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# REGULATION OF PLASMA CORTICOSTEROID-BINDING GLOBULIN IN ADULT CYNOMOLGUS MONKEY (*MACACA FASCICULARIS*) DURING DIFFERENT REPRODUCTIVE STATES

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**Summary**—The plasma concentration of the corticosteroid-binding globulin (mCBG) has been measured in *Macaca fascicularis*, during different stages of reproduction and under hormonal treatments. The mCBG level was determined by a specific electroimmunoassay. There was no difference between females in the follicular phase and intact males; mCBG concentrations were respectively (mean ± SEM) 469 ± 53 and 443 ± 25.6 nmol/l. The mCBG levels were similar during both the luteal (469 ± 33.5 nmol/l) and the follicular phase (469 ± 53 nmol/l). Compared to intact males, the mCBG levels were higher ($P < 0.05$) in castrated males (527 ± 6.6 nmol/l). During gestation, no systematic variations were found and the mCBG levels were not statistically different from the values found during the follicular phase. When estradiol benzoate was administered to castrated animals, the mCBG concentrations increased rapidly. In contrast, the values were reduced slightly by testosterone treatment. The sex-steroid action on the mCBG levels was discussed and compared with the mSBP levels. We question also, the mechanisms involved in the regulation of the mCBG levels during pregnancy.

## INTRODUCTION

Corticosteroid-binding globulin (CBG or transcortin) is a serum glycoprotein which binds glucocorticoids and progestins with high specificity and high affinity. Its presence in the plasma of many mammalian species, including humans (hCBG) and monkeys (mCBG), is now well established [1, 2]. The steroid binding characteristics and plasma concentrations of hCBG are similar to those attributed to mCBG in Old World monkeys [3–7]. Although its exact biological function is unknown, it is generally believed that CBG regulates the bioavailability of free steroids in the blood [8]. More recently, it has been demonstrated that various tissues contain specific binding sites for CBG [9–11]. Thus, control of the plasma concentration of CBG may have important regulatory functions governing corticoid actions. In humans, estrogens are a very potent stimulus to hCBG synthesis and high CBG values are observed during pregnancy [12–14]. Even though steroid-binding characteristics have been widely studied in monkeys, there are few reports on the hormonal regulation of mCBG. It has been shown that mCBG remains unchanged during pregnancy in rhesus monkeys [15, 16] suggesting that between humans and monkeys, the factors controlling the plasma concentration are different. Recently, in *Macaca fascicularis* (cynomolgus monkey), we have shown that such differences affect the hormonal regulation of another binding protein, the sex steroid-binding protein or mSBP: estrogens have none influence but androgens decrease the values like in humans [17].

The aim of this paper was: (i) to establish a specific and accurate immunoassay for mCBG and (ii) to investigate its hormonal regulation in *M. fascicularis*. The plasma mCBG levels were assayed in adults (males and females) in the basal state, after castration, after sex hormone treatments and during pregnancy.

## EXPERIMENTAL

### *Animals*

22 healthy, adult, laboratory-born monkeys (*M. fascicularis*) were studied. They were housed in individual cages under natural photoperiod in Paris. The laboratory conditions have been described elsewhere [18]. Blood samples were collected from males ($n = 9$) and females ($n = 13$) in different reproductive states: 6 normal and 3 castrated (for more than 3 yr) males, 1 ovariectomized and 5 cycling females (average length of the menstrual cycle ranging from 31 to 39 days). Blood samples were taken once during the follicular phase (days of the cycle, mean ± SEM =

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10 ± 1 day) and the luteal phase (21 ± 1.4 days) of the same cycle as determined by menstrual history. Pregnant females ($n = 7$, term mean = 168 ± 1.5 day) were time-mated and 4 (lactating) were studied 66 ± 4.5 day *post-partum*. Castrated animals were studied during 2 types of hormonal treatment; testosterone treatment: 2 males received 1 injection i.m. of 50 mg testosterone enanthate and the third animal received another injection 15 days later to increase hormonal response; estradiol treatment: 5 mg estradiol benzoate was injected i.m. once a week for 6 consecutive weeks to 2 males and 1 female.

*Blood samples*

Blood samples were collected usually at 11.00 h by venipuncture in tubes containing ethylenediamine tetra-acetic acid $Na_2$-salt (EDTA). Plasma, obtained after centrifugation, was frozen and stored at −20°C until steroid and protein concentrations were determined. To overcome fibrinogen precipitation during the immunological procedures, 1 ml of blood was allowed to clot and the serum was stored at −20°C.

During hormonal treatment, blood samples were collected before the injections once a week for 8 weeks.

A pool of monkey serum was prepared, one part of which was stored at −20°C and the remainder was stored at −80°C.

*Purification of human CBG*

Pure human CBG (hCBG) was obtained from late pregnancy serum by a modification of the procedure of Fernlund and Laurell[19] as described previously [20]. Briefly: dual ammonium sulfate precipitation, affinity chromatography on cortisol–sepharose 4B column and blue–sepharose CL6B column were carried out. The purity of hCBG was checked by SDS–Polyacrylamide gel electrophoresis.

*Antiserum*

An antiserum specific to native hCBG was prepared as described previously [20]. The specificity of this antiserum against *M. fascicularis* CBG was assessed by double immunodiffusion [21]. Specific anti-hSBP antiserum (10 μl) was put into the central well and serial doubling dilutions of human and monkey plasma were put into surrounding wells and were allowed to diffuse for 16 h at room temperature. Immunoprecipitation lines were visualized by staining with Coomassie brilliant blue R250.

*Binding assay procedure*

The CBG binding capacity of monkey serum was measured by steady-state polyacrylamide gel electrophoresis as described by Hansson *et al.*[22] except that the stacking gel was omitted. Endogenous steroids were removed by incubating overnight with Norit A charcoal (2.5% w/v) at 4°C, in 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 10% (v/v) glycerol (TEG buffer). The charcoal was removed by centrifugation at 11,000 *g* for 15 min at 4°C and by filtration (pore size 0.22 μm). The supernatant was incubated with 15 nM [$^3$H]cortisol (SA = 40 Ci/mmol, Amersham, Little Chalfont, England) for 2 h at 4°C. The mixture was then diluted in TEG buffer to a final dilution of 1:100 and applied to a 7% polyacrylamide gel (5 × 55 mm) containing 4.5 nM [$^3$H]cortisol. The electrophoresis was carried out at 4°C and at 1 mA per gel (constant current). The gel was then sliced and the slices were counted in pico-fluor (Packard, Zurich, Switzerland) after standing overnight at 4°C to improve the extraction of the radioactive steroid.

*Quantitative estimation of mCBG*

mCBG was quantified by electroimmunoassay (EIA) according to Laurell[23]. EIA was performed by electrophoresis in agarose gel containing the monospecific antiserum (4%, v/v). The half-diluted (in BSA) samples (5 μl) and the reference solutions were deposited in a row of wells punched along the cathodic edge of the gel. Electrophoresis was run overnight at 10 V/cm in a TGM buffer (Tris 58 mM, glycine 75 mM, morpholino-ethane sulfonic acid 82 mM) containing 0.3 mM Na azide. The remaining proteins were absorbed by applying filter papers. The gel was rinsed in saline and pressed again with filter papers, then air dried. Immunoprecipitation lines were stained by Coomassie brilliant blue R 250. Quantitation was based upon measurement of the heights of the rocket-shaped precipitates which were formed. The results were expressed in nmol/l by comparison with a pool of monkey serum calibrated by its binding capacity. The intra-assay variation was 5% and the inter-assay variation 8%. To minimize the inter-assay variations, all samples from the same animal were run in the same assay.

*Steroid concentrations*

Steroid concentrations were measured in duplicate by RIA following extraction and separation through Sephadex LH 20 (Pharmacia, Uppsala, Sweden) for estradiol [24] and through celite (BioMerieux, Charbonnières-les-Bains, France) for testosterone [25]. Cortisol was measured using a RIA kit (GammaCoat [$^{125}$I]cortisol, Baxter, Cambridge, Mass.).

*Plasma proteins*

Plasma proteins were quantitated using the Biuret technique (Technicon Auto Analyser).

*Statistics*

Differences between groups were evaluated by analysis of variance and Student–Fisher's *t*-test.

## RESULTS

*Binding assay*

As illustrated in Fig. 1 the peak represents the specific binding of [$^3$H]cortisol to mCBG, since an

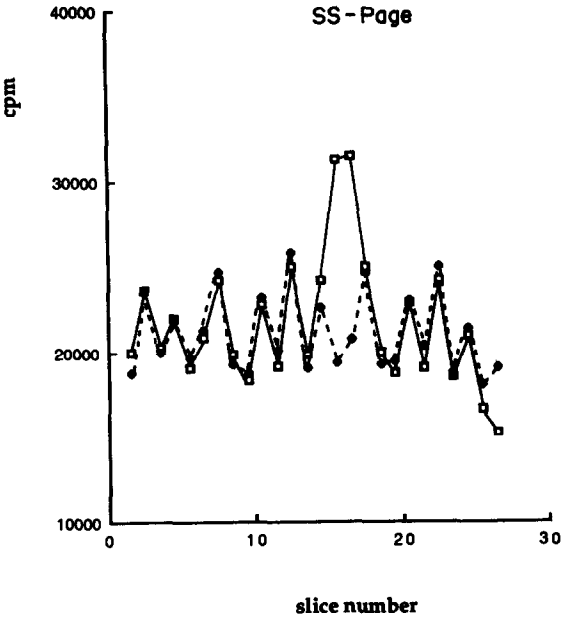



Fig. 1. CBG binding capactiy of monkey serum measured by steady-state electrophoresis. Serum was incubated with 15 nM [$^3$H]cortisol and submitted to electrophoresis in polyacrylamide gel (7%) containing 4.5 nM [$^3$H]cortisol. The gel was then sliced and the slices were counted (□). Same experiment, but the serum was incubated with an excess (1000-fold) of cold cortisol (■).

excess (1000-fold) of cold cortisol added at the time of incubation completely abolishes the binding. When the experiments were conducted in the same run, it was observed that the steroid binding capacity of the pooled monkey sera was lower in the samples stored at −20°C when compared with those stored at −80°C (molar concentration = 376.5 ± 46.7 nmol/l, $n = 3$ and 467 ± 42 nmol/l, $n = 7$, respectively).

*Specificity of the antiserum*

By double immunodiffusion, only one precipitin line was seen when anti-hCBG serum reacted with human and monkey serum (Fig. 2). This implies that antibodies cross-react with domains that are common to both hCBG and mCBG and gives evidence for the monospecificity of the antiserum against mCBG.

*Validation of the immunological assays*

The relationship between the peak heights and the values in a 3-fold range dilution of the pool of the monkey or the human serum was linear, which validated the EIA method (data not shown). By EIA, there was no difference between the peak heights of monkey serum stored at −20°C or −80°C. Because of its convenience and its specificity, the EIA was used to measure the mCBG concentrations in the cynomolgus monkeys in different physiological states and following hormonal treatments. The pool of monkey serum stored at −80°C was used as standard.

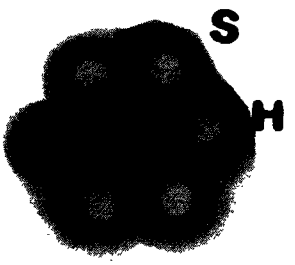



Fig. 2. Specificity of the antiserum. Specific anti-hSBP antiserum (10 µl) was put into the central well and serial doubling dilutions of human (H) and monkey (S) plasma were put into in the surrounding wells and allowed to diffuse for 16 h at room temperature.

*Concentration of mCBG during different reproductive states*

There was no difference beween females in the follicular phase and intact males. mCBG concentrations were respectively, (mean ± SEM) 469 ± 53 and 443 ± 25.6 nmol/l. The mCBG levels were similar during both the luteal (469 ± 33.5 nmol/l) and the follicular phase (469 ± 53 nmol/l). Compared to intact males, mCBG levels were higher ($P < 0.05$) in castrated males (527 ± 6.6 nmol/l) (Fig. 3).

Figure 4 illustrates the variations of proteins, steroids, and mCBG in 4 pregnant females through-

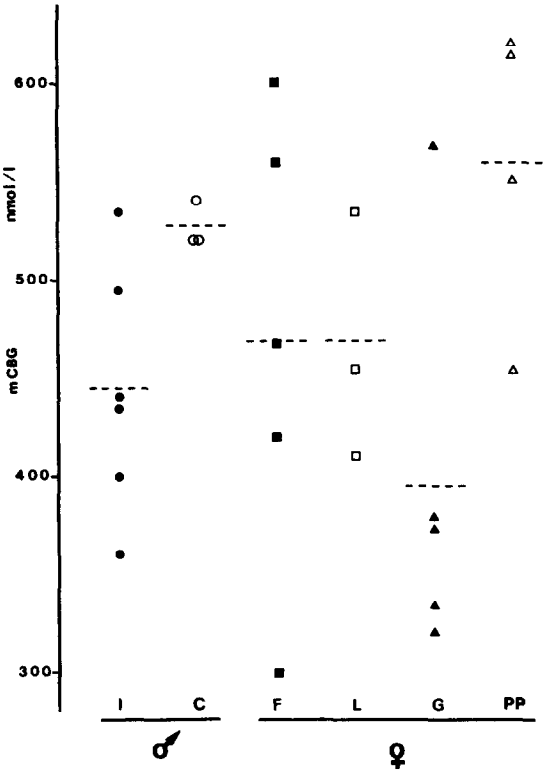



Fig. 3. mCBG concentrations during different reproductive states. (♂) adult males; I: intact; C: castrated. (♀) adult females; F: follicular phase (10 ± 1 day); L: luteal phase (21 ± 1.4 day); G: gestation (day 140 until term); PP: *post-partum* (66 ± 4.5 day).

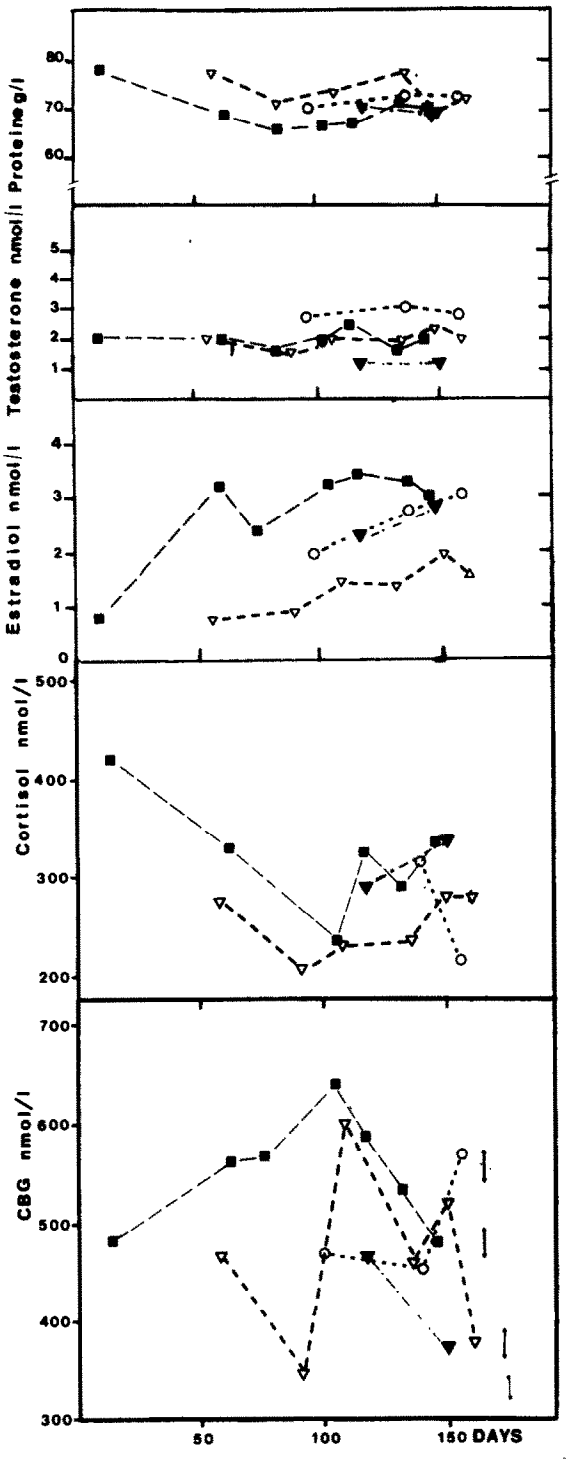



Fig. 4. Concentrations of plasma mCBG, cortisol, estradiol, testosterone and total proteins throughout gestation in 4 females (■, ○, ▽, ▼). Arrows indicate delivery.

out gestation. No systematic variations were found. In one case mCBG slightly increased then declined to the initial level. In late gestation (day 140 until term), the mCBG levels (395 ± 44.6 nmol/l) were not statistically different from the values found during the follicular phase. During lactation the mCBG increased so that the concentrations (550 ± 30.5 nmol/)

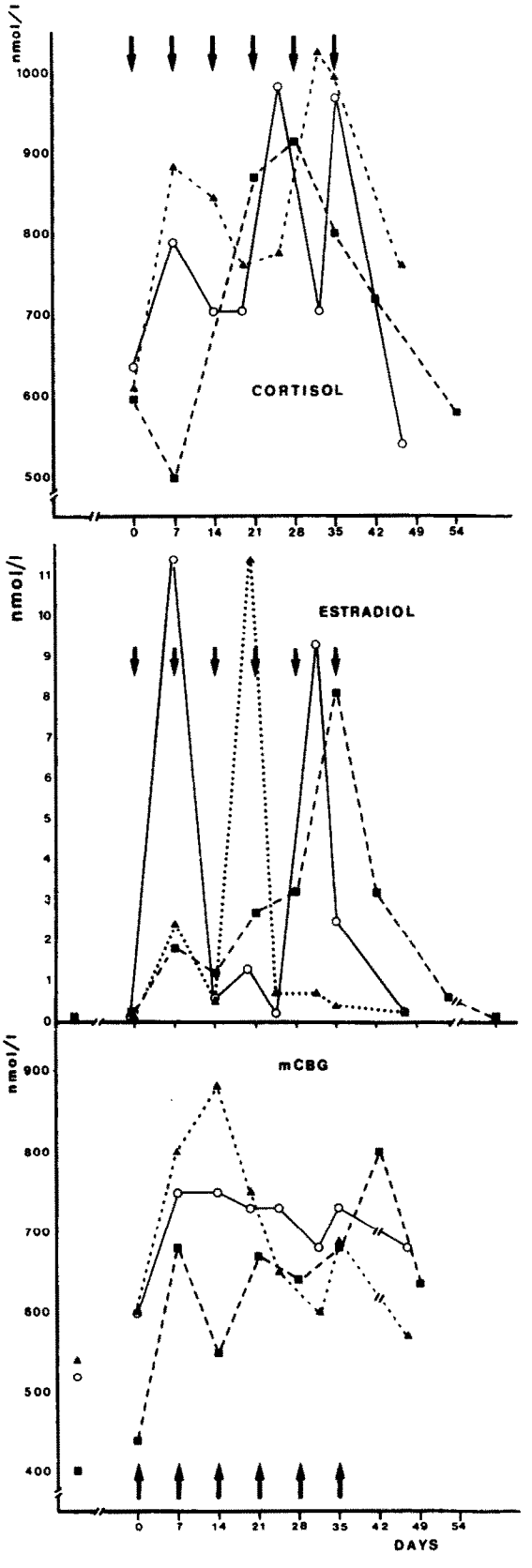
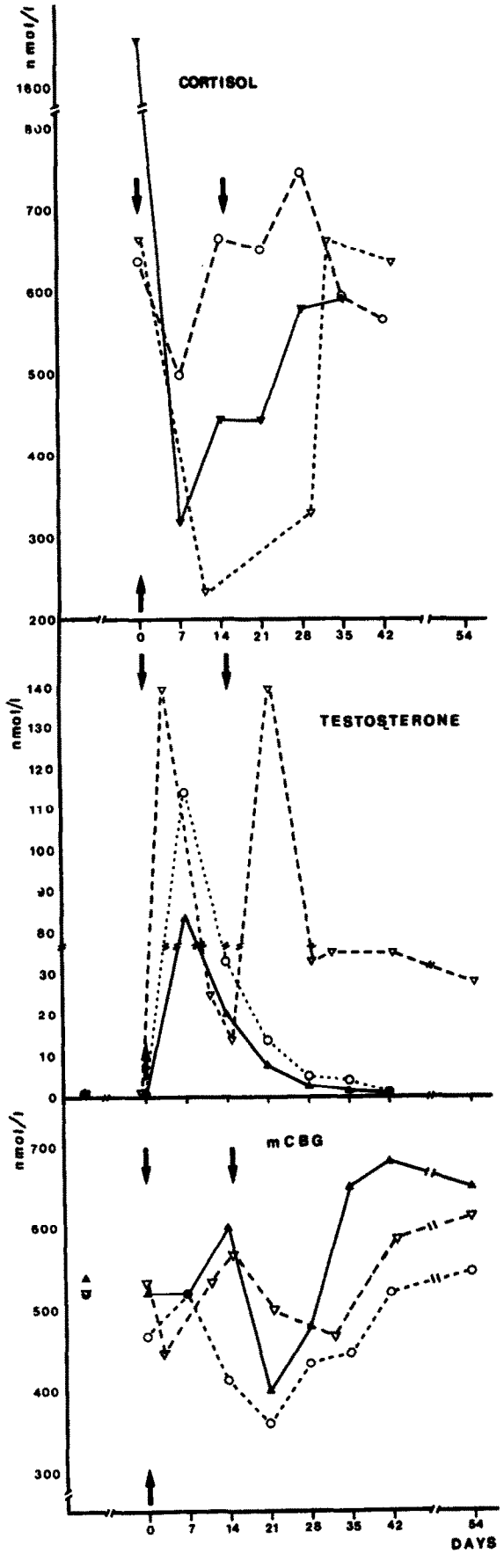



Fig. 5. Concentrations of mCBG, cortisol and estradiol during estradiol treatment in 2 castrated males (○, ▲) and 1 castrated female (■). Arrows indicate weekly injections of estradiol benzoate.




were higher ($P < 0.01$) than those found during the late gestation (Fig. 3). A 3–4-fold increase of the concentrations of estradiol in pregnant females was observed but testosterone and total plasma proteins remained at the same levels. Cortisol levels were within the normal range.

*Hormonal treatments*

When estradiol benzoate was administered to castrated animals (2 males and 1 female) (Fig. 5) high levels of estradiol were obtained over 7 weeks (mean = 2.9 nmol/l). The mCBG concentrations increased rapidly. Moreover, the response seemed to be higher in the female than in the males. A complete return to the baseline level did not occur, however, until 2 weeks. Cortisol levels parallel the changes in mCBG.

In contrast the mCBG levels decreased slightly within 2–3 weeks in the 3 animals that had received testosterone enanthate (Fig. 6). Testosterone levels remained within the physiological range [26] and there were broad and no systematic variations in cortisol levels.

## DISCUSSION

During primate evolution, a CBG-like protein has persisted [2, 4–7]. In all species the cortisol binding globulin exhibits similar electrophoretic mobilities in polyacrylamide gels [2, 3, 5]. We confirm here that plasma cortisol binding characteristics in *M. fascicularis* are similar to those of the other Old World primates [4, 5, 27]. Moreover, we show that the antigenic determinants which characterize the molecular configuration of hCBG are conserved in *M. fascicularis* during evolution [4, 6, 27]. These data indicate that the monkey may be useful for biological studies of CBG which are not possible to perform using human subjects.

Regulation of the mCBG concentration has been studied, but in monkeys all these determinations were usually performed by binding assay with [$^3$H]cortisol [5] or [$^3$H]corticosterone [3, 16, 28, 29]. Generally these methods require very stringent conditions (time and temperature of incubation). We have used a specific binding method which is not subject to interference by other cortisol binding proteins, such as albumin, to evaluate the binding capacity of mCBG. This may explain why the values that we have found are lower than those previously reported in monkeys [5, 27–29]. We have demonstrated in a pool of monkey serum, that binding capacity is modified by storage conditions. Thus, we have tried to develop a reliable method more suitable for the routine determination of mCBG. Nevertheless, we have only

Fig. 6. Concentrations of mCBG, cortisol and testosterone during testosterone treatment. Two males received one injection of testosterone enanthate (arrows) and the third animal (▽) received two injections.

investigated the immunological activity and we have quantified the concentration of the protein as an antigenic determinant.

In agreement with Koritnik[29] we have also failed to find a significant sex-difference in mCBG concentration as was suggested by Klosterman *et al.*[5]. Such a sex-difference has not been found in humans [1, 12, 14, 30]. It is surprising that mCBG levels are higher in castrated than in intact males thus suggesting an androgenic influence. This has not been reported in Old World primates. In contrast, in the Squirrel monkey—a New World primate—mCBG is higher in intact males than in castrated ones [31].

In order to try to understand the interaction between gonadal and adrenal physiology, we have studied the mCBG concentration during hormonal treatments. Our results confirm the estrogen stimulating effect on the mCBG concentration both in male and female cynomolgus monkey. The response is rapid and sustained. Of particular importance is the observation that the cortisol level is also increased. By using ethinyl estradiol (EE), an estrogenic effect was reported in the Green monkey [32] and in cynomolgus [29], but not in male rhesus monkeys [33]. Hence, regulation of CBG by estrogen seems to be a very common feature since it was found in humans [14], in rat [34] and in a New World monkey, the Squirrel monkey [31] that puts the CBG level in a position of a good indicator of estrogen influence. In cynomolgus, this regulation differs from the sex steroid-binding protein (mSBP) whose level was not affected by estrogen [17]. The fact that estradiol levels are very low during the menstrual cycle [35] cannot explain the lack of sex-differences in mCBG concentration since previous reports have demonstrated a gender difference in the mSBP level. Hormones other than estrogen may be implicated in mCBG regulation.

When testosterone is administered, the mCBG concentration decreases slightly but not as much as mSBP [17]. In monkeys, a small number of experiments using synthetic steroids have failed to demonstrate any influence [29] but in humans the results remain conflicting [12]. It is of interest to notice that antiandrogen does not affect the mCBG whereas it reduces mSBP in the rhesus monkey [36]. Thus, the mechanism and structural activity relationship for the steroidal action on mCBG and mSBP still remain unclear.

The results presented in this report demonstrate differences between CBG levels in macaques and humans during pregnancy. In the rhesus monkey, during the last trimester of pregnancy, the mCBG levels are similar to nonpregnant values [15] or decline before term [16]. These reports have investigated the binding capacity. We have demonstrated here, that the concentration of the protein as an antigenic determinant is not affected during gestation in cynomolgus monkey. During the first trimester, mCBG may increase, but it declines gradually by term to the initial levels. This feature is very surprising since we have demonstrated, firstly that mCBG was under estrogenic control and secondly that estradiol gradually increases 3–4-fold throughout pregnancy, while total testosterone was maintained. We have also eliminated plasma dilution since total plasma protein concentration is sustained. Nevertheless, it may be argued that sometimes estradiol levels were higher during estradiol treatment than during pregnancy so that a pharmacological effect of estradiol would be obtained. 2 months *post-partum*, the mCBG level is significantly higher than levels during the latter part of pregnancy. We have reported similar results for the mSBP: it declines gradually until the parturition, then increases again during the lactation [17]. We can conclude that the foeto-placental unit may play a role in the regulation of mCBG. In the hamster, it has been proposed that the decidualized uterus produces a signal that acts on the liver to increase production of CBG [37]. This model can be also proposed for the regulation of the human CBG, but needs to be demonstrated. Nevertheless, placental metabolism differs largely between human and nonhuman primates [38]. Compared to humans, monkeys have much lower levels of total estrogen and progesterone levels being one tenth or less of humans. Under these circumstances, the fact that mCBG levels do not increase during pregnancy in monkeys, does not modify the delivery of progesterone and cortisol to the target tissues. Another working hypothesis is that modification of the clearance rate may affect the CBG level. Two lines of evidence support our contention. Firstly, a pregnancy-associated variant of human transcortin has been demonstrated [39], and secondly an increase in the peripheral degradation, or elimination, of any glycoproteins may result from an alteration of the glycosylation [40].

The results presented in this report show a good correlation between the variations of the mCBG expressed by others in terms of binding capacity and by us in terms of immunological activity. However, on the one hand, determining why the estrogens have an important influence on the mCBG concentration and why the regulation throughout the gestation is quite different, as it is in humans, needs further explanation. On the other hand we show that in cynomolgus, the regulation of two steroid binding proteins, CBG and SBP, both synthesized by liver, are not similar.

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