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THE LOSS OF *IN VIVO* ACTIVITY OF RECOMBINANT HUMAN ERYTHROPOIETIN BY ACTIVE OXYGEN SPECIES

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The effects of active oxygen species on the *in vivo* activity of recombinant human erythropoietin (EPO) treated by Fenton system, xanthine (X) plus xanthine oxidase (XO) system and hydrogen peroxide (H_2O_2) has been studied by means of counting the increase in number of hemolyser-resistant cells (HRCs) in EPO-injected mice. The results showed that both Fenton and X plus XO systems caused a significant reduction of the activity in proportion to the concentration of generated active oxygen species. Meanwhile, the treatment of EPO with H_2O_2 alone resulted in a relatively slight reduction of the activity. Electrophoretic studies on the structure of EPO revealed that its main protein band on sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) disappeared in proportion with the extent of exposure to active oxygen generating systems. Both Fenton and X plus XO systems caused a significant loss of fluorescence in the pyridylamino (PA-) sugar chain in proportion to the concentration of generated active oxygen species, and no degradation products in the sugar chain part of the PA-sugar chain were detected. This showed that aromatic groups in EPO were sensitive to attack by active oxygen species. These results provide evidence that hydroxyl radical and other active oxygen species have a potential to react with EPO, leading to a reduction of its *in vivo* activity.

KEY WORDS: Erythropoietin, *in vivo* activity, active oxygen species, Fenton system, xanthine oxidase, pyridylamino sugar chain.

INTRODUCTION

Erythropoietin is a sialoglycoprotein produced mainly in the kidney, and it is the main regulator of erythropoiesis. Its molecular weight is about 35 kDa, and carbohydrates form about 40% of its structure.[1] It has been used successfully to treat dialysis anemia patients.[2] The level of erythropoietin in the blood and in the bone marrow is maintained mainly by the peripheral O_2 tension.[3] In red blood cells, oxygen radicals are produced continuously during hemoglobin autooxidation, and this process is accelerated by exposure to various xenobiotic agents.[4,5]

Active oxygen species are produced in the body in normal and pathological conditions by most, if not all cells.[6] They can damage many kinds of biochemical com-

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ponents, e.g., DNA/RNA, proteins, carbohydrates, and unsaturated lipids. They are implicated in the development of pathophysiological conditions such as ageing, cancer, heart disease, muscular dystrophy, and many others.[7] In recent years, proteins have received increasing attention as targets for active oxygen species damages.[8] Active oxygen species are known to cause certain structural changes in various proteins *in vitro* and *in vivo*.[9] However, little is known about their effects on glycoproteins.

In this study we investigated the effects of oxygen radical-generating systems such as Fenton system, xanthine plus xanthine oxidase system, and of hydrogen peroxide on the *in vivo* activity of recombinant human erythropoietin (EPO) which is a typical glycoprotein.

MATERIAL AND METHODS

Reagents

EPO (specific activity 2.2×10^5 IU/mg of protein) produced from CHO (Chinese hamster ovary) cells was a kind gift from Kirin Brewery, Japan. Pyridylamino (PA) derivative of N-acetyllactosamine, tetraantenary (PA-sugar chain "011") was obtained from Takara Shuzo Co., Ltd. Xanthine oxidase (EC 1.1.3.22, Grade III from butter milk: XO), xanthine (X), catalase (EC 1.11.1.6, from bovine liver), superoxide dismutase (EC 1.15.1.1, from bovine erythrocytes: SOD), deferoxamine mesylate (DFO) were purchased from Sigma. D-mannitol and hydrogen peroxide were purchased from Wako Pure Chemical Industries, Japan.

Treatment with Active Oxygen Species

1) Fenton system: EPO (0.098 mg/ml) or PA-sugar chain (1.67 nmol/ml) was mixed in a reaction mixture of 0.24 mM EDTA, 0.16 mM ammonium ferrous sulfate and 0.000016–0.16 mM H_2O_2 in 30 mM NaCl (pH 7.4).

2) Hydrogen peroxide: EPO (0.098 mg/ml) or PA-sugar chain (1.67 nmol/ml) was allowed to react with 0.016–1.6 mM H_2O_2 in 30 mM NaCl (pH 7.4).

3) Xanthine oxidase plus xanthine system: EPO (0.098 mg/ml) or PA-sugar chain (1.67 nmol/ml) was mixed with XO (0.001–1.0 unit/ml) and X (0.33 mM) as a substrate, and in 75 mM phosphate buffer (pH 7.4). An additional study was performed in the presence of 1.0 mM EDTA and 1.0 mM DFO to the reaction mixture to exclude the possible effects of metals.

The above reaction mixtures were incubated at 37°C for 30 min. For *in vivo* bioassay, an aliquot of reaction mixture was diluted with a diluting solution consisting of 0.9% NaCl, 5% mannitol and 0.05% bovine serum albumin. For the PA-sugar study, aliquots of the reaction mixture were analysed by HPLC.

In vivo Bioassay

Female mice (ICR) 8 to 10 weeks old weighing 20–30 g were used for the *in vivo* bioassay. Before the experiments, the mice were given ordinary chow and tap water *ad libitum* and were kept for at least one week in a room with a controlled environment. Three mice were treated with each dose of EPO-treated and untreated with

oxygen radical-generating systems. EPO solutions were diluted to an appropriate concentration with a diluting solution consisting of 0.9% NaCl, 5% mannitol and 0.05% bovine serum albumin. Then 0.2 ml of EPO solution was injected subcutaneously into each mouse once a day for three consecutive days. On day 4, blood was collected from the heart with an anticoagulant (EDTA-2Na), and it was treated as described before.[10] The blood was diluted 500 times with celluent (Sysmex). Three drops (100 μl) of hemolysing reagent was added to 10 ml of diluted blood. After 5 min, HRCs (hemolyser-resistant cells) were counted using an automated microcell counter (Sysmex F-300) set on leukocyte mode. The count of HRCs, immature reticulocytes resistant to hemolysis, was taken as an indication of *in vivo* activity and it was used to compare the estimated activity of both EPO-treated and untreated with oxygen radical-generating systems.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Fenton and X plus XO systems treated EPO samples were analyzed on 12.5% SDS-polyacrylamide gels as described by Laemmli.[11] Samples were incubated in the presence of 2-mercaptoethanol prior to electrophoresis and the gels were stained with Coomassie brilliant blue R-250.

Quantitative Assay of PA-sugar Chain by HPLC

A quantitative assay of PA-sugar was carried out by HPLC using a Hitachi L-6200 Chromatograph and a Shim-pack CLC-ODS column (0.6 × 15 cm). PA-sugar chain was detected by fluorescence (Hitachi F-1050) using an excitation and an emission at a wavelength of 320 nm and 400 nm, respectively. Elution was performed at a flow rate of 1.0 ml/min at 35°C using the following three solvents: Solvent A was 10 mM sodium phosphate buffer (pH 3.8), solvent B consisted of solvent A plus 0.5% 1-butanol, and solvent C was solvent A containing 35% acetonitrile. Two different mobile phase conditions were used. Condition 1: The column was equilibrated with a mixture of solvent B and A, 30:70 by volume. After injection of a sample, the percentage of solvent B was increased to 100% for 30 min with a linear gradient and then solvent B for 10 min. The retention time of the standard PA-sugar chain was 32.4 min. Condition 2: The column was equilibrated with a mixture of solvent C and A, 5:95 v/v. After injection of a sample, the percentage of solvent C was increased to 100% for 60 min with a linear gradient. The retention time of the PA-sugar chain was 11.5 min.

RESULTS

1. Loss of the In Vivo Activity of EPO by Fenton System and by H_2O_2

The *in vivo* activity of EPO-treated with Fenton system was significantly reduced in H_2O_2 which was concentration-dependent as compared to that of untreated EPO (21.6 units/mouse/day) as shown in Figure 1. The reduction of EPO activity caused by Fenton system reached to 21.5, 46.2, and 83.4% of control value when the H_2O_2 concentration was 0.0016, 0.016, and 0.16 mM, respectively. The inac-

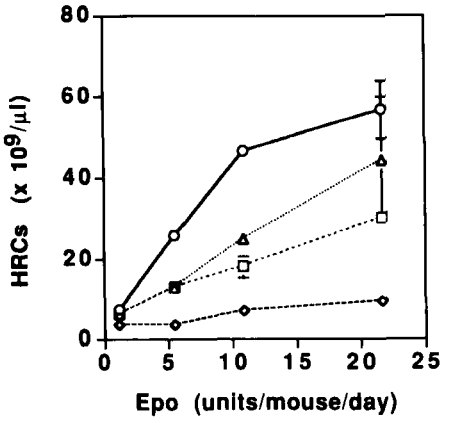

FIGURE 1 Effects of Fenton system on the *in vivo* activity of human recombinant erythropoietin (EPO). Intact EPO (0.098 mg/ml) was added to Fenton reaction mixture of the following composition: 0.24 mM EDTA, 0.16 mM ammonium ferrous sulfate and H_2O_2 of 0.0016 (Δ) , 0.016 (□), 0.16 (◇) mM in 30 mM NaCl (pH 7.4) at 37°C for 30 min. Untreated EPO (○). Values are mean ± S.E. of 3 mice.

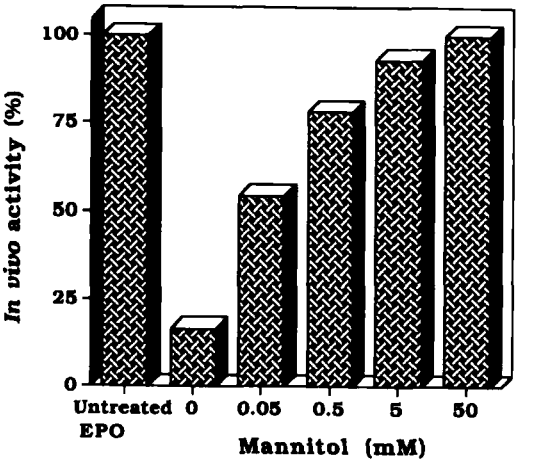

FIGURE 2 Effects of mannitol on EPO inactivation induced by Fenton system. Intact EPO was added to Fenton reaction mixture containing 0.16 mM H_2O_2 in the same buffer as Figure 1. Values are mean of 3 mice.

tivation of EPO caused by Fenton system was completely inhibited by mannitol (5 mM) as shown in Figure 2.

The treatment of EPO with H_2O_2 alone (up to 1.6 mM) induced a slight reduction of the activity as compared to its effect with Fenton system. The EPO inactivation caused by Fenton system was about two order magnitudes higher than that induced by H_2O_2 alone as shown in Figure 3.

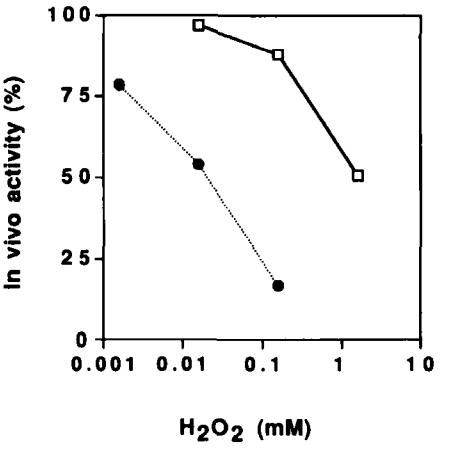

FIGURE 3 A comparison of effects of Fenton system (●) and H_2O_2 (□) on the *in vivo* activity of EPO. Values are mean of 3 mice.

2. *Loss of the In Vivo Activity of EPO by X plus XO system*

Figure 4 shows the effect of X plus XO system on the *in vivo* activity of EPO (21.6 units/mouse/day). Treatment of EPO with this system resulted in XO concentration-dependent reduction of its activity. The activity was completely lost when the concentration of XO reached 0.1 unit/ml. The loss of the activity induced by X plus XO system was slightly recovered by the addition of 1.0 mM DFO to the reaction mixture, but not by EDTA as shown in Figure 5. Catalase (0.02–20 units/ml), SOD

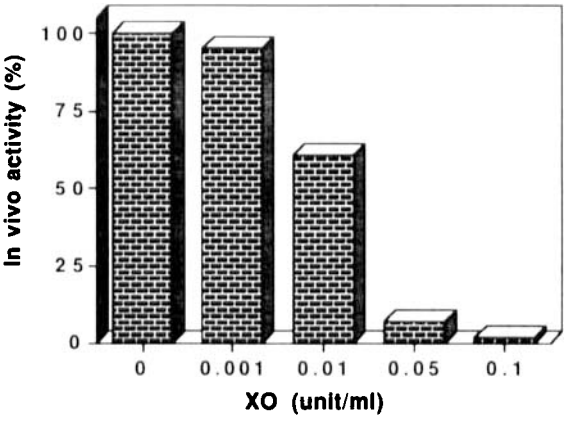

FIGURE 4 Effects of xanthine (X) plus xanthine oxidase (XO) system on the *in vivo* activity of EPO. Intact EPO (0.098 mg/ml) was added to a reaction mixture containing X (0.33 mM) and XO (0.001–0.1 unit/ml) in 75 mM phosphate buffer (pH 7.4) and incubated at 37°C for 30 min. Values are mean of 3 mice.

(0.025–33 units/ml), and mannitol (0.05–50 mM) did not exhibit any inhibitory effect on the inactivation of EPO caused by X plus XO system. The addition of 1 mM EDTA to SOD (33 units/ml) also did not show any degree of recovery from the inactivation (data not shown).

3. SDS-PAGE Following Exposure of EPO to Active Oxygen Species

As shown in Figure 6 the protein staining band of EPO at 35 kDa was decreased with the increase of H_2O_2 or XO concentration, respectively. The average loss of EPO band staining as performed by a densitometric analysis at 0.16 mM H_2O_2 and 0.1 unit/ml of XO was 20% and 50%, respectively. A dispersed pattern of low molecular weight protein fragmentation at 31–34 kDa was produced following the exposure of EPO to Fenton system, but not to X plus XO system.

4. Disappearance of PA-sugar Chain Peak in HPLC by Active Oxygen Species

Since tetraantenary sugar chain with fucose is the main component of both human urinary erythropoietin and a recombinant human erythropoietin, we selected a PA-sugar chain as a model compound. As shown in Figure 7 and 8, a remaining peak of PA-sugar chain in HPLC was decreased with the increase of H_2O_2 concentration in Fenton system or XO concentration in X plus XO system, respectively.

On the other hand, treatment with H_2O_2 alone induced a slight decrease of the HPLC peak (Figure 7). These phenomena were almost the same as the loss of *in vivo* activity of EPO by oxygen radical-generating systems.

In order to analyze the degradation products of active oxygen treated PA-sugar chain, two different HPLC mobile phases were used. We could not detect any degradation products of PA-sugar chain as shown in Figure 9. These results indicated that the active oxygen species selectively proceeded an attack on the aromatic ring of 2-aminopyridine and their fluorescence was decreased.

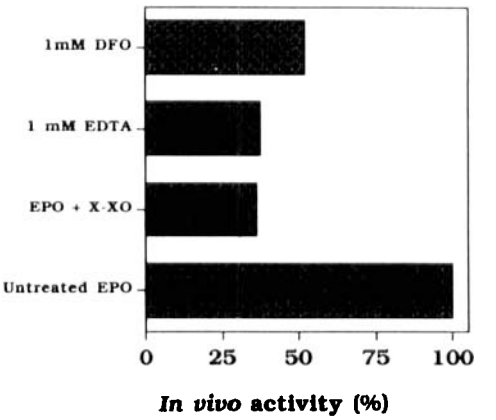

FIGURE 5 Effects of EDTA and DFO on loss of EPO *in vivo* activity following its exposure to X plus XO system. Intact EPO was added to a reaction mixture containing X plus XO (0.01 unit/ml) in the presence of 1 mM EDTA or 1 mM DFO in the same buffer as Figure 4. Values are mean of 3 mice.

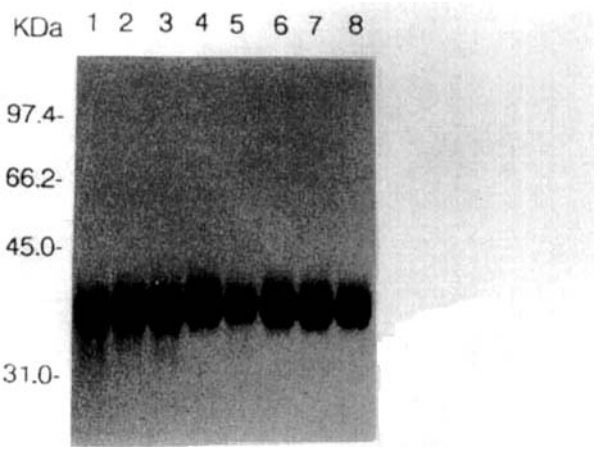

FIGURE 6 SDS-PAGE pattern following the exposure of EPO to active oxygen species. Lane 1-3 are Fenton system-treated EPO, incubated with H_2O_2 of 0.16, 0.016, and 0.0016 mM, respectively. Lane 5-7 are X plus XO system treated EPO, incubated with XO of 0.1, 0.01, and 0.001 unit/ml, respectively. Lane 4 and 8 were untreated EPO.

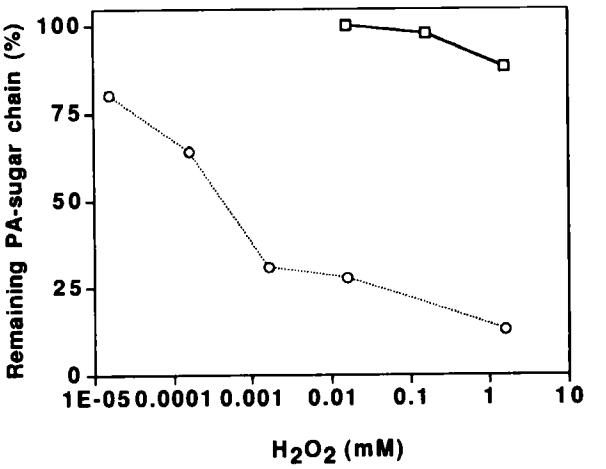

FIGURE 7 A comparison of loss of HPLC peak of PA-sugar chain following treatment with Fenton system (○) and H_2O_2 (□). PA-sugar chain (1.67 nmol/ml) was subjected to reaction conditions as those of Figure 1. Data show mean values obtained from two experiments.

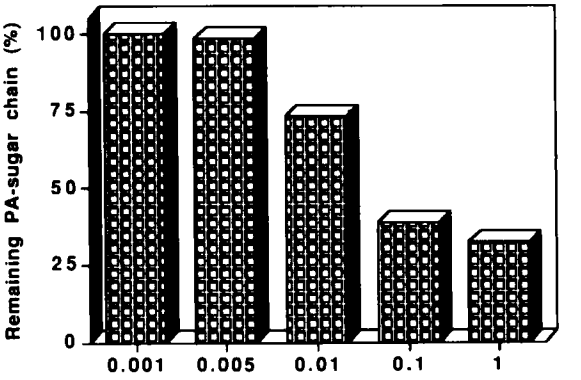

FIGURE 8 Loss of HPLC peak of PA-sugar chain following treatment with X plus XO system. PA-sugar chain (1.67 nmol/ml) was subjected to reaction conditions as those of Figure 3. Data show mean values obtained from two experiments.

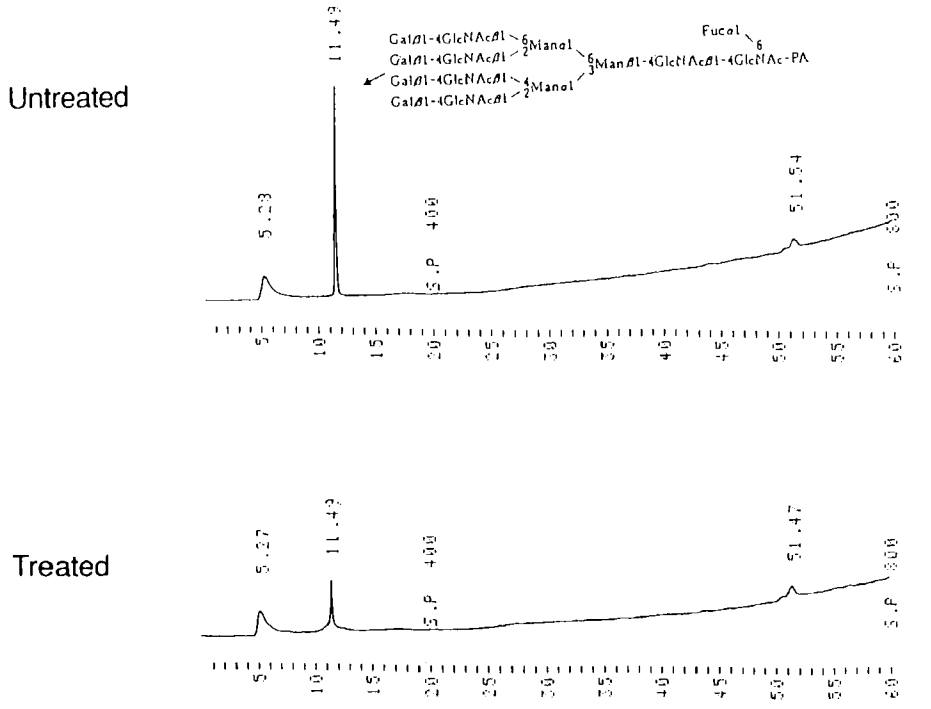

FIGURE 9 A comparison of HPLC profiles for Fenton system-treated and untreated PA-sugar chain. PA-sugar chain (1.67 nmol/ml) was added to Fenton reaction mixture containing 0.16 mM H_2O_2.

DISCUSSION

A large number of xenobiotics, including environmental agents[12] and medically useful drugs,[13] heavy smoking and diseases[7,14] exert their toxic effects by the abnormal increase in concentration of active oxygen species. Under oxidative stress, biological systems are exposed to a variety of active oxygen species. It is likely that pharmacuetical glycoproteins under various different conditions might be exposed to those active oxygen species. Active oxygen species have been reported to react readily with nucleic acids,[15] proteins,[8,9] lipids[16] and sugars,[17-19] but not with glycoproteins.

In this paper, we have examined whether active oxygen species cause any damaging effect on EPO by using Fenton system and X plus XO system as active oxygen generators. All of applied active oxygen species were able to induce a variable degree of reduction of *in vivo* activity of EPO. Fenton system significantly reduced the *in vivo* activity of EPO in H_2O_2 concentration-dependent manner, while H_2O_2 alone induced a slight reduction of the activity. It is strongly suggested that the inactivation is mediated by hydroxyl radicals in the case of Fenton system, because that the reaction was inhibited by mannitol.[6]

On the other hand, the inactivation produced by X plus XO system was not inhibited by SOD (33 units/ml) which is an effective O_2^- scavengers,[6] or catalase (20 units/ml), and mannitol (50 mM). At the same time, addition of EDTA (1 mM) and DFO (1 mM) did not produce any degree of inhibition of the inactivation. It is therefore likely that more than one kind of active oxygen species could be involved in this reaction. Suzuki *et al.*[20] reported that X plus XO reduced the activity of creatinine kinase. The loss of the activity was inhibited by catalse, but not by SOD or mannitol. They suggested that the inactivation may be due to the contribution of other different active oxygen species.

Electrophoretic studies on the structure of EPO revealed that the main protein band of EPO on SDS-PAGE disappeared in proportion to the extent of exposure to active oxygen generating systems.

The PA-sugar chain, which is a fluorescence labelling of sugar chain and a main sugar chain of EPO,[21] was applied for the study of sugar chain damage by active oxygen species. Hase[22] reported that the PA-group of PA-sugar chain is stable and its fluorescence does not disappear under the conditions used for structure elucidation, such as light, acid and alkaline hydrolysis, hydrazinolysis, or Smith periodate degradation. In our study, the PA-group of the sugar chain reacted with active oxygen species and lost its fluorescence, and their degradation products were not detected. Therefore active oxygen species might selectively attack the PA-group of the PA-sugar chain and not the sugar chain part. These results showed that the aromatic group in EPO, tryptophan, tyrosine and phenylalanine might be sensitive to attack by active oxygen species.

The most probable speculation concerning the inactivation of EPO caused by Fenton or X plus XO systems is that the protein part of EPO is damaged by active oxygen species. In conclusion, it is evident that active oxygen species have a potential to react with EPO, leading to the reduction of its *in vivo* activity.

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