results indicate that these liver functions are similar to control levels despite the addition of PCBs. Our results show that primary cultured hepatocytes mimic partially the intact liver in relation to some responses to PCBs. In isolated hepatocytes as well as *in vivo*, PCBs increased in cellular ascorbic acid level, aryl hydrocarbon hydroxylase and UDP-glucuronyl transferase activities. Thus, the present results may suggest that the metabolic changes *in vivo* concerning ascorbic acid induced by dietary PCBs were primarily related to the direct effects of PCBs *per se* on liver parenchymal cells.

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Immunoreactivity of endogenous digitalis-like factors

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Much evidence supports the view that the natural ligands of the digitalis receptor of the Na^+, K^+-ATPase may exist in the mammalian body. Such endogenous digitalis-like factors (EDLFs) may be physiological regulators of the enzyme and may play important roles in the regulation of sodium excretion and the pathophysiology of hypertension [1, 2]. Many investigators have chosen to use radioimmunoassay for digoxin to determine EDLFs on the assumption that an endogenous compound binding to a specific receptor may also bind to an antibody raised against the exogenous ligand [3]. However, this approach has many problems and has been a source of much controversy [4].

We have emphasized the digitalis-like biological activities to search for the EDLFs and were able to isolate two distinct EDLFs from human urine based on an inhibitory effect on [^{3}H]ouabain binding to intact human erythrocytes

[5–7]. In this study, we investigated whether the ouabain-displacing compounds we have purified are recognized by the antibodies toward digoxin and bufalin, which are specific Na^+,K^+-ATPase inhibitors of plant and toad origin, respectively. Further, we examined whether ouabain-displacing compounds are responsible for the inhibition of binding to anti-digoxin antibody.

Material and Methods

Preparation of two ouabain-displacing compounds. Approximately 120 L of human urine collected from normal male volunteers were processed as described previously [6, 7]. Human urine after filtration through a 0.45 μm membrane filter was applied to reverse-phase high performance liquid chromatography (D-ODS-5 column; YMC, Kyoto, Japan) and was fractionated with a gradient of acetonitrile in water (0–40%) over 80 min. One minute fractions were collected, freeze-dried and assayed for digitalis-like activity using the inhibitory effect on [^{3}H]ouabain binding to intact human erythrocytes. Polar ouabain-displacing compound-1 (ODC-1) and less polar ODC-2 were reproducibly eluted at 18 and 31% acetonitrile, respectively. Each ODC was separately purified on the same column under identical conditions three times and then used for experiments. One unit of ODC was defined as that amount to inhibit [^{3}H]ouabain binding by 50%.

Radioimmunoassay (RIA) for digoxin. RIA was mainly based on the method of Gruber *et al.* [8] using an anti-digoxin antibody (Miles, Elkhart, U.S.A.). We used 70 pg/tube [^{3}H]digoxin as a tracer. Incubation was carried out at 4° for 24 hr. Free digoxin was separated from antibody-bound digoxin by adsorption to dextran-coated charcoal. The sensitivity of the RIA was 15 pg/tube.

Enzyme immunoassay for bufalin. Antisera were raised in rabbits against bufalin conjugated with bovine serum albumin. Cross-reaction of the antiserum with digoxin was less than 0.2%. Anti-bufalin immunoglobulin G (IgG) was affinity-purified. Diluted anti-bufalin IgG (1:80000) (0.01 mL) and 0.1 mL of horse radish peroxidase (HRP)-labeled bufalin were added to 0.15 mL of sample solution and 0.15 mL of 0.05 M phosphate-buffered saline (pH 7.4). The mixture was allowed to stand at 4° for 18 hr. Free bufalin was separated from bound bufalin by polystyrene beads coated with second antibody toward rabbit IgG. The beads were preincubated with 0.6 mL of substrate solution containing 3,3',5,5'tetramethylbentidine and the reaction was started by addition of 0.2 mL of hydrogen peroxide. After 1 hr at 30°, the reaction was stopped by the addition of 2 M sulfuric acid and the absorbance was measured at 450 nm using a spectrophotometer.

Immunoprecipitation of ouabain-displacing compounds with anti-digoxin immunoglobulin G (IgG). Anti-digoxin IgGs were affinity purified from serum by Protein A Sepharose chromatography (Pharmacia). Anti-digoxin serum (6 mL) was loaded onto a Protein A Sepharose column that had been equilibrated with 0.1 M phosphate buffer (pH 7.4). The column was washed with phosphate buffer until the optical density at 280 nm ($O.D._{280}$) of the effluent was less than 0.1. IgGs were eluted in a 0.1 M acetic acid, pH 2–3, and fractions of 1 mL were collected and immediately neutralized with Tris base. The fractions containing greater than 0.1 $O.D._{280}$ were pooled, freeze-dried and stored at −20°.

Triplicate samples of ODCs were incubated with anti-digoxin IgG at 4 dilutions (1:5–100) and a buffer-only control, for 90 min at 25°. At no dilution, 1 mL of anti-digoxin IgG bound approximately 1 nmol digoxin. Equivalent dilutions of non-specific affinity-purified total rabbit IgG (Sigma) were also incubated with ODCs. A standardized suspension of *Staphylococcus aureus* cells (Pansorbin; Carbiochem), with a binding capacity of 2.1 mg IgG/mL, was added and the samples were incubated for an additional 5 min. IgG-complexed cells were removed by centrifugation and the supernatants were assayed by [^{3}H]ouabain radioreceptor assay with intact human red blood cells to determine the activity of ODCs.

Results and Discussion

As shown in Fig. 1A, ODC-2 at the concentrations between 0.05 and 12 units/mL had a prominent digoxin-like immunoreactivity and the dose–response curve of ODC-2 was almost parallel to that of digoxin. The IC_{50} value for the interaction of ODC-2 with the antiserum was around 0.5 units. In contrast, ODC-1 at the same concentrations showed a weak interaction with anti-digoxin antibody (Fig. 1A). High concentrations of ODCs (5–12 units/ml) showed cross-reactivities with anti-bufalin antibody in a dose-related manner. Again, effects of ODC-2 were more marked than those of ODC-1. However, none of these compounds inhibited binding by 50% or more (Fig. 1B).

Ouabain-displacing activity of ODC-2 was dose-dependently reduced by preincubation with anti-digoxin IgG and totally disappeared at two dilutions (1:5–10). Ouabain-displacing activity of ODC-1 was also significantly decreased by incubation with a large quantity of anti-digoxin IgG (Fig. 2). On the other hand, incubation with

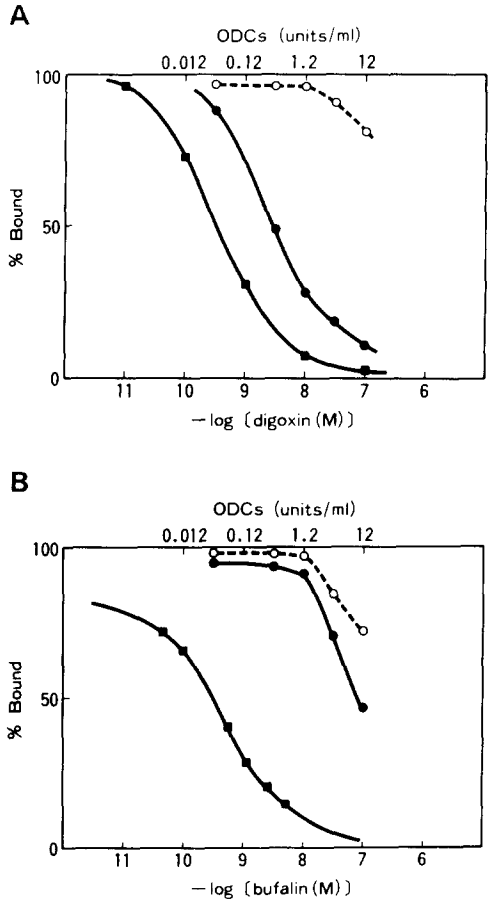

Fig. 1. Cross-reactivity of ouabain-displacing compounds (ODCs) with anti-digoxin (A) and anti-bufalin (B) antibodies. Effects of ODCs at a series of concentrations (0.05–12 units/mL) on binding of [^{3}H]digoxin (A) and horseradish peroxidase-labeled bufalin (B) to respective polyclonal antibodies were determined according to the methods described in Materials and Methods. (A) ■, digoxin; ○, ODC-1; ●, ODC-2. (B) ■, bufalin; ○, ODC-1; ●, ODC-2.

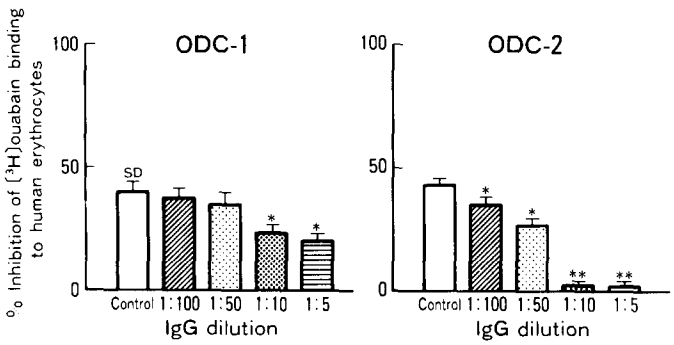

Fig. 2. Effects of anti-digoxin immunoglobulinG (IgG) on the activity of ouabain-displacing compounds (ODCs). Anti-digoxin IgGs were affinity-purified from serum by Protein A Sepharose column. Triplicate samples of ODCs were pretreated with anti-digoxin IgG at four dilutions. Total IgG was immunoprecipitated using Pansorbin cells and the supernatants were assayed for remaining activity of ODCs by standard [^{3}H]ouabain radioreceptor assay. *$P < 0.01$, **$P < 0.001$ as compared to the control.

equivalent dilutions of non-specific affinity-purified rabbit IgG has no effects on ouabain-displacing activity of either ODC (data not shown).

The results indicate that the two EDLFs based on the inhibitory effects on [^{3}H]ouabain binding to human erythrocytes may be recognized by highly specific anti-digoxin and anti-bufalin antibodies. However, it is noteworthy that the degree of cross-reaction markedly differ between two EDLFs. Because the interaction of polar ODC-1 with these antibodies is weak, it is possible that immunological methods alone may not be sensitive enough to detect the exact behaviour of ODC-1 [9]. Since the experiments described here involved the use of highly, but still partially, purified ODCs, it was critical to determine whether ODCs are responsible for the inhibition of binding to anti-digoxin antibody. Preincubation of crude ODCs with affinity-purified anti-digoxin IgG blocked the receptor competition. Thus, the ouabain binding-inhibitory activity seems to be caused by the same molecular species that cross-react with antibody toward digoxin.

In summary, highly specific antisera produced against cardenolide (digoxin) and bufodienolide (bufalin) may bind the endogenous ouabain-displacing compounds from human urine. However, it should be borne in mind that the degree of recognition by the antisera significantly differ between two ouabain-displacing compounds.

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