Axon-Tracing Properties of Indocyanine Green

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Objective: It has been shown recently that the application of indocyanine green (ICG) over the retinal surface is followed by prolonged staining of the optic disc. This study was performed to analyze the diffusion of ICG in the optic tract.

Methods: Anterograde diffusion of ICG was evaluated after injection into the vitreous of rabbits. Retrograde diffusion was evaluated after microinjection into the lateral geniculate nucleus of rats.

Results: Anterograde and retrograde diffusion occurred along the axons at a rate of about 2 mm per hour when ICG was injected. Anterograde staining of the visual pathway persisted for several weeks. After injection into the lateral geniculate nucleus, fluorescent retinal ganglion cells could be visualized for at least 7 days in conscious rats by conventional infrared photography. Microscopic examination findings of retrograde-labeled retinas showed the presence of ICG vesicles inside the axons, cytoplasm, and dendrites of retinal ganglion cells. No evidence of toxic effects was detected by optical microscopy.

Conclusions: Indocyanine green is a fast bidirectional axonal tracer. Injection into normal vitreous results in long-term staining of the visual pathway. In vivo counting of ICG-labeled retinal ganglion cells in rats can be performed for several days after injection. Indocyanine green is therefore potentially of interest for use in experimental neurophysiological studies.

Clinical Relevance: The present results suggest that in humans, epiretinal application of ICG results in prolonged staining of the visual pathway. Therefore, additional studies of long-term toxic effects of ICG on neural cells are warranted before recommending its use in humans as an intraoperative tool for vitreoretinal surgery.

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Indocyanine Green (ICG) is a tri-carbocyanine dye used in humans for the imaging of retinal and choroidal vessels and the measurement of cardiac output and cerebral blood flow. It was recently shown in humans that when ICG is directly applied over the retina during vitrectomy, it selectively stains the optic disc for several weeks. We postulated that this was due to intraneuronal diffusion of the dye. We thus performed the present study to explore the diffusion of ICG in the optic tract.

Methods

Rabbits and rats were provided by Elevage Janvier (Le Genest, France). All procedures were performed according to Association for Research in Vision and Ophthalmology statements on the care and use of animals in ophthalmic research.

The retrobulbar diffusion of ICG (Infra-cyanine; Société d’Etudes et de Recherches Biologiques, Paris, France) after intravitreal injection was evaluated in rabbits. Eight pigmented male rabbits weighing 1 to 2 kg were anesthetized by an intramuscular injection of ketamine hydrochloride, and additional topical anesthetics, oxybuprocaine chloride, was administered. Indocyanine green (250 µg in 50 µL of 5% glucose water) was injected into the vitreous of rabbits with a 30-gauge needle 2 mm from the limbus. One animal was humanely killed by an overdose of pentobarbital at each of the following points: 2, 3, 8, and 24 hours and 8, 15, 30, and 60 days after injection. The eyecups and brain were immediately isolated, fixed in 10% formalin in separate vials, and examined for ICG fluorescence. Other retinal and brain specimens were processed for conventional light microscopy after being dehydrated in ethanol, embedded in paraffin, and stained with hematoxylin-eosin.

The retrograde tracing following intracerebral injection into the lateral geniculate nucleus was analyzed in rats. Three adult male Wistar rats weighing 300 to 350 g were anesthetized with halothane (2.5% in 25% oxygen and 75% nitrogen) and mounted in a stereotaxic device. The skin of the skull was incised,
and the parietal bone was perforated with a dental drill at a point 4.4 mm caudal to the bregma and 3.8 mm lateral to the midline on the right side. Indocyanine green in 5% glucosed water (100 nL containing 5 µg of ICG) was stereotactically injected at a depth of 4.4 mm for 20 seconds with a glass micropipette. The skin was then closed with 6-0 silk sutures, and the animal was allowed to recover.

Macroscopic epifluorescence examination of stained tissues was performed using a digital fundus camera with built-in ICG filters (TRC 50 IA; Topcon, Tokyo, Japan). For fundus photography, the animal was conscious and was positioned in front of the camera by gentle bimanual handling, without mechanical constraint. Once the rat had become accustomed to this position, fundus photographs of both eyes were taken after pupil dilation at 2, 4, and 24 hours and 7 and 14 days after ICG injection. Flat-mounted retrograde-labeled retina specimens were examined with a Leitz fluorescence microscope equipped with a xenon lamp and an interference filter (CY7; Leitz Corporation, Germany; excitation 710-775 nm, emission 810-890 nm). The fluorescence images were captured with a cooled digital charged-coupled device camera (Princeton Instruments, Trenton, NJ) operated by computer software (WinView; Princeton Instruments).

To evaluate postmortem diffusion, ICG was applied over sectioned optic nerves of rabbits eyecups and on prechias-}

**RESULTS**

Macroscopic examination findings of injected rabbit eyes did not disclose gross abnormalities except for the presence of residual vitreal ICG in all specimens. In the brain, ICG staining was observed in the ipsilateral optic nerve, the chiasma, and in the contralateral superior colliculus (Figure 1) and lateral geniculate nucleus. There was no detectable staining of the adjacent neural structures. Euthanizing animals at different points made it possible to establish that ICG reaches the chiasma within 5 hours of injection and the contralateral colliculus within 8 hours, indicating an axonal transport velocity of about 2 mm per hour. Optical microscopic examination findings from retinal and brain sections of a rabbit examined 2 months after ICG injection were within normal limits (data not shown).

After ICG injection in the lateral geniculate nucleus, retrograde axonal tracing resulted in fluorescence of the contralateral retinal ganglion cells (RGCs) and of the disc
Retinal ganglion cell fluorescence appeared between 2 and 4 hours after injection. Staining was still visible at day 7 but was not detectable at day 14. In one animal, ICG was reinjected at day 21 on the same side, and RGC fluorescence was again observed. Ipsilateral RGC staining was limited to a few cells in the peripapillary area. Microscopic examination retinal findings viewed on flat-mounted slides revealed the presence of vesicles of ICG in the axons, cytoplasm, and dendrites of RGCs (Figure 3). There was notable heterogeneity in the aspect of labeled cells, suggesting that all populations of RGCs were labeled.

In postmortem samples, no diffusion of ICG was observed (data not shown).

**COMMENT**

Indocyanine green is an amphiphilic, ethanol-soluble tricarbocyanine dye that has been used in humans for more than 30 years. It was initially used to measure total blood flow from dye-dilution curves. Its medical and experimental use has since increased and presently includes ocular vessel angiography, liver function testing, and measurement of cerebral blood flow. In recent years, ICG has been used as an intraoperative tool because it selectively stains the internal limiting membrane when applied directly over the retina. In the postoperative period, prolonged and selective ICG staining of the optic disc was also observed. We postulated that this was due to intraneural diffusion of ICG. Although much is known about the pharmacokinetics of ICG and its plasma protein binding and fluorescence properties, little is known about its interaction with tissues. With regard to nervous tissues, selective ICG staining of cerebral tumors due to blood-brain barrier leakage has been reported. As other molecules of the carbocyanine family are known to possess axonal tracing properties, such as Di-I and Di-A, we therefore investigated the possibility that ICG was also an axonal tracer.

The results of the present study indicate that ICG undergoes fast bidirectional transport. Fluorescence of the colliculus was observed within 8 hours of ICG injection into the vitreous and persisted at least 2 months. The presence of ICG within RGCs was observed within 4 hours of ICG injection in the lateral geniculate nucleus, and persisted between 7 and 14 days. Microscopic examination findings of retrogradely labeled retinas revealed that ICG was present within vesicles in axons, cytoplasm, and dendrites of RGCs. The rapid diffusion into the visual pathway (about 2 mm per hour, anterograde and retrograde) and the absence of postmortem diffusion suggest that there is only active axonal transport as opposed to other tricarbocyanine dyes that also diffuse postmortem.

These results suggest that retrograde labeling of RGCs may be useful for experimental studies. The main interest of ICG as a retrograde tracer is that fluorescent RGCs can be counted in vivo in conscious rats for at least 7 days. Therefore, ICG makes it possible to monitor the short-term changes in RGC density in individual animals following experimental diseases. This may be of interest in studies addressing the effect of acute elevation in intraocular pressure or optic nerve ligation on the ganglion cell population in individual animals.

Indocyanine green appears to have a rate of diffusion comparable to Di-I and Di-A but without postmortem diffusion, which makes it unsuitable for axon tracing in the human brain. Additionally, ICG is relatively inexpensive. The possibility to convert ICG into an electron-dense product, as with Di-I, has not been documented to date.

In summary, we have shown that ICG in contact with neural tissues is actively transported via anterograde and retrograde means in axons vesicles. Since epiretinal application of ICG is currently used in humans, it is likely
that in patients undergoing surgery, ICG stains cerebral tissue as well. On the other hand, our results suggest that ICG is also a valuable tool for neurophysiological studies since ICG-labeled RGCs can be visualized in vivo. No evidence of neural toxic effects of ICG was detected, after either intravitreal injection or retrograde labeling. In particular, in animals examined 3 weeks after retrograde labeling, the ganglion cell layer had a normal appearance on histologic sections and by epifluorescence examination findings of flat-mounted slides. However, further exploration of the long-term neural toxic effects of ICG is warranted to assess its safety for human use during vitreoretinal surgery, because prolonged intracerebral staining is likely to occur.

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