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$1,25(OH)_2D$ Levels in Dihydrotachysterol-Treated Patients: Influence on $1,25(OH)_2D$ Assays

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ABSTRACT

Many clinicians continue to prefer dihydrotachysterol (DHT) as the initial vitamin D agent of choice in hypoparathyroidism and renal osteodystrophy because of its long history of efficacy and safety. Assessment of the factors influencing the clinical response to DHT treatment should include measurement of vitamin D metabolite profiles, but investigators have heretofore been unable to measure $1,25(OH)_2D$ because levels have been found to be falsely elevated when employing the chick intestinal cytosol receptor assay. After converting from the chick cytosol receptor assay to the calf thymus receptor assay for measuring $1,25(OH)_2D$, we did not note falsely elevated levels of $1,25(OH)_2D$ in DHT-treated patients. The design of this study, therefore, was aimed at determining whether or not the calf thymus receptor measured authentic $1,25(OH)_2D$ in such patients. We controlled for the possibility that freezing and thawing or prolonged storage might have either lowered $1,25(OH)_2D$ levels or degraded a metabolite(s) of DHT that would have otherwise been recognized as "$1,25(OH)_2D$" by the calf receptor. Similarly, technical differences between the two assays, source of thymus, and potential interference by other cytosolic proteins were eliminated as causes for the difference between the $1,25(OH)_2D$ levels in the two assays. Our experiments do not provide an explanation for why the thymus receptor does not "see" the interfering metabolite(s) of DHT. This could reflect either a tissue difference or perhaps a species difference. Our results do provide the first opportunity to expand the investigation of the metabolic effects of DHT therapy to include changes in intrinsic $1,25(OH)_2D$ metabolism.

INTRODUCTION

MANY CLINICIANS CONTINUE TO PREFER dihydrotachysterol (DHT) as the initial vitamin D agent of choice in hypoparathyroidism and renal osteodystrophy because of its long history of efficacy and safety.[1-5] Assessment of the factors influencing the clinical response to DHT or any vitamin D compound should include measurement of vitamin D metabolite profiles, but investigators have heretofore been unable to measure 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) in DHT-treated patients because levels of $1,25(OH)_2D$ have been found to be falsely elevated when employing the chick intestinal cytosol receptor assay.[6] This elevation has been thought to be due to a metabolite(s) of DHT co-purified with $1,25(OH)_2D$, which cross-reacts with the $1,25(OH)_2D$ receptor. We recently converted our method for measuring $1,25(OH)_2D$ from the chick cytosol receptor assay to the calf thymus receptor assay and did not note elevated levels of $1,25(OH)_2D$ in DHT-treated patients. This observation led us to investigate further whether the calf thymus receptor discriminates between authentic $1,25(OH)_2D$ and a metabolite(s) of DHT.

MATERIALS AND METHODS

Between 1980 and 1983, heparinized blood specimens were obtained from children with renal osteodystrophy and from a child with rickets before and after treatment with DHT_2,

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$25OHD_3$, or $1,25(OH)_2D_3$ after obtaining informed consent. The children with renal osteodystrophy were part of a comparative vitamin D therapy study which was approved by the Human Rights Committee at the Children's Hospital of Philadelphia. The plasma was frozen at −20°C until analyzed. Both the chick and thymus receptor assays for $1,25(OH)_2D$ were run on each individual patient's blood specimen but several years apart.

Normal adult volunteers, after giving informed consent and approval from the Human Rights Committee of the Alfred I. DuPont Institute, had blood drawn prior to taking DHT_2 and 5 and 8 days after starting daily therapy. Serum was stored at −20°C until analyzed. These specimens were collected in 1986.

Crystalline, pure $1,25(OH)_2D_3$ was a gift from Hoffman-LaRoche (Nutley, NJ). Tritiated $1,25(OH)_2D_3$ was obtained from Amersham (Arlington Heights, IL) (chick assay 180 Ci/mmol; thymus assay 85–107 Ci/mmol). Calf thymus 1 was obtained from a local slaughterhouse from a non-milk-fed veal calf. Calf thymus 2 was a gift from Dr. Timothy Reinhardt (Ames, IA).

The chick intestinal cytosol receptor binding assay was that of Shepard *et al.*[7] and the calf thymus receptor binding assay was that of Reinhardt *et al.*[8] The calf thymus buffer used differed from that stated in the Reinhardt paper. The buffer for the preparation of the receptor was 50 m*M* K_2HPO_4, 400 m*M* KCl, 1.5 m*M* Na_2EDTA, and 5 m*M* dithiothreitol, pH 7.5. The calf thymus receptor assay buffer was the same as above, except the concentration of KCl was 150 m*M*. Calf thymus cytosol receptor was prepared like the ammonium sulfate fractionated receptor, except that after the ultracentrifugation step the cytosol (minus the pellet and lipid layer) was aliquoted and frozen, deleting the fractionation step.

RESULTS

Table 1 shows the comparison between the chick cytosol and calf thymus receptor assays for five DHT_2-treated patients with renal osteodystrophy (#1–5). The $1,25(OH)_2D$ values were much lower for the calf thymus than for the chick assay and in the predictable range for such patients, suggesting that the calf thymus receptor did not recognize the interfering DHT-related substance. However, these specimens were several years old and had previously been thawed. To check for the possibility that storage and/or freezing and thawing had falsely lowered $1,25(OH)_2D$ levels when measured in 1986, specimens from $1,25(OH)_2D_3$- or $25(OH)D_3$-treated patients with renal osteodystrophy (#6–11), collected around the same time and also previously thawed, were run with the calf thymus assay. Most of these patients gave similar results for both the chick cytosol (measured in 1982–1983) and calf thymus assays (Table 1). Similar results were noted for a patient control group receiving no form of vitamin D. One patient in this group had rickets and the others had renal osteodystrophy. Another effect of storage might have been the breakdown of the interfering metabolite(s) of DHT_2, which in fact would have been recognized by the thymus receptor in fresh blood samples. Thus the thymus receptor assay would have overestimated $1,25(OH)_2D$ levels in fresh samples but would have yielded "normal" $1,25(OH)_2D$ levels in stored samples. As can be seen from Table 2, normal volunteers treated with DHT_2 had expected levels of $1,25(OH)_2D$ in fresh specimens.

TABLE 1. $1,25(OH)_2D$ LEVELS[a] IN DHT-TREATED PATIENTS AND CONTROLS

	D treatment	*Chick intestine (1982–83)*	*Calf thymus 1 (1986)*	*% Change*[d]	*Calf thymus 2 (1986)*
1	DHT_2	177	11	+1509	10
2	DHT_2	96	12	+700	22
3	DHT_2	81	16	+406	20
4	DHT_2	140	9	+1456	17
5	DHT_2	104	33	+215	44
6	$25(OH)D_3$	22	20	+10	26
7	$25(OH)D_3$	35	27	+30	—
8	$1,25(OH)_2D_3$	33	66	−50	—
9	$1,25(OH)_2D_3$	30	26	+15	—
10	$1,25(OH)_2D_3$	31	35	−11	—
11	$1,25(OH)_2D_3$	58	56	+4	—
12	None	30	32	+6	—
13	None	28	39	−28	—
14	None	38	25	+52	—
15	None	37	29	+28	31
16	None	24	25	−4	45
17[b]	None	34	33	+3	47
18[c]	None	—	22	—	32

[a]pg/ml.
[b]Rickets patient.
[c]Normal plasma control.
[d]Chick intestine from calf thymus 1.

TABLE 2. 1,25(OH)$_2$D LEVELS IN DHT-TREATED SUBJECTS: EFFECTS OF SAMPLE VOLUME AND EXTRACTION/PURIFICATION

Subject	Duration of treatment	1,25(OH)$_2$D levels (pg/ml)	
		1 ml[a]	5 ml[b]
1	pre	18	25
	day 5	17	14
	day 8	<10	11
2	pre	18	17
	day 5	21	18
	day 8	21	18

[a]Thymus extraction, thymus receptor.
[b]Chick extraction, thymus receptor.

To control for the possibility that a change in the DHT_2 preparation itself between 1983 and 1986 could have explained the difference in 1,25(OH)$_2$D levels when comparing the chick assay (1983) and the thymus assay (1986), both assays were run on aliquots of serum from two DHT_2-treated subjects (one volunteer, one patient) obtained in 1986. The results as shown in Table 3 reveal overestimation of 1,25(OH)$_2$D levels by the chick assay irrespective of the date of sampling.

Another possible explanation for the difference in 1,25(OH)$_2$D levels might relate to differences between the two assays. These would include (1) different extraction and chromatography methods; and (2) different sample volumes. The chick assay employs methanol:methylene chloride (2:1 v/v) extraction, Sephadex LH-20 chromatography, and high performance liquid chromatography (HPLC). The thymus assay employs acetonitrile extraction and chromatography with C_{18} and Silica sep-paks. Starting sample volume in the chick assay was 5 ml, in the thymus assay 1 ml. To investigate the possible influence of these technical factors, normal volunteers, referred to above, took DHT for eight days, and their 1,25(OH)$_2$D levels were quantitated using the calf thymus receptor. Samples were split. One ml volumes were extracted and chromatographed using the standard assay of Reinhardt. Five-ml volumes were extracted and chromatographed using techniques employed in the chick assay, including the HPLC step, and then subjected to the calf thymus receptor assay. The results in Table 2 show no difference in 1,25(OH)$_2$D levels.

TABLE 3. 1,25(OH)$_2$D LEVELS[a] IN DHT-TREATED SUBJECTS: EFFECT OF TIME (yr)

	Chick intestine	Calf thymus
Volunteer:		
Serum from 1983	62	25
Serum from 1986	85[b]	49
Patient:		
Serum from 1983	81	16
Serum from 1986	80[b]	<10

[a]pg/ml.
[b]Kindly performed in the laboratory of Dr. Francis Glorieux, Montreal, Quebec, Canada.

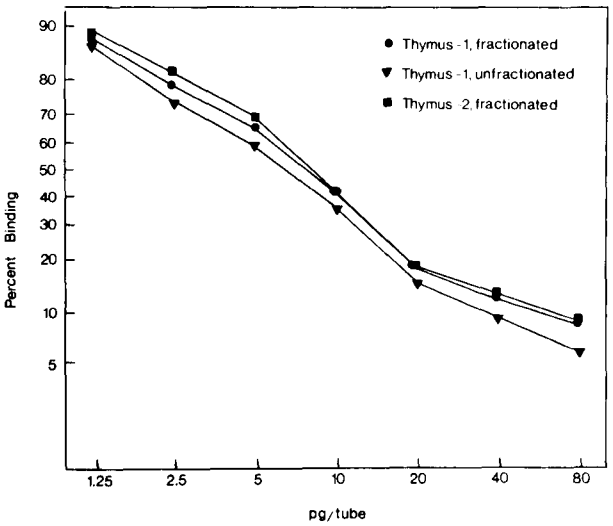

FIG. 1. Standard binding curves for 1,25(OH)$_2$D using thymus 1, fractionated; thymus 1, unfractionated; and thymus 2, fractionated. Percent binding is plotted on a percentage scale ($p = B - NSB \times 100/B_o - NSB$) and pg/tube is plotted on a logarithmic scale.

A second thymus was used for a few specimens to insure that the difference in the 1,25(OH)$_2$D levels was not due to a unique characteristic of our thymus. Results in Table 1 indicate that calf thymus results are independent of the calf thymus source. The standard curves were similar for both thymus assays (Fig. 1).

Binding proteins of various sedimentation rates have been detected in both chick intestinal and calf thymus cytosol.[9-11] These proteins can be removed from the cytosol by prior fractionation with ammonium sulfate. To examine the possibility that such proteins, if present in the calf thymus assay, might have bound an interfering metabolite(s) of DHT_2 and raised 1,25(OH)$_2$D levels comparable to those seen in the chick assay, some specimens were run in parallel, using fractionated and unfractionated calf thymus cytosol. 1,25(OH)$_2$D levels were similar for both (Table 4). The standard curves were similar for both preparations (Fig. 1).

DISCUSSION

The calf thymus receptor assay for quantitating 1,25(OH)$_2$D in plasma or serum has become the preferred method for measuring this metabolite in a number of laboratories. Advantages over the chick intestinal cytosol receptor assay include (1) smaller sample size; (2) shorter assay time; (3) elimination of an HPLC step; and (4) stability of the receptor on storage.[8] Our study has added yet another possible advantage of the calf thymus receptor, namely the ability to quantitate authentic 1,25(OH)$_2$D in samples taken from DHT_2-treated patients, a procedure heretofore unsuccessful when the chick receptor was used.[6] In the chick assay, a metabolite(s) of DHT presumably causes gross overestimation of 1,25(OH)$_2$D levels. This was not noted in the calf thymus assay. This study was performed

TABLE 4. EFFECTS OF THYMUS CYTOSOL FRACTIONATION ON $1,25(OH)_2D$ LEVELS

		1,25(OH)$_2$D levels (pg/ml)		
Patient	*Treatment*	*Thymus fractionated*	*Thymus unfractionated*	*Chick*
1	none	24	18	—
2	$1,25(OH)_2D_3$	35	26	26
3	$1,25(OH)_2D_3$	26	23	37
4	DHT_2	<14	<14	104
5	DHT_2	28	34	104
6	DHT_2	12	12	96
7	DHT_2	<11	<11	81

to control for the possibility that one or a combination of experimental artifacts might have explained the lower $1,25(OH)_2D$ levels when quantitated by the thymus receptor assay. We controlled for the possibility that freezing and thawing or prolonged storage of serum might have degraded a metabolite(s) of DHT that would have otherwise been recognized by the calf receptor. We also controlled for possible effects of different DHT_2 preparations. Similarly, technical differences between the two assays, source of thymus and potential interference by other cytosolic proteins were eliminated as causes for the differences between the $1,25(OH)_2D$ levels in the two assays.

Reinhardt *et al.* previously characterized a $1,25(OH)_2D_3$ receptor in calf thymus with properties indistinguishable from $1,25(OH)_2D_3$ receptors in other tissues.[11] Thus, he added another animal species whose tissues specifically bind $1,25(OH)_2D_3$ with high affinity and low capacity. Our experiments do not provide an explanation for why the calf thymus receptor fails to recognize a metabolite(s) of DHT that competes with $1,25(OH)_2D_3$ in the chick assay. This could reflect either a tissue difference or perhaps a species difference. Unfortunately, to our knowledge the literature fails to provide an answer to this question. The only reports examining the calcemic effect of dihydrotachysterol in cows employed A.T. 10, the forerunner of DHT. This compound was significantly different from DHT in its formulation and the amount of active sterol.[12]

Our results provide the first opportunity to expand the investigation of the metabolic effects of DHT therapy to include changes in intrinsic $1,25(OH)_2D$ metabolism. Such studies are currently underway in our laboratory.

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