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A New Tactic for the Treatment of Jaundice: An Injectable Polymer-Conjugated Bilirubin Oxidase (42747)

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Abstract. Bilirubin oxidase (BOX) derived from *Myrothecium verrucaria* was modified with polyethylene glycol (PEG). When the conjugated PEG-BOX was given intravenously to rats, its plasma half-life was 20 times longer than that of native BOX. In our preliminary investigations with experimentally jaundiced rats, the plasma bilirubin level dropped to normal after only one injection, and the low bilirubin level could be maintained for 12–48 hr; native BOX had a transitory suppressive effect that lasted only a few hours. The antigenicity of PEG-BOX was greatly reduced as expected. PEG-BOX appears to have potential value for the treatment of hyperbilirubinemia observed in such diseases as fulminant hepatitis and neonatal bilirubin encephalopathy.

Hyperbilirubinemia is associated with various liver disorders and hemolytic jaundice. Bilirubin, the end product of heme catabolism and lipid-soluble waste product, is generally regarded as a potentially cytotoxic agent that must be excreted. Because it affects all cellular activity—it uncouples oxidative phosphorylation in mitochondria (1)—its removal from the bloodstream is vitally important for patients with such diseases. Various methods are used for this purpose; plasma exchange, steroid therapy, and phototherapy have been advocated for severe jaundice, but none has proven to have therapeutic value as a first choice regimen.

Here we demonstrate a new method for treating jaundice by using the highly specific enzyme, bilirubin oxidase (BOX; M_r 50,000; EC 1.3.3.5), which is derived from the microorganism *Myrothecium verrucaria* MT-1 (2–6). Lavin and colleagues reported a treatment for severe neonatal jaundice with an immobilized BOX column system (7, 8). However, the column system has many inconveniences, such as physical limitations, clotting problems, and infections. We attempted to treat jaundice by making the BOX an injectable form with polymer conjugation.

In the past few decades the use of chemical modification of proteins (9, 10), particularly of enzymes, has grown. We and others have successfully synthesized a number of polymer-conjugated protein drugs, such as *smancs* (9–12), polyethylene glycol (PEG)-L-asparaginase (13–15), PEG-adenosine deaminase (ADA) (16), and PEG-interleukin 2 (17). We have now modified BOX with PEG to increase its plasma half-life, to diminish its immunogenicity, and to make it injectable. In the present paper we describe the possibility of the use of BOX as an injectable protein drug and problems associated with it.

Materials and Methods. BOX (3.5 units/mg) was obtained from Amano Pharmaceutical Co., Ltd., Nagoya, Japan. Diethylenetriaminepentaacetic acid (DTPA) anhydrate, was from Dojin Chemical Laboratories, Kumamoto, Japan. Radioactive $^{51}CrCl_3$ was from ICN Radiochemicals, California; and other chemicals were obtained from commercial sources.

Animals. Male Wistar rats, weighing about 200 g, and Gunn rats, weighing about 370 g, were used. To obtain the obstructive jaundiced rat model, the common bile ducts of Wistar rats were ligated and divided under ether anesthesia 3 days prior to the experiment. Gunn rats, which are congenitally icteric, were used as the constitutional jaundice model (18).

Preparation of PEG-BOX. BOX was conjugated with activated PEG (14, 15, 19, 20) through the use of putrescine (1,4-diaminobutane) (see Fig. 1). Native BOX (600 mg) was reacted with 1.86 g of putrescine by

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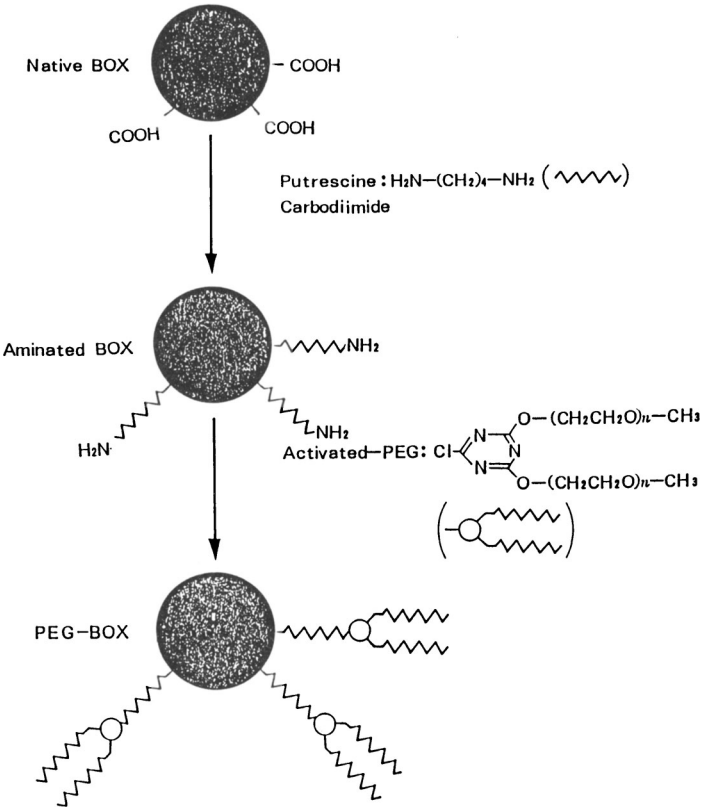

FIG. 1. Scheme of attachment of polyethylene glycol to BOX.

using 2.21 g of water-soluble carbodiimide·HCl (Dojin Chemical, Kumamoto, Japan) in 85 ml of 0.02 *M* sodium phosphate buffer, pH 7. The reaction was continued for 12 hr at 4°C and the conjugate was applied to a column of Sephadex G-25 (4.5 × 60 cm). After lyophilization of the void fraction, 30 mg of modified BOX was reacted overnight with 900 mg of activated PEG at 4°C, then dialyzed against 0.01 *M* phosphate-buffered 0.15 *M* saline (PBS) for 24 hr, and concentrated by Amicon ultrafiltration YM10 (Bedford, MA; cutoff M_r 10,000). The activity of PEG-BOX was about 30% that of the native BOX, and the apparent M_r of PEG-BOX was 110,000, determined by polyacrylamide gel electrophoresis in the presense of 0.1% sodium dodecyl sulfate (SDS).

Measurement of plasma BOX activity and plasma clearance in vivo. Rats were given 3.5 units of native BOX or PEG-BOX via the tail vein. At specific intervals, a 20-μl aliquot of blood was taken from the tail vein or the tail artery. The blood was added to 180 μl of PBS and was centrifuged at 9500*g* for a few seconds in an Ultrafuge (Kubota Ltd., Model 109-04, Tokyo, Japan). Then, 100 μl of supernatant was reacted at 37°C with 1.0 ml of substrate solution composed of 2.0 mg of bilirubin dissolved in 100 ml of 0.2 *M* Tris–HCl buffer at pH 8.4 in 9.0 m*M* SDS. Enzyme activity was determined as the decrease in absorbance at 440 nm for 1 min. A standard curve was obtained by using native BOX at different concentrations.

Treatment of jaundice with BOX in icteric rats; decrease in plasma bilirubin. Native BOX or PEG-BOX (70 units in 3 ml) was injected into the tail vein of icteric rats. Blood samples, 0.5–0.8 ml, were collected at varying time intervals by cardiac or tail artery puncture with a heparin-coated syringe fitted with a 26-gauge needle; samples were

immediately centrifuged at 9500*g* for 2–3 sec in an Ultrafuge (Kubota, Tokyo). The supernatant of each sample was frozen at −20°C until use but 0.1 ml was taken for bilirubin quantification by the standard alkaline azobilirubin method. This assay was performed immediately to avoid further enzyme reaction.

Tissue distribution of ^{51}Cr-labeled native BOX and PEG-BOX in rats. The radioactive BOXs were prepared by the method essentially described by Hnatowich *et al.* (21) and by us (22), which utilizes the bifunctional chelating agent DTPA, and the DTPA-tagged proteins were chelated with radioactive chromium (^{51}Cr) by using $^{51}CrCl_3$. The radiolabeled BOX (2×10^5 cpm in 1 ml) was injected into the tail vein of rats. The rats were killed under ether anesthesia at 48 hr after intravenous injection of BOXs, various tissues were removed, and specific radioactivity was determined. The radioactivity of the tissues was then counted by an auto-γ-counter (Packard, Model 5130, Downers Grove, IL). Mean values of four rats were used.

Immunogenicity. New Zealand White rabbits were immunized by intradermal administration as usual once every 2 weeks with 1.0 unit of native BOX or PEG-BOX together with Freund's complete adjuvant. Ten days after the last immunization, blood was withdrawn. Then Ouchterlony's immunodiffusion method was used and evaluated after 24 hr (23). We examined the antigenicity of native BOX and PEG-BOX by measuring the inhibitory rate of enzyme activities with those antisera. We also tested the immunogenicity when BOX or PEG-BOX was injected intravenously without adjuvant.

Results. *Plasma clearance and tissue distribution of BOX.* The activity of native BOX rapidly decreased after intravenous injection, whereas that of PEG-BOX decreased more slowly and was detectable even 48 hr after injection. Plasma half-lives of native BOX and PEG-BOX were about 15 min and 5 hr, respectively. The area under the curve of the activity of PEG-BOX was 26 times greater than that of native BOX (Fig. 2). The rapid drop of activity of native BOX from the circulation may indicate that it is trapped in some organs more readily than PEG-BOX.

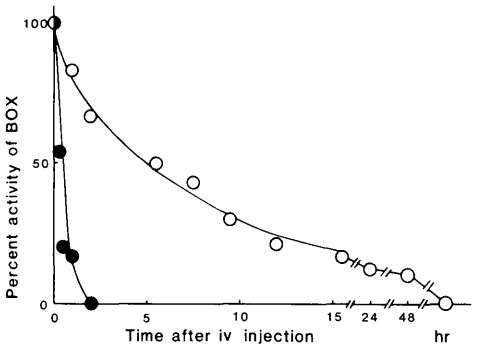

FIG. 2. Residual activities of intact bilirubin oxidase (●) and its polyethylene glycol conjugate (○) in rats. Data of each group represented by one rat (see text for details).

We then studied the accumulation of native BOX and PEG-BOX labeled with ^{51}Cr. The results are shown in Table I. Native BOX tended to be trapped more in the liver and kidney than PEG-BOX to a great extent. PEG-BOX accumulated more in the lung than native BOX although the total amount is much smaller.

Plasma bilirubin level after injection of BOX and its polymer conjugates. (A) Obstructive jaundice by ligation and dissection of the bile duct in rats. As shown in Fig. 3A, after intravenous injection of native BOX (70 units/rat), plasma bilirubin levels decreased immediately to about 17% of the pretreatment level (5–8 mg/dl), but began to increase rapidly and then 6–12 hr after injection became as high as the pretreatment level. In rats treated with PEG-BOX (70 units/rat) the bilirubin level dropped very rapidly and persisted at the low level for much longer than that of the native BOX.

(B) Congenital unconjugated hyperbilirubinemia model (Gunn rat). Plasma bilirubin levels were maintained at 6–8 mg/dl in 10-week-old Gunn rats without any treatment. Then native BOX and PEG-BOX were administered intravenously at 70 units/rat, respectively. Changes in plasma bilirubin levels are shown in Fig. 3B. PEG-BOX was more effective in both conjugated and unconjugated hyperbilirubinemia.

Immunogenicity. In Fig. 4 the results of the immunodiffusion method are shown; the PEG conjugate showed very weak antigeni-

TABLE I. ORGAN ACCUMULATION OF ^{51}Cr-LABELED NATIVE BOX AND PEG-BOX IN RATS[a]

Organ	Native BOX		PEG-BOX	
	cpm/g	% dose	cpm/g	% dose
Blood	110 ± 25[b]	0.05 ± 0.01	466 ± 89	0.23 ± 0.04
Liver	4340 ± 1470	2.29 ± 0.94	1360 ± 208	0.67 ± 0.11
Kidney	3437 ± 320	1.72 ± 0.16	1429 ± 243	0.71 ± 0.15
Spleen	2350 ± 1240	1.17 ± 0.62	2730 ± 235	1.36 ± 0.12
Lung	100 ± 51	0.04 ± 0.02	401 ± 10	0.02 ± 0.01

[a] Values are measured 48 hr after intravenous injection of ^{51}Cr-labeled native BOX or PEG-BOX into the tail vein of rats. Dose: 2×10^5 cpm/rat.
[b] Mean ± SD.

city against antisera raised in rabbits for native BOX with complete Freund's adjuvant. Native BOX showed a stronger precipitin line than the PEG conjugate. When native BOX and PEG-BOX were injected daily for 5 days into rats intravenously, only native BOX developed antibody (not shown). Table II summarizes the percentage inhibition of enzyme reaction between each BOX and respective antiserum. The results demonstrate the remarkable decrease in antigenicity of the PEG-BOX conjugate.

Discussion. The plasma bilirubin level is known to increase during various liver diseases and extensive hemolysis. The high level of bilirubin is generally regarded as cytotoxic, particularly in neural cells (24). Therefore, lowering the level of bilirubin is vital for such diseases as bilirubin encephalopathy. In this paper we report a new tactic for the treatment of jaundice by injecting a polymer-conjugated enzyme, PEG-BOX. Injection of PEG-BOX may be considerably superior to the extracorporeal systems, immobi-

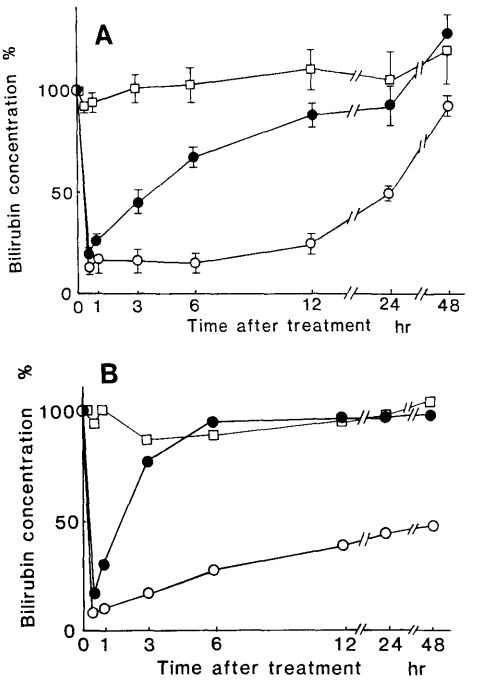

FIG. 3. Effect of intact bilirubin oxidase (●) and its polyethylene glycol conjugate (○) on plasma bilirubin level: (A) obstructive jaundiced (Wistar) rat (n = 3); each value represents the mean ± SD of three rats; (B) constitutional jaundiced (Gunn) rat (n = 1). Each rat from both groups was injected intravenously with 70 units of enzyme activity, respectively, at time zero. (□) Control group, injected with saline intravenously.

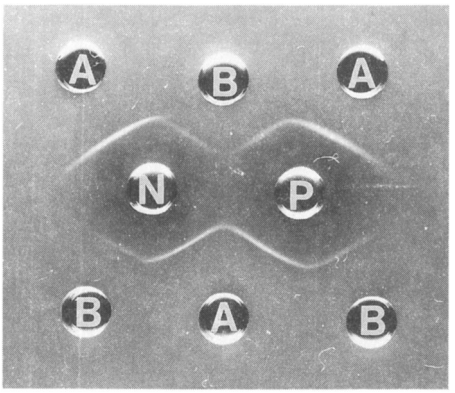

FIG. 4. Ouchterlony's agar gel diffusion. (N) Anti-native BOX antiserum; (P) anti-PEG-BOX antiserum. A and B are native BOX and PEG-BOX used as antigens, respectively. Each well contained the same amount of enzyme activity (0.035 unit per well). See text for details.

TABLE II. PERCENTAGE INHIBITION OF BOX ACTIVITY BY ANTISERUM AGAINST INTACT BOX OR PEG-BOX[a]

Antiserum raised in rabbit	Percentage inhibition of enzyme activity	
	Native BOX	PEG-BOX
Anti-native BOX	82.2	0
Anti-PEG-BOX	82.2	3.7

[a] Enzyme containing 0.35 unit/ml, 900 μl, was mixed with 100 μl of antiserum, and allowed to incubate at 37°C for 15 min. The enzyme activity was then measured as described under Materials and Methods.

lized BOX column (7, 8), activated charcoal column, membrane dialysis, or plasma exchange. These methods involve bleeding tendency because of extensive heparinization to prevent clotting, frequent possibilities of infection, physical confinement of patients, and time and expense. Despite these risks, in most cases these methods are not curative. Injectable PEG-BOX is much simpler than the presently available methods by any criteria.

The present data show that this tactic appears to be more effective therapeutically. The major reason for this is the prolonged plasma half-life of PEG-BOX (Fig. 2). We have demonstrated that a single intravenous injection of PEG-BOX can reduce the bilirubin value from 8 to 1.5 (normal range) in about 30 min, and low level is maintained for more than 12 hr. An additional injection resulted in normalization for about 48 hr (not shown). The reduced antigenicity of PEG-BOX was illustrated in Fig. 4 and Table II. However, the immunogenicity became nullified when PEG-BOX was given intravenously without Freund's adjuvant (not shown). Thus, a possible adverse effect, namely, immunological problems, appears to be controllable and clinical application of PEG-BOX seems feasible.

The presence of endogenous BOX *in vivo* has been shown: it was characterized by Billing and colleagues (25, 26) and Brodersen (27). In our limited studies of experimentally jaundiced rabbits and rats, the severity of the disease varied greatly: it was much more severe in rabbits than in rats. The reason may be correlated with the amount of endogenous BOX. It is, however, a feasible approach to utilize microbial BOX as an injectable drug for at least a limited time when patients are at a critical stage.

The detailed breakdown products of bilirubin by BOX are not known, although the first product of this enzymatic reaction was considered to be biliverdin, which is much less toxic than bilirubin (7, 8, 28); the next products were diazo-negative polar derivatives, which included dipyrroles and others (25–27). The final products of this oxidative reaction, however, need to be elucidated.

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