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A new less-invasive and more informative low-dose ACTH test: salivary steroids in response to intramuscular corticotrophin

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Summary

OBJECTIVE **The intravenous low-dose ACTH test has been proposed as a sensitive tool to assess adrenal function through circulating steroids. The aims of this study were to: (a) find the minimal intramuscular ACTH dose that induced serum and salivary cortisol and aldosterone responses equivalent to those obtained after a pharmacological dose of ACTH; and (b) define the minimum normal salivary cortisol and aldosterone responses in healthy subjects to that dose of ACTH. We also compared the performances of the standard- and low-dose ACTH intramuscular tests to screen patients with known hypothalamo–pituitary–adrenal impairments.**

DESIGN **Rapid ACTH tests were performed in individuals using various intramuscular doses (12·5, 25 and 250 μg) at 2-week intervals.**

SUBJECTS **Twenty-one healthy volunteers and 19 patients with primary (nine cases) and secondary (10 cases) adrenal insufficiency.**

MEASUREMENT **Serum and salivary cortisol and aldosterone concentrations were measured at baseline and after ACTH. Serum cortisol ≥ 552·0 nmol/l and aldosterone ≥ 555·0 pmol/l concentrations at 30 min after 250 μg of ACTH were defined as normal responses.**

RESULTS **In healthy volunteers cortisol and aldosterone responded to ACTH in a dose-dependent manner. The time to peak in saliva for each steroid was delayed as the dose of ACTH increased. The minimum ACTH dose that produced equivalent steroid responses at 30 min to 250 μg of ACTH (standard-dose test; SDT) was 25 μg (low-dose test; LDT). Saliva collection 30 min after LDT and SDT showed cortisol and aldosterone concentrations of at least 20·0 nmol/l and 100·0 pmol/l, respectively. These values were defined as normal steroid responses. Blunted salivary steroid responses to LDT and SDT were found in all patients with primary adrenal insufficiency. Subnormal salivary cortisol levels in response to LDT and SDT were found in all patients with secondary adrenal insufficiency. In five patients full recovery of adrenal function was demonstrated by both tests after steroid withdrawal. In the follow-up of four patients studied during the recovery period, subnormal SAF response after LDT and normal after SDT was demonstrated. Preservation of the adrenal glomerulosa was found in all the patients with secondary adrenal insufficiency through the normal rise in salivary aldosterone after both LDT and SDT.**

CONCLUSIONS **Adrenal function can be accurately investigated with simultaneous measurements of salivary cortisol and aldosterone in response to 25 μg of corticotrophin injected into the deltoid muscle. Our data suggest that this may become a useful and relatively noninvasive clinical tool to detect subclinical hypoadrenal states.**

Cortisol and aldosterone are neutral steroids and accumulated evidence (Riad-Fahmy *et al*., 1982; Read, 1989) indicates that their concentrations in saliva, like those in urine, reflect the free, nonprotein-bound fraction in plasma. Cortisol and aldosterone concentrations in saliva appear to accurately indicate adrenal activity, as do those in plasma (Riad-Fahmy *et al*., 1982; Few *et al*., 1984), suggesting that salivary assays could have an important role in screening adrenal function. Those reports described the responses of salivary cortisol and aldosterone to pharmacological ACTH stimulation; however, their clinical application was not fully investigated.

We recently demonstrated the clinical usefulness of simultaneous measurement of salivary cortisol (SAF) and salivary aldosterone (SAL) in response to the conventional high dose of ACTH

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(250 μg) injected directly into the deltoid muscle. We found blunted SAF and SAL responses after ACTH stimulation in all patients with primary adrenal insufficiency. By contrast, patients with secondary adrenal insufficiency had blunted SAF concentrations in association with normal SAL responses, demonstrating the utility of measuring steroids derived from both the adrenal fasciculata and glomerulosa (Cardoso *et al*., 2002).

The utility of a 'physiological' ACTH dose to assess hypothalamic–pituitary–adrenal axis function was proposed in 1991 (Dickstein *et al*., 1991, 1997; Daidoh *et al*., 1995; Laureti *et al*., 2002). However, it is not clear whether a lower dose of ACTH administered intramuscularly could induce a rise in cortisol equivalent to the standard 250 μg dose of ACTH. Rainis and Dickstein (2001) reported that administration of 20 μg of ACTH intramuscularly was sufficient to stimulate cortisol secretion in healthy subjects; however, serum cortisol levels did not increase as high as they did after 250 μg of ACTH, the standard clinical dose.

Therefore, the purpose of this study was to find the lowest intramuscular dose (LD) of ACTH that produced a cortisol rise at 30 min that was equivalent to that induced by the standard dose (SD) of ACTH. By using the LD we wanted to describe in healthy subjects a rapid, less-invasive testing procedure based on salivary steroid measurements; furthermore, repeated measurements of saliva after injections of low-dose ACTH were expected to distinguish between borderline and normal adrenal function. To investigate the clinical advantages of this test (LDT) compared to the standard ACTH test (SDT) we studied a small group of patients with known primary and secondary adrenal insufficiency.

Patients and methods

Control subjects and patients

Twenty-one healthy volunteers (nine women and 12 men, aged 22–54 years) constituted the control group. They were recruited from the staff, faculty and medical students of the Instituto de Investigaciones Médicas A. Lanari (University of Buenos Aires). They were using no medications and had normal sodium levels in their diets. Women were studied in the follicular phase.

Nineteen patients, aged 22–59 years, with known primary adrenal insufficiency (nine cases) or secondary adrenal insufficiency (10 cases) were also studied. Table 1 describes the aetiology of adrenal insufficiency in each case. Previous data showed that before glucocorticoid therapy ACTH levels ranged from 80 to 250 ng/l (normal ACTH values are below 50 ng/l) in patients with primary adrenal insufficiency. An insulin tolerance test (ITT) was performed, as described by Stewart *et al*. (1988), in three of the 10 cases (cases 1–3, Table 2). A normal cortisol response to ITT was defined as a maximum plasma cortisol level > 500 nmol/l with symptomatic hypoglycaemia (< 2·2 mmol/l).

Table 1 Diagnoses in 19 patients included in the study

	Number of cases
Adrenal disease	
Autoimmune adrenalitis	6
Tuberculous adrenalitis	2
Adrenoleukodystrophy	1
Treated pituitary disease	
Craniopharyngioma	1
Somatotrophic adenoma	2
Cushing's disease	1
Treated adrenal disease	
Cushing's syndrome	2
Glucocorticoid-treated patients	
Interstitial nephritis	1
Psoriasis	1
Kidney transplantation	1
Mixed connective tissue disease	1

Glucocorticoid-treated patients (cases 4, 6, 7 and 8, Table 2) and patients after successful surgical resection of an adrenal cortisol secreting adenoma (cases 5 and 9, Table 2) and pituitary ACTH microadenoma (case 10, Table 2) were followed only by ACTH stimulation tests (LDT and SDT) performed at variable intervals as described in Table 2.

In all cases hydrocortisone and prednisone were discontinued 24 and 48 h, respectively, before the testing procedures.

The study was approved by the Research Ethics Committee of the Instituto de Investigaciones Médicas A. Lanari, Faculty of Medicine, University of Buenos Aires, and all participants gave their informed consent.

Solution preparation

The solutions prepared under sterile conditions for injection (1 ml) in the deltoid muscle consisted of (1) a control saline solution (placebo) and (2) synthetic human β^{1-24} ACTH in doses of 12·5, 25 and 250 μg.

The content of a 250 μg/ml ampoule of ACTH (Synacthen; Novartis Pharma AG, Basle, Switzerland; provided through Novartis SA, Argentina) was added to 19·0 ml or 9·0 ml of 0·9% saline solution to prepare ACTH doses of 12·5 μg and 25 μg, respectively. For the SDT the entire contents of an undiluted ampoule containing 250 μg of ACTH/ml was injected. Each ACTH dose was prepared 30 min prior to injection.

Saliva samples

After overnight fasting, all individuals were instructed to collect 3·5 ml of whole saliva in polypropylene tubes. Two hours before the first sample collection, they brushed their teeth and rinsed

Table 2 Serum cortisol (F), salivary cortisol (SAF), serum aldosterone (A) and salivary aldosterone (SAL) levels* 30 min after ACTH stimulation with 25 μg (LDT) and 250 μg (SDT) in patients with secondary adrenal insufficiency

Patient no.	Diagnosis	Primary treatment	Testing time	F (nmol/l)		SAF (nmol/l)		A (pmol/l)		SAL (pmol/l)	
				LDT	SDT	LDT	SDT	LDT	SDT	LDT	SDT
1	Acromegaly	Pituitary surgery + radiotherapy	5 years after primary Rx	152	130	2	1·4	650	750	160	170
2	Craniopharyngioma	Pituitary surgery + radiotherapy	3 years after primary Rx	130	128	1	1·2	1250	1200	330	320
3	Acromegaly	Pituitary surgery + radiotherapy	3 years after primary Rx	70	70	1·2	1·2	668	650	130	125
4	Chronic renal failure	Kidney transplantation	12 months after Pred. 14·6 g	200	227	6	10	600	610	146	150
			12 months after Pred. 1·4 g	320	330	14	15	720	800	175	180
5	Cushing's syndrome	Adrenal surgery	Post Ax: 2 months after Pred. 0·45 g	125	110	1	0·5	780	800	139	139
			4 months after Pred. 0·45 g	290	315	10	12	880	880	170	170
6	Mixed connective tissue disease	Steroids	12 months after Pred. 14·6 g	325	336	12	13	940	1128	250	250
			12 months after Pred. 1·4 g	400	626	16	36	996	1000	220	248
			6 months after Pred. 0·15 g	632	700	55	56	1000	1100	230	226
7	Interstitial nephritis	Steroids	12 months after Pred. 4·0 g	340	350	13·5	14	580	570	152	250
			9 months after Pred. 1·3 g	428	650	16	22	800	900	170	150
			3 months after Pred. 0·18 g	790	820	33	35	810	876	160	165
8	Psoriasis	Steroids	12 months after Pred. 2·4 g	120	110	3	3	990	1000	160	170
			9 months after Pred. 0·675 g	330	605	18	32	1000	987	150	180
			6 months after steroid withdrawal	587	628	27	33	1384	990	175	180
9	Cushing's syndrome	Adrenal surgery	Post Ax: 3 months after Pred. 0·675 g	100	193	7·5	12	1000	1100	250	240
			6 months after Pred. 0·55 g	420	605	11	24	950	944	275	260
			3 months after Pred. 0·075 g	896	900	34	36	930	950	280	275
10	Cushing's disease	Pituitary surgery	Post surgery: 3 months after Pred. 0·375 g	313	315	12	12	820	970	156	175
			3 months after Pred. 0·15 g	697	700	20	22	830	700	162	170

*Normal response 30 min after LDT and SDT: F ≥ 552 nmol/l; SAF ≥ 20 nmol/l; A ≥ 555 pmol/l; SAL ≥ 100 pmol/l.
Rx, treatment; Ax, adrenalectomy; Pred, steroid replacement is expressed in equivalent doses of prednisone as a total dose (g).

their mouths with tapwater to eliminate food contamination. Salivary samples were centrifuged and the supernatants were kept at −20°C until steroids were assayed.

Procedure

At 08:30 h, after 30 min rest, an indwelling catheter was inserted in the antecubital vein of all healthy volunteers. Serum and saliva samples were obtained before and 30 min after intramuscular injection of placebo or 12·5, 25 or 250 μg ACTH. Further saliva samples were collected every 15 and 30 min after the 25 and 250 μg ACTH injections, during the next 120 min. We chose 30-min sampling in accordance with previous reports (Dickstein *et al*., 1991; Mayenknecht *et al*., 1998) showing that low doses of ACTH induced peak cortisol levels at 30 min that were not different from those found after the standard, high ACTH dose.

In patients with primary and secondary adrenal insufficiency, blood was drawn for serum cortisol and aldosterone before and 30 min after stimulation with 25 or 250 μg of ACTH. In addition, salivary samples were collected as in controls.

Cortisol assay

Total serum and SAF were assessed using a commercial solid-phase radioimmunoassay (RIA) kit (coat-a count; Diagnostic Products Corporation, Los Angeles, CA, USA). The minimal serum detectable cortisol dose was 6·0 nmol/l. The average intra- and interassay coefficients of variation (CVs) were less than 5·0% and 6·0%, respectively.

SAF was determined using modifications described previously (Raff *et al*., 1998) Intra- and interassay CVs were less than 6·0% and 13·0%, respectively. SAF was expressed in nmol/l. The

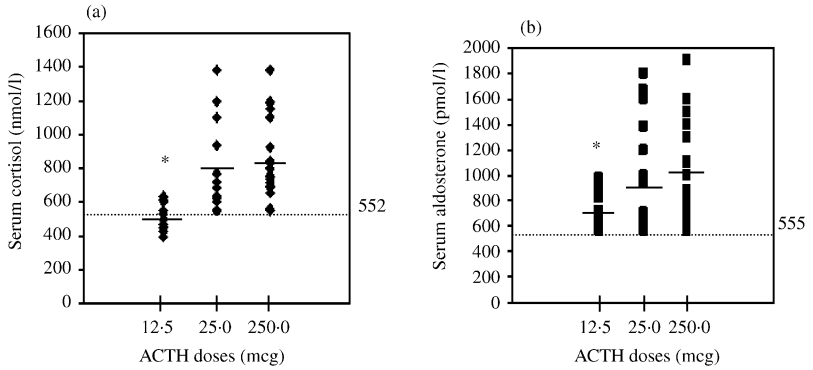

Fig. 1 Serum cortisol (a) and serum aldosterone (b) concentrations 30 min after intramuscular injection of different doses of ACTH (1–24) in 21 healthy volunteers. Horizontal bars represent means. Dotted lines indicate minimal serum steroid values obtained in response to 250 μg of ACTH. *$P < 0{\cdot}001$ compared to 250 μg of ACTH.

minimal SAF concentration detected was 0·5 nmol/l. The cross-reactivities of the assay for prednisolone, 11-deoxycortisol, prednisone and dexamethasone were 76%, 11·4%, 2·3% and 0·04%, respectively.

Aldosterone assay

Total serum and SAL concentrations were measured by competitive solid-phase RIA (coat-a-count; Diagnostic Products Corporation). Standard frozen plasma samples were used as control samples. The detection limit for the serum aldosterone assay was 33 pmol/l. The intra- and interassay CVs were less than 6·0% and 12·0%, respectively. SAL was measured using a modification of the same assay, as described previously (Cardoso *et al*., 2002). The minimal detectable concentration of SAL was 13 pmol/l. The intra- and interassay CVs were less than 8·0% and 12·0%, respectively. The assay had no cross-reactivity with cortisol and was negligible for other steroids.

Statistical analysis

Results are expressed as the mean ± SD and range. Results were analysed by analysis of variance (ANOVA) corrected for repeated measures. The differences between baseline and stimulated steroid concentrations were evaluated using the nonparametric Wilcoxon test. The steroid levels before and after the ACTH stimulus between the two subject groups were tested by the nonparametric Mann–Whitney test. Correlations were evaluated by Spearman analysis. *P*-values less than 0·05 were considered to be statistically significant.

Results

Serum cortisol response to stimulation with 12·5, 25 and 250 μg ACTH

Baseline serum cortisol levels from healthy subjects (302 ± 91; 139–442 nmol/l) did not change after 30 min of placebo (268 ± 84, 156–442 nmol/l; $P = 0{\cdot}419$). By contrast, serum cortisol concentrations rose significantly 30 min after 12·5, 25 and 250 μg of ACTH in comparison with basal levels ($P < 0{\cdot}001$) (Fig. 1a). Serum cortisol concentrations were 528 ± 72 nmol/l after 12·5 μg of ACTH, and were significantly lower ($P < 0{\cdot}001$) than those obtained after 25 or 250 μg of ACTH stimulation (792 ± 243 and 838 ± 230 nmol/l, respectively). Mean serum cortisol concentrations 30 min after 25 and 250 μg of ACTH were not different ($P = 0{\cdot}158$), reaching values of at least 552 nmol/l. This value at 30 min was defined as the minimum normal serum cortisol response to ACTH stimulus.

Serum aldosterone response to stimulation with 12·5, 25 and 250 μg ACTH

In healthy subjects, baseline serum aldosterone (316 ± 94; 138–475 pmol/l) did not change 30 min after placebo (278 ± 99, 112–420 pmol/l; $P = 0{\cdot}234$). However, significant increases were found 30 min after injections of 12·5, 25 or 250 μg of ACTH, reaching values of 695 ± 151, 952 ± 390 and 1054 ± 377 pmol/l, respectively, $P < 0{\cdot}0001$ (Fig. 1b). In all cases serum aldosterone concentrations at 30 min achieved values of at least 555 pmol/l; however, mean aldosterone levels were lower after 12·5 than 250 μg of ACTH ($P < 0{\cdot}0001$). By contrast, there was no difference between serum aldosterone mean levels 30 min after 25 or 250 μg ACTH ($P = 0{\cdot}318$). A level of at least 555 pmol/l at 30 min after ACTH was considered the lowest value of a normal serum aldosterone response. In view of these results we defined 25 μg of intramuscularly injected ACTH to be the lowest dose of ACTH that is comparable to the standard high dose, thus allowing comparisons among studies using LDT and SDT.

Salivary cortisol (SAF) responses to 25 and 250 μg of ACTH

During the testing procedures with placebo and ACTH doses of 25 and 250 μg, basal SAF concentrations did not show statistical

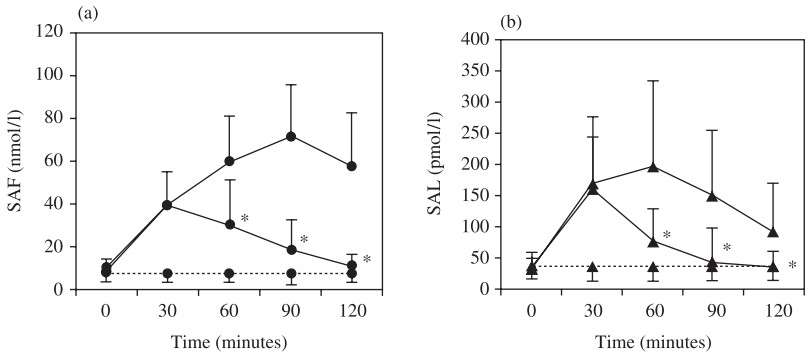

Fig. 2 Salivary cortisol (SAF, a) and salivary aldosterone (SAL, b) concentrations (mean ± SD) in 21 healthy volunteers before and after ACTH in doses of 25 μg (lower solid curves) and 250 μg (upper solid curves). Dotted lines show SAF and SAL levels in response to placebo. *$P = 0{\cdot}005$ compared to steroid concentrations after 250 μg of ACTH.

differences ($P \geq 0{\cdot}271$). Furthermore, there were no changes at any time after placebo injection ($P = 0{\cdot}281$, Fig. 2a). SAF levels were significantly elevated above baseline 15, 30, 45, 60, 90 and 120 min after injection of 25 μg ACTH ($P \leq 0{\cdot}022$) (Fig. 2a). All normal subjects achieved minimal SAF concentrations of at least 20 nmol/l at 30 min. Baseline SAF correlated significantly with basal serum cortisol levels ($r = 0{\cdot}800$, $P < 0{\cdot}0001$); however, there was no correlation between SAF and serum cortisol levels assayed after 30 min of ACTH stimulation ($r = 0{\cdot}099$; $P = 0{\cdot}665$). In response to SDT, SAF concentrations increased significantly above baseline levels at all times ($P < 0{\cdot}0001$) (Fig. 2a). As with serum cortisol, mean SAF levels at 30 min were not different from those after 25 μg of ACTH ($P = 0{\cdot}859$). The lowest SAF level reached after both ACTH doses was 20 nmol/l. However, after 250 μg of ACTH, SAF concentrations remained significantly higher at 60, 90 and 120 min than they did after 25 μg of ACTH ($P < 0{\cdot}0001$) (Fig. 2a). The peak SAF response depended on the ACTH dose, and became later as the dose increased (Fig. 2a).

In view of these data we defined a SAF value of at least 20 nmol/l, at 30 min after intramuscular administration of either 25 or 250 μg of ACTH, as a normal response.

Salivary aldosterone responses to 25 and 250 μg of ACTH

No statistical differences ($P = 0{\cdot}906$) were found among basal SAL concentrations before the different stimuli used (placebo and ACTH doses). After placebo injection SAL levels remained unchanged during 120 min ($P = 0{\cdot}900$, Fig. 2b). SAL levels increased significantly after 25 μg of ACTH at 15, 30, 45 and 60 min ($P \leq 0{\cdot}001$) (Fig. 2b). At 30 min SAL concentrations rose to 100 pmol/l. Baseline and 30 min-stimulated SAL concentrations correlated significantly with serum aldosterone levels ($r = 0{\cdot}74$ and $r = 0{\cdot}64$, respectively; $P \leq 0{\cdot}02$, in both).

Figure 2b shows SAL levels after administration of 250 μg of ACTH. SAL rose significantly during the test ($P \leq 0{\cdot}003$). Mean SAL concentrations achieved at 30 min were not different than those found after 25 μg of ACTH ($P = 0{\cdot}771$). At 30 min all subjects reached SAL levels of at least 100 pmol/l (after both 25 and 250 μg ACTH). SAL concentrations at 60, 90 and 120 min after 250 μg of ACTH were significantly higher than levels found after 25 μg of ACTH ($P \leq 0{\cdot}003$). As with SAF, the SAL peak response was delayed after high-dose ACTH in comparison with low-dose ACTH (Fig. 2b).

These data show why we consider SAL levels of at least 100 pmol/l at 30 min after either 25 or 250 μg of ACTH a normal response.

Steroid responses to ACTH stimulation tests in patients with primary and secondary adrenal insufficiency

Primary adrenal insufficiency. In nine patients with primary insufficiency basal serum cortisol (150 ± 31 nmol/l) and aldosterone (242 ± 38 pmol/l) concentrations before LDT were similar to those obtained before SDT (170 ± 17 nmol/l and 250 ± 25 pmol/l, respectively, $P \geq 0{\cdot}170$ for both). These basal values were significantly lower than controls ($P \leq 0{\cdot}017$).

In response to LDT and SDT serum cortisol levels at 30 min were 161 ± 43 (108–260) and 162 ± 25 (120–192) nmol/l, respectively. These values were significantly lower than those found in controls ($P < 0{\cdot}0001$). Serum aldosterone responses were blunted at 30 min after 25 and 250 μg of ACTH. Mean levels were 242 ± 29 (200–285) and 255 ± 26 (210–290) pmol/l, respectively. These values were significantly lower than controls ($P < 0{\cdot}0001$). Serum cortisol and aldosterone did not show significant changes at any time of the test after stimulation with either LDT or SDT ($P \geq 0{\cdot}125$).

In all patients basal SAF and SAL levels were significantly lower than controls ($P < 0{\cdot}0001$). Basal SAF levels before LDT (150 ± 31 nmol, 100–200) and SDT (170 ± 17 nmol/l, 145–192) were not different ($P = 0{\cdot}147$). These values did not show changes after either 25 or 250 μg of ACTH at any time ($P = 0{\cdot}162$ and $P = 0{\cdot}171$, respectively). At the same time basal SAL levels before LDT (242 ± 38, 200–290 pmol/l) and SDT (250 ± 25, 220–290 pmol/l) were similar ($P = 0{\cdot}282$). No significant differences were found in SAL values after stimulation with LDT and SDT at any time ($P = 0{\cdot}453$ and $P = 0{\cdot}107$, respectively).

Secondary adrenal insufficiency. Initial evaluation of 10 patients with known secondary insufficiency demonstrated that basal serum and salivary cortisol before LDT (161 ± 57 nmol/l and 3·0 ± 2·1 nmol/l) and SDT (159 ± 53 and 3·0 ± 2·1 nmol/l) were significantly lower than controls ($P = 0·0001$ for all). In addition, baseline serum and salivary cortisol levels were similar when compared for each patient before LDT and SDT ($P = 0·373$ and $P = 0·11$, respectively). At the same time basal serum and salivary aldosterone levels did not show differences from controls ($P = 0·385$ and $P = 0·157$). When basal values before LDT and SDT were compared, no statistically significant changes were found ($P = 0·224$ and $P = 0·865$). Serum and salivary cortisol and aldosterone concentrations at 30 min after LDT and SDT are shown in Table 2. The first evaluation of all 10 patients showed blunted serum cortisol and SAF responses to LDT and SDT at 30 min. Three patients with treated pituitary disease (cases 1–3) failed to increase serum and salivary cortisol normally after either 25 or 250 μg of ACTH, both tests performed 3–5 years after primary pituitary therapy. At the same time, these patients demonstrated subnormal serum cortisol levels (149, 135 and 75 nmol/l, respectively) at 30 min after ITT.

Patients in whom adrenal suppression was secondary to endogenous or exogenous glucocorticoid excess were evaluated at different time-periods during their follow-up. Patients 4 and 5 were tested after 24 months of oral steroid reduction and 6 months after successful adrenal adenomectomy for Cushing's syndrome, respectively. Both patients showed an absence of a normal rise in serum cortisol and SAF levels after LDT and SDT. These patients will be followed clinically and instructed to reduce oral steroid doses to the minimum tolerated. The next adrenal evaluation will be scheduled in 6 and 4 months, respectively. Patients 6–9 had a second evaluation after reduction of initial total prednisone oral doses that, at the time of the study, were 1·4, 1·3, 0·675 and 0·55 g, respectively. In these patients, serum cortisol and SAF levels rose normally after SDT but remained subnormal after LDT. These patients were retested 6 and 3 months later after having reduced and/or discontinued oral steroid therapy. A final LDT was performed in all of these patients, and serum and salivary cortisol reached normal levels in response to ACTH, thus showing full recovery of cortisol secretion. One female patient (case 10) with Cushing's disease demonstrated identical serum cortisol and SAF responses after either LDT or SDT in her second evaluation, 6 months after successful transsphenoidal pituitary adenomectomy.

All of these patients demonstrated a normal serum and salivary aldosterone rise 30 min after LDT and SDT (data shown in Table 2).

Discussion

In this study we have demonstrated that 25 μg of synthetic corticotrophin (LDT) injected directly into the deltoid muscle of healthy volunteers was the lowest dose that induced a rise of cortisol and aldosterone in serum and saliva after 30 min that was not different from levels achieved after a standard dose of 250 μg ACTH (SDT). Our preliminary clinical data strongly suggest the usefulness of assessing adrenal function through the response of salivary steroids after LDT. This test clearly identifies patients with partial ACTH deficiency states, is relatively noninvasive and simple to perform in ambulatory patients. Moreover, repeated use of the test allows the clinician to titrate steroid doses in glucocorticoid-treated patients.

In all healthy volunteers after LDT, serum cortisol achieved concentrations of at least 552 nmol/l, a cut-off that is accepted for the SDT as the minimal normal rise (Streeten *et al*., 1996, 1999; Thaler & Blevins, 1998). At the same time serum aldosterone levels of 555 pmol/l or more were achieved, slightly lower than the 579 pmol/l described after intravenous stimulation with 1 μg of ACTH (Mancini *et al*., 2003).

This is the first report that validates LDT to assess adrenocortical function measuring salivary steroids. Comparison of salivary steroid concentrations in response to LDT and SDT in healthy subjects showed that although at 30 min there were similar SAF responses, the time of peak salivary steroid responses depended on the dose, and appeared later as the dose of ACTH increased. These findings agree with Daidoh *et al*. (1995), who reported that the time of the peak of serum cortisol and aldosterone was dependent on the dose of ACTH, and was delayed as the dose increased. Although the reason for this is unclear, the authors suggested that continuously high plasma ACTH levels may result in the accumulation of plasma steroid and thus the delay in the time of the peak; the shorter half-life of plasma aldosterone (15 min) than cortisol (80–120 min) might also contribute to this result.

In healthy volunteers basal SAF and SAL values correlated positively with total serum steroid levels, although after LDT or SDT the correlation for cortisol disappeared. We ascribe this to saturation of transcortin and albumin binding sites (Read, 1989) leading to amplification of the increments shown in saliva after stimulation not visible in plasma (Umeda *et al*., 1981; Cardoso *et al*., 2002). On the other hand, SAL remained positively correlated with serum levels at all ACTH doses, in accordance with Atherden *et al*. (1985).

It is well known that in unstressed subjects with symptoms suggesting adrenal dysfunction, a rapid ACTH test is a simple and valuable first step. Although there is evidence that the LDT is more successful than the 250 μg ACTH test in identifying patients with functional adrenal impairment, larger studies are needed (Weintrob *et al*., 1998; Tordjman *et al*., 2000; Nye *et al*., 2001; Laureti *et al*., 2002; Dickstein, 2003). Once we established the fact that LDT was sufficient to cause a steroid response of similar magnitude as SDT at 30 min, we wanted to determine if the low-dose SAF test might be clinically useful. When evaluating

patients with primary adrenal insufficiency we found blunted SAF responses to both ACTH doses in accordance with previous clinical data of cortisol deficiency. In three cases with proven pituitary disease, both LDT and SDT reconfirmed the adrenal hypofunction demonstrated by the gold standard test (ITT), probably due to the longstanding and severe disease. An ITT was not performed in the rest of the patients because it was either contraindicated (three cases) or refused by the patients (four cases). We analysed and compared SAF responses to LDT and SDT head-to-head in seven patients with secondary adrenal insufficiency during the recovery period. In four of these patients mild adrenal cortisol suppression was detected by our more sensitive test (LDT) through the subnormal response of SAF to LDT. Complete recovery of adrenal function was finally demonstrated by both LDT and SDT.

As expected, patients with primary adrenal insufficiency failed to increase SAL levels in response to LDT and SDT. By contrast, in all patients with secondary adrenal insufficiency normal SAL responses to LDT and SDT were detected, revealing the integrity of adrenal glomerulosa.

In conclusion, we propose a new, rapid, low-dose intramuscular ACTH test using salivary steroid determinations that has the following advantages: (1) intramuscular synthetic ACTH is less invasive than ACTH administered intravenously; (2) salivary steroids reflect biologically active serum steroids and this stress-free and noninvasive methodology allows multiple sampling collection in ambulatory patients; and (3) the low-dose corticotrophin stimulus allows the identification of mild adrenal hypofunction. This new test may contribute to the development of noninvasive tests easily performed in outpatients.

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