

Testosterone-induced inhibition of spermatogenesis is more closely related to suppression of FSH than to testicular androgen levels in the cynomolgus monkey model (*Macaca fascicularis*)

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Abstract

We have investigated the antigonadotropic and anti-spermatogenic effects of exposure to a long-acting testosterone ester in the cynomolgus monkey model. Groups of five adult animals were exposed either to vehicle or to 10 mg/kg or 20 mg/kg testosterone buciclate (TB) over a 26-week period with injections given in weeks 0, 11 and 18. In week 26, testicular biopsy tissue was collected. Serum testosterone levels were in the upper normal range with 10 mg/kg TB and were approximately twofold higher with 20 mg/kg TB. The estradiol pattern followed that of testosterone and body weights increased in a testosterone-dependent manner. TB completely abolished serum LH bioactivity. Serum concentrations of FSH and inhibin- α were suppressed in a TB dose-dependent manner. During weeks 4–8 after the first injection, a rebound of FSH and inhibin but not bioactive LH secretion occurred. This rebound was followed immediately by a restimulation of testis size and sperm numbers. After the next TB injections these parameters

were once again suppressed. Nadir testis size was 30–40% of baseline and animals were severely oligozoospermic or transiently azoospermic. Consistent azoospermia was not achieved. Quantitation of serum inhibin B, proliferating cell-nuclear antigen staining and flow cytometric analysis of germ cell populations revealed pronounced suppression of spermatogenesis in both TB-treated groups whereas androgen receptor expression remained unchanged. Testicular androgens levels, determined in week 26, did not differ among all three groups and did not correlate with sperm numbers, histological and immunocytochemical findings. All suppressive effects were fully reversed during the recovery period. We have concluded that pronounced suppression of primate spermatogenesis seemingly requires inhibition of FSH rather than testicular androgen levels, at least in this preclinical non-human primate model. For the purpose of male contraception, FSH inhibition appears mandatory.

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Introduction

Spermatogenesis in primates is primarily driven by the gonadotropic hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Androgens and FSH are capable of stimulating the entire process of spermatogenesis as suggested by complete spermatogenesis observed in patients with activating mutations of gonadotropins and receptors (Nordhoff *et al.* 1999) or by the results from administration of the respective endocrine factors in experimental studies (Weinbauer & Nieschlag 1998, Nieschlag *et al.* 1999). However, these effects are frequently not very pronounced in quantitative terms, and – in most instances – fail to provide quantitatively normal germ cell numbers. Thus, it is generally agreed that the combination of testosterone/LH and FSH activity is needed for stimulating primate spermatogenesis in quantitative terms.

Conversely, male contraceptive studies have revealed that the suppressive effects of gonadotropin-releasing hormone (GnRH) analogs, androgens and gestagens on testicular function are generally related to the extent of suppression of both LH and FSH secretion. This is evident from studies using GnRH antagonists (Bastias *et al.* 1993, Behre *et al.* 1997, Swerdloff *et al.* 1998), androgens alone (Behre *et al.* 1995, Zhang *et al.* 1999) and androgens in combination with gestagens or estrogens (Handelsman *et al.* 1996, 2000, Meriggiola *et al.* 1998, Kamischke *et al.* 2000).

FSH seems to be of particular importance for the spermatogenic process of primates. FSH specifically stimulates spermatogonial proliferation and survival in rhesus and cynomolgus monkeys (Weinbauer *et al.* 1991, Marshall *et al.* 1995), and a key role for FSH in primate fertility is suggested by studies in bonnet monkeys (*Macaca radiata*) demonstrating that immunization against FSH

abolished fertility in mating studies (Moudgal *et al.* 1997). Conversely, spermatogenesis can be clearly reduced in non-human primates and in man by immunoneutralization of FSH (Moudgal & Sairam 1998). Patients with inactivating mutations of the FSH receptors were reported to produce sperm and can be fertile (Tapanainen *et al.* 1998). It is not entirely clear whether the biological activity was totally abolished in those patients with sperm production and fertility. However, two men bearing a defective FSH- β subunit and suffering from azoospermia have been described (Lindstedt *et al.* 1998, Phillip *et al.* 1998), suggesting that FSH is necessary for the complete initiation of spermatogenesis in man. Earlier studies demonstrated that adding FSH treatment to androgen-exposed men yielded quantitatively normal sperm numbers (Matsumoto & Bremner 1989).

It is also interesting to note that even in the intact adult cynomolgus and rhesus monkey testes, sperm production is not maximal under physiological conditions. Direct administration of FSH (van Alphen *et al.* 1988, Lindstedt *et al.* 1998, Phillip *et al.* 1998) or hemicastration associated with hypersecretion of FSH (Ramaswamy *et al.* 2000) provoke a clear-cut increase in the number of differentiating spermatogonia, spermatocytes and spermatids. Circumstantial evidence for such effects has also been obtained for bonnet monkeys (Medhamurthy *et al.* 1995). On the other hand, predominant FSH action can substantially maintain the spermatogenic process after inhibition of endogenous gonadotropin secretion. This is suggested by the description of a hypophysectomized patient in whom an activating mutation of the FSH receptor co-existed with normal spermatogenesis in the absence of LH but with detectable serum testosterone levels (Gromoll *et al.* 1996). Although the intratesticular testosterone concentrations were not known, the case of this patient suggests that the constitutive activation of the FSH receptor is associated with normal spermatogenesis.

Despite the well-documented fact that exogenous testosterone administration – depending on the dose administered – can either suppress gonadotropin secretion and testicular functions or activate the spermatogenic process, surprisingly little is known about the precise relationship between testicular androgen levels and spermatogenesis among primates. Initiation of spermatogenesis in the immature gonad apparently requires very high local androgen concentrations (Steinberger *et al.* 1973, Chemes *et al.* 1982, Bidlingmaier *et al.* 1983, Marshall *et al.* 1984). The available evidence, however, is very limited and also rather puzzling for the adult testis. Exposure of hypophysectomized cynomolgus monkeys to 7- to 12-fold elevated peripheral testosterone concentrations partially maintained spermatogenesis but testicular testosterone levels were 50% lower than normal (Marshall *et al.* 1986). In the GnRH antagonist-suppressed and testosterone-supplemented macaque, testicular testosterone levels were lowered in the absence of bioactive LH

in all animals but were not different between animals given GnRH antagonist alone and those receiving GnRH antagonist plus testosterone although spermatogenesis was maintained in the latter (Weinbauer *et al.* 1988).

The present work was undertaken to investigate the suppressive effects of testosterone buciclate (TB) in a non-seasonal non-human primate model, the cynomolgus monkey. TB provided stable testosterone concentrations over prolonged periods in non-human primates (Weinbauer *et al.* 1986, Behre *et al.* 1995, Rajalakshmi *et al.* 1995) and suppressed spermatogenesis in contraceptive studies (Behre *et al.* 1995). Gonadotropic hormones, testosterone, estradiol, inhibins, testicular androgens, testis size, semen parameters, germ cell production and germ cell proliferation (proliferating cell-nuclear antigen (PCNA), androgen receptor expression and testicular androgen levels were evaluated in a control group and two groups exposed to TB. Analysis of PCNA was chosen since PCNA serves as a testicular marker of germ cell proliferation (Steger *et al.* 1998) and is gonadotropin dependent in the primate testis (Schlatt & Weinbauer 1994). The data suggest that FSH suppression is critical for testicular suppression whereas testicular testosterone levels, unexpectedly, did not correlate at all with various testicular and spermatogenic parameters.

Materials and Methods

Animals

Fifteen adult cynomolgus monkeys (*Macaca fascicularis*) (4.5–6.5 kg body weight) were maintained under a 12 h day:12 h night regimen in a temperature-controlled environment. All animals had access to pelleted food and tap water, and monkey chow was supplemented daily with fresh fruit. All experimental studies were undertaken in accordance with German Federal Law on the Care and Use of Laboratory Animals.

Experimental design and sample collection

Animals were randomly assigned to three groups of five animals. The experimental protocol consisted of a baseline period, a 26-week exposure period and a 14-week recovery period. Preparations were injected intramuscularly in weeks 0, 11 and 18. Group 1 animals received diluent, group 2 animals were given 10 mg/kg TB, and group 3 animals were treated with 20 mg/kg TB on each occasion. During the exposure period, body weight, testis size, semen parameters and serum concentrations of bioactive LH, testosterone, estradiol, FSH and inhibin- α were determined at weekly intervals thereafter during weeks 30, 34 and 40. FSH and inhibin- α were measured until week 34. In week 26, a testicular biopsy was performed on all animals. Tissue was processed for histological analysis

and for immunocytochemical localization of PCNA and androgen receptor. A second tissue sample (0.7–5.8 mg) was used for flow cytometrical analysis, and a third piece (2.3–23 mg) for determination of testicular androgen levels.

Ejaculates were collected by an established procedure using rectal probe electrostimulation of animals sedated with ketamine hydrochloride (10–15 mg/kg). Serum samples were collected from sedated animals by venipuncture of the cubital or saphenous vein, and cooled blood was allowed to clot overnight, centrifuged twice and stored at -20°C until analysis. Testicular biopsies were collected under sodium pentobarbital anesthesia (10–20 mg/kg) and processed as described earlier (Weinbauer *et al.* 1998b). Tissue destined for histological and immunohistochemical analysis was fixed in Bouin's solution, dehydrated, embedded in Paraplast (Clancer, Oxford, UK) and sectioned at 5 μm . For flow cytometric analysis, tissue was suspended and homogenized in 0.1 mol/l citric acid containing 0.5% Tween-20, and fixed in 96% ethanol until analysis. For determination of tissue concentrations of androgens, tissue was snap-frozen on dry ice and stored at -20°C until analysis.

Hormone determinations

Bioactivity of LH was measured using an established mouse Leydig cell-based bioassay. Assay sensitivity was 5.4 IU/l (78/549 NIBSC standard preparation; Potters Bar, Herts, UK) and intra- and interassay variations were 10% and 14.3% respectively. Testosterone was determined from ether-extracted serum using an established radioimmunoassay. Detection limit was 0.68 nmol/l and intra- and interassay variations were 5.2% and 8.8% respectively. For determination of testicular androgen levels (the antiserum also cross-reacts with dihydrotestosterone), the tissue samples were extracted twice with ether and the extracted samples were assayed at three dilutions. Recovery of tritiated testosterone during extraction was 80–100%. Estradiol levels were assessed by a commercial radioimmunoassay kit (Sorin Biomedica, Düsseldorf, Germany). The limit of detection was 18.5 pmol/l and intra- and interassay coefficients of variation were 5.7% and 6.3%. Inhibin- α was determined by the 'Monash' assay validated for this macaque species (Fingscheidt *et al.* 1989). This assay was used specifically for this study since the antibody detects FSH-dependent forms of inhibin in the cynomolgus monkey (Weinbauer *et al.* 1991, 1994b) and thus serves as an indicator of endogenous FSH activity. Assay sensitivity was 270 U/l and intra- and interassay variations were 3.1% and 5.6%. Inhibin B was determined by a two-site enzyme-linked immunoassay as reported earlier (Foppiani *et al.* 1999). Assay detection limit was 7.8 ng/l and intra-assay coefficient of variation was 4.2%. FSH levels were determined by a heterologous radioimmunoassay using human tracer, cynomolgus monkey

standard and ovine antiserum as previously described (Khurshid *et al.* 1991). Detection limit was 0.156 mg/l and intra- and interassay variations were 4.2% and 6.7%.

Testis size and ejaculate parameters

Testicular volume determinations were made from caliper measurements of testicular width and length using the formula for a regular ellipsoid for volume calculation. Data are expressed as combined volume of left plus right testis. For determination of sperm numbers, enumerations were performed in the liquid and solid portions of the ejaculate and numbers are expressed per whole ejaculate sample. Gross sperm motility was determined by counting the number of motile sperm per 100 sperm twice. Testis biopsy sections were stained by periodic acid-Schiff's base and hematoxylin for histological evaluation of the spermatogenic status.

Immunocytochemistry

Immunocytochemical localization of PCNA was performed as described previously (Schlatt & Weinbauer 1994). Immunocytochemical localisation of PCNA was performed by the alkaline-phosphatase-anti-alkaline-phosphatase technique. In brief, the sections were deparaffinized and rehydrated. Tris-buffered saline (TBS; 50 mM, 150 mM NaCl, pH 7.6) was used as washing buffer. For suppression of unspecific binding, bovine serum albumin (BSA; 0.1%) was added during antibody incubation and a blocking step was performed using 5% normal goat serum. As described before (Schlatt & Weinbauer 1994), a monoclonal mouse antiserum anti-PCNA (Dako Diagnostika GmbH, Hamburg, Germany; code no. M 879; dilution 1:50) was used as primary antibody. The label was visualized as a red precipitate (Fast Red) using the alkaline phosphatase anti-alkaline phosphatase kit and the instructions of the supplier (Dako Diagnostika GmbH; code no. D 0651). This analysis was chosen since the number of PCNA-expressing germ cells is gonadotropin dependent in the cynomolgus monkey (Schlatt & Weinbauer 1994). For control strains, the primary antibody was replaced by buffer.

The protocol for immunocytochemical localization of the androgen receptor on cryosections was described previously (Dankbar *et al.* 1995). Here, we used the same approach on paraffin-embedded material which required the introduction of microwave-assisted antigen retrieval. A monoclonal mouse antibody (MAB 394.1, diluted 1:2000 in TBS plus 0.5% BSA) was allowed to bind to its antigen during overnight incubation at 4°C after antigen retrieval in 0.05 M glycine-HCl buffer (10 min in microwave oven at 100°C) of deparaffinized and rehydrated sections. This antibody was raised against the human DNA-binding domain. Peroxidase-anti-peroxidase (PAP) was used for visualization of the label. Successive incubations were performed using rabbit anti-mouse antiserum (Dako

Diagnostika GmbH; code no. Z 456) and the PAP complex (Sigma, Deisenhofen, Germany; code no. 3039; dilution 1:25). Diaminobenzidine was used as substrate to obtain an insoluble brown reaction product. For control strains, the primary antibody was replaced by buffer.

For quantitative analysis of PCNA from immunocytochemical stains, an image analysis system (VIDAS; Zeiss, Oberkochen, Germany) and a procedure similar to that described previously (Weinbauer *et al.* 1998a) were used. Parameters analysed were the area occupied by PCNA-stained cells and the average integrated staining density. Interactive mode threshold setting was used for discrimination of PCNA-stained cells from unstained cells. The optimized sequence of commands and settings was applied to all sections at a defined microscope light intensity and all sections were analysed in one session. Twelve randomly selected areas were analysed for each biopsy and the average taken as the value per animal entering statistical analysis. Since the system was not equipped with calibration software, values obtained are relative and expressed in arbitrary units based upon equipment-inherent grey scales.

Flow cytometry

For flow cytometric analysis, 0.7–5.7 mg testicular tissue was prepared as previously described (Weinbauer *et al.* 1991). Following mincing with surgical blades and dispersion in 0.1 M citric acid containing Tween-20, cells were fixed in ethanol. After resuspension in pepsin, cells were stained with 4,6-diamidino-phenyl-indole/sulforhodamine 101-staining solution; analysis was done on the PAS II sorter (Partec GmbH, Münster, Germany). Different cell populations were separated according to DNA content, i.e. elongated spermatids (HC), round spermatids (1C), spermatogonia and somatic cells (2C), and tetraploid cells comprising primary spermatocytes and G2-spermatogonia (4C).

Statistical analysis

Data obtained from flow cytometry and for tissue androgen levels were analyzed using ANOVA followed by Duncan's test. All data sets comprising longitudinal data for individual animals were analyzed by MANOVA followed by Duncan's test. For the multiple comparison analysis, data from weeks 1–26 (exposure period) were pooled for each experimental group. Percent data were arcsin-transformed prior to statistical analysis. Data for sperm number are expressed as median levels because of skewed data distribution and data were analysed using the Kruskal–Wallis test. The level of significance was set at 5%.

Results

Hormones

In group 1, LH bioactivity (Fig. 1) ranged, on average, between 10 and 35 U/l throughout the entire observation

period. Among groups 2 and 3, bioactive LH levels dropped significantly ($P < 0.05$) to close to or below the assay detection limit within 1 week after the first injection of TB and remained in that range until week 26 with no significant differences between the two groups ($P > 0.05$). LH activity recovered slightly earlier in group 2 (10 mg/kg TB) compared with group 3 (20 mg/kg TB) as judged during week 30 but thereafter this effect was not clear-cut. Levels of LH bioactivity attained the baseline range during the recovery phase.

Group 1 serum testosterone levels (Fig. 1) were 53.0 ± 15.5 nmol/l at baseline and ranged from 20 to 40 nmol/l throughout the study period. Among group 2 animals, TB administration raised testosterone levels two- to threefold over baseline and for group 3 the increments were four- to fivefold. Overall, testosterone concentrations were significantly different between all groups ($P < 0.05$). Based upon serum testosterone levels, the interval of 11 weeks was too long and, hence, the third injection was given 7 weeks later. Serum testosterone levels returned to the baseline range during the recovery period.

Serum concentrations of estradiol (Fig. 1) fluctuated between 50 and 100 pmol/l in the control group. For groups 2 and 3, the estradiol pattern correlated to that of testosterone, and levels were significantly higher with the higher dose of administered testosterone ($P < 0.05$). Estradiol levels were significantly higher in the TB-treated groups compared with the control group ($P < 0.05$) but attained the baseline range during recovery.

Serum concentrations of FSH (Fig. 2) were rather variable between animals with two animals showing clearly higher values than the other three resulting in substantial variation around mean values in group 1. Administration of TB reduced FSH concentrations in both groups and FSH levels were close to or undetectable in group 3 during weeks 3–7. During weeks 6–11 FSH escaped suppression in group 2 followed by suppression after the next TB injection and remained low thereafter. An FSH rebound was seen in group 3 during weeks 10–11, followed by a decline and sustained suppression of FSH secretion. Suppression of FSH levels was reversible by week 34 of the study. Overall, FSH levels were significantly different between the three groups ($P < 0.05$).

Inhibin concentrations (Fig. 2) remained within the normal range (3000–6000 U/l) in group 1 except for an isolated drop to 2582 ± 337 U/l in week 20. In group 2, inhibin dropped initially but rebounded during weeks 5–11, declined again subsequent to the next TB injection, and remained below 3000 U/l until week 26, and recovered fully by week 30 of the study. In group 3, exposed to higher testosterone levels, a far less pronounced rebound of inhibin was encountered in week 11, but inhibin also returned to baseline values by week 34 of the study. Overall, inhibin levels were significantly ($P < 0.05$) different among the three groups.

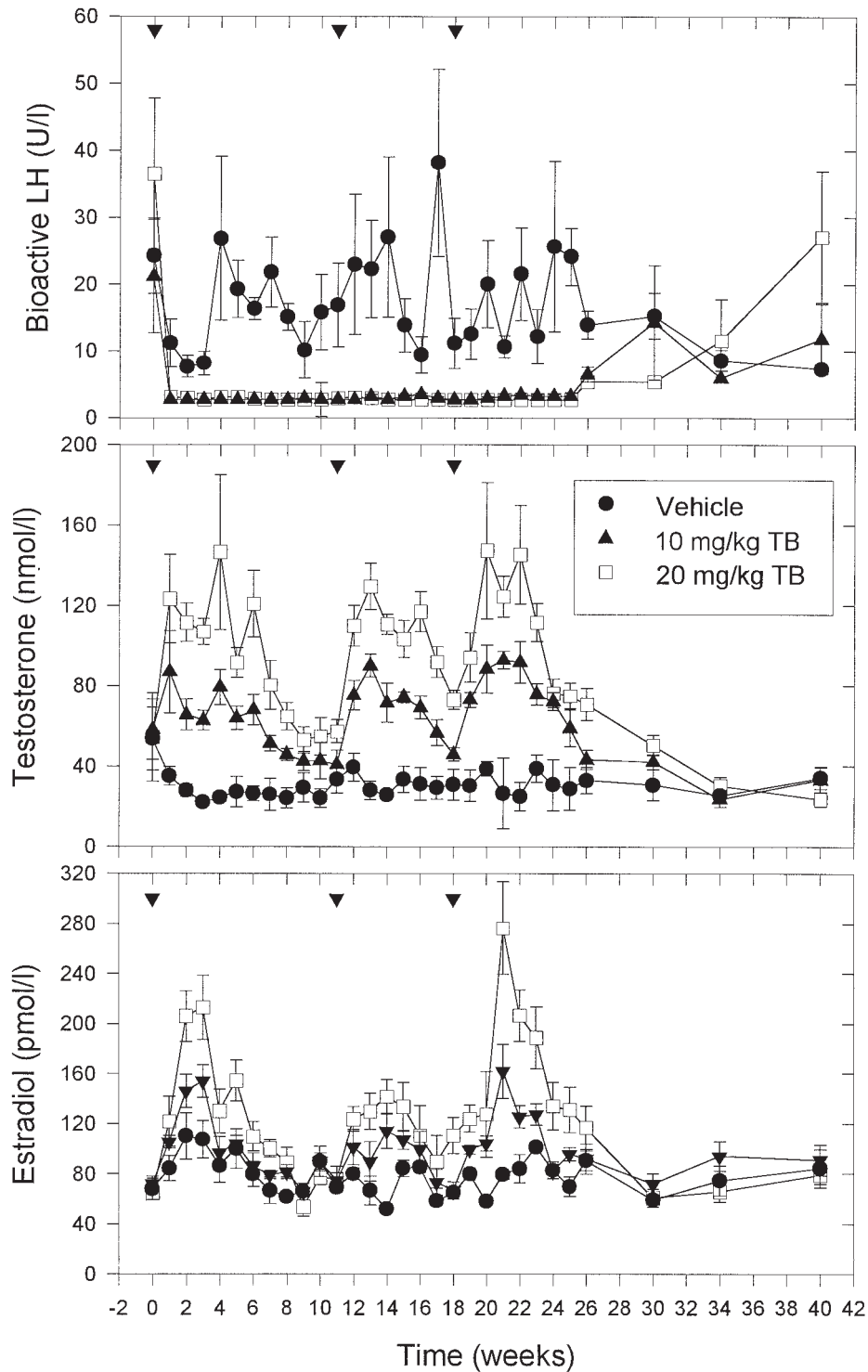


Figure 1 Serum concentrations of bioactive LH (top panel), testosterone (middle panel) and estradiol (bottom panel) of cynomolgus monkeys injected (arrowheads) with vehicle, 10 or 20 mg/kg TB. For the multiple comparison analysis, data from weeks 1–26 (exposure period) were pooled for each experimental group. For LH, differences are significant between vehicle and TB ($P < 0.05$) but not between TB groups ($P > 0.05$); for testosterone and estradiol, all groups are significantly different from each other ($P < 0.05$). Values are mean \pm S.E.M.

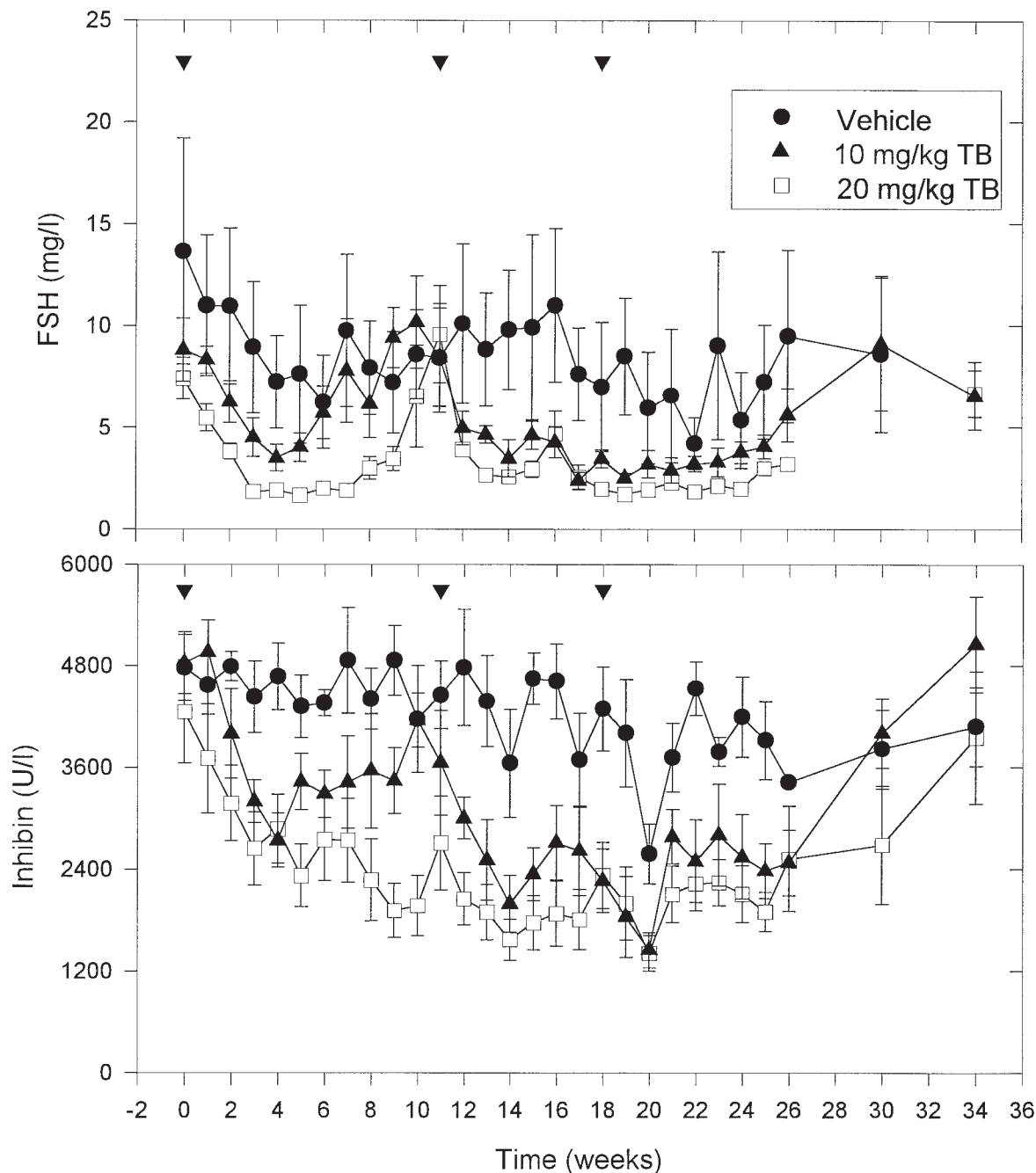


Figure 2 Serum concentrations of FSH (upper panel) and inhibin- α (lower panel) of cynomolgus monkeys injected (arrowheads) with vehicle, 10 or 20 mg/kg TB. For the multiple comparison analysis, data from weeks 1–26 (exposure period) were pooled for each experimental group. For FSH and inhibin- α , all groups are significantly different from each other ($P < 0.05$). Values are mean \pm S.E.M.

Body weights, testis size and semen parameters

Body weights (Fig. 3) increased significantly ($P < 0.05$) by 2–3% in group 2 and by 5–10% in group 3 over baseline. This effect became even more pronounced as group 1

animals lost up to 8% of body weight during the course of the study. The differences for body weights between groups 2 and 3 were also significant ($P < 0.05$).

Average testis size (Fig. 4) increased slightly and transiently during the initial 4–6 weeks but remained constant

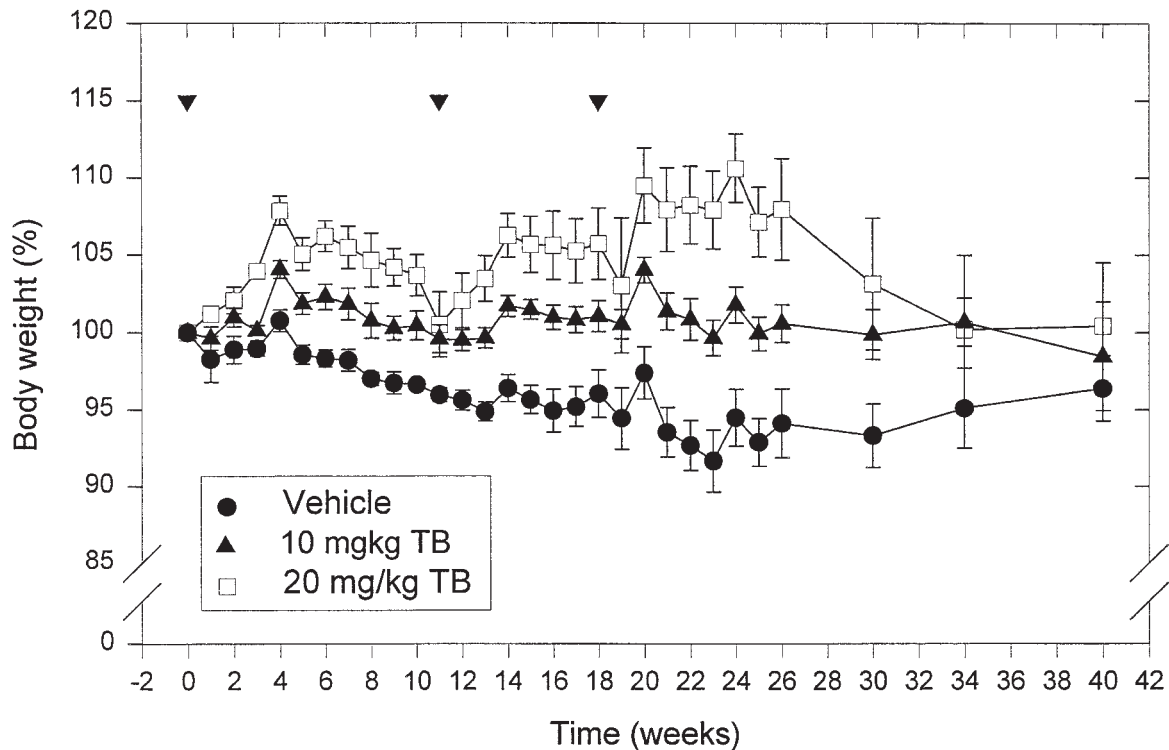


Figure 3 Body weight changes in cynomolgus monkeys injected (arrowheads) with vehicle, 10 or 20 mg/kg TB. For the multiple comparison analysis, data from weeks 1–26 (exposure period) were pooled for each experimental group. All groups are significantly different from each other ($P < 0.05$). Values are mean \pm S.E.M.

($\pm 10\%$ variation) throughout the study among group 1 animals. In contrast, testicular volumes decreased continuously in groups 2 and 3 ($P < 0.05$ vs group 1). During weeks 10–15, testicular size decline stopped in group 2 and a rebound was seen followed by a continuation of testicular involution. Based upon the entire set of values during the exposure period, testis size was smaller in group 3 compared with group 2 animals ($P < 0.05$). Nadir values of testis size were attained by week 16 in both groups and ranged between 35% and 45% of baseline dimensions.

Sperm numbers (Fig. 4) remained stable throughout the evaluation period in group 1. Baseline sperm numbers ranged from 73 to 114×10^6 sperm/ejaculate. Sperm numbers dropped precipitously in groups 2 and 3 until week 14 of the study to values below 2×10^6 sperm/ejaculate. Thereafter, sperm numbers transiently but substantially rebounded until week 21 and thereupon remained below 4×10^6 sperm/ejaculate. Only three azoospermic ejaculates (from the same animal) could be collected in this group. For group 3, the rebound was minimal and azoospermia was achieved in three animals, though no longer than throughout a 3-week period, and severe oligozoospermia ($< 0.5 \times 10^6$ sperm/ejaculate) was attained in the remaining two animals. At the time of collection of testicular biopsies, median sperm numbers

were 25×10^6 sperm/ejaculate in group 1, 0.64×10^6 sperm/ejaculate in group 2, and 0.16×10^6 sperm/ejaculate in group 3.

Ejaculate weights were significantly higher in groups 2 and 3 as compared with group 1 ($P < 0.05$) but did not differ significantly between groups 2 and 3 ($P > 0.05$) (data not shown). Sperm motility ranged between 60 and 100% in group 1 ejaculates. Motility analysis was only possible on a restricted set of samples owing to the low numbers of sperm and the small amount of exudate. In those cases in which analysis was possible, sperm motility appeared unaltered in group 2 and group 3 animals (data not shown).

Testicular histology, flow cytometry, androgen receptor expression, and PCNA staining

Complete and intact spermatogenesis was seen in tissue from control animals. Specimens from both TB-exposed groups revealed various degrees of spermatogenic involution and qualitative reduction/loss of advanced germ cells. Flow cytometry indicated a decrease in the proportion of HC cells, 1C cells, 4C cells and a corresponding increase of 2C cells in the TB-treated groups as compared

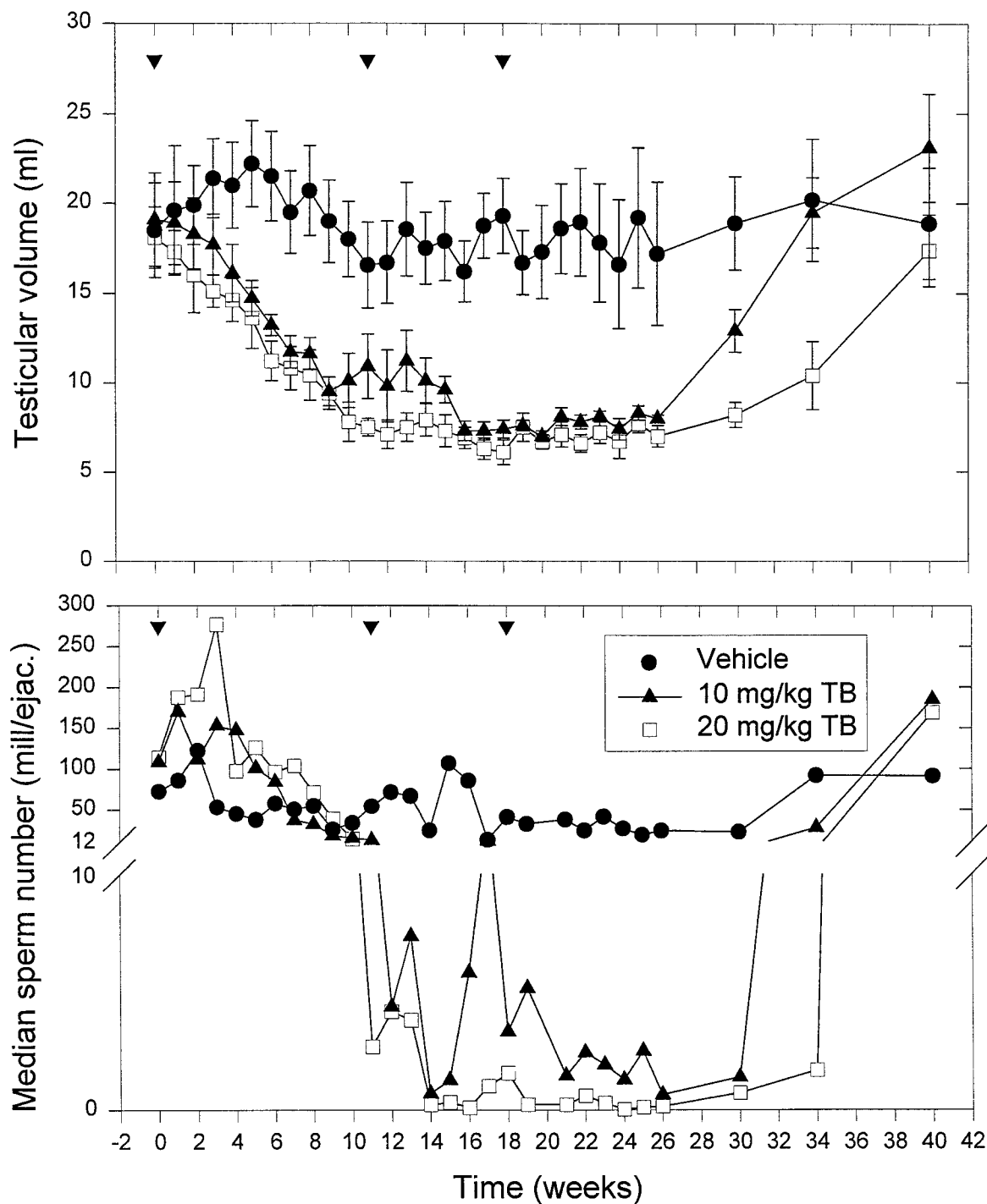


Figure 4 Testis size (upper panel) and sperm numbers (lower panel) of cynomolgus monkeys injected (arrowheads) with vehicle, 10 or 20 mg/kg TB. For the multiple comparison analysis, data from weeks 1–26 (exposure period) were pooled for each experimental group. For testis size, all groups are significantly different from each other ($P < 0.05$), for sperm numbers, differences are significant between vehicle and TB ($P < 0.05$) but not between TB groups ($P > 0.05$). Values are mean \pm S.E.M.

Table 1 Flow cytometric analysis of testicular cell populations in cynomolgus monkeys exposed to vehicle or to 10 mg/kg or 20 mg/kg TB. Values are means \pm S.E.M. of four to five animals per group

Treatment	HC cells1	IC cells	2C cells	4C cells
Vehicle	20.7 \pm 4.4	38.8 \pm 4.9	31.5 \pm 3.3	8.2 \pm 0.7
10 mg/kg TB	14.3 \pm 2.5	25.2 \pm 5.5	52.5 \pm 8.9	6.5 \pm 1.1
20 mg/kg TB	10.2 \pm 3.9	21.0 \pm 6.4	62.1 \pm 10.6	6.7 \pm 0.5

Differences are not significant ($P > 0.05$).

with the control group (Table 1). The observed effects were TB dose-dependent but did not attain statistical significance ($P > 0.05$).

Androgen receptor signals (Fig. 5) were observed in somatic cells and peritubular cells, Sertoli cells and Leydig cells. Cells were identified by nuclear size and shape, and localization. Androgen receptor staining was also seen in some elongating spermatids. This stain was unspecific and also detectable in control sections. However, this phenomenon precluded random-based interactive image analysis for the quantitation of androgen receptor expression since elongated spermatids – though only some of them stained – were present on many sections.

PCNA (Fig. 5) was expressed in spermatogonia and early primary spermatocytes with a pattern similar to that reported earlier for this species (Schlatt & Weinbauer 1994). Cells were identified by nuclear size and shape, and by the relative position within the seminiferous epithelium. Following administration of TB, the abundance

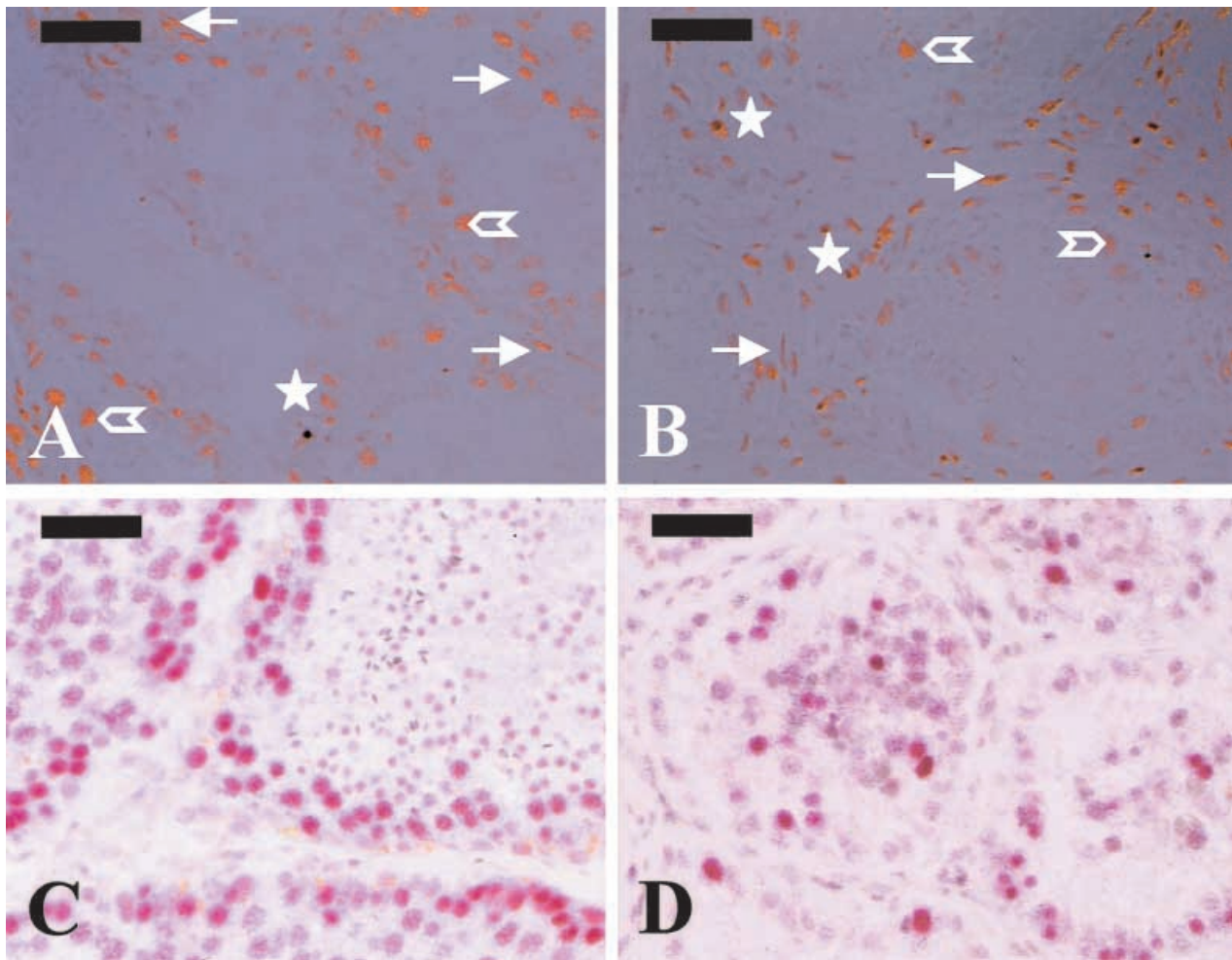


Figure 5 Immunolocalization of androgen receptor on cryosections in (A) control testis and (B) following 26 weeks of exposure to 20 mg/kg TB. Leydig cells (stars), peritubular cells (arrows) and Sertoli cell nuclei (open arrowheads) are stained. Panels C and D display immunolocalization of PCNA in (C) control testis and (D) in testis following 26 weeks of exposure to 10 mg/kg TB. Spermatocytes and spermatogonia are stained (red colour). Note the clear reduction of cell numbers and numbers of PCNA-stained cells. Bar represents 50 μ m for all figures.

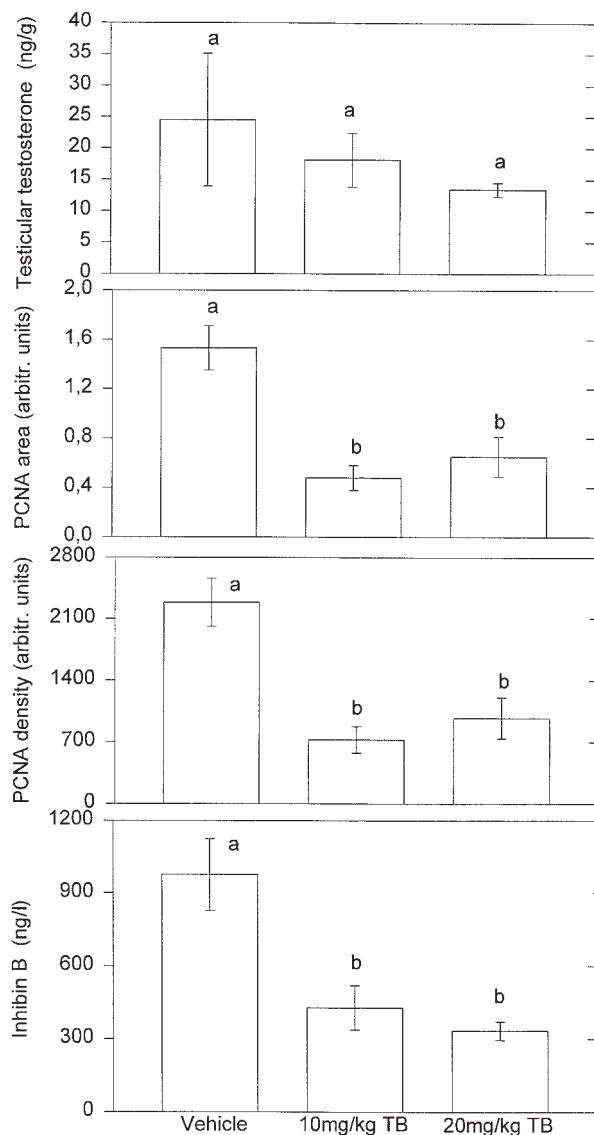


Figure 6 Testicular androgen (top panel), PCNA area and density (two middle panels), and serum inhibin B concentrations (bottom panel) following 26 weeks of exposure of cynomolgus monkeys to vehicle, 10 mg/kg or 20 mg/kg TB. Values are mean \pm S.E.M. Different superscripts indicate significant differences between groups ($P < 0.05$). Note that the large variation of testicular androgen levels in the vehicle group is due to one animal with a value of 60.9 ng/g.

of PCNA staining and the number of PCNA-positive cells appeared reduced on qualitative histological inspection. Quantitative analysis demonstrated that the area of PCNA staining as well as the average staining intensity (density) were significantly lowered compared with the control group ($P < 0.05$) without a statistically significant difference between the TB-treated groups ($P > 0.05$) (Fig. 6).

Intratesticular androgens

Levels for testicular androgens (Fig. 6) ranged from 5.5 to 60.9 nmol/l in group 1, from 4.4 to 26.6 nmol/l in group 2 and from 10.2 to 16.1 in group 3. Differences among groups did not differ statistically ($P > 0.05$). No correlation (Fig. 7) was apparent between intratesticular androgen levels and serum testosterone concentrations ($r = -0.362$, $P > 0.204$), area of PCNA stain ($r = 0.200$, $P > 0.493$) or density of PCNA stain ($r = 0.201$, $P > 0.490$). A statistically significant ($r = 0.733$, $P < 0.001$) but biologically insignificant correlation (Fig. 7) was seen for testicular androgen concentration and sperm number. It was noticed that in three TB-exposed animals (one in group 2 and two in group 3) with lowest sperm counts at the time of biopsy, testicular androgen levels were in the upper range of the respective group.

Discussion

Supranormal serum testosterone levels were used in the present study to evaluate the antispermatogenic and contraceptive potential of TB and permitted analysis of the relationship between spermatogenic activity, FSH/inhibin and testicular androgens in a non-human primate species, which serves as a well-suited preclinical animal model for the endocrine control of testicular function (Weinbauer & Nieschlag 1999). Administration of the long-acting TB ester profoundly raised serum testosterone concentrations in a strictly dose-dependent manner. In the higher dose group (20 mg/kg), animals were exposed to approximately four- to sixfold higher peripheral androgen levels than control animals over a period of about 26 weeks. Originally >10-week injection intervals were anticipated. However, at the lower dose of 10 mg/kg, a rebound was seen for FSH, inhibin, testis size and sperm number. Based upon serum testosterone levels, the injection interval was shortened to 7 weeks for the last injection. The duration of testosterone release from the ester had previously been tested in castrated (Weinbauer *et al.* 1986) and in GnRH antagonist-suppressed, i.e. castrate-like serum testosterone levels, cynomolgus monkeys (Weinbauer *et al.* 1986, 1989, 1994a). On a body weight basis, a duration of >10 weeks was expected from these studies for the present investigation. The decreased duration of action in animals with normal serum testosterone levels at the time of TB administration could be related to circumstances in which suppression of endogenous testosterone elicits an enhanced GnRH release and drive to the pituitary that could not be overcome by the lower dose of TB (10 mg/kg).

The unwanted rebound effect, however, provided interesting insights into the endocrine regulation of spermatogenesis in the non-human primate model. At first, bioactive LH secretion remained suppressed uniformly throughout the entire period of exposure to higher than normal androgen concentrations. In sharp contrast,

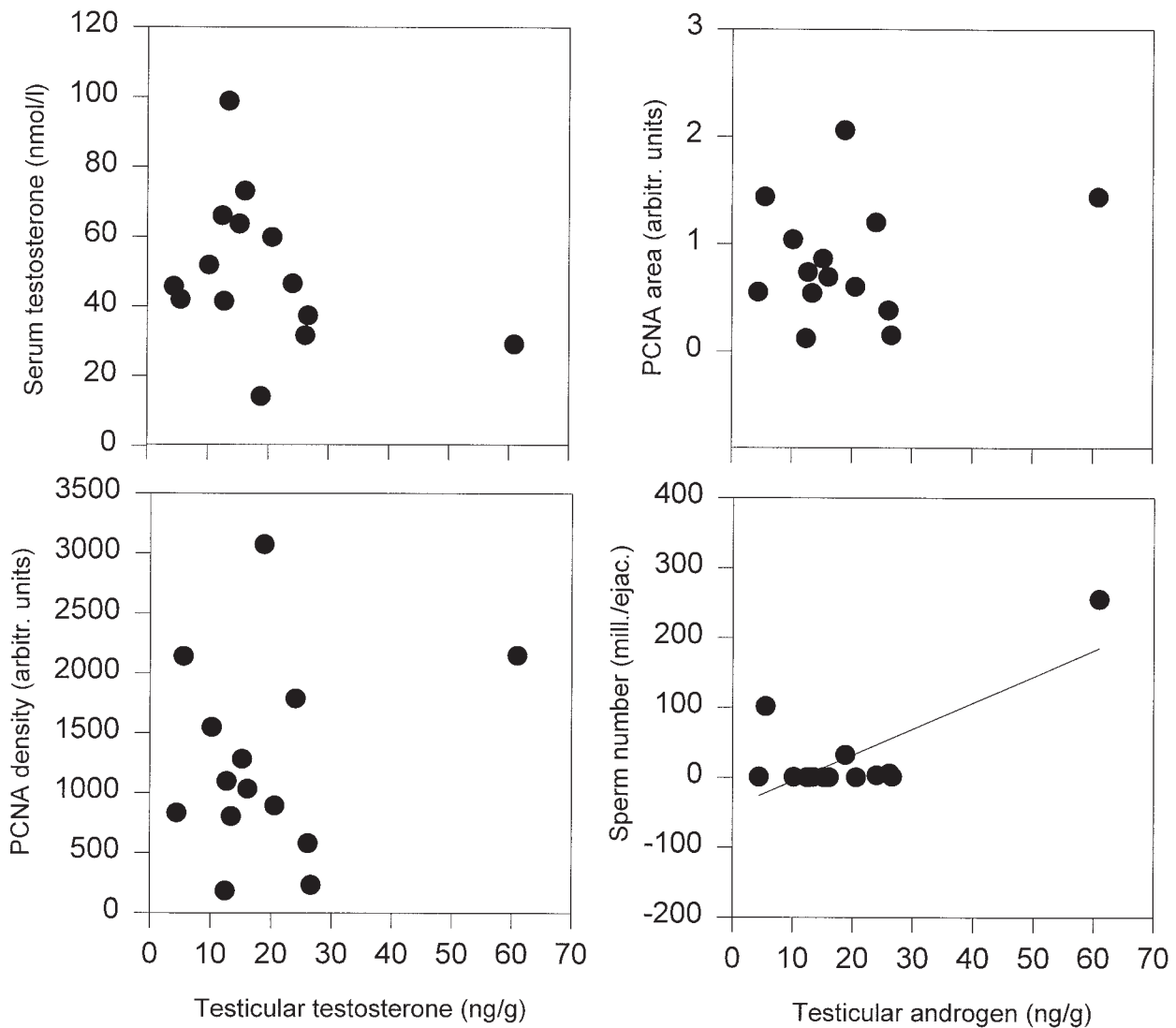


Figure 7 Correlations between testicular androgen levels and PCNA area and density, and sperm number following 26 weeks of exposure of cynomolgus monkeys to vehicle, 10 mg/kg or 20 mg/kg TB.

FSH-dependent inhibin- α , followed by testis size and sperm numbers, transiently escaped suppression until serum testosterone levels could be restored into the supranormal range. Thereafter, these parameters declined again. We estimate from the pattern of testosterone and inhibin- α levels that an approximately twofold elevation of serum testosterone concentrations is required for complete and sustained suppression of FSH secretion in the adult and intact male cynomolgus monkey. These observations suggest that complete suppression of FSH secretion might be mandatory in order to achieve suppression of spermatogenesis, at least in this macaque model. It had been suggested earlier from studies in other macaque species (bonnet and rhesus monkey) that had been immunized actively or passively against FSH or FSH

receptor, that this gonadotropin is a prime regulator of spermatogenesis (Moudgal & Sairam 1998, Nieschlag *et al.* 1999). Administration of exogenous FSH to macaques also supports this view (van Alphen *et al.* 1989, Weinbauer *et al.* 1991). Similarly, recent studies on endocrine male contraception suggest the need for profound suppression of FSH (Büchter *et al.* 1999, Handelsman *et al.* 2000, Kamischke *et al.* 2000). Suhana *et al.* (1999) reported that in the cynomolgus monkey exposed to Western or Asian diet the antispermatogenic effects of an androgen/gestagen combination were more pronounced in the group with lower FSH levels. The present work extends these observations, since it provides further data indicating that endogenous FSH was capable of stimulating Sertoli cells (as evidenced by inhibin- α), testis size and sperm

production in the absence of detectable LH bioactivity in a physiological setting.

The time-lag between the rebound of inhibin- α levels and testis size was 5–6 weeks. This corresponds closely to the time required for differentiated spermatogonia to develop into sperm in this macaque species (Weinbauer *et al.* 1998b). It is known that gonadotropin deficiency specifically affects premeiotic and differentiated spermatogonia (Weinbauer *et al.* 1998b, Zhengwei *et al.* 1998) but does not affect the duration of spermatogenesis (Aslam *et al.* 1999) in the macaque. Hence, it is reasonable to assume that during the rebound spermatogonia were activated immediately to enter meiosis leading to resumption of the spermatogenic process. This assumption further implies the need for complete and sustained inhibition of FSH and FSH-dependent testicular factors for the purpose of endocrine male fertility control.

Overall, the higher dose of TB provoked a more profound reduction of inhibin- α secretion, testis size and sperm counts when compared with the effects of the lower dose. Data obtained from flow cytometric analysis of testis tissue, albeit not statistically significant, lend further support to this view. It was of interest to note, however, that the higher dose of TB did not produce intratesticular androgen levels higher than from those seen with the lower dose. No relation was apparent between the suppression of spermatogenesis as seen from PCNA and inhibin B and testicular androgen measurements. These observations also would support the view of a pivotal role of endogenous FSH secretion for macaque spermatogenesis. In addition, however, these data suggest that the dosage window between gonadotropin suppression and direct spermatogenic stimulation by testosterone might be rather large in primates. On a theoretical basis, high doses of testosterone alone might well provide full and sustained inhibition of sperm production in primates. Notwithstanding, from a practical point of view, high-dose testosterone would carry the risk of untoward androgen-related side effects in volunteers during contraceptive studies such as altered lipid metabolism, gynecomastia or acne.

To our surprise, testicular testosterone levels in the TB-treated groups did not differ significantly from those measured in the control group despite the abolition of detectable bioactive LH secretion. Also, androgen receptor protein expression, as revealed by immunocytochemical analysis, was retained at least to a qualitative extent. Knowledge about the relationships between gonadotropins, testicular androgens and spermatogenesis is very limited for the primate. In the GnRH antagonist-suppressed cynomolgus monkey, testicular testosterone levels were reduced to about one-third of baseline within 15 weeks of bioactive LH deficiency (Weinbauer *et al.* 1988). Since LH bioactivity was assessed by the same assay procedure and the period of lost LH bioactivity was actually substantially longer in the present study, a decrease

of testicular androgen secretion was expected for the current study. Possibly, TB supplementation maintained local androgen levels although it remains to be explained why a double dose failed to deliver higher testosterone amounts to the testis. On the other hand, it must be recalled that substitution with either 6 mg/kg or 30 mg/kg TB over a period of 15 weeks did not significantly alter testicular testosterone levels in the GnRH antagonist-treated animals (Weinbauer *et al.* 1988). It must also be mentioned that the assessment of testicular androgen levels in the present study relies on a single measurement. Hence, we cannot rule out entirely the possibility of changes in testicular androgen levels at times prior to termination of the exposure phase.

Alternatively, one could speculate that the entry of testosterone into the testicular microcirculation is dependent on whether hypogonadotropic hypogonadism had been induced by the suppressive effects of androgens or GnRH antagonist. In the rat testis, LH or GnRH are required to maintain blood flow, and GnRH directly affects blood flow via specific testicular GnRH receptors (Bergh & Damber 1993). In the primate, the presence of functional GnRH receptors has been considered unlikely since GnRH analogs failed to exert a direct effect on Leydig cell function as judged by steroid hormone production. Also, Northern blot analysis did not reveal GnRH receptor transcripts in the human testis (Chi *et al.* 1993). However, other reports suggest specific binding and localization of GnRH receptor in the interstitial area of the human testis (Bourne *et al.* 1980, Bahk *et al.* 1995). More recently, two transcripts of the GnRH receptor were detected by RT-PCR in the human testis (Botte *et al.* 1999, Kottler *et al.* 1999). Hence, the primate testis, like the human ovary, may well contain GnRH receptors, and testicular blood flow and microcirculation might be affected also by local GnRH. However, data on the relationship between hormones, local GnRH and blood are lacking for the primate testis, and the view presented here must remain speculative at present.

Vehicle-exposed animals lost body weight throughout the study phase. This loss of body weight is most probably related to a reduction of testosterone concentrations observed in this study. Testosterone deficiency is associated with body weight loss in the cynomolgus monkey (Weinbauer *et al.* 1988, 1994a). The reason for the reduction of testosterone concentrations remains unclear. Seasonal effects are unlikely since this equatorial macaque species is not considered to exhibit seasonality of reproduction. Possibly, the weekly sedation throughout a continued period of time might have caused reduction of testosterone levels and subsequently body weights.

In conclusion, our data suggest that FSH rather than testicular testosterone suppression is critical for testicular involution. By inference for the clinical situation, complete and sustained FSH suppression is mandatory for the purpose of male contraception.

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