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human chorionic gonadotropin
Leydig cells

luteinizing hormone
testosterone

Kinetics of the HCG-induced Steroidogenic Response of the Human Testis. III. Studies in Children of the Plasma Levels of Testosterone and HCG: Rationale for Testicular Stimulation Test

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Summary

Little information is available regarding the time, rhythm, number, and appropriate dosage of human chorionic gonadotropin (HCG) for adequate testing of testicular function in human. The time course of the effect of two, three, or seven HCG injections at intervals of one, five, and two days, respectively, on the plasma levels of testosterone was studied in 11 boys. The first injection induced a progressive and modest rise of T. The second given one day later had little additive effect, maximal values being seen 72 to 120 hr later. In the prepubertal boys to whom several HCG injections were given, testosterone levels reached comparable levels after four injections every five days or seven injections every other day. Although the number of subjects studied was relatively small, these results give some rational basis for the following HCG test: two or four injections at four-day intervals.

Speculation

In adult men or rats, human chorionic gonadotropin induces a rapid increase in testosterone biosynthesis, followed by a steroidogenic desensitization phenomenon and a trophic effect. The recruitment of new functioning Leydig cells probably occurs parallel to the steroidogenic refractoriness of the cells when gonadotropic stimulation reaches a certain level.

During pubertal development, luteinizing hormone also induces the differentiation of existing nonsteroidogenic interstitial cells into Leydig cells. Meanwhile, circulating levels of testosterone progressively increase. Assuming that endogenous luteinizing hormone has the same actions as human chorionic gonadotropin, it is speculated that if a possible induction of refractoriness of the Leydig cells is present at this stage, it might "modulate" the testicular response to the increasing levels of luteinizing hormone and partially explain the relatively slow achievement of a fully active testicular secretion in humans.

The stimulatory effect of gonadotropin on testicular endocrine function is well established (4, 23, 24, 26, 27, 34, 47). Early studies have shown that in children, like in adults, the parenteral administration of human chorionic gonadotropin (HCG) is followed by an increase in the urinary excretion (7, 22, 25, 51) or the plasma levels (13, 27) of testosterone.

In the last decade, numerous studies attempting to test testicular function in children in normal or pathologic conditions appeared in the literature (1–3, 5, 6, 8, 10, 11, 14, 15, 29–32, 35, 36, 39, 42, 48–50, 52). They have used protocols varying in the dose, number, and time of HCG injections as well as in the time of blood sampling for determination of plasma testosterone levels. Therefore, it is not possible to compare testosterone levels observed at the end of HCG tests between studies. Moreover, except for one report (51) of the daily excretion of testosterone following one IM injection of 5000 IU/m^2 of HCG, no study of the dynamics of response of plasma testosterone to single or repeated HCG stimulations in children is available in the literature.

On the other hand, it has been shown recently that in adult rats (45) as well as in men (12, 38), a single injection of HCG at respective doses of 40 to 2000 and 90 ± 8 IU/kg of body weight after an initial acute stimulation of plasma testosterone induces a temporary state of steroidogenic unresponsiveness to further HCG stimulation. This phenomenon appears to be time- and dose-dependent (40, 46) and is also observed in the immature rat (43).

The present study had several purposes: first, to document the sequence of the changes in plasma levels of testosterone in response to one or repeated HCG stimulations during childhood, second, to investigate whether or not HCG induces a steroidogenic desensitization of the Leydig cell in children, and finally, to try to establish a rational protocol for HCG stimulation test in both prepubertal and pubertal boys.

PATIENTS AND METHODS

SUBJECTS AND PROTOCOLS

Eleven children were studied. Their ages, body surface areas, pubertal stages, and the protocols used for each subject are listed in Table 1. Subjects 1, 4, 6, 7, 9, 10, and 11 were unilateral cryptorchid boys who were otherwise normal. Subject 2 had simple hypospadias, and subjects 3 and 5 presented with male pseudohermaphroditism but had apparently normal testicular biosynthesis and other normal endocrine functions, as evidenced by studies made 1 to 2 and 8 to 9 years before the present one. Subject 8 was referred for small testis and genitalia for age. At the time of study, subjects 5 and 8 were at the early stage (P_2) of pubertal development as indicated by enlarged testicular volume and increased basal testosterone levels as compared to those of prepubertal boys. Test of testicular function was requested by the children's physicians in all cases and performed with the informed consent of the parents.

Testicular response to HCG was studied under three different protocols using the same batch of HCG (Pregnyl; Organon). In protocol I, at 8 AM, subjects received an IM injection of 1500 IU (82 ± 22 IU/kg) of HCG which was repeated seven times at 48-hr intervals. Blood was obtained before the first HCG injection and 4 hr after each HCG injection, except for subject 5 in whom blood was collected before and 4 hr after each HCG injection. In protocol II, at 8 AM, subjects were given two (IM or IV) injections of 1050 to 2000 IU of HCG (83.5 ± 33.5 IU/kg) at 24-hr intervals. Blood was drawn before, and 2, 4, and 8 hr after each injection,

Table 1. *Subjects and protocols*

Subject	Diagnosis[2]	Age (yr)	Body surface area	Pubertal stage[1]	Protocol	HCG Dose per injection (IU)	HCG Route
1	UC[a]	3 5/12	0.605	I	I	1500	IM
2	H	4 6/12	0.6	I	I	1500	IM
3	MPH	5 4/12	0.76	I	I	1500	IM
4	UC	9 10/12	0.88	I	I	1500	IM
5	MPH	11 10/12	0.98	2	I	1500	IM
6	UC	1 6/12	0.435	I	II	1050	IV
7	UC	11 7/12	0.805	I	II	2000	IV
8	SG	12 8/12	1.14	2	II	1500	IM
9	UC	1 9/12	0.42	I	III	1500	IM
10	UC	3 5/12	0.61	I	III	1500	IM
11	UC	4 6/12	0.6	I	III	1500	IM

[1] According to Tanner (44).

[2] UC, unilateral cryptorchidism; H, hypospadias; MPH, male pseudohermaphroditism; SG, small genitalia.

and then every morning at 8 AM for seven days. Subjects studied with protocol III received 1500 IU of HCG (99 ± 51 IU/kg) IM on days 0, 5, 10, and 15. Blood was sampled before, and 2, 4, and 8 hr after each injection.

METHODS

Plasma testosterone (T) was measured in triplicate by a specific radioimmunoassay after purification by celite chromatography as previously described (9). To minimize interassay variations, all samples from a single subject were run in the same assay. Blanks were negligible; sensitivity of the assay was 7.5 pg. Intraassay and interassay variations were 5.7 and 9%, respectively.

Plasma HCG levels were measured by radioimmunoassay (28) in a single series. The reagents were purchased from CEA (Gif-sur-Yvette, France). The sensitivity of the assay was 0.75 to 1 mIU/ml.

Statistical analysis of the results (given in the text as mean ± S.D.) was done using both paired and unpaired Student's *t* test.

RESULTS

PROTOCOL I

Testosterone Levels. Mean basal plasma T levels in the 4 prepubertal boys were 0.079 ± 0.038 ng/ml, similar to values established in normal children (9). Plasma T concentrations seen in subjects 2 and 4 at the end of the test (3.4 and 4.8 ng/ml) were in the range of the response previously observed in 2 groups of normal boys submitted to the same test, *i.e.*, 5.5 ± 1.2 (37) and 6.1 ± 1.4 ng/ml, (10), respectively. In the two other boys, end-test values (2.2 and 2.5 ng/ml) were comprised between 2 and 3 standard deviations below the mean normal response. Nevertheless, the pattern of the plasma T response to repeated HCG stimulations had a similar profile in all subjects, an apparent progressive increase throughout the test (Fig. 1, *bottom*).

In the pubertal boy (subject 5), after the first HCH injection plasma T rose in 4 hr by 2- to 7-fold more sharply than in prepubertal boys (Fig. 1) and continued to rise until the 48th hr, being at that time in the range of basal adult T levels (Table 2). The early responses (as defined by the changes in plasma T seen 4 hr after HCG injection) to repeated stimulations were also studied. Values observed before each HCG injection did not vary significantly after the second injection. The early responses to subsequent HCG injections varied; T levels decreased somewhat within the 4 hr following the second, third, fifth, and sixth injections, whereas a small rise was observed after the fourth and seventh injections (Table 3). Thus, T levels observed 4 hr after each HCG injection decreased somewhat after the fourth HCG injection, but reincreased on day 13 (Fig. 1).

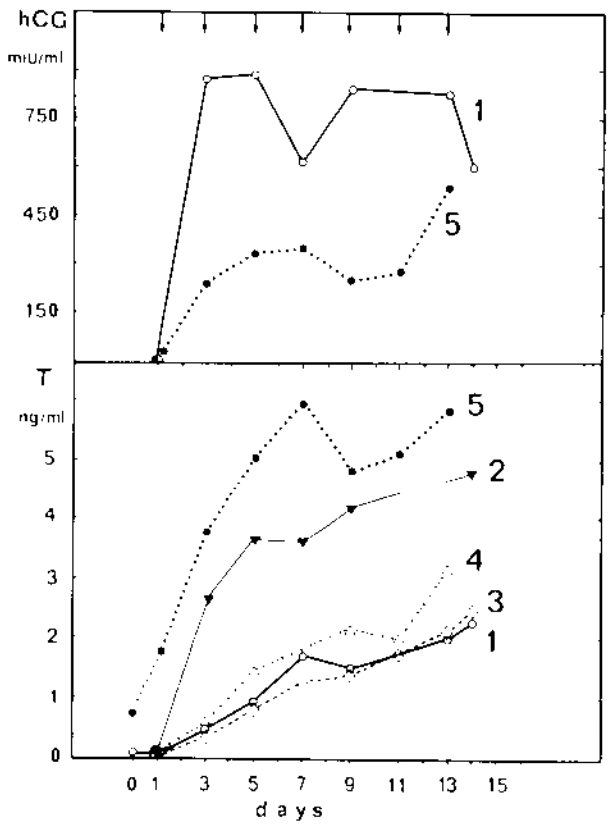

Fig. 1. Effect of repeated gonadotrophic stimulation (1500 IU IM every other day for seven days) on the plasma levels of HCG (*top*) and T (*bottom*) measured before the test (day 0) and 4 hr following each injection of HCG (*arrows*) (days 1 through 14).

The Plasma Concentrations of HCG. Plasma concentrations of HCG increased rapidly after the first injection of the hormone; HCG levels observed 4 hr after any of the further HCG injections reached values comparable in each subject (Fig. 1; *top*) and were not correlated to the amplitude of the T response.

PROTOCOL II

Prepubertal Boys (Subjects 6 and 7). Following the first HCG injection (whether administered IM or IV), testosterone levels were unchanged for the first 8 hr in the 2 prepubertal boys studied. As illustrated in Figure 2, HCG levels increased sharply within 2 hr and then declined rapidly in subject 7 to whom HCG was

administered IV. Meanwhile, T levels only began to rise slowly. Values observed at 24 hr were significantly higher than basal levels but remained rather low (Table 4).

After the second HCG injection, T levels increased within 4 hr by 2-fold ($P < 0.05$) and continued to rise thereafter. Maximal values were observed at 96 hr, plateauing or declining until 144 hr. The late rise in plasma T occurred at the time when plasma HCG levels were considerably reduced (Fig. 2). In subject 7, disappearance of HCG from plasma had at least 2 slopes with half-lives of 5 and 24 hr, respectively. These values are similar to those reported in adults (33).

Table 2. *Time course of the effect of a single injection of HCG on the plasma levels of T (ng/ml) in two pubertal boys and in adult men*

Hr after HCG	Pubertal[1] Subject 5	Pubertal[1] Subject 8	Adult[2]
0	0.67	0.81	6.1 ± 1.3[3]
4–8	1.77	1.16	9.9 ± 2.2[4]
24		2.37	8.0 ± 1.6[4,5]
48	4.38		
120			12.3 ± 2.6[4,5]

[1] Subject number at stage P_2 of Tanner (44).
[2] Values obtained in 13 normal adult men. From Saez and Forest (38).
[3] Mean ± S.D.
[4] $P < 0.001$ *versus* basal.
[5] $P < 0.001$ *versus* preceding line.

Table 3. *Plasma testosterone levels (ng/ml) before and 4 hr after each of seven HCG injections given at 48-hr intervals in a pubertal boy*

	No. of injections 1	2	3	4	5	6	7
Before	0.67	4.38	5.23	5.83	5.82	5.60	5.38
Four hr after	1.77	3.76	5.02	6.82	4.79	5.06	6.14

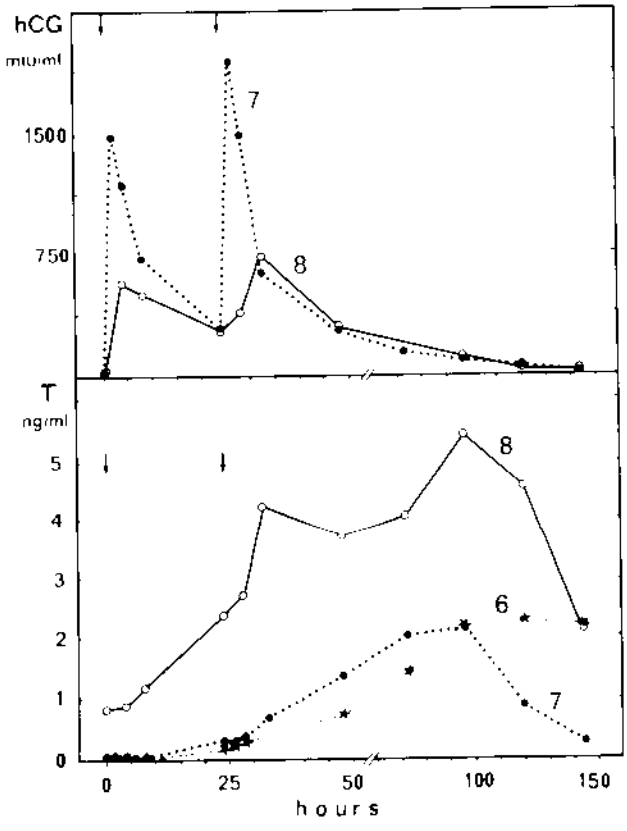

Fig. 2. Effect of 2 injections of ~100 IU/kg of HCG given at 24-hr intervals on the sequence of the changes in plasma concentrations of HCG (*top*) and testosterone (*bottom*). T levels in subject 6 (★). HCG and T were both measured in subjects 7 (●) and 8 (○).

Table 4. *Time course of the effect of a single injection of HCG on plasma T levels in prepubertal children*

Hr after HCG	Protocol	n[1]	T (ng/ml)
0	I, II, III	9	0.07 ± 0.03[2]
4–8	II, III	5	0.09 ± 0.045
24	II	2	(0.14–0.31)[3]
120	III	3	2.58 ± 0.31[4,5]

[1] n, number of subjects.
[2] Mean ± S.D.
[3] Single values.
[4] $P < 0.001$ *versus* basal.
[5] $P < 0.001$ *versus* 24 hr

Pubertal Boy (Subject 8). In the pubertal boy (subject 8), the pattern was quite different: following the first HCG injection, the increase in plasma T occurred earlier and was greater than in prepubertal boys (Fig. 2). After the second HCG injection, T also increased within 8 hr by about 2-fold, reaching levels almost 10 times higher than those seen in the youngest boys, but it decreased thereafter. Plasma T reincreased again, and a second and larger peak was observed at 96 hr at the time when maximal T values were also seen in the youngest boys.

Finally, the pattern in plasma T and the absolute T levels seen in this subject after 1 and 2 HCG injections resembled that observed in subject 5, who, however, received the first two HCG injections at 48-hr intervals (Figs. 1 and 2).

The sequence of the changes in plasma HCG levels in subject 8 (Fig. 2, *top*) had an overall pattern similar to that of the prepubertal boy (subject 7) except that T peak levels were 3 times lower and that their disappearance from plasma was slower (half-life of about 31 hr). These differences are likely related to the mode of administration, IM injection, resulting in a slow release of HCG into the circulation.

PROTOCOL III

Testosterone Levels. The plasma concentrations of T found before and 4 to 8 hr after the first HCG injection in subjects 9 to 11 were not different from those observed at the same times in subjects submitted to protocol II. Values were therefore pooled and are given in Table 3. Likewise, T levels (2.58 ± 0.31 ng/ml) found in this protocol 120 hr after the first HCG injection were similar to those (2.15 ± 0.03 ng/ml) obtained at the same time but after 2 injections of HCG in protocol II (Table 5).

On day 5, after the second HCG injection, plasma T increased significantly ($P < 0.05$) within 4 hr by 1.4 ± 0.12-fold (Fig. 3). In contrast, on day 10, T levels did not vary significantly during the 8 hr following the third HCG injection. Furthermore, the "late" post-HCG rise apparently did not occur or did not occur at the same time because the levels of T observed 120 hr after the third HCG injection (4.97 ± 2.17 ng/ml) were not significantly different from those (4.6 ± 1.6) observed 120 hr after the second injection (Table 4; Fig. 3, *open columns* on days 15 and 10).

On the other hand, on day 15, an acute and significant ($P < 0.05$) T response to the fourth HCG injection was observed within 8 hr (1.4 ± 0.05-fold increase). Mean T levels (6.5 ± 1.4 ng/ml observed at that time were identical to those observed after 7 HCG injections (protocol I) in normal boys (6.1 ± 1.4 ng/ml) using the same radioimmunoassay technique (10). Both end-test values are those of adult men in basal conditions (9).

HCG Plasma Levels. They were measured in 2 subjects (Fig. 3, *top*). Values observed before or after each HCG injection were similar in each subject but varied between subjects, as previously

Table 5. *Effect of repetition of HCG injections at 1-, 2-, or 5-day intervals on the plasma concentration of T in prepubertal boys*

Hr after first HCG injection	Hr after second HCG injection	Hr after third HCG injection	Protocol	T (ng/ml)	n[1]
24			II	(0.14–0.31)[2]	2
28	4		II	(0.28–0.66)[2]	2
52	4		I	1.10 ± 1.09[3, 4]	4
48	24		II	(0.70 1.36)[3]	2
96	72		II	(2.16 2.12)[3, 5]	2
120	96		II	(2.24–0.84)	2
240	120		III	4.60 ± 1.61[3, 4]	3
100		4	I	1.84 ± 1.05	4
360		120	III	4.97 ± 2.17[6]	3

[1] n, number of subjects.
[2] Single values: left for subject 6, right for subject 7.
[3] $P < 0.01$ *versus* levels at 24 hr.
[4] Mean ± S.D.
[5] $P < 0.001$ *versus* the preceding column.
[6] $P < 0.0001$ *versus* levels at 24 hr.

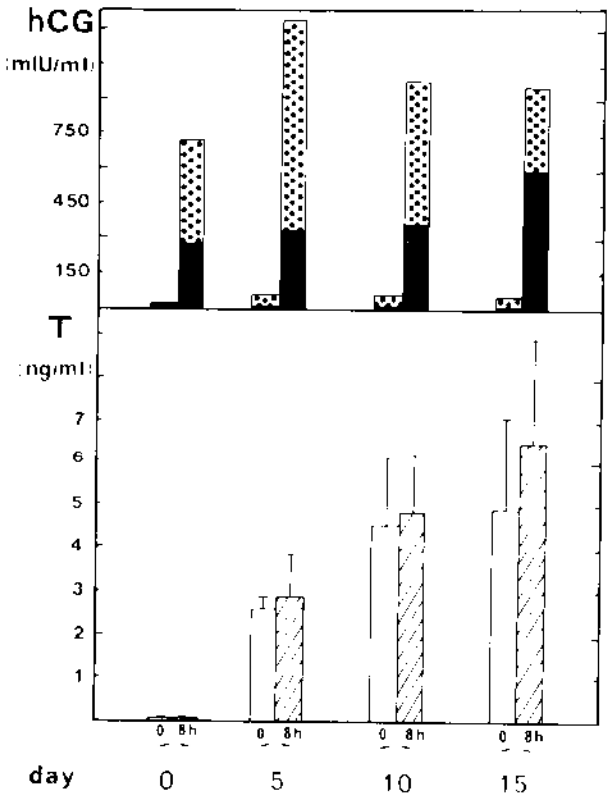

Fig. 3. Effect of four injections of HCG (~100 IU/kg) given at 5-day intervals on the plasma levels of testosterone of subjects 9 to 11 (*bottom*). Mean ± S.D. of values obtained before (*open columns*) and 8 hr after (*hatched columns*) each HCG injection. *Top*, corresponding plasma levels of HCG observed before and 8 hr after each HCG injection in subjects 9 (*dotted columns*) and 10 (*black columns*).

observed in protocol I. They obviously did not correlate with the testosterone responses (Fig. 3).

DISCUSSION

This study has obvious limitations regarding the number of subjects studied and the frequency of the blood sampling which was restricted to a reasonable number for ethical considerations. However, because this study was focused on the dynamic of the T response to HCG, some conclusions can be drawn.

Whatever the protocol used, it is obvious that there was no correlation between the plasma levels of HCG and T. This was more apparent in protocol II (Fig. 2); maximal plasma levels of T were observed when those of HCG were decreasing. Using dosages of HCG comparable to most protocols currently used, the HCG concentrations reached in plasma are enormous and even 100 hr later still are far higher than any physiologic levels of luteinizing hormone (LH) seen in normal adults (assuming a potency ratio of 2:1 between HCG and LH). Thus, during most of the period studied, the Leydig cells are exposed to very high concentrations of gonadotrophin. However, if testicular stimulation only depends upon the circulating levels of HCG, there would have been a sharp increase in plasma T after each HCG injection. It was not the case (Fig. 3; Table 3).

The T response to HCG appeared to be rather related to the time elapsed either after the initial stimulation or between two stimulations because maximal T responses were observed 72 to 120 hr after either one or two HCG stimulations (Figs. 2 and 3). Furthermore, the T values observed after the second or third HCG injection administered at 5-day intervals (protocol III) were in the range of those previously observed in normal boys given seven injections every other day (10, 37).

In adult rat, an HCG refractoriness of the Leydig cell steroidogenesis has been described. This is a complex phenomenon (see reviews, Refs. 18 and 45). It is reflected by the time course of the T response to HCG, *i.e.*, after one HCG injection, plasma T rises sharply (early response) and then declines. A spontaneous and paradoxical peak of T (late response) is observed 50 to 100 hr later. A second injection of HCG is uneffective in modifying T levels for approximately 3 to 5 days. A quite similar pattern of T response to HCG has been recently described in adult men (Table 2; Refs. 12 and 38).

In the prepubertal subjects hereby studied, although the late paradoxical rise (protocols II and III) occurred at about the same time (72 to 120 hr) as in adult men studied with the same protocol conditions (HCG dose/kg body weight and rhythms of injections), the early T response to HCG differed. Thus, if the HCG-induced steroidogenic refractoriness of the Leydig cells occurs in children, it is not apparent for at least the first 24 hr following HCG stimulation. Between children and adults, there are obvious differences of testicular steroid production, endogenous LH levels, and Leydig cell maturation. The HCG-induced Leydig cell desensitization also occurs in immature rat (43) or in animals deprived of endogenous LH (19). In hypophysectomized rats, early T response to HCG is blunted, whereas the delayed peak is seen at about the same time as in intact animals (19). This pattern strikingly resembles that observed in prepubertal children (protocol II).

On the other hand, besides its acute effect on steroid biosynthesis, HCG has "trophic" effects on the Leydig cell itself, inducing protein synthesis, histologic changes, and enzyme activities (17, 41). Enzyme induction seems to be a slow process requiring about 48 hr before it is expressed (17, 20). It is possible that this long-term effect on the enzyme activities of the testicular steroidogenic pathway may account for the late T response to HCG. The fact that in all protocols used there was a progressive rise in T levels would at first support this view. However, the profiles of the T response observed in the two pubertal boys and the slight decrease in T levels 4 hr after 4 subsequent HCG injections in subject 8 or the blunted early response observed on day 10 in prepubertal boys submitted to protocol III cannot then be fully understood.

It is possible that in prepubertal children as in the rat, high doses of HCG might induce a steroidogenic desensitization phenomenon parallel to its trophic effect, the latter being responsible for increasing testosterone production at the time of recovery from desensitization. This hypothesis remains however to be demonstrated.

Whatever the exact mechanism responsible for the patterns of the T response to HCG stimulation, our results give some basis for the use of a more rational HCG stimulation test. Various protocols are currently in use. They all vary in the dosage of each HCG injection (500 to 5000 IU), the number (1 to 7), and the rhythm (1or 2-day intervals) of the injections. Moreover, in most instances, the single blood sampling made after the last HCG injection is not rigourously timed. Each of these parameters has its own or mutual importance.

From the present results and data obtained in the rat (17, 20), it appears evident that daily injections of HCG are not necessary because basal T levels are increased by almost 30- to 50-fold the fifth day after a single injection. That the HCG-induced steroidogenic refractoriness recovers in children after the same lapse of time as in rats (after 3 days) (18) remains to be established, but it is suggested by the profiles of plasma T after HCG. If so, an earlier repetition of HCG stimulation is useless and might even be harmful (26, 51) with plasma concentrations of HCG reaching huge and unphysiologic levels after each injection.

Should even HCG stimulation be repeated? Some of the protocols in current use (1, 3, 37) led to an increase in plasma T levels to the range of adult basal values. Are those absolute criteria? In testing testicular response to HCG, the major needs are to evidence Leydig cell endocrine function and to recognize pathologic conditions. As clearly pointed out by Zachman (51) who several years ago has proposed a single dose HCG stimulation test, attainment of adult T levels might not be a preemptory criterion because full maturation of interstitial tissue does not seem to be achieved prior to the occurrence of a steroidogenic testicular response to HCG.

On the other hand, there are pathologic conditions such as hypopituitarism (4, 15, 30, 32) or hypogonadotrophic hypogonadism (4, 15, 30), abnormal testicular biosynthesis or dysgenetic testis (14, 29, 35, 39) in which substantial increase in plasma T levels may require prolonged HCG stimulation. Cryptorchidism (2, 6, 8, 11, 31, 48) is another situation in which HCG is used both for investigation of normal testicular endocrine capacity (20) and for therapeutic purpose (3, 8, 10). Prolonged HCG stimulation seems to be required for positive effects, whether or not the hormone acts directly or indirectly by a still unknown mechanism (16, 21). Therefore, repetitions of HCG stimulations might nevertheless be necessary.

Which dose should be used? There are no data yet to answer this question. *In vitro* production of T by testes from rats given injections of 10 or 100 IU of HCG (about 50 to 500 IU/kg) seems to be impaired to the same extent during the refractoriness period. However, both steroidogenic response and steroidogenic desensitization of the Leydig cell are dose related (40, 46). Which predominates over which and at which times is totally unknown in the human. Because HCG would appear to affect both sensitivity and responsiveness in the immature rat testis (43), it seems mandatory to use a constant dose for body size (per kg, for instance) in a given protocol, which is not by far a current general use.

From previous data in adults (38) and as suggested by the results obtained in children submitted to protocols II and III, the mode of injection of HCG does not seem to influence the pattern of the T response to HCG.

Finally, almost all authors have favored the easiest sampling of blood over urine collection and used plasma T to estimate testosterone secretion. As is clearly shown in this preliminary study, timing of blood sampling after the last HCG injection is most important. When carefully reinvestigated, it might improve the homogenity so often looked for (see review in Ref. 10) of the results obtained with a given protocol.

In conclusion, from our results and the above-mentioned data in the rat, in children we propose an HCG stimulation test consisting of 2 to 4 HCG injections at a body size-related dosage (50 to 100 IU/kg body weight) administered at 4-day intervals. Blood should be samples before the test and 4 to 6 and 72 hr after the last injection. It is, however, evident that standardization of this stimulation test will greatly benefit from careful evaluation of optimal dose and times of administration of the hormone and of the blood sampling.

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