The Effects of Myristyl γ-Picolinium Chloride on the Rabbit Retina: Morphologic Observations

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Purpose. This study was designed to localize the site of action of myristyl γ-picolinium chloride (MGP) in the rabbit retina and to evaluate the extent of the structural damage induced by the drug.

Methods. The structural damage was assessed at the light microscopic level in eyes treated with various concentrations of MGP at different time intervals after intravitreal injection of the drug. Glial fibrillary acidic protein (GFAP) immunoreactivity was tested in the same eyes and served as an index of retinal damage.

Results. The rabbit retinas, examined about 1 mo after MGP injection, exhibited loss of photoreceptors and thinning of the retina in the regions close to the site of injection; remote retinal areas appeared morphologically intact or only slightly affected. Immunocytochemical analysis demonstrated the presence of GFAP in Müller (glial) cells throughout the entire retina. When the effects of MGP were examined at short time intervals (24 and 72 hr) after injection, severe morphologic damage in areas adjacent to the site of drug injection developed in parallel with the electroretinographic findings. However, GFAP could not be demonstrated.

Conclusions. MGP, the preservative used in Depo-Medrol (Upjohn, Kalamazoo, MI), is highly toxic to the rabbit retina. Invest Ophthalmol Vis Sci. 1993;34:2360-2366.

Depo-Medrol (depot form of methylprednisolone, Upjohn, Kalamazoo, MI) is a corticosteroid-containing drug that is often used in the treatment or prevention of ocular inflammation. In cases where high doses are required, it is administered pericaremally. However, with this mode of drug application, the risks of inadvertent intravitreal injection should be considered. We have recently demonstrated, using electroretinograms (ERG), that Depo-Medrol does not produce any detectable functional damage to the albino rabbit reti-
utilized to localize precisely the site of action of toxic drugs. A reduction in the amplitude of the ERG responses can reflect a variety of mechanisms that act on different ocular structures. The ERG decreases when the intensity of the light reaching the retina is reduced as a result of opacities in the optic media or failure to dilate the pupil completely. Any inherited or acquired pathologic process that directly acts on the outer retinal layers will be reflected in the ERG responses. Drugs that exert a toxic action on nonneural cells (i.e., Müller cells and pigment epithelial cells) will also reduce the ERG. The Müller cells are responsible for a large part of the ERG b-wave, and thus, any change in their function will be reflected in the ERG. The pigment epithelial layer contributes a large electrical resistance to current flow and thus determines the magnitude of the corneal ERG. Therefore, to localize the site of action of any agent that impairs visual function, histologic techniques must accompany functional tests.

The current study was designed to localize the site of MGP-induced functional damage in the rabbit retina. The retinas of the control and experimental eyes were prepared for light microscopy to identify the retinal structures that exhibited morphologic changes and to assess the degree of these effects and their spread from the site of drug injection to more peripheral retinal loci. An immunocytochemical technique was applied to identify the sites at which glial fibrillary acidic protein (GFAP) could be detected. In normal rabbit retinas fixed as in our study, only astrocytes are labeled for GFAP; hence, only the regions around the medullary rays exhibit GFAP immunoreactivity. In the retinas of rats and rabbits, Müller cells always exhibited GFAP immunoreactivity after an injury was inflicted to the photoreceptors, to the ganglion cells, to the optic nerve, or to the Müller cells themselves. Because GFAP can be demonstrated in Müller cells even in cases where the retina appears morphologically normal, it can be used as a sensitive cellular marker for retinal injury. We also compared the short-term with the long-term effects of MGP on the functional and morphologic status of the rabbit retina. This goal was achieved by conducting ERG measurements and by preparing the eyes for histologic and immunocytochemical analysis at different time intervals (24 hr, 72 hr, 4 weeks, and 6 weeks) after the intravitreal injection. The rabbit was anesthetized with sodium pentobarbital (30 mg/kg body weight) injected intravenously. After applying topical anesthesia to the cornea (benoxinate hydrochloride 0.4%), the ERG responses were recorded in the light- and dark-adapted state. Then, a 25-gauge needle attached to a 1.0-ml tuberculin syringe was inserted into the eye, 3 mm posterior to the limbus. The needle was directed through the vitreous under visual control with the aid of an indirect ophthalmoscope toward the region of the optic disc as close as possible to the retina. A volume of 0.1 ml of solution was then slowly injected. The experimental solutions were prepared by dissolving MGP in saline in one of the following concentrations: 0.38, 0.76, or 1.52 mg/ml. These levels are respectively two-, four-, and eightfold the concentration found in Depo-Medrol (0.19 mg/ml). It should be noted that the change in osmolality of the most concentrated MGP solution used in this study (1.52 mg/ml) was too small to produce retinal detachment. In each rabbit, the experimental eye was injected with 0.1 ml of the MGP solution, and the control eye was injected with a similar volume of saline solution. Approximately 5–10 min after the injection, the light-adapted ERG responses were recorded to verify that no retinal damage was inflicted during the injection.

At different time intervals (24 hr, 72 hr, 4 weeks, and 6 weeks) after the intravitreal injection, the rabbits were killed by an overdose of sodium pentobarbital. Both eyes were enucleated and immersed in Karnovsky’s medium for fixation. Before dissection for histologic analysis, the eyes were examined with a binocular dissecting microscope for gross abnormalities. All experimental procedures conformed to the ARVO Resolution on the Use of Animals in Research.

Histologic Analysis

The eye was soaked for 10 min in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer (pH 7.4). It was opened, 2 mm posterior to the limbus, to facilitate fixation. After 72 hr, the anterior segment of the eye was removed by a circumferential incision 2 mm posterior to the limbus. After removal of the vitreous, the posterior eye cup was bisected at the level of the optic disc. One half of each eye cup was rinsed in water, dehydrated twice in 70% alcohol for 3 hr, and twice in 96% alcohol, for another 3 hr. Embedding was done in a JB-4 resin (Bio-Rad, Wadford, England). The tissue sections were cut by a microtome (Reichard Jung, Heidelberg, Germany) at a 2-μm thickness and mounted onto slides. For light microscopy, the sections were stained with Richardson’s stain.
Immunocytochemical Analysis

Slides with 2-μm sections of the eye tissues were treated with sodium ethoxide for 15 min (prepared as a saturated NaOH solution in absolute alcohol, diluted 1:1 with absolute alcohol) to promote the penetration of the chemicals through the JB-4 resin. After rinsing twice in distilled water, the slides were soaked for 5 min in 0.01 mol/l phosphate-buffered saline (PBS), pH 7.4, and then incubated overnight at 4°C in a moist chamber with primary antibody to GFAP (Boehringer, Mannheim, Germany), diluted in PBS at 1:10. For immunofluorescence visualization, the slides were rinsed three times in PBS and then incubated for 40 min in goat anti-rabbit fluorescein isothiocyanate-labeled antibody. Then, the slides were washed in PBS 0.01 mol/l, pH 7.4, and mounted in glycerol diluted with PBS at a ratio of 1:9. Visualization was done by a Zeiss (Oberkochen, Germany) fluorescence microscope.

RESULTS

We have previously shown that MGP exerts profound effects on the ERG of the albino and pigmented rabbits. Functional damage was evident as early as 1 week after injection and developed with time to a stable level that was reached approximately 4 weeks after the drug was injected into the vitreous of the experimental eye.1 In this study, we examined the morphologic effects of MGP at shorter postinjection time intervals and compared them with the long-term effects.

Short-Term Effects of MGP

The short-term effects of MGP were studied with the average b-wave ratio (MGP-injected eye/saline-injected eye) for the dark-adapted ERG responses was 0.39 ± 0.07 (standard deviation).

The morphologic effects of the MGP solution (1.52 mg/ml) that developed within the first 24 hr after drug injection were compared in Figure 1 with the effects of saline solution injected into the control fellow eye (left and right columns, respectively). Light micrographs from three different retinal loci are shown. The area adjacent (2 mm) to the site of drug injection was characterized by severe morphologic damage, which was expressed in disarrayed and swollen retinal layers (Fig. 1A). These effects could not be attributed to the injection procedure because a similar site in the control eye appeared normal (Fig. 1B). In a more remote area, approximately 10 mm from the site of injection, the retina from the experimental eye retained its layered structure, although the density of the photoreceptor nuclei seemed to be reduced compared with the section from a comparable area in the control eye (Figs. 1C and 1D, respectively). Areas in the far periphery (approximately 20 mm from the site of injection) of the experimental eye exhibited minimal changes and could not be distinguished from similar sites in the normal control eye (Figs. 1E and 1F, respectively).

The retinas of these experimental and control eyes were also tested for GFAP immunoreactivity because this protein serves as a cellular marker for retinal injury even in normal-looking retinal areas.12,14 Contrary to the severe morphologic abnormalities that developed within 24 hr in the eye treated with the MGP solution (Fig. 1A), the immunocytochemical labeling for GFAP showed no significant differences between the experimental and control eyes. Figure 2 shows GFAP immunoreactivity of two different retinal areas in the experimental eye (the same ones used in Fig. 1). The area adjacent (2 mm) to the site of injection (Fig. 2A) exhibited severe morphologic damage; a more remote (20 mm) region (Fig 2B) appeared undisturbed. Neither of these sections exhibited GFAP immunoreactivity. All sections from the control eye, regardless of their locus relative to the site of saline injection, were similar to the one shown in Figure 2B and are, therefore, not shown here. Retinal sections of either eye (experimental or control), obtained from areas adjacent to the medullary rays, exhibited heavy staining for GFAP because of the presence of astrocytes (not shown here).

The eyes that were tested for morphologic damage and GFAP immunoreactivity 72 hr after drug injection exhibited similar findings to those described for the 24-hr postinjection interval (Figs. 1, 2). Therefore, these data are not shown here.

Long-Term Effects of MGP

The MGP-induced functional damage to the rabbit retina, as assessed from the ERG responses, stabilized within 2 weeks after injection and showed no recovery until at least 8 weeks after injection.1 Therefore, the long-term morphologic effects of the drug were determined at either 4 or 6 weeks after injection. Because the histologic and immunocytochemical data did not differ between the retinas prepared 4 or 6 weeks after injection, only data from an eye prepared 6 weeks after injection will be shown. In Figure 3, light micrographs of retinal sections from an eye injected with a solution containing the smallest concentration of MGP (0.38 mg/ml) and from the control eye injected with saline are compared (left and right columns, respectively). Similar to the short-term (24 hr) effects of MGP (Fig. 1), the long-term (6 weeks) MGP-induced
FIGURE 1. Short-term (24 hr) effects of MGP solution (1.52 mg/ml) on the rabbit retina. Corresponding retinal areas from the experimental (left column) and the control eyes (right column) are shown. The light micrographs demonstrate retinal areas 2 mm (A and B), 10 mm (C and D), and 20 mm (E and F) from the site of injection. Calibration bar = 50 μm.

FIGURE 2. Short-term (24 hr) effects of MGP solution (1.52 mg/ml) on GFAP immunoreactivity in a rabbit retina. The sections were obtained from a morphologically damaged area, 2 mm from the site of drug injection (A) and from a peripheral area (20 mm from the site of injection) that appeared morphologically undamaged (B). In each section, the vitreous is in the upper side. All retinal areas from the control eye, irrespective of their locus relative to the site of injection, appeared similar to the one shown in B and are, therefore, not shown here. Calibration bar = 25 μm.
morphologic damage varied across the retina. Severe structural damage was seen in areas adjacent to the site of injection (Figs. 3A, 3B). The retina in these regions was thin, lacking most of its layers, and only a few unidentified cell nuclei could be occasionally seen. The degree of damage gradually was reduced in areas further away from the site of injection. The section in Figure 3A, showing a retinal area 2 mm from the site of injection, demonstrates the transition from severe damage (left side) to a less affected region in which typical organization into layers can be seen. At 10 mm from the site of injection (Fig. 3C), the retina from the experimental eye retained its organized structure, and all layers could be identified. However, even in this remote region, the retina of the experimental eye exhibited abnormal structural changes compared with a similar area from the control fellow eye (Figs. 3C, 3D).

The retina in more peripheral regions (15 mm from the site of injection) of the experimental eye appeared indistinguishable at the light microscopic level from corresponding areas in the control eye (Figs. 3E and 3F, respectively). All eyes treated with MGP solution of any concentration and prepared for histologic examination either 4 or 6 weeks after drug injection (n = 11) exhibited similar pattern of retinal damage.

The retinas of the experimental and control eyes were also tested for GFAP immunoreactivity (Fig. 4). These eyes were prepared for GFAP immunoreactivity 6 weeks after MGP solution (0.38 mg/ml) or saline were injected into the experimental and control eyes, respectively. Every retinal area from the experimental eye, regardless of its eccentricity, demonstrated GFAP immunoreactivity. Heavy labeling was seen in the region of the medullary rays (not shown here), mainly as
FIGURE 4. GFAP immunoreactivity of an experimental eye injected with MGP solution (0.38 mg/ml, A–C) and a control eye injected with saline (D). The eyes were prepared for GFAP immunoreactivity 6 weeks after injection. Two sections are shown for the experimental eye: one taken 10 mm and the other 20 mm from the site of drug injection (A and B, respectively). Immunocytochemical control was done by treating a section from the experimental eye for GFAP immunoreactivity without the GFAP antibody (C). Peripheral retinal areas from the control eye showed no GFAP immunoreactivity (D). Calibration bar = 25 μm.

a result of staining of astrocytes. Remote retinal areas (10 and 20 mm from the site of drug injection), which exhibited minor structural damage, demonstrated GFAP immunoreactivity (Figs. 4A and 4B, respectively). The morphology of the stained structures is typical for retinal Müller cells because they span most of the retinal depth (from the inner to the outer limiting membranes). This staining was specific for GFAP because a similar section treated without the GFAP antibody showed no staining (Fig. 4C). In the control eye, GFAP expression was seen only in the region of the medullary rays where astrocytes were found (not shown here). However, unlike the experimental retina, GFAP labeling was not seen in any section of the peripheral retina (Fig. 4D), consistent with previous reports. Similar patterns of GFAP immunoreactivity were seen in all the experimental and control eyes (n = 11) tested for GFAP immunoreactivity at long time intervals after intravitreal injections (4 or 6 weeks).

DISCUSSION

The histologic and immunocytochemical findings discussed here support our previous electrophysiologically based conclusion that MGP, the preservative used in Depo-Medrol, is highly toxic to the rabbit retina. MGP-induced retinal damage developed very fast and could be demonstrated histologically as soon as 24 hr after intravitreal injection of the drug. At this short time interval after drug injection, the MGP-induced damage was expressed in a swollen retina with disarrayed nuclear and plexiform layers (Fig. 1A). At longer times after injection (6 weeks), the retina in the affected areas lost most of its components, and only a few unidentified cell nuclei could be seen (Fig. 3A).
Although the ERG measurements provide only a general assessment of the degree of functional damage, the morphologic observations also provide information on the spatial distribution of the damage. Short- and long-term MGP-induced damage did not spread uniformly across the retina (Figs. 1, 5). The most severely affected areas were those adjacent to the site of injection (approximately 2 mm). The longer the distance between the site of injection and the retinal area examined was, the smaller was the degree of the damage. At the far periphery, approximately 20 mm from the site of injection, the retina seemed to retain its normal structure although it appeared slightly swollen.

Retinal damage was also assessed by testing the experimental and control retinas for GFAP. This protein is always found in astrocytes but is only expressed in Müller (glia) cells after retinal insults. All retinal pieces obtained from eyes tested for the long-term effects of MGP exhibited positive staining for GFAP in Müller cells, regardless of the degree of morphologic damage and eccentricity (Fig. 4). Thus, even areas that appeared structurally unharmed at the light microscopic level seemed to suffer some degree of damage. These findings suggested that MGP induced widespread retinal damage, which expanded beyond the site of injection. This diffuse retinal damage could account for the severe reduction in the ERG responses. These observations support the use of immunocytochemical techniques for identifying cellular components, such as GFAP, a sensitive marker for retinal damage. These techniques can identify damaged retinal regions that are not seen by the light microscope but can be detected with the aid of functional tests, such as ERG measurements.

The deleterious effects of MGP on the rabbit retina developed very fast. Twenty-four hours after injection of the drug, the ERG responses indicated an average functional damage of 60%. This functional deficit was accompanied by severe morphologic abnormalities, which were most apparent in retinal areas adjacent to the site of drug injection (Fig. 1). However, at this short postinjection period, GFAP immunoreactivity was seen only in the region of the medullary rays where astrocytes were abundant. Müller cells in the peripheral retina did not stain for GFAP even when tested at a longer time interval, 72 hr, after drug injection. These observations indicate that the expression of GFAP in Müller cells lags behind the development of retinal damage and, therefore, cannot be used to follow the time course of progression of drug-induced retinal toxicity.

Key Words
electroretinogram, myriistyl γ-picolinium, glial fibrillary acidic protein (GFAP), Müller cells, retina

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