Binding Properties of Indocyanine Green in Human Blood

Shin Yoneya,1 Tamiya Saito,1 Yoshiko Komatsu,1 Iwao Koyama,2 Keiichi Takabashi,1 and Josephine Duvall-Young2

PURPOSE. Binding properties of indocyanine green (ICG) to human plasma proteins were identified using electrophoresis and a fundus video system.

METHODS. Blood samples were obtained from three healthy volunteers after intravenous administration of ICG. The resulting plasma samples were fractionated by agarose gel immunoelectrophoresis and polyacrylamide gel DISC electrophoresis. In the former, antisera, anti-apolipoprotein (Apo)-A, and anti-Apo-B antibodies were used to identify all classes of plasma proteins, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), respectively. In the latter method, plasma samples could be separated into chylomicron, very low-density lipoprotein, LDL, and HDL. The electrophoretic pattern obtained by each method was observed with an ICG fundus video system. Furthermore, we studied the affinity of ICG for lipids that were common molecular components of HDL and LDL. Four kinds of ICG solutions mixed with phospholipid, free cholesterol, esterified cholesterol, and triacylglycerol were observed with the ICG fundus video system.

RESULTS. Both electrophoretic studies showed that ICG bound intensely to HDL and moderately to LDL, and only the solution with phospholipid fluoresced brightly when observed with the ICG fundus video system.

CONCLUSIONS. These findings indicated low vascular or tissue permeability of ICG, which is caused by the larger molecular size of HDL and LDL. Also noted was that the ICG fluorescence observed in the angiogram may be equivalent to the hemodynamics of HDL alone or in combination with LDL in the bloodstream. This biochemical consideration may be a basis for the further understanding of ICG angiography. (Invest Ophthal Vis Sci. 1998;39:1286–1290)

Recently, there has been renewed interest in indocyanine green (ICG) angiography, because of its potential usefulness in examining choroidal hemodynamics and choroidal vascular disorders. ICG video angiography now has been applied not only to investigate choroidal neovascularization, but also to study a wider spectrum of choroidal diseases, including choroidal tumors, vascular disorders, and uveitis.

ICG emits near-infrared fluorescence, which penetrates the retinal pigment epithelium when stimulated with adequate exciting light. We already have shown that the peak absorption of aqueous ICG is 780 nm and that it shifts to 805 nm after binding to plasma protein. The binding process takes longer for ICG to achieve than it does for fluorescein sodium. However, no one has yet properly applied any of them, especially those concerning the binding properties of ICG to plasma protein. When ICG angiography was introduced to ophthalmology clinics, it was thought that more than 98% of ICG bound to plasma albumin and that free components were minimum. Subsequently, Baker pointed out that ICG bound to alpha-1 lipoprotein but not to albumin. Because vascular permeabilities may vary greatly, this difference is clinically important, and this issue should be resolved soon to further the understanding of ICG angiography. The present study aimed to identify the binding properties of ICG to plasma proteins in detail, by combining present electrophoretic methods and an ICG fundus video system.

MATERIALS AND METHODS

Binding of Indocyanine Green to Plasma Proteins

All subjects were treated in accordance with the tenets of the Declaration of Helsinki, and informed consent was obtained after the nature of the study was explained. Accordingly, blood samples were obtained from three healthy volunteers, 1 minute after the intravenous administration of 50 mg/2 ml ICG in distilled water. EDTA was used as an anticoagulant, and the resulting plasma samples were subjected immediately to immunoelectrophoresis and polyacrylamide gel DISC electrophoresis.

Agarose Gel Immunoelectrophoresis. Plasma samples were loaded on the agarose gel and were incubated for 24 hours with anti-human whole serum polyclonal antibody (The Binding Site, Birmingham, UK), anti-human apolipoprotein (Apo)-A, and anti-human Apo-B polyclonal antibodies (The Binding Site), to identify all classes of plasma proteins, high-density lipoprotein (HDL), and low-density lipoprotein (LDL), respectively. Precipitin bands were observed using the ICG fundus video system (TRC-50IA; Topcon, Tokyo, Japan) and with an immunoviewer (Joko, Tokyo, Japan). The results were compared among these plates with different antibodies.

Polyacrylamide Gel DISC Electrophoresis. Using an LDL system (Lipoprint; Quantimetrix, Hawthorne, CA), plasma samples were separated in a gel tube directly into chylomicron, very low-density lipoprotein (VLDL), LDL, and HDL, from the top of the tube to the bottom, in order of molecular size and net charge. Samples were prestained

From the 1Department of Ophthalmology, 21st Department of Biochemistry, 4th Department of Internal Medicine, Saitama Medical School, Japan; and the 3Department of Ophthalmology, Wycombe Hospital. Supported by a Grant-in-Aid of Scientific Research (C) of The Ministry of Education, Science, Sports and Culture, Japan. Submitted for publication April 23, 1997; revised September 29, 1997; accepted January 27, 1998. Proprietary interest category: N. Reprint requests: Shin Yoneya, Department of Ophthalmology, Saitama Medical School, Morohongo 38, Moroyama, Iruma, Saitama, 350–04, Japan.
TABLE 1. Components of Plasma Lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Chylomicron</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL2</th>
<th>HDL3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific gravity</td>
<td>&lt;0.96</td>
<td>0.96–1.006</td>
<td>1.006–1.019</td>
<td>1.019–1.063</td>
<td>1.063–1.125</td>
<td>1.125–1.21</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>1–10 × 10^5</td>
<td>5–100 × 10^5</td>
<td>3–4 × 10^6</td>
<td>2–3 × 10^6</td>
<td>18–36 × 10^6</td>
<td>15–18 × 10^6</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>80–1000</td>
<td>30–75</td>
<td>22–30</td>
<td>19–22</td>
<td>8.5–10</td>
<td>7.0–8.5</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>Neutral</td>
<td>Pre-β</td>
<td>Intermediate</td>
<td>β</td>
<td>α</td>
<td>α</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>85</td>
<td>55</td>
<td>24</td>
<td>10</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Ch-F</td>
<td>5</td>
<td>12</td>
<td>33</td>
<td>37</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>Ch-E</td>
<td>2</td>
<td>7</td>
<td>13</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>PL</td>
<td>6</td>
<td>18</td>
<td>12</td>
<td>22</td>
<td>29</td>
<td>23</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>2</td>
<td>8</td>
<td>18</td>
<td>23</td>
<td>42</td>
<td>58</td>
</tr>
</tbody>
</table>

* Values in parentheses are percentages.

RESULTS

Binding of Indocyanine Green to Lipid Moieties

Plasma lipoprotein is a molecular complex composed of apolipoproteins and lipids. The surface structure of lipoprotein consists of several apolipoproteins, phospholipids, and free cholesterol. Esterified cholesterol and triacylglycerol are within the complex. HDL and LDL have different kinds of apolipoproteins but have the same lipid components (Table 1). Therefore, the lipid portion is assumed to be the binding site. Consequently, we studied the affinities between ICG and lipids. Four different types of lipids were extracted and separated from human plasma by thin-layer chromatography. After removing the organic solvent completely, each lipid was emulsified with a sonicator and mixed with 0.5 μg/ml ICG. Each emulsion was observed with the ICG fundus video system and compared with aqueous ICG at the same concentration as a control.

Polyacrylamide Gel Disc Electrophoresis. With this method, a ladder pattern of Sudan black staining could be appreciated in the order of molecular size and net charge with chylomicron, VLDL, LDL, and HDL. When viewed with the ICG fundus video system, intense fluorescence was observed at the area corresponding to HDL. Fluorescence was moderate at the area of LDL (Fig. 5, right). No fluorescence was observed in either the chylomicron or the VLDL strips. These findings also suggested that ICG bound mainly to HDL and LDL in the blood.

Binding of Indocyanine Green to Lipid Moieties

As a control, aqueous ICG was observed to fluoresce faintly. When ICG was mixed with free cholesterol, esterified cholesterol, and triacylglycerol, the fluorescent intensities of the substrates were nearly the same as the control. Only the phospholipid solution mixed with ICG fluoresced intensely (Fig. 6), although all solutions contained the same concentration of the dye.

CONCLUSION

In the ophthalmic literature, the conventional view has been that ICG binds preferentially to albumin. However, Baker first demonstrated that ICG bound to alpha-1 lipoprotein and not to albumin. Her work also was corroborated by other authors. Since then new biochemical techniques have
become available, and we used agarose gel immunoelectrophoresis and polyacrylamide gel DISC electrophoresis to completely fractionate plasma samples into all unitary proteins or unitary lipoproteins. The distribution of ICG-binding plasma proteins could be detected directly and easily by observing them through the ICG fundus video system. Also, in our method, ICG was injected before blood sampling was performed, whereas in earlier methods ICG was mixed with plasma samples in vitro.

Our experimental results were summarized as follows: An agarose gel immunoelectrophoresis study showed that ICG bound to HDL and LDL. The ratio of free (nonbinding) ICG could not be measured in our experiment. However, we expected that it would be low, because intense ICG fluorescence could be detected only at HDL and LDL. Based on our findings, the binding site of ICG may be assumed to be the portion of lipids that are common molecular component in all plasma samples.
FIGURE 4. Agarose gel immunoelectrophoresis by anti-apoli-
ipoprotein-B observed with indocyanine green fundus video
system. Top: overview of the plate. Middle: close-up view of
the alpha (A) area. No fluorescent precipitin bands are de-
tected. Bottom: close-up view of the beta (B) area. Precipitin
band of low-density lipoprotein fluorescence (short arrow).

FIGURE 5. Polyacrylamide gel DISC electrophoresis. Left: stain-
ing with Sudan black. From top to bottom, chylomicron (C),
very low-density lipoprotein (V), low-density lipoprotein (LDL)
(L), and high-density lipoprotein (HDL) (H) were differenti-
ated. Right: Using the indocyanine green fundus video system,
intense fluorescence bands could be observed at the area cor-
responding to HDL (H) and moderate bands could be ob-
served at the area corresponding to LDL (L).

FIGURE 6. A solution of indocyanine green mixed with lipid
emulsions. From left to right are the control (cont), free chlo-
esterol (Ch-F), esterified cholesterol (Ch-E), phospholipid
(PL), and triacylglycerol (TG). Only the solution with PL fluo-
resces brightly.

lipoproteins. We also think that the hydrophilic portion
of the phospholipid is a candidate for that site. Molecular
arrangements of phospholipids are similar between plasma
lipoproteins and the emulsion (liposome) of phospholipids,
with the hydrophilic portion directed outside. The reason
that ICG fluorescence cannot be detected from chylomicron
and VLDL may be that relatively small amounts of phospho-
lipids are included in these lipoproteins,7 and that the
plasma concentrations of VLDL and chylomicron are lower
than those of HDL or LDL. Chylomicron is almost entirely
occupied by triacylglycerol and tends not to form complete
miscell as a result of its very large molecular size.

Consequently, we suspect that ICG binds intensely to HDL
and moderately to LDL in the blood, and that phospholipid is
the binding site. At present, we do not know whether LDL and
HDL would fluoresce with the same intensity after binding
with ICG. Further study may be required to evaluate the inten-
sity of ICG-bound lipoproteins quantitatively.

On the basis of these experimental results, we think
that most ICG fluorescence observed in the angiograms
demonstrate the hemodynamics of plasma lipoproteins, es-
specially that of HDL alone or in combination with LDL in the
bloodstream. The lower vascular or tissue permeabilities of
ICG also can be supported because lipoproteins have much
larger molecular sizes than other plasma proteins such as
albumin. Furthermore, the hyperfluorescence that was seen
in the late phase might exhibit phospholipid deposits similar
to some drusen or lipid accumulated in Bruch’s membrane
as a result of aging.8 Small amounts of free ICG also may
affect the result of the ICG angiogram and may diffuse into
the interstitial space and bind to the phospholipid deposit
by its high affinity. Thus, the hyperfluorescence in the late
phase may illustrate a sequel of accumulated plasma HDL
alone or in combination with LDL or phospholipid deposits
in ocular tissues. In conclusion, knowledge of the binding
properties of ICG can help us to interpret correctly the
angiographic findings and perhaps to understand the patho-
logic process at a biochemical level.
The Organophosphate Pesticide Chlorpyrifos Affects Form Deprivation Myopia

Andrew M. Geller, Ali A. Abdel-Rahman, Robert L. Peiffer, Mohamed B. Abou-Donia, and William K. Boyes

PURPOSE. The effects of the anti-cholinesterase organophosphate pesticide chlorpyrifos (CPF) on the refractive development of the eye were examined. Form deprivation was used to induce eye growth to address the previously reported relationship between organophosphate pesticide use and the incidence of myopia.

METHODS. Chickens, a well-established animal model for experimental myopia and organophosphate neurotoxicity, were dosed with chlorpyrifos (3 mg/kg per day, orally, from day 2 to day 9 after hatching) or corn oil vehicle (VEH) with or without monocular form deprivation (MFD) over the same period. The set of dependent measures included the refractive state of each eye measured using retinoscopy, axial dimensions determined with A-scan ultrasound, and intraocular pressure.

RESULTS. Dosing with CPF yielded an inhibition of 35% butyrylcholinesterase in plasma and 45% acetylcholinesterase in brain. MFD resulted in a significant degree of myopia in form-deprived eyes resulting from significant lengthening of the vitreal chamber of the eye. CPF significantly reduced the effect of MFD, resulting in less myopic eyes (mean refraction: VEH-MFD = −16.2 ± 2.3 diopters; CPF-MFD = −11.1 ± 1.8 diopters) with significantly shorter vitreal chambers. Nonoccluded eyes were, on average, slightly hyperopic. Treatment with CPF for 1 week in the absence of MFD led to no significant change in ocular dimensions or refraction relative to controls.


An epidemic of ocular toxicity reported in agricultural regions of Japan during the late 1950s, 1960s, and 1970s was attributed to heavy regional use of organophosphate insecticides. A high incidence of myopia was prominent among a variety of other visual symptoms and classical cholinergic signs and symptoms. Subsequent experimental studies in Japan reported degeneration of the retina, optic nerve, and extraocular muscles in addition to myopia. The Japanese reports are controversial, however, because similar cases of ocular toxicity have not been reported elsewhere, and many of the Japanese reports do not meet currently accepted standards of experimental design, methodology, and reporting of results. In support of the Japanese findings, retinal and optic nerve lesions have been seen after the feeding of organophosphate pesticides to rats in studies reported to the US Environmental Protection Agency by pesticide manufacturers. In addition, a variety of biochemical, neuropathologic, and electrophysiologic changes have been noted in the retina and optic nerve of adult rats treated with the organophosphate pesticide thionin. To date, we know of no investigations outside of the early Japanese reports that address the issue of myopia after developmental organophosphate pesticide exposure.

The study of experimental myopia has shown that the growth of the axial length of the eye is governed by visual input and that a cholinergic system is involved in the regulation of this process. Deprivation of patterned visual input during development results in an increased axial length of the eye (i.e., axial myopia). Treatment with the muscarinic blocking agents atropine or pirenzepine prevents the development of experimental axial myopia independent of effects on accommodation, implicating retinal processes. Areas of local pat-