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TESTOSTERONE BINDING OF MATERNAL PLASMA IN TWIN PREGNANCY

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SUMMARY

Binding of testosterone by plasma proteins has been studied by equilibrium dialysis in late single and twin pregnancies. The binding has been studied both before and after delivery. It was higher in late twin pregnancies, with a testosterone binding capacity $7{\cdot}3 \times 10^{-7}$ M/l as compared with $4{\cdot}4 \times 10^{-7}$ M/l for simple pregnancies. These values suggest that this plasma may be a good source of testosterone binding globulin(s). Results of serum protein electrophoresis and oestriol determinations of the studied subjects are also included.

INTRODUCTION

IT HAS BEEN established that steroids circulating in the blood are bound in varying degrees to plasma proteins. In early studies of the binding of testosterone (17β-hydroxy-4-androsten-3-one) to plasma proteins, the high capacity of albumin to bind this steroid was observed. Later it was suggested by Chen *et al.*[1] that other plasma proteins besides albumin might bind testosterone, and the existence of a specific testosterone-binding globulin (TBG) was first reported by Mercier *et al.*[2]. The properties of TBG have been studied with different techniques[3–11]. It has been shown that the testosterone-binding affinity of plasma is lower in males than in females and increases after oestrogen treatment and during pregnancy.

We determined the testosterone binding of maternal plasma in five twin pregnancies at the 38th week of pregnancy and found it to be higher than in normal pregnancies. This is due to a relative increase of some binding protein(s) other than albumin, probably TBG. It seems that twin pregnancy plasma would be a good source of TBG for determination of testosterone, using competitive binding methods[12–15].

MATERIALS AND METHODS

[1,2^{3}H] Testosterone with a specific activity of 153 mC/mg was obtained from Amersham, England. Before use its radiochemical purity was verified by thin layer and paper chromatography. Non-labeled testosterone was purchased from Ikapharm, Ramat-Gan, Israel. The purity of the compound was verified by gas-liquid chromatography (F&M, Avondale, Pa. U.S.A., Model 400) on a 1% SE-30 liquid phase, using the free steroid and its trimethylsilyl ether.

All solvents were of analytical grade and used without further purification.

The plasma samples were obtained after overnight fasting from three non-pregnant healthy women, five normal pregnant women and five having twin pregnancy. All the pregnant subjects were in the 38th week of pregnancy. Heparinized blood samples were drawn from a peripheral vein at 9 a.m. They were centrifuged immediately, and the plasma separated and either processed on the same day or kept frozen at -20°C.

A cellophane dialyzing tube about 0·6 cm dia. (Arthur H. Thomas Co. No. 4465-A 2, Philadelphia, Pa., U.S.A.) was cut into segments 17 cm long and immersed in distilled water for 30 min. The

segments were dried and 1 ml of plasma diluted with 0·9% saline solution was placed inside the tube. The tube was secured with double knots and placed in a 50 ml Erlenmeyer flask. Previously the cold and labelled steroids were separately added to the flask in an ethanol solution. The total volume of ethanol containing the hot and cold testosterone was less than 0·1 ml in order to avoid protein denaturation. After the addition of 10 ml of 0·9% saline solution and thorough mixing the flask was closed with Parafilm and shaken in a horizontal incubator at 25°C for 22 hr. Previous experiments showed that this period of time is adequate to reach equilibrium. After this period 0·5 ml of the solution in the tube was diluted to 2 ml with saline. This diluted sample and 2·0 ml from the solution in the flask were extracted twice with 10 ml of ethyl ether. The ether was transferred to a counting vial and evaporated to dryness under a stream of nitrogen. Recovery was quantitative. No increase in volume was detected in the solution inside the dialysis tube and there was no adsorption to the flask or the dialyzing membrane. The dry residue was dissolved in 0·5 ml of methanol and 10 ml of a scintillation mixture (PPO 4 g, POPOP 0·1 g, toluene ad 1000 ml) was added. Radioactive samples were counted in a Packard TRI-CARB model 3003 (LaGrange, Illinois, U.S.A.)—only two experiments—and in a WALLAC NTL 314 (Turku, Finland) liquid scintillation spectrometer. The efficiency for tritium in unquenched samples in the WALLAC NTL 314 was 48 per cent with a background of 25 cpm. The quenching effect due to the presence of methanol and of the biological material was about the same in both the inside and outside phase, the difference in counting efficiency being less than 0·5 per cent in all experiments. The correction was made by the external standard channels ratio method. The amount of radioactivity added to the external phase was 55,000 dpm. The percentage of radioactive steroid bound was calculated by the formula

$$\% \text{ steroid bound} = 100\left(1 - \frac{\text{cpm outside phase} \times \text{vol. inside phase}}{\text{cpm inside phase} \times \text{vol. outside phase}}\right) \quad [16].$$

All the experiments were performed in duplicate or triplicate and the values represent the mean values. The standard deviation of duplicates was ± 0·48 per cent. Total plasma proteins were measured with the biuret reaction, using the Technicon N-method in an Auto analyzer. The fractionation of serum proteins was performed electrophoretically with a Beckman-Spinco apparatus using cellulose acetate slides in barbiturate buffer of pH 8·6 containing calcium chloride (0·002 M).

The testosterone binding capacity of plasma was obtained by determining the specific binding of testosterone at 25°C in diluted plasma to which 1, 3, 5, 8, 12, 20, 30 ng of testosterone respectively were added. (Experimental conditions as described above.) Results were plotted on a Scatchard type plot with Bound/Unbound on the ordinate and the molar concentration of bound testosterone on the abcissa. In the calculation of binding capacity, the concentration of endogenous steroid was not taken in account, being of very small quantitative importance. The weight of the radioactive steroid (0·16 ng) was neglected when the added steroid exceeded 8 ng.

RESULTS

With a 1 in 5 dilution of plasma samples and the same experimental conditions as Rivarola *et al.*[16], the percentage of radioactive steroid bound was 96·7 per cent for non-pregnant subjects. This is in good agreement with the value of 95 ± 1·2 per cent reported by these authors. They also observed that the binding capacity of plasma from late pregnant subjects, under these experimental conditions, is 99·0 ± 0·2 per cent.

For comparison of the binding affinity of plasma of normal pregnant women with that of women with twin pregnancy, a dilution of the plasma proteins to 1 mg/ml was used. This dilution is about 1 in 100, so any differences should be apparent, but as Pearlman[17] has pointed out, owing to the great difference in protein content during pregnancy TBG studies must be done on plasma dilutions with the same protein content. Table 1 shows the results of the serum protein electrophoresis. Table 2 shows the percentage binding in the samples studied with progressive addition of cold testosterone to the outside phase. Table 3 shows the percentage binding in 3 cases of twin pregnancy studied before and after delivery.

Table 1. Results of serum protein electrophoresis in the 5 normal and 5 twin pregnancies used for the testosterone binding studies (G) = twin pregnancy

Subject	(g/100 ml)	Albumin (g/100 ml)	α_1-glob (g/100 ml)	α_2-glob (g/100 ml)	β_1-glob (g/100 ml)	β_2-glob (g/100 ml)	γ-glob (g/100 ml)
Hay	6·8	3·72	0·20	0·91	0·65	0·46	0·85
Hie	7·8	3·86	0·29	1·14	0·93	0·64	0·93
Nis	6·7	3·59	0·22	0·83	0·66	0·44	0·94
Juu	6·3	3·14	0·29	0·98	0·59	0·49	0·78
Gro	6·6	3·65	0·30	1·00	0·50	0·40	0·85
(G) Sal	5·1	2·93	0·13	0·64	0·51	0·25	0·63
(G) Ruu	5·8	2·90	0·22	0·65	0·73	0·43	0·87
(G) Ham	6·1	3·38	0·33	0·94	0·55	0·44	0·44
(G) Sin	6·8	3·76	0·07	0·79	0·94	0·51	0·72
(G) Aht	6·0	3·15	0·23	0·98	0·53	0·45	0·68

Table 2. Percentage binding of testosterone in 10 plasma samples obtained from pregnant women and diluted to a protein concentration of 1 mg/ml (G) = twin pregnancy

	Addition of cold testosterone (ng)			
Subject	0	1	3	5
Hay	90·0	86·8	84·9	80·7
Hie	94·7	89·9	83·9	82·3
Nis	93·4	91·4	85·6	80·9
Juu	94·5	91·1	85·9	80·6
Gro	90·7	88·6	82·6	78·8
(G) Sal	95·9	96·2	94·2	92·6
(G) Ruu	93·1	92·7	85·8	81·7
(G) Ham	94·2	94·3	90·2	88·8
(G) Sin	94·2	94·2	90·7	87·3
(G) Aht	94·9	93·9	90·5	85·0

Note 1. Subject Hie was later found to have had slight intrahepatic cholestatic jaundice. Liver dysfunction may enhance testosterone binding [6].

Note 2. In the case of subject Ruu plasma was taken six hours after delivery; this may have resulted in slightly lower testosterone binding [17].

In order to have a more comparable numerical expression for the testosterone binding capacity in single and twin pregnancies, the binding capacity was determined with use of a Scatchard type plot. The mean value for single pregnancies in the 38th week was $4{\cdot}4 \times 10^{-7}$ M/l. This is in a good agreement with the results of Vermeulen [8], who reported values up to $3{\cdot}6 \times 10^{-7}$ M/l in the second and third trimester, (no indication of what week) using Sephadex filtration. In three twin pregnancies the results were 6·0, 7·7 and $8{\cdot}2 \times 10^{-7}$ M/l., the mean thus being $7{\cdot}3 \times 10^{-7}$ M/l.

DISCUSSION

Following the discovery by Mercier *et al.*[2] that a specific globulin binding testosterone is present in plasma, increased interest has been shown in the sub-

Table 3. Percentage binding of testosterone in 3 twin pregnancies before and after delivery

Week	Addition of cold testosterone (ng)			
	0	1	3	5
		Ham (G)		
38th	94·2	94·3	90·2	88·8
40th	95·2	94·7	92·5	89·8
		Delivery		
1st	92·9	90·1	85·0	77·9
3rd	88·2	82·6	77·6	71·7
5th	86·2	80·5	71·7	66·2
		Sin (G)		
38th	94·2	94·2	90·7	87·3
		Delivery		
1st	90·7	89·5	83·3	78·2
3rd	86·1	81·7	75·3	69·4
		Ras (G)		
35th	90·7	90·6	85·8	81·2
37th	93·1	90·8	86·5	82·2
		Delivery		
1st	89·0	80·8	74·7	70·6

ject. Pearlman and Crepy[3] have pointed out that the measurement of endogenous testosterone and the testosterone-binding capacity is desirable for a proper assessment of circulating androgenicity. The order of testosterone-binding activities in plasmas has previously been demonstrated to be as follows: hypogonadal male–male < hypogonadal female–female < oestrogen-treated female < pregnant female. TBG activity remains low and relatively constant during the menstrual cycle, but rises sharply and steeply during the first trimester of pregnancy and gradually thereafter. To our knowledge, TBG activity in twin pregnancies has not been investigated hitherto. The results of the present study seem to demonstrate that in twin pregnancies the affinity of plasma for testosterone is even higher than in normal pregnancies. Oestrogens are generally thought to provoke a rise in TBG levels. Our study supports the theory that oestrogens enhance TBG production. It is known that in twin pregnancy oestrogen production is much higher than in normal pregnancy. In our cases the urinary total phenolic steroids in mg/24 hr were: in single pregnancies: Hay: 35, Hie: 21, Nis: 29, Juu: 30, Gro: 33, and in twin pregnancies: Sal: 65, Ham: 67, Sin: 47 (the method is used for routine clinical purposes and is rather nonspecific). (The individual role of each oestrogen in increasing TBG production is not yet known and studies in this laboratory are therefore being carried out in order to correlate the TBG with excretion of 12 to 14 oestrogens.) There are individual variations in the testosterone binding capacity in the different late pregnancy plasma both in twin and in simple pregnancies but as group it is higher in the cases of twins. The existence of one or two placentas in a twin pregnancy may play a role. There is steady increase of the TBG until delivery in the twin pregnancies as already reported in simple pregnancies. Thus no upper limit could be detected in the experiments in the prepartum period as exemplified by the three cases studied before and after delivery. These results are in accordance with the similar

studies in simple pregnancies of Pearlman *et al.*[17]. The time of return to non-pregnant levels would be of value for the establishing of the half life of the protein.

Elevated testosterone levels in pregnancy have lately been reported by two groups of researchers[18, 19]. It is possible that the enhanced TBG during pregnancy eliminates the biological effect of high testosterone level. No permanent masculinization of the mother is observed during the pregnancy and this binding may serve also as a second protective barrier for the foetus in addition to the placental aromatization of testosterone in a manner similar to that suggested for cortisol by de Moor[20].

Elevated TBG levels have been reported in pregnancy. However, in these reports only a comparison of TBG per unit of volume was calculated. In fact the total amount of TBG is much greater since we have to take into account the increase in blood volume which occurs in pregnancy (up to 25 per cent).

The results of the electrophoretic studies are shown in Table 1. The typical pattern seen in pregnancy is noticeably accentuated in twin pregnancies. A lower albumin and an increase in α-globulins can be seen. There is no definite increase in β-globulins in twin pregnancies as compared to single pregnancies as might be expected, since the TBG is a minor component of this fraction.

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