Enzymatic Removal of Bilirubin Toxicity by Bilirubin Oxidase *In Vitro* and Excretion of Degradation Products *In Vivo* (43119)

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Abstract. **The toxic effects of the degradation products of bilirubin that were formed by reaction with bilirubin oxidase were investigated with the C 1300 mouse neuroblastoma cell line by examining the following parameters: growth inhibition, morphologic characteristics, membrane transport, DNA synthesis, and protein synthesis. The addition of bilirubin to the cells resulted in definite cytotoxic effects on all of these parameters in a dose-dependent fashion; the addition of bilirubin oxidase reversed the toxic effects on the C 1300 cells *in vitro*. Furthermore, we found that most of these enzymatic degradation products of bilirubin were excreted by the kidney into the urine in a few hours after intravenous injection of the degradation products; in contrast, no intact bilirubin was excreted. Thus, these findings suggest that hyperbilirubinemia in newborn infants (kernicterus) may be prevented by administering polyethylene glycol-conjugated bilirubin oxidase, with a longer plasma half-life which has been reported previously to oxidize bilirubin to its nontoxic components in the bloodstream.** [P.S.E.B.M. 1990, Vol 195]

Bilirubin, the end product of heme catabolism, is generally regarded as a potentially cytotoxic agent, especially in newborn infants. Bilirubin encephalopathy (kernicterus) is usually considered to be caused by the entry of circulating free (albumin-unbound) unconjugated bilirubin into the cerebral tissue (1, 2); this effect has been reproduced in experimental animals (3–5). Bilirubin conjugation with glucuronic acid takes place in the liver and the process is impaired in the liver diseases. The conjugate is excreted into the bile duct.

We recently demonstrated a new tactic for the treatment of jaundice by using the highly specific enzyme bilirubin oxidase (BOX), which oxidized free bilirubin 100 times more than albumin-bound bilirubin. Modifying BOX with polyethylene glycol (PEG) resulted in the polymer-conjugated enzyme (PEG-BOX) having a remarkably prolonged plasma half-life and reduced antigenicity. It thus became possible to administer PEG-BOX intravenously (6). The final products of this enzymatic reaction have not been clarified completely, although the first products are considered to be biliverdin and diazo-negative polar agents containing dipyrrols (7–9).

For clinical application, it is essential to confirm that these final products are less toxic than bilirubin or are nontoxic to the major vital organs and that they will be excreted rapidly from the bloodstream. In this study, we clarified the characteristics of the degradation products *in vitro* and *in vivo*. To demonstrate the toxic effects of bilirubin in the live animal model is complicated, so we examined bilirubin cytotoxicity and elucidated detoxification by BOX *in vitro*.

Toxicity of bilirubin to cellular activities has been investigated by many workers: it uncouples oxidative phosphorylation (10–12); it depresses protein synthesis (13, 14); it inhibits DNA synthesis (15, 16); it decreases ATP content (14, 17); and it increases potassium leakage (14) in various tissue culture cells. It is also recognized to be potentially immunosuppresive and immunotoxic by its direct cytotoxic effects on human lymphocytes (18), granulocytes (18), and macrophages (19). Furthermore, bilirubin-mediated hemolysis resulting from bilirubin-erythrocyte membrane interaction has been reported (20, 21).

These cytotoxic effects of bilirubin are greater with

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a higher bilirubin to albumin molar ratio, a higher absolute concentration of bilirubin, and a longer exposure (15, 22). To our knowledge, no studies have been done on the cytotoxicity of the degradation products that result from treatment of bilirubin with BOX, and these results will be useful for the therapeutic rationales described previously (6). In this study, we examined the cytotoxicity of these breakdown products on the C 1300 neuroblastoma cell line. Urinary excretion of the degradation products was also elucidated in a rat model.

Materials and Methods

Reagents. Crystalline bilirubin was obtained from Sigma Chemical Co., St Louis, MO. Bilirubin oxidase (BOX), 3.5 units/mg, was obtained from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. [^{3}H]Thymidine (6.7 Ci/mmol) and [^{3}H]leucine (130 Ci/mmol) were from ICN Radiochemicals, California, fluorescein diacetate (FDA) was obtained from Dojin Chemical Co. Ltd., Kumamoto, Japan, 20% mannitol solution was from Nikken Kagaku Co. Ltd., Tokyo, Japan, and other chemicals were from commercial sources. The bilirubin solution was prepared according to the methods described by Sugita *et al.* (23, 24), with a slight modification; bilirubin, used as unconjugated form, was dissolved in 0.1 *N* NaOH at various specific concentrations and then was passed through a 0.2-μm pore filter (Corning) for sterilization immediately before use.

Preparation of Bilirubin. Culture medium containing bilirubin was prepared as follows: bilirubin was dissolved in 0.1 *N* NaOH at various concentrations, and 10 μl of the solution were added to 2 ml of RPMI 1640 medium with 10% heat-inactivated fetal calf serum (FCS) with or without BOX (final concentration, 50 μg/ml). The final bilirubin concentration was in the range of 0–2.5 mg/dl and the pH was 7.2–7.4. All reactions were conducted in the dark to avoid photooxidation.

Cell Culture. C 1300 mouse neuroblastoma cells were maintained and grown in monolayer culture in RPMI 1640 medium supplemented with 10% FCS at 37°C under a humidified atmosphere containing 5% CO_2. Single-cell suspensions were obtained by treatment with 0.05% trypsin-0.02% EDTA at 37°C. These suspensions were used for cell counting or inoculation.

Assay of Growth Inhibition. Growth inhibitory activity was measured according to the method of Notter and Kendig (13). Briefly, C 1300 mouse neuroblastoma cells were suspended in RPMI 1640 medium containing 10% FCS and were plated in 16-mm wells (Falcon 24-well plates) at a concentration of 1.6×10^5 cells/well in 1 ml of culture medium. After overnight incubation at 37°C, samples of medium containing bilirubin solution as described above, bilirubin plus BOX, and 0.1 *N* NaOH as control were added to the cells. At given intervals, control and treated cells were trypsinized and counted with a hemocytometer; the medium containing bilirubin was removed by suction and the cells were treated with 0.05% trypsin-0.02% EDTA. Trypsin activity was then neutralized by adding RPMI 1640 medium containing 10% FCS. Cell number and viability were determined by the trypan blue dye exclusion method using a hemocytometer. C 1300 mouse neuroblastoma cells were quite easy to detach from the bottom of the well, so we did not replace the medium by suction but instead added the medium containing specific experimental factors.

Fluorescence Microscopy. This procedure is used to evaluate the membrane transport by using FDA as a probe, which becomes fluorescent after uptake into the cell; the method is described in detail elsewhere (25,

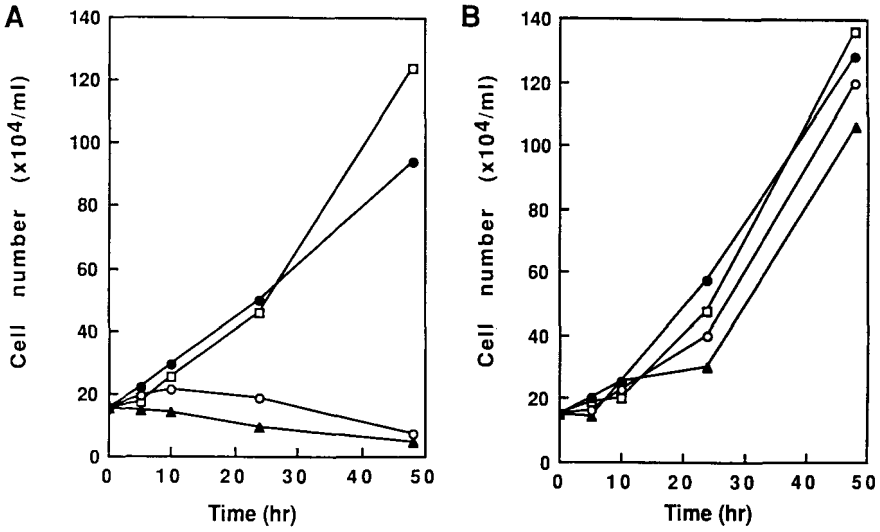

Figure 1. Growth inhibition of C 1300 neuroblastoma cells by bilirubin (A) and its degradation products formed by reaction with BOX (B). The concentrations of bilirubin were (□), 0, (●) 0.625, (○) 1.25, and (▲) 2.5 mg/dl and BOX was 50 μg/ml (0.175 units/ml) in B.

26). Under the conditions described above cells were washed twice with phosphate-buffered saline, and 0.05 ml of FDA (25 μM in phosphate-buffered saline) solution was added to each well, after which fluorescence microscopy was done at room temperature.

DNA Synthesis Assay. The effect of bilirubin on DNA synthesis was quantified as incorporation of [^{3}H] thymidine into DNA according to the method of Schiff *et al.* (15). C 1300 cells were seeded at a concentration of 1×10^4 cells/well in a 96-well dish and were allowed to grow overnight in RPMI 1640 medium containing 10% FCS. Then, to the medium was added a solution (experimental media) containing bilirubin or bilirubin treated with BOX (50 μg/ml); this mixture was incubated for specified intervals. The cells were then pulse labeled with [^{3}H]thymidine, 0.25 μCi/dish for 3 hr. Triplicate analyses were performed for each experimental point. At the end of the incubation period, the cells were collected onto glass microfiber mats (Lab Science, Tokyo, Japan), and thymidine incorporation was determined by liquid scintillation spectrometry.

Protein Synthesis Assay. The effect of bilirubin on protein synthesis was quantified by incorporation of [^{3}H]leucine into cultured mouse neuroblastoma C 1300 cells according to the method of Notter and Kendig (13). C 1300 cells were plated at 3.6×10^5/35-mm well dish. After overnight incubation, the cells were treated with the experimental medium as described above for

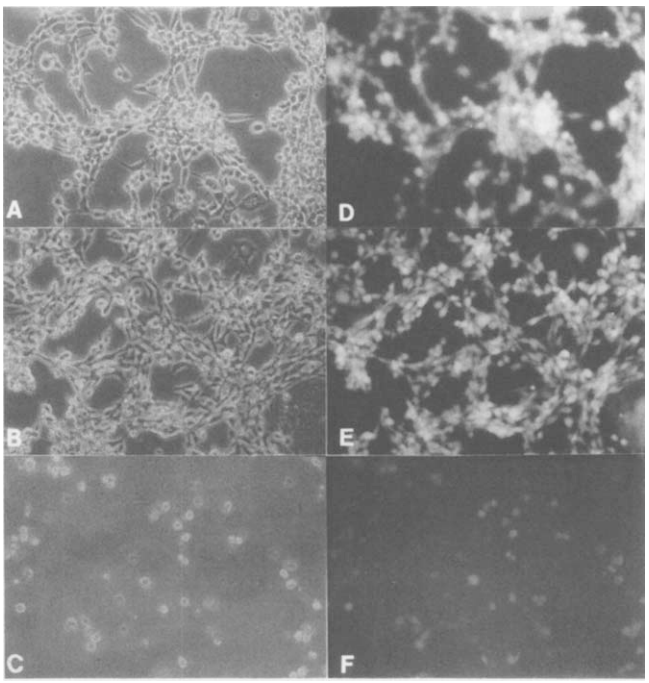

Figure 2. Photomicrographs of C 1300 cells treated for 24 hr with bilirubin or with the degradation products produced by BOX under phase contrast (A–C) and fluorescence (D–F) conditions. (A and D) Control (no bilirubin or its degradation products added). (B and E) Bilirubin, 2.5 mg/dl plus BOX, 50 μg/ml. (C and F) Bilirubin, 2.5 mg/dl with no BOX (original magnification ×200).

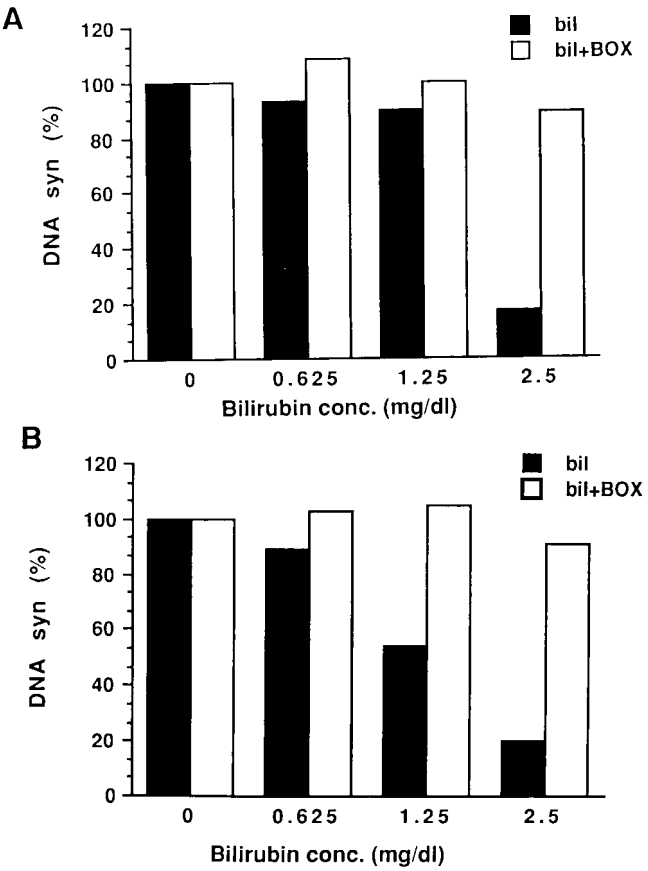

Figure 3. Effect of bilirubin or degradation products on DNA synthesis in C 1300 cells. The cells were incubated with various concentrations of bilirubin (■) or degradation products (□) for 1 hr (A) and 6 hr (B) and were treated with [^{3}H]thymidine (0.25 μCi/well) for 3 hr. Each value represents a mean of triplicate assays (percentage to control).

3 hr, and then 0.1 ml of [^{3}H]leucine (5 μCi/ml) was added to half of the cultures for 3 hr. The other cultures were exposed to experimental medium for 6 hr and were used to measure total protein according to the method of Lowry *et al.* (27), with bovine serum albumin as the standard.

Incorporation of [^{3}H]leucine was determined by precipitating protein with cold 10% trichloroacetic acid. The cells were collected onto glass microfiber filter mats (GF/C2.5 cm; Whatman Ltd., Maidstone, England), and leucine incorporation was determined by liquid scintillation counting. The results were expressed as average counts of duplicate samples.

Excretion of the Degradation Products of BOX-Treated Bilirubin into Urine. Male Wistar rats each weighing about 300 g were obtained from SLC (Shizuoka, Japan) and fed with regular feeds *ad libitum* (F-2; Funabashi Farm, K.K., Funabashi, Japan) until use. With the rats under general anesthesia (40 mg/kg of pentobarbital sodium given intraperitoneally), a polyethylene tube (0.8 mm in diameter) was inserted into the urinary bladder via the urinary tract for collecting urine samples at specific intervals. Rats were injected

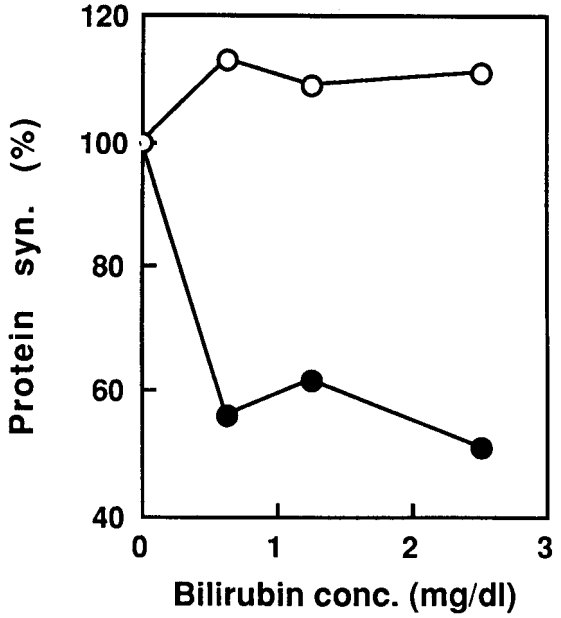

Figure 4. Effect of bilirubin or its degradation products on protein synthesis of C 1300 cells. The cells were incubated for 3 hr with bilirubin (●) or its degradation products (○) and then were pulse labeled with [^{3}H]leucine for another 3 hr. The points are mean values from two wells.

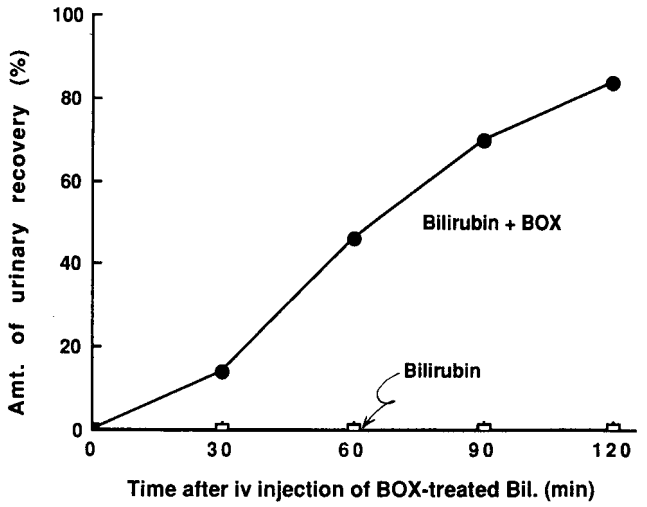

Figure 5. Urinary excretion of free bilirubin or BOX-treated bilirubin in the rat. Wistar rats were injected intravenously with free bilirubin (3 mg/rat) (□) or BOX-treated bilirubin (3 mg of bilirubin plus12 mg of BOX/rat) (●) and urine samples were taken via catheter of the urinary bladder every 30 min for 2 hr. The concentrations of bilirubin and degradation products in the urine were measured by absorbance at 440 and 320 nm, respectively, and the values represent a percentage of the amount of urinary recovery against the injected dose. Mannitol (20% solution, 3 ml) was administered intravenously in both groups to facilitate urination (see text). This figure shows data of duplicate experiments.

intravenously with 3 ml of 20% mannitol solution to facilitate urinary output; 10 min later 3 ml of bilirubin (1 mg/ml in a 0.2 *M* borate buffer, pH 8.0) or the degradation products (1 mg of bilirubin plus 4 mg of BOX in the same borate buffer) were injected via the tail vein. The urine samples in plastic conical centrifuge tubes (Eppendorf, Hamburg, West Germany) were centrifuged at 9500*g* for a few seconds in an Ultrafuge (model 109-04; Kubota Co. Ltd., Tokyo, Japan). The supernatant was diluted with 0.2 *M* borate buffer, and the absorbance was measured spectrophotometrically (bilirubin at 440 nm; degradation products at 320 nm, usually after about a 10-fold dilution). The standard curve was prepared for each rat by dilution of bilirubin or the degradation products with pretreated urine.

Results

Growth Inhibition. The effects of various concentrations of bilirubin on growth of C 1300 neuroblastoma cells are shown in Figure 1. Growth of the cells was affected by the exposure to bilirubin in a dose-dependent fashion (Fig. 1A); growth of cells treated with bilirubin was inhibited significantly more at higher concentrations and longer exposure times. After 24 hr, the cells were swollen and detached from the surface of the culture dish (Fig. 2C). In contrast, when bilirubin and BOX were added simultaneously to the medium, the growth inhibitory effects were reduced markedly, even at the highest concentration of bilirubin (2.5 mg/dl) (Figs. 1B and 2B), and the cells remained small, elongated elements that crowded the growth surface (Fig. 2B), similar to untreated cells (Fig. 2A).

To confirm this cytotoxic effect obtained by the trypan blue dye exclusion test, we examined FDA uptake by fluorescence microscopy. As shown in Fig. 2D–F, FDA was rapidly taken up by the attached cells, especially undamaged cells compared with damaged ones.

Effect of Bilirubin on DNA and Protein Syntheses and Reversal by BOX Treatment. Figure 3 shows the toxic effects of bilirubin on [^{3}H]thymidine incorporation into C 1300 cells and reduction of these effects by BOX treatment. The toxicity of bilirubin to [^{3}H]thymidine incorporation was rapid and marked compared with the effects on growth and morphology. At the higher concentration of bilirubin and the longer treatment time, more suppression of [^{3}H]thymidine incorporation was observed. However, BOX treatment, at 0.125 units/ml, normalized [^{3}H]thymidine incorporation to the level of control. When the FCS concentration was reduced from 10% to 7.5% or 5.0%, the suppression of DNA synthesis increased markedly.

When the cells were cultured for 6 hr, protein synthesis was inhibited in a dose-dependent manner, similar to the situation for cell growth and DNA synthesis (Fig. 4). By adding BOX, protein synthesis was restored at any dose of bilirubin to the level of the bilirubin-untreated control.

Excretion in the Urine of the Degradation Products of Bilirubin Produced by BOX. Urine samples were collected every 30 min for 2 hr after injection of

bilirubin or the degradation products. The excretion of the final products in urine is summarized in Figure 5. About 80% of the degradation products were excreted in the urine in 2 hr; in contrast, free bilirubin was not excreted at all in the urine.

Discussion

We have recently developed a new method of treatment of jaundice by using an enzyme, BOX; large amounts of which can be obtained from the microorganism *Myrothecium verrucaria* MT-1. The enzyme possesses a specific activity for oxidative degradation of bilirubin, and this activity was retained even after conjugation with polymer (6). Our next step toward clinical application was to confirm the elimination of toxicity of bilirubin and the urinary excretion of the degradation products of bilirubin by the use of this enzyme. In this study, we investigated the cytotoxicity of bilirubin and its BOX-treated products to a neural cell line by studying several parameters. Bilirubin had toxic effects on C 1300 neuroblastoma cells in a concentration-dependent fashion, as expected (13, 15). On the other hand, the toxic effects were nullified by the addition of BOX. The breakdown products are quite hydrophilic, like biliverdine, which may be less toxic to cell membranes than lipophilic bilirubin. There may also be less internalization of bilirubin (3) or less interaction (perturbation) with the cell membranes by reduced lipophilicity (28).

It is well known that serum proteins bind to unconjugated bilirubin and reduce the toxic effect of bilirubin on tissues *in vivo* and cells *in vitro*. In our experiments, when the concentration of FCS was reduced from 10 to 7.5%, and then to 5.0%, the toxic effects of bilirubin became markedly intensified (data not shown). This observation is similar to the clinical manifestation in neonatal or premature kernicterus, in which the plasma albumin concentration is below 3%. These results confirmed that the toxicity was related to the concentration of free bilirubin rather than protein-bound bilirubin. When higher concentrations of bilirubin were used with a low percentage of FCS in long-term incubation, some bilirubin precipitates were formed in the medium. When such precipitation occurred, the toxicity of bilirubin seemed to be reduced, as Lie and Brantlid (22) reported. Schiff *et al.* (15) reported that pH, oxidation, and uptake into the cell influenced the concentration of bilirubin in the media during the course of experiments with cells.

Recently, the possible cause of neonatal jaundice (kernicterus), free unconjugated bilirubin at a level higher than 0.5 mg/dl, has been shown to be an important parameter clinically (29). Bilirubin value has been estimated by use of BOX in a clinical laboratory (30, 31). It was also known that BOX oxidized free unconjugated bilirubin 100–200 times more than albumin-bound bilirubin *in vitro*. Thus, BOX may be able to reduce the toxicity of free unconjugated bilirubin and salvage neonatal jaundiced patients.

Bilirubin is generally metabolized by the liver and excreted into the bile. In the case of obstructive jaundice, conjugated free bilirubin, but not unconjugated free bilirubin, is excreted by the kidney (32). In this study, we investigated the renal excretion of unconjugated bilirubin and the oxidized products, and we confirmed that unconjugated bilirubin is not excreted by the kidney. On the other hand, soon after intravenous injection of bilirubin that had been treated with BOX, the color of urine changed to brown, the same color of the end products. This result indicates that the end products are more water soluble and thus could be excreted by the kidney.

These results of detoxification of bilirubin and excretion of the end products of BOX-treated bilirubin indicate the possible clinical application of administering PEG-BOX for the treatment of neonatal jaundice. A different approach reported by Berglund (33, 34), in which tin-protoporphyrin was used to inhibit heme oxygenase and thus suppress production of bilirubin, can be used in conjunction with the present method in order to enhance the therapeutic efficacy.

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