Effect of Thyroxine-Binding Globulin (TBG) on Thyroxine (T_4) uptake by Human Peripheral Mononuclear Cells: Evidence of TBG-Dependent uptake of T_4

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Abstract

The effect of sialylated TBG and desialylated TBG on thyroxine (T_4) uptake by human peripheral mononuclear cells was investigated *in vitro*. [125 I]- T_4 uptake was observed when the cells were incubated with free [125 I]- T_4 . The uptake was inhibited in a concentration dependent manner when TBG was added. During the incubation, [125 I]- T_4 binding to TBG was observed. [125 I]- T_4 incorporation into cells was also observed when the cells were incubated with [125 I]- T_4 -sialylated TBG or with [125 I]- T_4 -desialylated TBG complex. The uptake was related to the temperature and length of time of the incubation. The amount of [125 I]- T_4 incorporated into the cells incubated with [125 I]- T_4 -sialylated TBG was greater than that into the cells incubated with [125 I]- T_4 -desialylated TBG during the early 0-20 min. incubation, whereas the amount of [125 I]- T_4 incorporated into the cells incubated with [125 I]- T_4 -desialylated TBG became greater than that into the cells incubated with [125 I]- T_4 -sialylated TBG after 20 min. of incubation. Pretreatment of the cells with methylamine blocked [125 I]- T_4 uptake in both cases, i.e. incubated with [125 I]- T_4 -sialylated TBG and incubated with [125 I]- T_4 -desialylated TBG. The results suggest that TBG plays a role not only as a carrier protein for T_4 in circulation but also as a protein which can transport T_4 from the extracellular into the intracellular space, so that the mechanism of T_4 transport mediated by desialylated TBG is different from that mediated by sialylated TBG, and that the T_4 transport system in both cases, mediated by sialylated TBG and by desialylated TBG, may be related to the internalization of T_4 -TBG-TBG receptor complex or of T_4 - T_4 receptor complex if TBG receptors are present in the outer surface of the cell membrane.

It is well known that more than 99% of circulating thyroxine (T_4) is bound to serum proteins (Oppenheimer 1968 and Refetoff *et al.*, 1970). There are three thyroxine-binding proteins in serum, viz., thyroxine-binding globulin (TBG), thyroxine-

binding prealbumin (TBPA) and albumin (Oppenheimer 1968). The chief determinant of the amount of free T_4 that can be transported into the cells is believed to be the concentration of TBG in serum (Freinkel *et al.*, 1957 and Refetoff *et al.*, 1972(a)). It is thus postulated that TBG plays a role not only as a carrier protein but also as a

regulator protein for the T_4 transport system in target tissues.

Recently, it has been demonstrated that two distinct TBGs which were separated from serum by electrophoresis are present (Blumberg & Robbins 1960, Blumberg *et al.*, 1961, Thorson *et al.*, 1966, Premachandra *et al.*, 1970, Marshall *et al.*, 1972 and Refetoff *et al.*, 1975). One is sialylated and the other is desialylated TBG. Refetoff and his coworkers (1975) found that desialylated TBG is rapidly cleared from serum by the liver and suggested that conversion of TBG to desialylated TBG is the limiting step in the regulation of TBG metabolism. On the other hand, TBG, as one of the glycoproteins in serum, can bind to its specific receptor in peripheral tissue cells and be internalized with its receptor into cells (Ashwell & Harford 1982 and Stahl & Schwartz 1986). Although it is not known whether the internalization of T_4 -TBG-TBG receptor complex plays a role in the T_4 transport system, rapid clearance of desialylated TBG and slow clearance of sialylated TBG indicates different roles in T_4 moving across cell membrane. In this study, we aimed to investigate the effect of TBG on T_4 transport into cells by using human mononuclear cells *in vitro*.

Materials and Methods

Preparation of human mononuclear cells

Human mononuclear cells were obtained from a normal man who was fasted prior to preparation of the cells overnight. The cells were prepared from 50 ml blood samples by Ficoll-Hypaque density gradient centrifugation (Boyum 1968).

Preparation of TBG

TBG was prepared from serum of normal man. The serum (50 ml) was incubated in the presence of 10% charcoal (Aktivkohle) (Merck, Darmstadt, West Germany) for 30 min. at 4°C to remove free hormones. The supernatant after centrifugation at $27,000\times g$ for 30 min. at 4°C

was applied to a column (4.0 $\times$ 60 cm) of DEAE Sephadex A-50 (Pharmacia Fine Chemical Inc.) which had been previously equilibrated with 10 mM sodium phosphate buffer pH 7.5, containing 5% glycerol, and proteins were eluted with a gradient of NaCl (0-0.4 M) in the same buffer. A single peak of immunoreactive TBG (TBG concentration in each fraction was determined by RIA (Hoechst Japan, Tokyo) was obtained. The fraction containing TBG was applied to a T_4 affinity column (1.0 $\times$ 20 cm), and proteins were eluted with 0.002 M KOH pH 9.6, as described by Pensky and Marshall (1969). CNBr-activated Sepharose 4B for T_4 affinity column chromatography was obtained from Pharmacia, and the T_4 -bound CNBr-activated Sepharose 4B was prepared as previously described (Hashizume *et al.*, 1986). Sialylated protein was prepared by using Concanavalin A (Con A) Sepharose 4B (Pharmacia) column chromatography as previously described (Hashizume *et al.*, 1978). The TBG obtained by T_4 affinity column chromatography was applied to a column (2.0 $\times$ 10 cm) of Con A Sepharose 4B which had been equilibrated with 10 mM sodium phosphate buffer pH 7.5. After washing the gel with the same buffer, proteins were eluted with 10% 1-*O*-methyl- α -D-glucopyranoside (α -MGP) (Sigma Chemical Co., St. Louis, MO) in the same buffer. TBG which was eluted after loading the sugar was further purified with Sephadex G-100 (Pharmacia) to remove sugar and was used as a sialylated TBG. Desialylated TBG was prepared as previously described (Marshall *et al.*, 1972 and Pensky & Marshall 1969) with minor modifications. Sialylated TBG (20 μ g protein) was incubated with 25 mU of neuraminidase (Worthington Biochemicals) in 1.0 ml of 0.1 M Hepes pH 6.2 for 3 hrs at 37°C. To terminate the reaction, 0.2 M Tris pH 8.9 was added, and pH was adjusted to 7.5. The mixture was applied to a column of Con A Sepharose 4B as described. TBG which was eluted before loading α -MGP was further purified by Sephadex G-100 column chromatography and was used as desialylated TBG.

Preparation of [125 I]- T_4 -TBG complexes

TBG (sialylated and desialylated) containing fraction (1.0 ml) (25 μ g protein) was incubated in the presence of 5×10^{-8} M [125 I]- T_4 (specific activity; 1,250 μ Ci/ μ g) (New England Nuclear, Boston, MA) for 30 min. at 34°C. After incubation, the mixtures were separately applied to a column (2.0 $\times$ 15 cm) of Sephadex G-25 (Phar-

macia) which had been equilibrated with phosphate buffered saline to remove free radioactive hormone, and the complexes were eluted with the same buffer. The fraction which contained radioactivity was used as $[^{125}\text{I}]\text{-T}_4\text{-TBG}$ complexes. The specific activity in $[^{125}\text{I}]\text{-T}_4\text{-TBG}$ complexes was 2.5×10^4 cpm/ μg protein in sialylated TBG and 2.3×10^4 cpm/ μg protein in desialylated TBG. $[^{125}\text{I}]\text{-T}_4$ bound to TBG was calculated to be 0.2–0.3 $[^{125}\text{I}]\text{-T}_4$ per molecule of TBG. During the incubation of $[^{125}\text{I}]\text{-T}_4\text{-TBG}$ complexes at 34°C for 40 min., $[^{125}\text{I}]\text{-T}_4$ was not dissociated from $[^{125}\text{I}]\text{-T}_4\text{-TBG}$ complexes.

The assay of $[^{125}\text{I}]\text{-T}_4$ uptake by mononuclear cells

The obtained mononuclear cells were suspended in modified Krebs-Ringer phosphate (KRP) buffer (pH 7.4) (Hashizume *et al.*, 1983) which contained $[^{125}\text{I}]\text{-T}_4\text{-TBG}$ complex or free $[^{125}\text{I}]\text{-T}_4$ (12,500–150,000 cpm), and were incubated for various incubation times at 15 or 34°C in 450 μl of incubation medium. After incubation the mixture was centrifuged at $700 \times g$ for 5 min. at 4°C . The packed cells were washed with 1.0 ml phosphate buffered saline and the radioactivity in the pellet after centrifugation at $700 \times g$ for 5 min. was measured with a Packard Autogamma-spectrometer (efficiency = 74%).

Treatment of the cells with methylamine

The cells obtained were incubated with 10 mM methylamine (Sigma) in KRP buffer at 34°C for 30 min. as previously described (Hashizume

et al., 1983). After incubation the cells were sedimented by centrifugation at $700 \times g$ for 5 min. and were washed with KRP buffer (1.0 ml). The final packed cells were suspended in KRP buffer and used as methylamine-treated cells.

Analytical procedures

The protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) (Sigma) as a standard. The binding of $[^{125}\text{I}]\text{-T}_4$ to serum protein was estimated by the charcoal method described previously (Hashizume *et al.*, 1986). Statistical analysis of the significance between groups was done by means of Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

Results

Preparation and properties of sialylated TBG and desialylated TBG

TBG purified by T_4 affinity Sepharose 4B column chromatography was applied to a Con A Sepharose 4B column. The TBG eluted after loading α -MGP was incubated, in the presence of 0.1 M Hepes pH 6.2 which did not contain neuraminidase, for 3 hrs at 37°C . After incubation, pH was adjusted to 7.5 by adding Tris pH 8.9, and the material was applied to a Con A Se-

Fig. 1. Con A Sepharose 4B column chromatography of sialylated and desialylated TBG.

Sialylated TBG (●) and desialylated TBG (○) which were prepared as described in the text were applied to a column (2.0 $\times$ 60 cm) of Con A Sepharose 4B. After washing the gel with 10 mM sodium phosphate buffer (pH 7.5), protein trapped by gel were eluted with 10% α -MGP. The arrow indicates the position of α -MGP loading.

pharose 4B column. After washing the gel with 10 mM phosphate buffer, TBG was eluted with α -MGP (10%). As shown in Fig. 1, 70–75% of TBG was eluted after loading α -MGP. The TBG obtained was used as sialylated TBG in the following studies. On the other hand, neuraminidase-treated TBG was eluted before loading α -MGP (approximately 60–70% of TBG was eluted before α -MGP). The TBG which was not trapped by Con A Sepharose 4B had slow electrophoretic mobility and was used as desialylated TBG. $[^{125}\text{I}]\text{-T}_4$ -sialylated TBG and $[^{125}\text{I}]\text{-T}_4$ -desialylated TBG were prepared as described in *Materials and Methods*. Fig. 2 shows typical elution profiles of both $[^{125}\text{I}]\text{-T}_4$ -TBG complexes in Sephadex G-25 column chromatography.

Effect of TBG on $[^{125}\text{I}]\text{-T}_4$ uptake into human peripheral mononuclear cells

Fig. 3 shows the effect of TBG on $[^{125}\text{I}]\text{-T}_4$ uptake by human mononuclear cells. When the cells (1×10^6 cells) were incubated

Fig. 2. Sephadex G-25 column chromatography of $[^{125}\text{I}]\text{-T}_4$ -TBG complexes.

$[^{125}\text{I}]\text{-T}_4$ -sialylated TBG (●) and $[^{125}\text{I}]\text{-T}_4$ -desialylated TBG (○) were prepared as described in *Materials and Methods*. Radioactivity in each fraction is shown.

with free $[^{125}\text{I}]\text{-T}_4$ (150,000 cpm) at 34°C for 30 min., $6,800 \pm 340$ cpm (4.50% of radioactivity added) was incorporated. The amount of $[^{125}\text{I}]\text{-T}_4$ incorporated into cells was diminished by adding TBG. Both sialylated and desialylated TBG decreased $[^{125}\text{I}]\text{-T}_4$ incorporation in a concentration dependent manner. The decrease observed in the cells incubated with desialylated TBG, however, was greater than that in the cells incubated with sialylated TBG. On the other hand, the amount of TBG-bound $[^{125}\text{I}]\text{-T}_4$ in the medium incubated with sialylated TBG was greater than that in the medium incubated with desialylated TBG. The de-

Fig. 3. Effect of TBG on $[^{125}\text{I}]\text{-T}_4$ uptake by human peripheral mononuclear cells.

Human peripheral mononuclear cells (10^6 cells) were incubated with free $[^{125}\text{I}]\text{-T}_4$ (150,000 cpm) in the presence of various concentrations of sialylated TBG (●) (▲) or desialylated TBG (○) (△) for 30 min. at 34°C. Radioactivity incorporated into cells (●) (○) was measured as described in *Materials and Methods*. Radioactivity bound to TBGs (▲) (△) was assessed by the charcoal method of Hashizume *et al.* (1986, Endocrinology). Each point and vertical bracket indicates the mean $\pm$ SEM of five determinations.

crease in the amount of $[^{125}I]-T_4$ incorporated was also observed by adding human serum albumin but not by adding human immunoglobulin G (Data not shown).

$[^{125}I]-T_4$ uptake by human mononuclear cells during incubation with $[^{125}I]-T_4$ -TBG complex

In order to estimate whether TBG plays a role in transporting T_4 from the circulation to cells, we employed $[^{125}I]-T_4$ -TBG

complexes in the studies on T_4 uptake. As shown in Fig. 4, when $[^{125}I]-T_4$ -sialylated TBG complex or $[^{125}I]-T_4$ -desialylated TBG (both 25,000 cpm) was added to the incubation medium, radioactivity was incorporated into cells in a time dependent manner. When 1×10^5 cpm of $[^{125}I]-T_4$ -sialylated TBG complex or $[^{125}I]-T_4$ -desialylated TBG complex was added to the incubation medium, radioactivity was also incorporated

Fig. 4. $[^{125}I]-T_4$ uptake by human mononuclear cells incubated with $[^{125}I]-T_4$ -TBG complexes.

Human mononuclear cells (10^6 cells) were incubated with 25,000 cpm of $[^{125}I]-T_4$ -sialylated TBG (●) or $[^{125}I]-T_4$ -desialylated TBG (○) for various incubation times at 34°C or 15°C. The radioactivity incorporated into the cells was determined as described in *Materials and Methods*. Each point and vertical bracket indicates mean $\pm$ SEM of five determinations.

Fig. 5. $[^{125}I]-T_4$ uptake by human peripheral mononuclear cells incubated with $[^{125}I]-T_4$ -TBG complexes.

Human peripheral mononuclear cells (10^6 cells) were incubated with 100,000 cpm of $[^{125}I]-T_4$ -sialylated TBG (●) or $[^{125}I]-T_4$ -desialylated TBG (○) for various incubation times at 34°C or 15°C. The radioactivity incorporated into cells were determined as described in *Materials and Methods*. Each point and vertical bracket indicates mean $\pm$ SEM of five determinations.

into cells in a time dependent manner (Fig. 5). These incorporations were observed to be related to temperature. As shown in Fig. 4, however, the uptake of radioactivity was greater in the cells incubated with [125 I]-T $_4$ -sialylated TBG than that in the cells incubated with [125 I]-T $_4$ -desialylated TBG when the cells were incubated with 25,000 cpm of radioactive materials at 34°C. Similarly, when the cells were incubated with 100,000 cpm of [125 I]-T $_4$ -TBG complexes, the amount of radioactivity incorporated into the cells was greater in the cells incubated with [125 I]-T $_4$ -sialylated TBG than that in the cells incubated with [125 I]-T $_4$ -desialylated TBG during 0–20 min. incu-

bation at 34°C. However, the amount of radioactivity incorporated into the cells incubated with [125 I]-T $_4$ -desialylated TBG was greater than that into the cells incubated with [125 I]-T $_4$ -sialylated TBG during incubation for 20 min or longer. As shown in Fig. 6, the K_m value for [125 I]-T $_4$ -TBG association to the cells in 10 min. incubation at 34°C was significantly lower in the cells incubated with [125 I]-T $_4$ -sialylated TBG than that in the cells incubated with [125 I]-T $_4$ -desialylated TBG. During the incubation of the cells with [125 I]-T $_4$ -TBG complexes, free [125 I]-T $_4$ which was dissociated from [125 I]-T $_4$ -TBG complexes was less than 0.1%.

Fig. 6. Double reciprocal analysis of [125 I]-T $_4$ uptake by human mononuclear cells.

The experimental conditions were the same as mentioned in Fig. 4 and 5. Incubation was performed in the presence of various concentrations of [125 I]-T $_4$ -sialylated TBG (—●—) or of [125 I]-T $_4$ -desialylated TBG (—○—). Each value was calculated from the data for 10 min. incubation at 34°C. Each point and vertical bracket indicates mean $\pm$ SEM of five determinations.

Fig. 7. Effect of pretreatment of the cells with methylamine on [125 I]-T $_4$ uptake.

The cells were incubated with buffer (KRP) alone (—●—) (—○—) or with buffer containing 10 mM methylamine (—■—) (—□—) for 30 min. at 34°C. Each cell (10^6 cells) was incubated with [125 I]-T $_4$ -sialylated TBG (25,000 cpm) (—●—) (—■—) or with [125 I]-T $_4$ -desialylated TBG (25,000 cpm) (—○—) (—□—) for various incubation times at 34°C. After incubation radioactivity incorporated into the cells was determined. Each point and vertical bracket indicates mean $\pm$ SEM of five determinations.

Effect of pretreatment of the cells with methylamine on [¹²⁵I]-T₄ uptake

In order to examine whether the uptake of radioactivity by the cells was mediated by internalization of TBG receptor or of T₄ receptor, the uptake was assessed in the cells pretreated with methylamine which is known as an inhibitor of internalization (Chaung 1981). As shown in Fig. 7, pretreatment of the cells with 10 mM methylamine significantly decreased the amount of radioactivity incorporated into cells. In this study, the diminution of cell activity for [¹²⁵I]-T₄ uptake was observed in both conditions, incubated with [¹²⁵I]-T₄-sialylated TBG and incubated with [¹²⁵I]-T₄-desialylated TBG.

Discussion

TBG is known to act as a reservoir for thyroid hormone in serum, as a carrier protein of the hormone from thyroid gland to target tissues, and as a regulator of thyroid hormone uptake in target tissues (Woeber & Ingbar 1968, Nicoloff 1978 and Refetoff 1979). In this study, we also observed that uptake of free T₄ by human peripheral mononuclear cells was dramatically inhibited by TBG. The inhibition might be produced by competition in T₄ binding between TBG and T₄ receptor on the cell surface *in vitro*. These observations of ours and others (Dowling *et al.*, 1960, Braverman & Ingbar 1967, Refetoff *et al.*, 1972(b) and Robbins 1973) suggested that TBG plays a key role in regulating the concentration of free thyroid hormone which can be incorporated into peripheral cells (Frenkel *et al.*, 1957, Refetoff *et al.*, 1972 (a)). This hypothesis is supported by several clinical findings. In subjects with a high concentration of serum TBG, such as in pregnancy (Robbins & Nelson 1958 and Man *et al.*, 1969) and certain condition of liver dysfunction (Refetoff *et al.*, 1972 (c)),

free hormone concentrations are maintained at constant levels even though the thyroid functions, such as thyrotropin response to thyrotropin releasing hormone, and radioactive iodine uptake by thyroid, are not different from those of normal subjects.

In a physiological condition, TBG is the protein that has the highest affinity constant for thyroid hormone (especially T₄) binding among binding proteins in serum, and 70–75% of total T₄ in serum is considered to be bound to TBG (Refetoff 1979). In a euthyroid subject with a normal TBG concentration, about one-third of TBG is saturated with thyroid hormone, and when fully saturated, TBG can carry about 20 µg of T₄/100 ml of serum (Refetoff 1979). These observations suggested that tightly and abundantly bound T₄ is also incorporated into the cells. Recently, TBG was divided into two subclasses; one is sialylated and another is desialylated TBG (Blumberg & Robbins 1960, Blumberg *et al.*, 1961, Thorson *et al.*, 1966., Premachandra *et al.*, 1970, Marshall *et al.*, 1972 and Refetoff *et al.*, 1975). These proteins are also felt to bind to their specific receptors (Ashwell & Harford 1982), and can be internalized with their receptors by target tissues (Stahl & Schwartz 1986). Based on these findings and considerations, it is possible to speculate that TBG acts not only as a determinant of free hormone concentration in serum but also as an active transporter of thyroid hormone from serum to cell across cell membrane.

In this study, we have demonstrated that T₄ can be transported into cells, and this is mediated by preceding binding of T₄ to TBG. In the early phase of incubation (0–20 min.), T₄ transport mediated by sialylated TBG was prominent, whereas with longer incubation, T₄ transport mediated by desialylated TBG was conspicuous. The results suggested that although the precise mechanism(s) is not certain, the mechanism of T₄ transport mediated by

desialylated TBG is different from that mediated by sialylated TBG. The findings that desialylated TBG-mediated incorporation was dependent on temperature and was blocked by pretreatment of the cells with methylamine indicated that, although we could not produce evidence of the presence of TBG receptor, the mechanism of desialylated TBG-mediated T_4 uptake is physiologically present and that the desialylated TBG-mediated T_4 incorporation is produced by an internalization of T_4 -desialylated TBG-TBG receptor complex. Desialylated TBG is rapidly cleared from serum, whereas clearance of sialylated TBG is extremely slower (Refetoff *et al.*, 1975), suggesting that T_4 incorporation mediated by sialylated TBG may not be induced by internalization of T_4 -TBG-TBG receptor complex. Nevertheless, T_4 incorporation mediated by sialylated TBG, occurred in earlier phase of incubation was temperature dependent and was blocked by pretreatment of the cells with methylamine in our study. The results suggested that this mechanism for T_4 transport is also related to internalization. There are many studies reported the evidence for the presence of thyroid hormone receptor on the cell surface (Chabri & Toressani 1979, Chabri & Toressani 1981, Maxfield *et al.*, 1981 and Segal & Ingbar 1982) and for the presence of receptor-mediated uptake of thyroid hormone (Cheng *et al.*, 1980). Thus it is likely that T_4 transport induced by sialylated TBG is mediated by transfer of T_4 to its receptor on the cell surface from T_4 -sialylated TBG complex, and the receptor-bound T_4 is internalized with its receptor. During this process, the affinity of T_4 binding to TBG may be related to T_4 uptake into the cells since the affinity of T_4 binding to sialylated TBG is higher than that to desialylated TBG (Refetoff *et al.*, 1975). However, we did not show direct evidence that T_4 -sialylated TBG complex binds to its receptor. Thus whether T_4 transfer from T_4 -sialylated

TBG complex to T_4 receptor in the membrane occurs after the binding of the complex to the membrane is not certain.

In this report, we provided evidence that TBG plays not only as a determinant of the concentration of serum free hormone but also as a transporter for the hormone at the plasma membrane levels. It appears that the structure (sialylated and desialylated) dependent mechanism for T_4 uptake is present. While the role of these TBGs in the uptake of T_4 by the cells is no doubt important, the precise mechanism remained to be elucidated.

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