb under the various adaptation conditions tested. The blank columns represent the spr-values of the control eyes and the hatched columns the spr-values of the Eb-injected eyes. Spr-values are clearly lower in Eb-treated retinas when Eb is administered in the light-adapted state (L) and dramatically so when administered during the development of spinules going from dark to light.

The reduction of the spr-values in light-adapted retinas required high doses of Eb. The same doses were much more effective in suppressing the outgrowth of new spinules during light adaptation; under these conditions, spinule formation was vigorously inhibited by approximately 75%. Correspondingly, inhibition of spinule outgrowth was observed with lower doses of Eb: concentrations of 1 mM and 0.1 mM caused an inhibition of approximately 50% of the spr-values (1 mM: 1.05 ± 0.22; 0.1 mM: 1.17 ± 0.24; paired difference tests against controls, \( P < 0.001 \)). When
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**FIGURE 4.** Ethambutol (Eb) effects are dose related. (A) Histograms summarizing the high-dose effects of intravitreally applied Eb. In the Eb experiments, 10 mg of Eb were injected into the vitreous. Blank columns represent the control retinas, hatched columns the treated retinas. The number of spinules-to-ribbons is normalized to the light control value. Error bars denote standard deviations. Injections were administered in light-adapted state (L), dark-adapted state with subsequent light adaptation (DL), dark-adapted state (D), light-adapted state with subsequent dark adaptation (LD). (B) Dose-response data for Eb injected into the vitreous of light-adapted fish (open circles) and dark-adapted fish undergoing subsequent light adaptation (filled circles). In Eb-treated animals, the Eb concentration at the retinal surface ranged from approximately 10 mM (log 0) to 0.01 mM (log −3); eyes of control animals were injected with saline. ***Highly significant at \( P < 0.001 \). Error bars indicate standard deviation.

During light adaptation under physiological conditions, vesicles in the cone terminal fuse with the pedicle membrane; this fusion was abolished by Eb. In an additional series of experiments, cobalt (CoCl₂) was used in light- and dark-adapted retinas to exclude indirect effects of Ethambutol on spinules, for example, through interplexiform cells or by an endogenous spinule trigger. None of the Eb effects on spinules were altered by cobalt treatment in these preparations. Because cobalt by itself affects spinule outgrowth and retraction (Kohler, unpublished data), it was not used in those experiments involving a change in adaptation conditions.

**Cone-Specific Neurotoxicity**

Besides the described alterations at the spinule-type synapses, a cone-specific neurotoxicity was observed in some of the Eb-treated animals. The cytoplasm of cone pedicles and their telodendria stained intensely dark and were filled with debris after exposure to Eb (Fig. 5). Vesicles lost their normal shape and aggregated. The cytoplasm within the pedicles was microvula-culated, and several large, swollen, empty vacuoles and some smaller vacuoles were filled with electron-dense material. The regular arrangement of the vesicles surrounding the synaptic ribbons was destroyed, but the synaptic ribbons themselves were unharmed by the drug (Fig. 5A). The intercellular space was extremely widened along the seam of the pedicle cavity, but the dendritic terminals of second-order neurons within the central cavity appeared normal and did not have darkened cytoplasm. Horizontal cell spinules behaved as seen in non-neurotoxic Eb preparations (Fig. 5A). Degenerated electron-dense cone pedicles were invariably surrounded by healthy, unaffected rod spherules (Fig. 5B), and the dendritic network in the outer plexiform layer did not contain necrotic processes. Obviously, the cytoplasm of the cone pedicles was particularly vulnerable to the toxic effects of Eb. Some of the cone nuclei in the affected retinas were also electron-dense and pycnotic (not shown).

The toxic effects in the pedicles were not uniformly distributed over the entire piece of retinal tissue selected for examination (see Materials and Methods). Areas without degeneration and those with different amounts and degrees of necrosis were to be found in the same retinal piece. This is probably because of the location of the intravitreal injection. If the tip of the syringe is near the retinal surface, extremely high amounts of Eb may reach certain parts of the retina before the drug is homogeneously distributed within the vitreous.

Degeneration was not a general symptom but occurred occasionally in retinas treated with Eb concentrations of 10 mM (2 of 5 retinas) and 1 mM (1 of 5 retinas). Only retinas exposed to light were affected.

The vulnerability of cone pedicles to the toxic...
effects of Eb was enhanced by cobalt (10 mM and 1 mM each in 3 of 5 retinas). With cobalt, pedicle necrosis was also observed in a dark-adapted retina.

When necrosis was present, all pedicles in that area were affected; there was no preference in degeneration for a special chromatic type of cone. Rod spherules were always unaffected.

**DISCUSSION**

Behavioral experiments in fish showed that Ethambutol administered with the diet induces deficiencies in red-green color vision similar to those observed in humans. In the fish retina, the first color-coding step occurs distally in horizontal cells. The color-opponent behavior of

**FIGURE 5.** Electron micrographs showing Eb-caused cone specific neurotoxicity. (A) Light-adapted retina after exposure to cobalt and 10 mM Eb. Spinules are retracted and bent away from the synaptic ribbon, and the interstitial space between the invaginating dendritic terminals is widened at the base of the ribbon synapse but not between dendrites located more centrally. Horizontal cell terminals bear membrane densities, and their cytoplasm appears unharmed. The cytoplasm of the cone pedicle is dark, electron dense, and necrotic, even in the telodendron; it contains vacuoles of different sizes and large, swollen vesicles. The synaptic ribbon is unaffected. (B) Light-adapted retina after exposure to high concentrations of Eb (10 mM injected). Degenerated electron-dense cone pedicles are surrounded by unaffected rod spherules (asterisks). Calibration bar = 500 nm.
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If such synaptic impairments are incremental from horizontal to ganglion cell, then even low doses of Eb may result in severe reduction of color discrimination in ganglion cells. A different vulnerability to Eb in the inner and outer retina was recently shown by Witasma and Spekreijse. They found that HC responses, and also spinules, appeared normal but that color-opponent ganglion cells lost the inhibition between red and green color processes in fish treated by mouth with Eb. Unfortunately, we do not know to what degree Eb accumulates in the retina when it is administered systemically, because we do not know to what extent it is able to penetrate the blood–retina barrier. Systemic drug application is handicapped further by the fact that both eyes are affected, and there is no control.

Can we infer from the data found in fish possible pathologic mechanisms in humans? When Eb is administered systemically during tuberculosis therapy, the local concentration within the retina may depend on the degree of vascularization, thus producing a gradient of intoxication along the vessels. If the toxicity is dose related, all toxic alterations, from mild to serious, could occur within a single retina. Severe deficiencies in visual processing, like optic neuropathy or central scotomas, will occur in areas well supplied with blood, such as around the optic disk. Indeed, retinal hemorrhages between disk and macula have been described in patients treated with Eb. The ganglion cell layer is also well streaked with vessels and may be more exposed to blood-mediated intoxication than the distal retina and the avascular fovea. In humans treated with Eb and in whom there is clear development of color deficiency, signals of all three types of spectrally different cones are present. This means that the retinal Eb concentration in these patients is too low to be toxic for all, or at least the majority of cones but is high enough to affect second-order neurons in the outer retina or, even more pronounced, color coding of third-order neurons in the ganglion cell layer of the inner retina.

Only 3% of the patients treated with Eb have been reported to have visual side effects. However, in this 3%, the side effects were so severe that the patients sought help. It may be assumed that the actual number of Eb-caused color vision deficiencies is much higher but goes unnoticed. It is well known that Eb side effects in humans are dose dependent; however, with respect to the variance of Eb side effects caused by equal doses, there also may be a certain genetic disposition to the vulnerability of Eb.

By which molecular mechanisms does Eb act in the fish retina to induce alterations in spinules and degeneration of cone pedicles? Eb is known to be a potent agent for chelating endogenous intracellular zinc. It is intriguing to speculate about the molecular...
mechanisms of Eb, on the assumption that Zn$^{2+}$-dependent mechanisms in photoreceptors and second-order neurons are disturbed by Eb in the outer retina. Intracellular Zn$^{2+}$ is involved in the translocation and catalytic activity of protein kinase C (PKC), and Zn$^{2+}$ also plays a role in the attachment of PKC to a component of the cytoskeleton, which may be actin.\(^{18}\)

We have shown\(^{19}\) that PKC is a vigorous trigger for spinule formation and, furthermore, that the outgrowth of spinules is actin dependent.\(^{20,21}\) However, we showed that inhibition of actin polymerization only suppressed the protrusion of fingers, whereas submembranous densities, normally characterizing the spines, were still formed within the dendritic terminals. As seen in this study, Eb suppressed primarily the PKC-triggered, actin-dependent outgrowth of the finger-like processes but did not prevent the formation of membrane densities (Fig. 3). This could indicate an Eb-effect on PKC activity, actin polymerization, or both. The common source may be an Eb-induced deficit of available intracellular zinc.

It remains unclear what happened in fully light-adapted retinas in which only the highest Eb concentration was able to decrease slightly the number of spinules. The mechanism for spinule retraction might not require an intact actin network within the HC terminal\(^{22}\) but, instead, passive forces from outside the dendritic terminal. It is reasonable to suppose that these forces are exerted by the pedicle membrane. Possibly, in Eb-treated retinas, outgrowing spinules cannot penetrate in the region of the synaptic ribbon during light adaptation because the surface of the adjacent pedicle membrane cannot yield. This may occur in synergism with the Eb effect on the actin network in the HC terminals. Spinules already formed, however, are stable and cannot be altered easily without an appropriate signal, such as darkness or glutamate.\(^{22}\) That is why spinules are still present, though occasionally bent and folded, at higher Eb concentrations in light-adapted retinas. This is obviously caused by alterations of the pedicle membrane and not by a disruption of the retracting mechanism in the HC terminals. The data obtained from Ethambutol-treated animals provide the first hint that membrane rigidity, a mechanism not before considered, may play an essential role in synaptic plasticity.

With respect to the tuberculostatic effects of Ethambutol, we can now speculate, based on this interpretation of what is happening to spinules, that its effect is to diminish penetration of bacteria into cells because of altered membrane rigidity.

Here it is appropriate to think of zinc-dependent mechanisms in the retina. Zinc plays an essential role in the cellular machinery of photoreceptor terminals. In the tiger salamander retina, an intracellular pool of zinc is located in regions near the photoreceptors' synaptic terminals, and Zn$^{2+}$ hyperpolarizes horizontal cells comparably to the way bright light does, indicating a suppressive action of zinc on presynaptic calcium currents.\(^{23}\) Thus, lowered zinc content in pedicles, caused by the chelating effects of Eb, may be responsible for disturbed vesicle fusion and subsequent disturbed membrane turnover. Moreover, Eb in high concentrations exerts a cone-specific neurotoxicity under photopic conditions. Notably, this Eb toxicity is enhanced by cobalt. This points to a disturbed balance of divalent ions: Co$^{2+}$ may occupy those binding sites normally held by Zn$^{2+}$, thus increasing the necrosis induced by a reduced zinc level. However, zinc is present in cone pedicles and rods spherules in the tiger salamander retina.\(^{29}\) We do not know why there is such a specific degeneration of pedicles with Eb in carp.

In conclusion, Eb vigorously suppresses the outgrowth of spinules either by affecting the actin network in HC terminals or by altering the membrane rigidity of the pedicle or by both mechanisms synergistically. Ethambutol does not inhibit the formation of spinule-characteristic membrane densities in the HC terminal, and it gives at most only a weak signal for the HC component of spinule retraction. In high concentrations, Eb exerts a cone-specific toxicity and leaves the rods unharmed, and it affects the synaptic arrangement of the light-adapted ribbon synapse, presumably by altering the membrane rigidity of the pedicle.

**Key Words**
cones, horizontal cells, synapse, synaptic plasticity, zinc chelating agent

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