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EFFECTS OF ROSE BENGAL ON SERUM LEVELS OF THYROID HORMONES AND THYROID PEROXIDASE ACTIVITY IN MALE MICE.

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Abstract.....The thyrotoxic effect of Rose bengal (RB) (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein disodium salt ; Food Red No. 105) was examined in male (C57BL/6N × C3H/N) F₁ mice. They were given drinking-water containing RB at levels of 0 (control), 0.125 and 0.250% for 2 weeks. The effect resulted in decreases in serum levels of 3,5,3'-triiodothyronine (T₃) and thyroxine (T₄), and slight increases in serum 3,3',5'-triiodothyronine (rT₃) levels and thyroid weight, but no difference in the values for the body-weight gain, serum thyroid stimulating hormone (TSH) levels and thyroid peroxidase (TPO) activities. However, the *in vitro* inhibitory effect of RB on TPO activity was observed by addition of RB to the TPO-catalyzed guaiacol oxidation. These results suggest that RB might have weak goitrogenic properties, inhibiting the peripheral conversion of T₄ to T₃ and/or inhibiting TPO to lead a decrease of T₄ and T₃ formation.

Key words : Rose bengal, T₄, T₃, rT₃, thyroid peroxidase, hypothyroidism, male mice.

INTRODUCTION

Of many fluorescein dyes, erythrosine (ER) (2',4',5',7'-tetraiodofluorescein disodium salt ; FD&C Red No.3) was reported to produce an increased incidence of

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thyroid follicular cell hyperplasia and adenomas in male rats (CCMA 1983), and to affect rat thyroid hormones (Ruiz & Ingbar, 1982 ; Minegishi et al., 1986). However, ER is considered to be non-mutagenic (CCMA 1984 ; Lin & Brusick, 1986).

Rose bengal (RB) (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein disodium salt ; Food Red No.105) is a red fluorescein-dye used in foods and cosmetics among East Asian countries including Japan. RB is known to be unstable under the light or in the acid phase. In photodynamic genetic damage of transforming DNA, the inactivation by RB was increased under a light (Bellin & Oster, 1960 ; Yoshikawa et al., 1978). RB induced photosensitivity in mice under a natural and artificial light (Nishie & Keyl, 1962). But, no adverse effect was noted in any organs except the weight increase of thyroid glands on a chronic test of RB in Wistar rats (Ikeda 1968).

Recently, Ito et al. (1986) reported that RB induced colloid goiters and thyroid adenomas in male (C57BL/6N $\times$ C3H/N) F₁ (B6C3F₁) mice given 0.5% RB in the drinking water for 18 months. We were interested in RB on the action for the male thyroid, because a common structure between RB and ER is fluorescein having iodine elements. This study was conducted to investigate an early effect of RB on the thyroid function in B6C3F₁ mice and an inhibitory effect of RB on TPO activity *in vitro*. This study will participate in an understanding of the effects of RB in producing thyroid adenomas in rodents.

MATERIALS AND METHODS

Animals and Treatment : Male (C57BL/6N $\times$ C3H/N) F₁ (B6C3F₁) mice (6 weeks of age) weighting 20–25 g were obtained from Charles River Japan Inc. (Atsugi, Japan). Twenty-five to thirty mice were randomly assigned to three groups. Five mice were housed in a plastic cage, fed a diet (MF) obtained from Oriental Yeast Co. Ltd. (Tokyo) and given tap water *ad libitum*. Animal room was maintained at 22–25°C and artificially illuminated for 12 hr/day. After acclimation for two weeks, mice were given for 2 weeks the drinking-water containing 0 (control), 0.125, and 0.250% rose bengal (RB) *ad libitum*, which was obtained from Wako Pure Chemical Industries. Ltd. (Osaka, Japan). The mice were observed daily for general appearance, behavior and survival. Body weights were measured on alternate days. After 2 week-treatment, animals were lightly anesthetized with ether and armpit incision was made. The branchial artery was cut to collect the blood. The serum was prepared by centrifugating the blood at 3,000 g for 5 min, and used for determination of the levels of T₄, T₃, TSH and rT₃ ; this order was precedence.

Assay of thyroid peroxidase (TPO) activity : Thyroids were collected and weighed individually. The thyroids from 4 mice of a group were pooled in one homogenizer and homogenized. The microsomal fractions were prepared by a slightly modified method of Nagataki et al. (1973) and used for measuring TPO activity. TPO activity was measured by the method of Hosoya & Morrison (1967), using a guaiacol assay (nmole/min/mg protein) on a Shimadzu UV-260 recording

spectrophotometer at 470 nm. TPO-catalyzed oxidation of guaiacol was carried out by the addition of 270 nmole of H_2O_2 to the enzyme fraction (49–225 $\mu\text{g/ml}$ of protein) and 33 mM guaiacol in 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 1 ml. The *in vitro* inhibition of RB on TPO activity was observed by the addition of RB solution to the guaiacol oxidation system. In the case of preincubation with H_2O_2 and RB, the reaction was started by the addition of guaiacol solution.

The protein concentrations were measured by the method of Lowry et al. (1951).

Enzyme- and radio-immunoassay: Serum T_3 and T_4 concentrations were measured by enzyme immunoassay using standard kits from Boehringer Mannheim Co. Ltd. (Germany). Serum rT_3 and TSH concentrations were determined with radioimmunoassay kits obtained from Dainabot Co. Ltd. (Tokyo, Japan) and Daiichi radioisotope Ltd (Tokyo, Japan), respectively.

Data analyses: Statistical analyses were carried as described previously (Yamazaki et al., 1981; Ged & Weil, 1982). Analyses of factors were evaluated using Bertlett's test for homogeneity of variance, analysis of variance (ANOVA) (or Kruskal-Wallis Nonparametric ANOVA when the variances were not homogeneous), and Scheffe's Multiple Comparison test (or Scheffe's type mean rank test when Kruskal-Wallis ANOVA was used). The correlation coefficient procedure was used to determine the degree of linear correlation among serum thyroid hormones.

RESULTS

Animal behavior and physical appearance were normal for all mice during the study. The feces of animals administered with RB were colored red during the treatment. There was a water-feed refusal at 0.250% dose level (Table 1). The consumption of drinking-water in the 0.250% dose group was significantly ($p < 0.01$) reduced in comparison with other groups. The total RB intake per mouse in the 0.250% dose group was only 1.6 times as large as that (265 mg/kg/day) in the 0.125% dose group. The body-weight gain did not show any difference between the control and treated groups (Table 2). The absolute and relative thyroid weights of RB-treated mice appeared to be somewhat higher than the control, though these were

Table 1. Effect of two-week Rose bengal (RB) intake on consumption of drinking-water in male B6C3F₁ mice

Concn of RB (% in drinking water)	Drinking-water consumption		RB consumed	
	(g/5 mice/day)		mg/mouse/day	mg/kg/day
0 (control)	28.56 ± 2.75 ^a	(24) ^b	0	0
0.125	29.26 ± 4.78	(20)	7.31	265
0.250	23.26 ± 2.74 ^{*c}	(20)	11.63	423

a: Values are means ± SD.

b: Number of measurement.

c: Significantly different from the control (*, $P < 0.01$).

not statistically significant ($p > 0.05$).

The serum T_4 levels in the RB-treated groups were significantly decreased to 91% of the controls ($p < 0.01$ for both dose groups) (Table 3). The serum T_3 levels were significantly lower in the RB-treated mice (73% : mean of both groups) than

Table 2. Effects of two-week Rose bengal intake on body weights and thyroid size of male B6C3F₁ mice

Concn of RB (% in drinking water)	No. of animals	Body weight		Thyroid weight	
		Starting (g)	Terminal (g)	Absolute (mg)	Relative (mg/g body wt)
0 (control)	30	26.23±0.23	28.42±0.26	2.98±0.85	0.108±0.031
0.125	25	26.46±0.27	28.92±0.32	3.52±0.82	0.125±0.033
0.250	25	26.26±0.24	28.76±0.30	3.38±0.83	0.121±0.029

Values are given as means ± SD.

Table 3. Effects of two-week Rose bengal intake on serum levels of T_4 , T_3 , rT_3 , TSH and thyroid peroxidase activity in male B6C3F₁ mice

	Control		Rose bengal-treated group			
			Concentration of Rose bengal			
	0 %		0.125%		0.250%	
T_4 ($\mu\text{g}/100\text{ml}$)	5.00±0.56 ^a	N=30 ^b	4.56±0.51 ^{***e}	N=25	4.55±0.43 ^{**}	N=25
	((100) ^c		(91)		(91)	
T_3 (ng/ml)	0.90±0.21	N=30	0.62±0.18 ^{**}	N=24	0.69±0.15 ^{**}	N=25
	(100)		(69)		(77)	
rT_3 (pg/ml)	235.9±57.7	N=13	243.3±62.4	N=16	294.7±65.1	N=9
	(100)		(103)		(125)	
T_4/T_3	58.9±17.2	N=30	80.4±27.3 ^{**}	N=24	69.8±20.1	N=24
	(100)		(137)		(119)	
T_4/rT_3	219.2±62.3	N=13	192.5±37.8	N=16	153.2±32.4 ^{**}	N=9
	(100)		(88)		(70)	
T_3/rT_3	4.23±1.49	N=13	2.57±0.41 ^{**}	N=15	2.53±0.56 ^{**}	N=9
	(100)		(61)		(60)	
TSH ($\mu\text{u}/\text{ml}$)	1.63±0.34	N=30	1.60±0.21	N=23	1.58±0.28	N=24
	(100)		(98)		(97)	
TPO ^d	18.53±3.56	N=8	18.84±4.19	N=6	17.26±2.32	N=6
	(100)		(102)		(93)	

a: Values are means ± SD.

b: Number of measurement of experimental animals.

c: Relative values in parentheses compared with control (100%)

d: TPO activity; oxidation of guaiacol nmol/min/mg protein of microsomal fraction.

e: Significantly different from control group (0%), (** $P < 0.01$).

those in the control ($p < 0.01$ for both dose levels). The serum rT_3 levels appeared to be slightly higher in the RB-treated mice. There were positive correlations between T_4 and T_3 levels ($r = 0.396$, $p < 0.01$), and between T_4 and rT_3 levels ($r = 0.343$, $p < 0.05$), but not between T_3 and rT_3 levels ($p > 0.05$).

The ratio of T_4/T_3 was higher in the RB-treated mice ($p < 0.01$ for the 0.125% group) than in the control. The ratio of T_4/rT_3 was lower ($p < 0.01$ for 0.250% dose) and the ratio of T_3/rT_3 was also lower ($p < 0.01$ for both doses) in the RB-treated mice than in the controls (Table 3). In the RB-treated mice, serum T_3 levels were more markedly decreased than serum T_4 levels, and serum rT_3 levels were relatively increased in comparison with decreases in serum T_3 and T_4 levels.

Serum TSH level and TPO activity of guaiacol oxidation in the RB-treated mice showed no difference among the groups (Table 3).

An inhibitory effect of RB was examined *in vitro* on TPO-catalyzed guaiacol oxidation. Clear inhibition was observed by addition of RB in the course of guaiacol oxidation (Fig. 1A). When the reaction was started after addition of RB, the inhibitory effect was moderately observed (Fig. 1B). The preincubation of TPO fractions with RB and hydrogen peroxide for 10 minutes inhibited greatly the guaiacol oxidation (Fig. 1C). The re-addition of hydrogen peroxide did not recover TPO activity. TPO-catalyzed oxidation of guaiacol was greatly inhibited by addition of RB (3–20 μM) and 50% inhibition seemed to occur at about 3 μM of RB.

DISCUSSION

The significant decrease in serum T_3 levels (–27%) and the decrease in serum T_4 levels (–9%) were observed in the RB-treated mice. The absolute and relative thyroid weights of the RB-treated mice were slightly higher than the control value. The results showed a hypothyroidal effect of RB and suggest that RB is a very weak goitrogen. Elevated TSH is the common denominator for goiter, but RB did not cause the elevated serum TSH levels.

In general, the excess iodide causes the hormonal imbalance. Since RB has the high iodine content (50%) as well as ER (58%), when iodine was released from RB *in vivo*, the excess iodide would have caused the Wolff-Chaikoff effect so long as serum iodide was over 30 $\mu g/100$ ml (Wolff & Chaikoff, 1948). If the escape mechanism (Braverman & Ingbar, 1963) failed, the excess iodide would induce goiter, often with hypothyroidism. Until now, there are no data on metabolism and distribution of RB in mice. In rats, the orally administered ^{131}I -labelled RB was slightly absorbed, and the radioactivity was distributed in liver and kidneys at 2 hr and extremely in thyroid at 12 hr after administration (Onoda et al. 1973). The liberated ^{131}I , RB or its metabolite should have accumulated in thyroid glands. Iodine might have been released from ^{131}I -labelled RB, since it was suggested ER had been deiodinated in rats (Vought et al. 1972; Minegishi et al. 1986).

Almost all compounds with antithyroid activity were found to inhibit TPO-

Fig. 1 *In vitro* inhibition of RB on TPO-catalyzed guaiacol oxidation.

A : (—), TPO-catalyzed guaiacol oxidation was started by the addition of H_2O_2 (0.27 mM) to the enzyme fraction (205 μ g of protein/ml) and guaiacol (33 mM) in 0.1 M potassium phosphate buffer (pH 7.4). The inhibition was observed by the addition of RB (18 μ M) in the course of the reaction indicated by arrow. B : (---), the measurement was started by the addition of H_2O_2 after the addition of RB. C : (- - -), the measurement of guaiacol oxidation was started by the addition of guaiacol after preincubation of the enzyme fraction with H_2O_2 and RB for 10 min.

catalyzed iodination (Coval & Taurog, 1967; Taurog, 1976). Many of these compounds also inhibited TPO-catalyzed oxidation of guaiacol, a reaction that does not involve iodide (Taurog, 1976; Hosoya, 1963). Though TPO activity was not decreased in the RB-treated mice, RB inhibited TPO-catalyzed guaiacol oxidation *in vitro*, and the ^{131}I uptake by the thyroid gland was decreased in the RB-treated mice (Ito et al., 1986). These results suggest that RB could have inhibited TPO-catalyzed organification of iodide *in vivo*, since the *in vivo* inhibition on TPO-catalyzed iodination does not always accompany with the decrease in TPO activity of guaiacol

oxidation. The administration of thiourea to rats inhibited the iodination of protein in thyroid, but not guaiacol oxidation of TPO (Davidson et al., 1979).

The levels of serum rT_3 were relatively increased in contrast with severely decreased T_3 levels by RB intake. This result suggests that RB might have inhibited some of modulating effects of T_4 on hepatic enzymes by *in vivo* blockade of extrathyroidal T_4 to T_3 conversion. This is consistent with the result that ER inhibited the hepatic conversion of T_4 to T_3 *in vitro* (Ruiz & Ingbar, 1982). It is likely that the changes in circulating thyroid hormones are caused by alterations in peripheral hormone.

Several disturbances of thyroid hormone metabolism have been described in chronic liver disorders. A goitrogenic substance, propylthiouracil was shown to decrease serum T_3 by blocking peripheral T_4 5'-deiodinase (Chopra 1977 ; Saberi et al., 1975). Amiodarone is a potent inhibitor of T_4 to T_3 conversion in liver (Jonckheer et al., 1978 ; Sogol et al., 1983). Some goitrogenic substances demonstrate inhibitory effects on extrathyroidal biochemical events, notably reducing conversion of T_4 to T_3 , i. e., 5'-deiodination.

RB is sometimes used as a specific modifying agent for a number of enzymes. RB might have inhibited T_4 5'-deiodinase and/or TPO activity in a similar way to these facts. RB was found to bind tightly to apo-hemoproteins including apocytochrome c peroxidase (Coulson & Yonetani, 1972). Also, RB binds covalently to the essential histidyl residue at or near the active sites of some redox and hydrolytic enzymes (Hederstedt & Hatefi, 1986).

It is reported that ER induced thyroid tumors in male Sprague-Dawley rats after life-time feeding diet containing 4% ER (CCMA, 1983). But, ER was excluded as a genotoxic inhibitor (CCMA, 1984 ; Lin & Brusick, 1986) and it is suggested that some other mechanism is responsible for the increase in tumors (CCMA, 1984). We reported two-week-feeding effects of ER on the serum levels of T_3 , T_4 and TSH, and TPO activity in male rats (Minegishi et al., 1986), and the decreases in serum T_3 levels and TPO activity were observed in the ER-treated rats. In connection with the results of ER, we have studied here the effects of RB on thyroid function of male B6C3F₁ mice, and summarized them in Fig. 2. We have presumed that RB might inhibit the peripheral conversion of T_4 to T_3 in liver, and/or inhibit thyroid function to lead a decrease of serum T_3 and T_4 levels. It is anticipated that the more precise studies will facilitate an understanding of the effects of RB in producing thyroid adenomas in rodents.

Fig. 2 Presumed action of RB *in vivo*. TRH, thyrotropin-stimulating hormone; TSH, thyroid-stimulating hormone; TPO, thyroid peroxidase; T₄, thyroxine; T₃, 3,5,3'-triiodothyronine; ⁴rT₃, 3,5,3'-triiodothyronine; RB, rose bengal.

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