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INCREASE IN PLASMA 5α-ANDROSTANE-3α,17β-DIOL GLUCURONIDE AS A MARKER OF PERIPHERAL ANDROGEN ACTION IN HIRSUTISM: A SIDE-EFFECT INDUCED BY CYCLOSPORINE A*

PATRICK VEXIAU,[1]† JEAN FIET,[2] PHILIPPE BOUDOU,[2] JEAN-MARIE VILLETTE,[2] GILLES FEUTREN,[3] NOAH HARDY,[2] RENE JULIEN,[2] CLAUDE DREUX,[2] JEAN-FRANCOIS BACH[4] and GERARD CATHELINEAU[1]

[1]Diabetology and Endocrinology Department, Hôpital Saint-Louis, [2]Biochemistry and Neuroendocrinology Laboratory, Hormone Biochemistry Unit, Hôpital Saint-Louis, [3]Sandoz Laboratory, Rueil-Malmaison and [4]Immunology Department, Hôpital Necker, Paris, France

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Summary—Dose-dependent hypertrichosis is a common dermatological side-effect affecting the majority of patients treated with cyclosporine A (CSA). Previous studies have not demonstrated the influence of CSA on specific sex hormone levels. The aim of this study is to investigate whether CSA increases the activity of 5α-reductase, an enzyme which transforms androgens into dihydrotestosterone in peripheral tissues. The metabolite which best reflects this activity is 5α-androstane-3α,17β-diol glucuronide (Adiol G). The study was carried out on 49 insulin-dependent diabetes patients particpating in the double-blind "*Cyclosporine-Diabète-France*" clinical trial, of which 28 were treated with CSA (16 males and 12 females), and 21 received only placebo (10 males and 11 females). All patients underwent extensive clinical and laboratory evaluations prior to and during the present study. In addition to Adiol G, testosterone (T), dehydroepiandrosterone sulfate (DHEA S) and sex hormone-binding globulin (SHBG) were assayed. Levels of Adiol G increased significantly in CSA-treated groups: males, 11.86 ± 2.58 vs 7.83 ± 2.30 nmol/l; females, 4.48 ± 2.70 vs 2.10 ± 1.22 nmol/l; $P < 0.02$ (comparison of means). There were no significant differences in this parameter before and during treatment in either the male or female placebo groups (paired t-test). During the treatment period, T, DHEA S, SHBG and the T/SHBG ratio did not significantly change with respect to their baseline values in any of the groups studied (comparison of means). Comparison (using paired t-test) showed a significant increase of DHEA S in CSA-treated groups: males, $\delta = 3.08 \pm 3.33$ nmol/l, $P < 0.01$; females, $\delta = 0.98 \pm 1.13$ nmol/l, $P < 0.05$. In conclusion, it is possible that CSA induces hypertrichosis or hirsutism by increasing 5α-reductase activity in peripheral tissues. Nevertheless the role of increased DHEA S as a possible Adiol G precursor cannot be excluded.

INTRODUCTION

Cyclosporine A (CSA) is an immunosuppressor which has been widely used since 1977. Originally administered in organ heterograft cases, since 1983 it has been used in clinical trials with patients suffering from autoimmune disease such as insulin-dependent diabetes (IDD) [1].

A nearly constant side-effect occurring in both sexes is the appearance of hypertrichosis or hirsutism, involving normally hairless areas [2, 3]. Nude mice treated with CSA become hairy, an effect which is reversed by interrupting CSA administration [4].

Several studies of androgenizing steroids have failed to demonstrate increased serum androgen levels in humans treated with CSA [3, 5, 6]. These results seem to show the absence of effects on ovarian, testicular and adrenal androgen production. In fact, with high CSA doses, testicular testosterone secretion is inhibited in the animal [7]. These studies also failed to demonstrate a decrease in sex hormone-binding globulin (SHBG) levels, thus eliminating the hypothesis that an increase in free androgens is responsible for the observed hypertrichosis and hirsutism. In addition, the results of studies undertaken on allograft recipients treated with CSA are difficult to interpret because such patients often receive high doses of corticosteroids, which interfere with the metabolism of endogenous androgens.

Considering that there is no change in androgen production and transport (as there is in idiopathic

*From the Cyclosporine/French Study Group (see acknowledgements).

†Author to whom correspondence should be addressed: Dr P. Vexiau, Hôpital Saint Louis, 1, rue Claude Vellefaux, 75475 Paris Cedex 10, France.

hirsutism) [8] we measured 5α-androstane-3α,17β-diol glucuronide (Adiol G) in order to study cutaneous 5α-reductase activity [9]. Indeed, Adiol G is a major dihydrotestosterone metabolite which results from the 5α-reduction of testosterone in peripheral target cells, especially those located in the skin.

The present prospective study was carried out on IDD patients treated with CSA, but without corticosteroids. Since the study was conducted in double-blind (CSA vs placebo), it included a control group receiving only placebo and subjected to the same conditions as the experimental group.

PATIENTS AND METHODS

Patients

Since the study was conducted on samples obtained from a bank of sera collected during the "*Cyclosporine-Diabète-France*" clinical trial (CDF) [10], the number of assays was limited by the small quantity of serum in each sample we obtained. The following experimental and control groups, consisting of recent IDD patients (less than 6 weeks since the start of insulin therapy) aged between 15 and 40 yr, were constituted and the patients treated under the following double-blind conditions:

Treatment group 1—16 men receiving CSA;
Treatment group 2—12 women receiving CSA;
Control group 3—10 men receiving placebo;
Control group 4—11 women receiving placebo.

All patients were aggressively treated with insulin and trained in glucose self-monitoring. Insulin therapy consisted of intermediate plus regular insulin or a combination of long-acting and regular insulin.

Contraceptive therapy agents were prescribed as a precaution in female patients. Of the 12 in the CSA-treated group, 5 received microprogestogens, 5 received macroprogestogens and 2 no oral contraceptive; in the control group, 4 received microprogestogens, 5 received macroprogestogens and 2 no oral contraceptive.

Methods

Control samples were drawn from patients prior to beginning CSA or placebo administration. A sample was also drawn from each patient between the 3rd and 6th month of treatment. Additional samples were drawn from 28 patients, in the four groups, between the 6th and 18th month of treatment.

During the first few months, patients in the treatment groups received CSA doses of either 7.5 or 10 mg/kg/day. Daily doses were later adapted to individual patients' cyclosporinemia, not exceeding 300 ng/ml whole blood residual CSA levels. After 6 months of treatment, CSA was progressively diminished. Whole blood residual CSA levels were determined using the Sandoz CSA radioimmunoassay kit.

In order to eliminate interseries variation, all samples drawn from a given patient were assayed in the same series. Plasma Adiol G was measured according to the following procedure [9, 11]: One ml of plasma was extracted with a cyclohexane/ethyl acetate mixture (50/50, v/v) in order to remove free steroids. A sample (0.1–0.3 ml) of the plasma was then incubated at 45°C for 48 h with 50 IU (one IU corresponds to 200 Fishman units) of *Escherichia coli* beta-glucuronidase (Pasteur Institute, Paris, France) plus 0.1 ml of phosphate buffer (0.1 M; pH 6.35). After incubation, 1800 cpm of [^{3}H]5α-androstane-3α,17β-diol was added to the plasma 30 min prior to extraction with 10 ml of cyclohexane/ethyl acetate (50/50, v/v).

The dried residues re-dissolved in 1.5 ml of pure isooctane were transferred into 5 ml siliconized pipettes packed with 0.75 g of a Celite-ethylene glycol mixture (1 g/0.5 ml) up to the 3.2 ml mark.

The steroids were then eluted, using solvents of increasing polarity (isooctane/dichloromethane) under positive pressure. 3α-adiol was collected in a 6 ml isooctane/dichloromethane (64/30, v/v) fraction (Adiol-fraction) and clearly separated from the less-polar steroids (testosterone, DHT, androsterone, delta 4-androstenedione and DHEA), which were eluted in a less-polar isooctane/dichloromethane fraction (80/20, v/v).

The Adiol-fraction was evaporated to dryness then redissolved in 1.7 ml of phosphate/gelatin buffer [12]. An aliquot of the Adiol-fraction was then transferred to a counting vial for determination of tritiated Adiol recovery. The remainder of this fraction was used for Adiol RIA, employing a specific rabbit antibody against 5α-androstane-3α,17β-diol-15 CMO: BSA, developed in our laboratory [13].

The main cross-reactions of this antibody were: 5α-androstane-3α,17β-diol: 100%; 5α-DHT: <0.01%; testosterone: <0.01%; 5α-androstane-3β,17β-diol: <0.01%; androstenedione: <0.1%; DHEA: <0.1%; 11β-hydroxy-4-androstenedione: <0.1%; androsterone: 0.6% and 5-androstene-3α,17β-diol: <0.02%. After overnight incubation of the samples with [^{3}H]Adiol (10,000 CPM/tube) and the antibody, the bound and free fractions were separated, using a dextran-coated charcoal suspension. Recovery of [^{3}H]Adiol after extraction and chromatography was 80–85%. Intra- and interassay variations of Adiol G assay were respectively 8.5 and 11%. In order to determine recovery of Adiol G from plasma, known amounts of 5α-androstane-3α-17β-diol 17β-D glucuronide (Research Plus Inc. Bayonne and Denville, N.J., U.S.A.) were added prior (to hydrolysis) to a plasma sample whose level of Adiol G has been previously measured. The following percentages of recovery were observed for the corresponding concentrations of Adiol G: 89% (1.31 nmol/l), 98% (3.57 nmol/l), 101% (5.84 nmol/l), 96% (8.10 nmol/l) and 98% (10.36 nmol/l). Using this method, the mean value found among 37 non-hirsute, control women (age range: 25–40 yr) was 3.32 ± 1.45 nmol/l (range: 1.20–7.94). Among 23

control men (age range: 22–45 yr) it was 9.82 ± 2.21 nmol/l (range: 4.33–15.08). Plasma testosterone was measured by means of a radioimmunoassay kit ("SB-Testo", Oris/CEA, Gif-sur-Yvette, France) using an iodinated tracer and a specific antitestosterone 19-hemisuccinate/BSA antiserum. The specificity of this assay, checked by comparison with classical methods using tritiated testosterone, extraction and a chromatographic step, was excellent (unpublished data). Intra- and interassay variations were 5.5 and 8.9%, respectively. Plasma DHEA S was assayed with a DHEA S RIA kit (Biomerieux, Marcy-l'Etoile, 69752 Charbonnière-les-Bains, France) for which the intra- and interassay variations were 8.5 and 12.5%, respectively. SHBG was assayed using a competitive radioimmunological method commercially available as a kit (Milab, Immunolaboratorium, Malmö, Sweden, obtained from Mallinckrodt, Evry-Lisses, France), with an anti-SHBG antiserum and [^{125}I]SHBG. Intra- and interassay variations were 9 and 12%, respectively.

The results obtained were expressed as means ± SD. Statistical analysis was carried out using a parameteric test, the Student's t-test, for mean comparisons and matched pair analysis (paired t-test).

RESULTS

The onset and duration of diabetes among the various groups were comparable, as were control steroid levels in the CSA and placebo groups of both sexes. In the CSA-treated groups, clinical hypertrichosis or hirsutism was observed in all but one female patient. The Ferriman and Gallway score was lower than 10 in all patients before treatment, but increased to 18 ± 3 during treatment. 56% of the men had some increase in trichosis, half of which were moderate and the other half severe. Each patient was clinically evaluated before and during treatment by the same clinical investigator.

5α-androstane-3α,17β-diol glucuronide

In group 1 (male CSA group), mean Adiol G levels were significantly higher during the treatment period than before treatment (11.86 ± 5.58 nmol/l vs 7.83 ± 5.30 nmol/l; $P < 0.02$). In group 3 (male placebo group), mean Adiol G levels did not vary significantly before and during treatment (5.47 ± 3.31 nmol/l vs 7.00 ± 4.55 nmol/l). During treatment, the Adiol G values ($n = 28$) in the CSA-treated patients were higher than those ($n = 38$) of the placebo group before and during treatment plus those of the CSA-treated group before treatment (11.86 ± 5.58 nmol/l vs 7.00 ± 5.30 nmol/l; $P < 0.001$). Although variations in Adiol G levels in samples drawn before and during treatment were significant in the male CSA-treated group, such variations were not statistically significant in the male placebo group. Those men in the study among whom increased trichosis ($n = 9$) was noted had significantly higher incremental Adiol G levels than men whose hairness did not increase ($n = 7$) (5.69 ± 4.48 nmol/l vs 1.68 ± 1.76 nmol/l; $P < 0.01$) (Fig. 1). In the CSA-treated group, whole blood residual CSA levels were significantly higher among those ($n = 9$) with hypertrichosis than in those without hypertrichosis ($n = 7$) (396 ± 229 ng/ml vs 173 ± 107 ng/ml; $P < 0.01$).

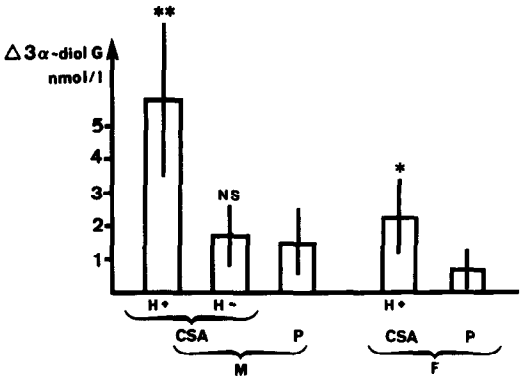

Fig. 1. Incremental 5α-androstane-3α,17β-diol glucuronide. M = male, F = female, CSA = patients with Cyclosporine A, P = patients receiving placebo, H+ = patients with hypertrichosis, H− = patients without hypertrichosis (hypertrichosis was observed in all but one female patient). Comparisons were done placebo vs CSA groups: $^{**}P < 0.01$, $^{*}P < 0.02$, NS = not significant.

Among the women in group 2 (CSA-treated), mean Adiol G levels were significantly higher during than before treatment (4.48 ± 2.70 nmol/l vs 2.10 ± 1.22 nmol/l; $P < 0.01$). In group 4 (female placebo group), mean Adiol G levels before and during treatment were not significantly different (1.27 ± 0.58 nmol/l vs 2.06 ± 1.42 nmol/l). Variations in Adiol G levels were significant in female CSA-treated group, but not in the placebo group. Finally, the values of the female CSA-treated group's Adiol G levels ($n = 21$) during treatment were significantly higher than those of the female placebo group during treatment added to the values of both female groups before treatment ($n = 39$) (4.48 ± 2.70 nmol/l vs 1.85 ± 1.20 nmol/l; $P < 10^{-5}$). Among CSA-treated female patients, there was no correlation between the Ferriman and Gallway scores and blood CSA levels.

Testosterone (T)

Mean plasma T levels did not significantly increase in group 1 or in group 3. Among the women, plasma T levels did not change in group 2. In addition, the variations in all groups were not significant.

Dehydroepiandrosterone sulfate (DHEA S)

Mean plasma DHEA S levels were not significantly different before and during treatment in group 1 or in group 3. Among the women, mean DHEA S levels were not changed in group 2 or in group 4. However, DHEA S variations were significantly increased in CSA-treated groups (male, $\delta = 3.08 \pm 3.33$, $P < 0.01$; female, $\delta = 0.98 \pm 1.13$, $P < 0.05$) and were

Table 1.

	Group	Adiol G	T	SHBG	T/SHBG	DHEA-S
1	Before	7.83 ± 5.30	19.00 ± 6.69	21.29 ± 8.23	1.13 ± 0.57	6.53 ± 3.03
	During	11.86 ± 5.58*	21.91 ± 6.34	21.82 ± 29.63	1.27 ± 0.99	9.52 ± 6.23
	Variation	4.44 ± 4.21****	2.11 ± 6.13	0.42 ± 8.13	0.06 ± 0.97	3.08 ± 3.33**
2	Before	2.10 ± 1.22	1.73 ± 0.76	28.78 ± 14.87	0.12 ± 0.18	4.21 ± 1.81
	During	4.48 ± 2.70**	1.77 ± 0.76	21.29 ± 9.84	0.14 ± 0.14	5.03 ± 1.96
	Variation	2.23 ± 2.21***	0.04 ± 0.87	−5.02 ± 15.19	−0.002 ± 0.14	0.98 ± 1.13*
3	Before	5.47 ± 3.31	20.77 ± 8.46	29.85 ± 14.98	0.83 ± 0.31	6.81 ± 3.82
	During	7.00 ± 4.55	26.14 ± 8.45	30.60 ± 15.19	1.04 ± 0.54	6.77 ± 3.95
	Variation	1.46 ± 1.98	4.54 ± 7.48	1.49 ± 9.30	0.21 ± 0.36	0.56 ± 4.43
4	Before	1.27 ± 0.58	1.63 ± 0.66	35.36 ± 23.43	0.03 ± 0.03	3.73 ± 1.84
	During	2.06 ± 1.42	1.66 ± 0.80	32.69 ± 29.53	0.05 ± 0.03	3.69 ± 1.96
	Variation	0.71 ± 1.24	0.04 ± 0.69	−2.28 ± 26.21	0.007 ± 0.03	−0.07 ± 1.22

The results obtained were expressed as means ± SD. Statistical analysis was carried out using the Student's t-test, for mean comparisons and using matched pair analysis (paired t-test) for the variations. 3α-androstane-3α,17β-diol glucuronide (nmol/l) (Adiol G); Testosterone (nmol/l) (T); Sex hormone-binding globulin (nmol/l) (SHBG); Testosterone/sex hormone-binding globulin ratio (T/SHBG); Dehydroepiandrosterone sulfate (nmol/l) (DHEA-S).

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

significantly different from those of the placebo groups (male, $\delta = 0.56 \pm 4.43$; female, $\delta = -0.07 \pm 1.22$).

Sex hormone-binding globulin (SHBG)

Among the males, plasma SHBG levels did not change in either CSA-treated group or in the placebo group. The SHBG variations were not significant in either of these groups.

Similarly, among the women, plasma SHBG values did not change in the CSA-treated group or in the placebo group. The SHBG variations were not significantly different between these two groups.

Testosterone–SHBG ratio (T/SHBG)

The ratio of plasma testosterone to SHBG was not changed in male CSA-treated patients or in the placebo group. Variations in the T/SHBG ratio were not significant in either group.

Similarly, among the women, the T/SHBG ratio was not changed in the CSA-treated women, or in the placebo group. Variations in the T/SHBG ratio were not significant in either group.

DISCUSSION

The use of prospective study methodology permitted us to evaluate variations in individual parameters, each patient acting as his own control. Double-blind study of treatment and placebo groups allowed comparison of the specific effect of CSA with the effects due to metabolic changes associated with the underlying pathology. Since the patients in this study were all insulin-dependent diabetics in the CDF clinical trial, which proscribed corticosteroid therapy (in contrast with other studies which included organ transplant patients), it was possible to eliminate the effects of corticosteroids on the endocrine system. The use of progestogens might have modified Adiol G levels in the CSA-treated group, but since micro- and macro-progestogens were also used in the placebo group with the same distribution pattern without increasing Adiol G levels, it would seem that progestogens were not responsible for the increase observed in the female-treated group. Moreover, increased Adiol G levels were also found among the male CSA-treated patients. In studies using organ-transplant patients, hypertrichosis and hirsutism, which are dose-dependent side-effects of CSA therapy, have been reported [3, 5, 10]. Our study showed smaller increases in degree of hypertrichosis, but the doses of CSA administered were lower than in organ-transplant cases [1, 10].

In addition, those male patients with the highest plasma CSA concentrations also developed hypertrichosis and had the highest plasma Adiol G levels. In men, chest-hair density has been correlated to serum Adiol G [14]. Thus, it is not surprising to note an increase of Adiol G levels in patients with hypertrichosis. Among the CSA-treated women, this comparison was not possible because all but one presented uniform hirsutism with comparable Ferriman and Gallway scores; it was therefore not possible to identify various sub-groups of trichosis increase. Many studies in women have demonstrated that Adiol G assay is a very good marker of hirsutism [9, 11, 15]. In "idiopathic hirsutism," while plasma androgen levels are normal, only Adiol G is increased, demonstrating its specificity as a marker for cutaneous 5α-reductase activity [11].

Earlier CSA treatment studies have failed to show changes in the androgen precursors: androstenedione, testosterone and DHEA [3, 5, 6]. We also were unable to detect an increase in the main androgens and a decrease in SHBG, thus probably eliminating increased free active testosterone as the source of increased Adiol G levels. It seems possible that the slight increase in DHEA S (revealed only with the paired t-test) does participate in the increase in Adiol G level, but no correlation between Adiol G level and DHEA S has been previously described [16]. Moreover the absence of increase in T levels or in the T/SHBG ratio did not substantiate such a hypothesis. The demonstration of a significant increase in Adiol

G and of a somewhat lesser but still significant increase in DHEA S among these diabetic patients rendered hypertrichosis by CSA administration, favors hypothesis implicating CSA in the mechanism of such hypertrichosis. Our results lead to hypothesize an increase in cutaneous 5α-reductase activity in the hair follicle under the influence of CSA. Paulson *et al.*[17] have recently demonstrated that the plasma Adiol G level was well-correlated with cutaneous 5α-reductase activity.

The observed increase in plasma Adiol G does not seem related to a hypothetical decrease in urinary Adiol G clearance, which is a possible consequence of a decrease in the glomerular filtration rate, classically described in CSA therapy [18]. Indeed, urinary Adiol G excretion measured in three of the female patients in the CSA-treated group was greater than that found among normal subjects (unpublished personal data).

In conclusion, we show for the first time, to our knowledge, that the appearance of hypertrichosis or hirsutism during CSA treatment is associated with increased plasma of Adiol G concentrations, due to increased local androgen metabolism, probably caused by increased 5α-reductase activity in hair follicles.

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Local coordinators. C. Boitard (Paris, Hôpital Necker), P. Bertault-Peres (Marseille, Faculté de Pharmacie), M. Hournant (Nantes, Hôtel-Dieu), A. Wagner (Montpellier, CDTS).

Monitor. J. Sirmai.

Sandoz. A. Lallemand and M. J. Barros (Rueil-Malmaison), B. von Graffenried (Basel).

Coordination. G. Feutren and J. F. Bach (chairperson) (Paris, Hôpital Necker).

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