Acute toxic effects of chloroquine on the cat retina

Eliot L. Berson

Intravitreal administration of 0.5 mg. per 0.1 c.c. of chloroquine hydrochloride produces an irreversible toxic effect to the cat photoreceptors within 40 hours. Of 24 cats that received 0.5 mg. per 0.1 c.c., 19 showed an average reduction of 50 per cent in the amplitude of the electroretinogram (ERG), whereas doses less than 0.5 mg. produced no ERG changes. When the near-threshold dose of 0.5 mg. per 0.1 c.c. is used, the inner retinal layers, examined with the light microscope, appear preserved even though the drug must have passed through these layers to reach the photoreceptors. Simultaneous reduction of a-wave and b-wave amplitude in the ERG as well as light microscopic studies indicate that this near-threshold dose initially damaged the photoreceptors, although an additional primary toxicity to the pigment epithelium cannot be excluded. Comparable destruction was present in retina overlying the pigmented (nontapetal) and nonpigmented (tapetal) pigment epithelium in the same eye. Twenty times the near-threshold dose of chloroquine required for acute photoreceptor damage by the intravitreal route results in no ERG changes when administered by the long posterior ciliary artery (LPCA). Results of LPCA injections of chloroquine hydrochloride and sodium iodoacetate are compared.

Key words: Chloroquine, cat retina, electroretinogram, long posterior ciliary artery, photoreceptors, a-wave, b-wave, amplitude, light microscope, intravitreal, sodium iodoacetate, pigment epithelium, toxicology.

A minimum total dose of 100 to 200 Gm. of chloroquine ingested chronically over a one-year period has been considered necessary before signs of drug-induced retinopathy can begin to develop in man. The length of time between onset of chloroquine therapy and the onset of visual loss and the low incidence of retinopathy in patients receiving chloroquine have made it difficult to undertake prospective studies on ways to influence this side effect. Laboratory investigations on the compound have also been difficult because prolonged systemic administration is necessary to produce retinal damage in animals. Therefore, there is a need to produce acute chloroquine retinal lesions experimentally that could serve as a manageable test system for evaluation of the toxic effects of this drug.
The variable retinal sensitivity to chloroquine of different species was considered in the selection of an animal for the present experiments. Previous work in pigmented and albino rabbits showed that chronic administration of chloroquine for 10 to 16 months was required to produce a retinopathy. Amopyroquin, a compound related to chloroquine, was given to monkeys for over one year without effect on the retina, but it did cause retinal atrophy in 5 months in albino rats and beagle dogs. In swine, lesions in the nerve fiber layer were visible histologically after only 2½ weeks of chloroquine ingestion; however, pathologic changes did not involve photoreceptors and pigment epithelium, whereas this occurs in man. In cats, chloroquine administered systemically did damage the pigment epithelium and photoreceptors after only 4 to 7 weeks of ingestion. These observations suggested that the initial experiments to produce acute chloroquine retinopathy should be attempted in the cat.

The unusual anatomy of the uveal vasculature of the cat also made this animal appropriate for these studies. In the cat the central retinal artery is vestigial and branches of the posterior ciliary arteries supply the retina. Wong and Macri have shown that the ophthalmic artery bifurcates into two long posterior ciliary arteries (LPCA), that the temporal LPCA sends branches to form a vascular annulus around the distal part of the optic nerve, and that the blood supply of both the retina and choroid arise from this annulus (Fig. 1). This vascular pattern differs from that of primates, in which the ophthalmic artery branches into the LPCAs, the short posterior ciliary arteries that supply the choroid and outer retina, and a central retinal artery that provides a separate circulation for the inner retina. In the cat the temporal LPCA is accessible under the lateral rectus muscle and anatomical studies, as well as pilot investigations with sodium iodoacetate and quinine, indicated that drugs injected retrograde into this artery would be pushed forward by the ophthalmic artery pressure head into both the choroidal and retinal circulation (see inset). Electroretinograms are recorded from both eyes simultaneously using corneal contact lens electrodes (C).
the retinal and choroidal circulation (Fig. 1).

In the present investigations, chloroquine hydrochloride was locally administered into the eyes of cats either intravitreally or by the LPCA route. Electroretinograms (ERGs) were used to detect the near-threshold dose sufficient to produce acute toxicity to the cat retina. Some animals received LPCA injections of sodium iodoacetate, a metabolic poison with known toxicity to the photoreceptors,20-22 for comparison with the effects of chloroquine.

Methods

The route chosen for administration of chloroquine hydrochloride, sodium iodoacetate, or normal saline was either intravitreal (route A, Fig. 1) or intra-arterial (route B, Fig. 1). All cats were anesthetized with Nembutal administered by the intraperitoneal route. Intravitreal injections were made through a one-half inch 24 gauge needle that was inserted through the pars plana 1 mm. above the horizontal meridian in front of the insertion of the right lateral rectus muscle. The needle was directed toward the optic nerve, and placement near the retina was controlled under visualization with the indirect ophthalmoscope. Animals with vitreous hemorrhage following insertion of the needle were excluded. Intra-arterial injections were made through a microcanula (tapered polyethylene No. 10 tubing) directed posteriorly into the temporal LPCA (B, Fig. 1). The artery can be easily exposed by separation of the fibers of the right lateral rectus muscle. For both routes of injection the syringe containing drug was driven automatically to regulate the rate of drug administration to 0.1 c.c. per minute. Thirty-one adult cats received chloroquine by the intravitreal route in the right eye, 7 cats, chloroquine by the LPCA; 6 cats, sodium iodoacetate by the LPCA; 3 cats, normal saline by the LPCA; and 3 cats, normal saline by the intravitreal route.

Prior to drug injection each anesthetized cat was dark adapted for one hour, and ERGs were recorded simultaneously from both eyes as a baseline. After the injection into the right eye, ERC testing was performed on both eyes immediately and then every hour for 2 to 12 hours. In the intravitreal studies, recordings were usually done on day 1, 3, 5, and/or 7 after the injection. Four cats were followed for 6 weeks after the intravitreal injection. In the intra-arterial studies, ERGs were obtained on day 1 and day 7. On all animals ERGs were recorded just prior to enucleation of both eyes. The eyes of the majority of the animals were fixed in Bouin's solution, embedded in paraffin, and sectioned horizontally. Sections were stained with hematoxylin and eosin. Several eyes were fixed for electron microscopy and are the subject of a separate report.

The ERGs were recorded with a contact lens electrode on each eye and a reference electrode on the scalp. The animal's position was secured with a bite board and head holder to insure a 6 inch distance between the photostimulator (Grass PS II) and both eyes for all experiments. Red (Wratten No. 26) and white test flashes, 10 sec. in duration, were presented at a minimum interval of 2 seconds. Responses from each eye were amplified by a preamplifier (Tektronix 122 camera (Grass C-4). In these experiments relative ERG amplitude is designated as the amplitude of the response from the right eye compared to that from the left eye expressed in per cent. ERG amplitudes were measured from the peak of the cornea-negative phase or a-wave to the peak of the cornea-positive phase or b-wave for each response. Responses to single flashes of red light, presented at 2 second intervals over 20 seconds, were measured each hour, and amplitudes were averaged. A difference of greater than 25 per cent in amplitude between eyes was considered clear evidence for the onset of retinal damage in the right eye (see Results).

One milliliter of the stock solution of drug (Aralen, Winthrop Laboratories, New York, N. Y.) contains 50 mg. of chloropaine dihydrochloride salt equivalent to 40 mg. of base. This stock solution had a pH of 5.8 contained no preservatives and as a rule was diluted with normal saline to obtain 0.5 mg. per 0.1 c.c. (0.0125M) of chloropaine hydrochloride or lower concentrations. In some instances the stock solution was used. For comparison with the intra-arterial injections of chloroquine, sodium iodoacetate was dissolved in normal saline to achieve 0.268 mg. per 0.1 c.c. (0.0125M) concentration and was administered into the right temporal LPCA. This concentration of sodium iodoacetate was selected as equinolar to the "near-threshold toxic dose" of chloroquine required for photoreceptor damage when chloroquine was administered intravitreally (see Results). In two animals sodium iodoacetate was injected intra-arterially in a dose of 0.54 mg. per 0.1 c.c.

As controls for these experiments, three animals received 0.1 c.c. of normal saline over one minute by the intravitreal route in the right eye. Three other animals were given 0.3 c.c. of normal saline over one minute by the LPCA route. These animals received no drugs. They were followed with
ERG recordings weekly for 6 weeks after these injections and then killed.

Results

Electroretinograms to the same light stimuli recorded simultaneously from both eyes of 50 cats prior to the injection of any drug showed identical waveforms and almost identical amplitudes in both eyes; the maximum difference in ERG amplitude between two untreated eyes of the same cat was always less than 10 per cent (right eye relative to left eye). Injections of normal saline by either the intravitreal or the intra-arterial route produced no change in the ERG.

Table I summarizes the effects of local chloroquine injections on the cat retina based on ERG and histopathologic evaluation. In each group the injected right eye of each cat was compared to the non-injected left eye of the same cat. In every cat the response amplitude of the right eye relative to the left eye to single flashes of red light, presented successively at 2 second intervals over 20 seconds, varied within a 5 per cent range. Of 24 cats that had received 0.5 mg. of chloroquine by the intravitreal route in the right eye, 19 showed greater than 25 per cent reduction in relative ERG amplitude within 6 to 24 hours. The average reduction in this group was 50 per cent. Intravitreal chloroquine injections of less than 0.5 mg. did not produce changes detectable with the ERG or the light microscope while doses greater

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>No. of cats</th>
<th>Dose (mg./total volume injected OD)</th>
<th>Relative ERG amplitude* (OD/OS per cent)</th>
<th>Pathology (light microscopy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Intravitreal chloroquine (above threshold)</td>
<td>4</td>
<td>1-5 mg./0.1 c.c.</td>
<td>5-15</td>
<td>All layers of 60 to 90 per cent of retina damaged</td>
</tr>
<tr>
<td>II. Intravitreal chloroquine (near threshold)</td>
<td>19</td>
<td>0.5 mg./0.1 c.c.</td>
<td>25-65†</td>
<td>Selective damage to 50 per cent of photoreceptors</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.5 mg./0.1 c.c.</td>
<td>100</td>
<td>No retinal damage</td>
</tr>
<tr>
<td>III. Intravitreal chloroquine (below threshold)</td>
<td>3</td>
<td>0.1-0.25 mg./0.1 c.c.</td>
<td>100</td>
<td>No retinal damage</td>
</tr>
<tr>
<td>IV. Intra-arterial chloroquine</td>
<td>6</td>
<td>0.5 mg./0.1 c.c. to 10.0 mg./0.4 c.c</td>
<td>100</td>
<td>No retinal damage</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>45 mg./2.7 c.c.</td>
<td>50</td>
<td>Photoreceptor damage†</td>
</tr>
<tr>
<td>V. Intra-arterial sodium iodacetate (for comparison with group IV)</td>
<td>4</td>
<td>0.268 mg./0.1 c.c.</td>
<td>40-50</td>
<td>Loss of 25 to 35 per cent of photoreceptors</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.54 mg./0.2 c.c.</td>
<td>50-60</td>
<td>Loss of 40 to 50 per cent of photoreceptors</td>
</tr>
<tr>
<td>VI. Intravitreal normal saline (control for groups I to III)</td>
<td>3</td>
<td>0.1 c.c.</td>
<td>100</td>
<td>No retinal damage</td>
</tr>
<tr>
<td>VII. Intra-arterial normal saline (control for group IV)</td>
<td>3</td>
<td>0.3 c.c.</td>
<td>100</td>
<td>No retinal damage</td>
</tr>
</tbody>
</table>

*ERG amplitude recorded one week after injection.
†Range refers to ERG changes seen in different cats, usual reduction 50 per cent.
††See text for description of this animal.
L/V

Fig. 2A. ERG tracings from a dark-adapted cat response to red light prior to injection (left column) and from the same cat 24 hours after an intravitreal injection of 0.5 mg. per 0.1 c.c. of chloroquine in the right eye (right column). The ERGs were recorded simultaneously from both eyes (left eye top, right eye bottom for each pair) and stimulus flash intensity was the same for corresponding pairs in each column. Responses to 4 stimulus flash intensities of decreasing brightness (second to fifth pair) are shown, as well as recordings studied at faster sweep speeds (top pair). Splitting of the earlier cone from later rod b-wave can be seen (see arrows). Stimulus onset is vertical hatched line. Calibration symbol, lower right corner, is 50 msec. horizontally for the lower 4 pairs and 25 msec. horizontally for the uppermost pair in each column, and 50 μV vertically for all tracings. Cornea positivity is an upward deflection.

Fig. 2B. ERG tracings from two dark-adapted cats in response to white light prior to injection (left column) and 24 hours after an intravitreal injection of chloroquine in the right eye (right column). The ERGs were recorded simultaneously from both eyes for each cat (left eye top, right eye bottom for each pair). Upper tracings (right column) are from cat that received 0.5 mg. per 0.1 c.c. in the right eye and lower tracings (right column) are from a cat that received 5 mg. per 0.1 c.c. in the right eye. Reduction of amplitude of the early cornea-negative a-wave and later cornea-positive b-wave (see arrows) can be seen in both animals. Stimulus onset is vertical hatched line. Calibration symbol, lower right corner, is 50 msec. horizontally and 100 μV vertically.

tudes of both the cornea-negative a-wave and cornea-positive b-wave responses are reduced (Fig. 2B, right column). Toxicity could often be detected with the ERG within 6 hours after injection and the a-wave and b-wave amplitudes became diminished at the same time. A much larger change in ERG amplitude followed injection of 5 mg. of chloroquine (Fig. 2B, lower right) compared with the reduction produced by 0.5 mg. of chloroquine (Fig. 2B, upper right).

The toxic effect to the retina of 0.5 mg. of chloroquine hydrochloride administered by the intravitreal route appeared maximum within 24 hours and unchanged in animals followed for 8 weeks after the injection (Fig. 3). The per cent reduction in ERG amplitude at 24 hours varied in this group from 25 to 65 per cent but remained the same (± 5 per cent) at separate testing periods for a given cat over the eight
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Fig. 3. Effects of chloroquine (0.5 mg. per 0.1 c.c.) administered intravitreally in the right eye on the relative ERG amplitude (O.D. as a per cent of O.S. for each cat) of 4 cats (△, ◯, ●, ■). Recordings from each cat were done on day 1, 7, 14, 42, and 56 after injection. Hatched line is the average for the 4 cats.

Weeks. Response amplitudes were reduced similarly to both red and white test flashes.

Light microscopic studies showed that the per cent reduction in ERG amplitude was correlated with the area of visible receptor damage 40 hours or later after injection (see Table I). Fig. 4 (B, C, D, and E) illustrates representative pathologic changes from cats given 0.5 mg. of intravitreal chloroquine compared with a normal control (Fig. 4, A). Although ERG changes were detectable 6 to 24 hours after injection, no abnormalities were visible with the light microscope until 40 hours after injection, at which time clear definition of the outer segments was lost and the adjacent pigment epithelium appeared swollen. Fig. 4, B demonstrates that 3 days after injection of chloroquine the outer segments are destroyed and in some areas the inner segments are disrupted. Although the drug was administered in the vitreous, the inner layers of the retina as well as the outer nuclear layer appear normal at the time when the photoreceptors are damaged. Fig. 4, C illustrates loss of inner and outer segments as well as pyknosis of nuclei in the outer nuclear layer 5 days after injection. A higher power view (Fig. 4, E) indicates these changes as well as vacuoles in the cytoplasm of pigment epithelial cells. Seven days after injection (Fig. 4, D), there is loss of nuclei in the outer nuclear layer. In all eyes, the pigment epithelial layer appeared uninterrupted and the underlying tapetum was normal. Chorioretinal adhesions were rarely seen. Animals given 0.5 mg. of chloroquine had photoreceptor damage in both tapetal and nontapetal areas that was usually most extensive on the temporal side of the eye. Animals given doses greater than 0.5 mg. showed damage not only to the photoreceptors but also to the ganglion and bipolar cell layers in many areas and varying amounts of retinal destruction represented by Fig. 4, B to E, could be seen in the same eye.

When chloroquine was administered by the LPCA route, 20 times the intravitreal "near-threshold toxic dose" failed to produce retinal abnormalities detectable with the ERG or the light microscope. One animal received 90 times the "near-thresh-
Fig. 4, A to E. Normal cat retina (A) and representative sections from cats that received intravitreal chloroquine (0.5 mg. per 0.1 c.c.) 3 days (B), 5 days (C), and 7 days (D) after injection. (Hematoxylin and eosin; x100.) Higher power view of the retina 5 days after injection (E) shows loss of inner and outer segments with vacuoles (see arrow) in the cytoplasm of a macrophage or displaced pigment epithelial cell. (x300.) Seven days after injection (D) separation of retina from pigment epithelium was seen in some sections.

A

B

C

D

E

old dose" (45 mg. of chloroquine hydrochloride) by the LPCA route and developed a 50 per cent reduction in the amplitude of the a-wave and b-wave in the right eye within 24 hours that remained the same for one week. In this animal, one week after the injection, folds in the retinal tissue with swelling of the outer and inner segments (Fig. 5) could be seen in all histologic sections.

Fig. 6 illustrates pathologic changes in a cat that received sodium iodoacetate (0.0125M) by the LPCA route one week prior to enucleation. The ERG in this ani-
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Fig. 5. Section of cat retina one week following LPCA injection of 45 mg. of chloroquine. Folding of tissue and swelling of outer segments were seen in all sections. Adherence of pigment epithelium to areas of damaged outer segments was also visible in some areas. (Hematoxylin and eosin; ×100.)

Fig. 6. Representative section of cat retina one week following LPCA injection of 0.268 mg. per 0.1 c.c. of sodium iodoacetate. (Hematoxylin and eosin; ×100.)

Systemic administration of chloroquine for months to years is necessary to produce retinal damage in man1-6 and in animals.7-12 In the present experiments one intravitreal injection of chloroquine (0.5 mg. per 0.1 c.c.) resulted in an irreversible toxic effect to large areas of photoreceptors of the cat within 40 hours. Simultaneous reduction of both a-wave and b-wave amplitude in the ERG indicated that this "near-threshold toxic dose" of chloroquine initially caused damage to the photoreceptors themselves.24-26 Results of light microscopic studies corroborated the ERG findings and revealed disruption of inner and outer segments and later loss of the outer nuclear layer.

In contrast to man, the acute drug-induced injury in the cat was not initially limited to the central retina; therefore, the electroretinogram, recorded at the cornea, could be used effectively to monitor the early widespread involvement of the photoreceptors in the experimental animal. Hommer27 demonstrated that 10^{-3} and 10^{-4}M chloroquine hydrochloride applied directly on the isolated rabbit retina produced a rapid diminution of the amplitude of the electroretinogram within minutes. In Hommer's experiments prompt replacement of the chloroquine with plasma reversed the changes. In the present investigations, chloroquine hydrochloride (0.0125M) placed in the vitreous was not removed, and the signs of damage proved to be permanent.

Although chloroquine undoubtedly passed from the vitreous through the in-
ner retinal layers to reach the photoreceptors, the inner retinal layers appeared preserved under the light microscope when near-threshold doses were used. An additional primary toxic effect on the pigment epithelium could not be excluded with near-threshold doses because these cells were altered wherever areas of damaged photoreceptors were visible. Nevertheless, the acute retinal lesion in the cat could not depend on the known binding of chloroquine to melanin in the pigment epithelium because comparable destruction was present in the retina overlying both the pigmented (nontapetal) and nonpigmented (tapetal) pigment epithelium in the same eye. The exact pathogenetic events produced by chloroquine remain unknown although several possibilities have been adequately reviewed.

Autoradiographic studies on normal outer segments containing labelled amino acids and electron microscopic examination of both light-induced retinal degenerations and experimental retinal detachments have shown that the pigment epithelium can engulf portions of photoreceptor outer segments. The appearance on light microscopic examination of vacuoles in the cytoplasm of pigment epithelial cells and in macrophages in the subretinal space combined with the rapid disappearance of damaged outer and inner segments suggested a similar mechanism for removal of the photoreceptor cell remnants in the present experiments. The findings on electron microscopic examination of these animals will be contained in a separate report.

Twenty times the near-threshold dose of chloroquine necessary for causing acute damage to the photoreceptors and pigment epithelium by the intravitreal route resulted in neither detectable ERG changes nor histologic signs of damage when administered by the LPCA route. In contrast, concentrations of iodoacetate, equimolar to the "near-threshold toxic dose" of chloroquine, consistently produced acute monocular toxicity to the photoreceptors and pigment epithelium when injected by this arterial route. These findings with LPCA injections of iodoacetate were comparable to the acute changes previously described in animals that received larger doses of iodoacetate introduced through a limb vessel. The iodoacetate experiments also suggested that chloroquine administered through the LPCA was perfused through the choroidal and/or retinal vessels even though no acute toxicity to the photoreceptors occurred following these chloroquine injections. Further experiments with radioactive labeled chloroquine would help to demonstrate the distribution of this drug in the cat eye and perhaps explain the apparent difference between the intravitreal and intra-arterial near-threshold toxic dose. The distribution of radioactive labeled chloroquine placed in the vitreous might also explain the reason for the greater retinal damage observed on the temporal side of the eye in these experiments. One animal given 90 times the near-threshold intravitreal toxic dose of chloroquine by the LPCA route did develop ERG and histologic changes so that further studies should be done on the effect of high doses of chloroquine injected by this route.

The present investigations demonstrate an in vivo approach to evaluate the near-threshold effects of drugs that are potentially retinotoxic. This experimental model allows injection of small doses of drugs relative to the entire animal but large concentrations of drugs relative to the tissues of the injected eye. Previous attempts to produce chloroquine retinopathy in monkeys and cats with prolonged oral administration of this drug did result in systemic effects, and this systemic toxicity could modify any interpretation of ocular changes. Large doses of chloroquine administered in a single injection through a limb vessel can also be lethal to the cat. In the present studies the problem of systemic side effects has been obviated, and the dose of chloroquine required to pro-
duce damage to the photoreceptors and pigment epithelium had no detectable effects on the other eye or the general health of the animal. The author thanks Peter Gouras, M.D., for his advice and Ludwig von Sallmann, M.D., for review of the manuscript. The author is also grateful to Mrs. Eleanor Collins who prepared the histologic sections and Miss Kay Boetker who provided technical assistance.

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