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Resistance to 1,25-dihydroxyvitamin D. Association with heterogeneous defects in cultured skin fibroblasts.

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Research Article

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Resistance to 1,25-Dihydroxyvitamin D

ASSOCIATION WITH HETEROGENEOUS DEFECTS IN CULTURED SKIN FIBROBLASTS

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ABSTRACT We evaluated the interaction of $[^3H]1,25(OH)_2D_3$ with skin fibroblasts cultured from normal subjects or from affected members of six kindreds with rickets and resistance to 1-alpha, $25(OH)_2D$ $[1,25(OH)_2D]$. We analyzed two aspects of the radioligand interaction; nuclear uptake with dispersed, intact cells at 37°C and binding at 0°C with soluble extract ("cytosol") prepared from cells disrupted in buffer containing 300 mM KCl and 10 mM sodium molybdate.

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In conclusion: (*a*) Fibroblasts cultured from human skin manifest nuclear uptake and cytosol binding of $[^3H]1,25(OH)_2D_3$ that is an expression of the genes determining these processes in target tissues. (*b*) Based upon data from clinical evaluations and from analyses of cultured fibroblasts, severe resistance to $1,25(OH)_2D$ resulted from five or six distinct genetic mutations in six kindreds.

INTRODUCTION

In 1937 Albright and co-workers suggested that rickets might arise in some cases not from deficiency of vitamin D but from inadequate action of vitamin D (1). Deficiency rickets is associated typically with hypocalcemia and secondary hyperparathyroidism. Although resistant rickets in Albright's and in many other patients has been associated with hypophosphatemia but not hypocalcemia,[1] Prader reported that some pa-

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The opinions expressed herein are those of the authors and are not to be construed as reflecting the views of the Navy Department, of the Naval Service at large, or of the Department of Defense.

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[1] The terms resistance, insensitivity, and unresponsiveness are sometimes used interchangeably. We use resistance in its broadest sense to describe a state where normal levels of a factor are not associated with a normal biologic effect. Responsiveness is the maximal attainable affect from high dosage of a factor. Sensitivity varies as inverse of the dosage of a factor to give half of the maximal attainable effect.

tients exhibited early onset hypocalcemic rickets without the common causes (D deficiency, renal failure, intestinal malabsorption) (2). This has been termed vitamin D-dependency or pseudo-vitamin D-deficiency, and it sometimes occurs on a hereditary basis. Vitamin D action requires metabolism by hydroxylations at positions 25 and 1-alpha to yield $1,25(OH)_2D$ (3). Many patients with hereditary vitamin D-dependency are thought to have a defect in 1-alpha-hydroxylation of 25(OH)D (4); however, some have been recognized to exhibit long-term and profound resistance to $1,25(OH)_2D$ itself (5–15). The following features have characterized patients with severe resistance to $1,25(OH)_2D$: hypocalcemia, secondary hyperparathyroidism, osteomalacia or rickets with normal vitamin D intake, and normal or high circulating levels of $1,25(OH)_2D$. In most of these latter cases there has been data suggesting autosomal recessive transmission of the resistance. However, subtle clinical and biochemical findings have suggested heterogeneity in the underlying defects. Alopecia is found in affected members of some (7, 9, 10, 12, 13, 15) but not all (5, 6, 8, 11, 14) kindreds; in one patient there was hyperphosphatemia and undetectable levels of $24,25(OH)_2D$ (10); and one patient failed to achieve eucalcemia even with serum $1,25(OH)_2D$ at concentrations 100 times normal for 12 mo.[2]

Like the true steroidal hormones, $1,25(OH)_2D$ acts in the nucleus of target cells (16); this requires a multistep process involving hormone receptors, localization of receptor and hormone in the nucleus, and induction of specific messenger RNA synthesis. In theory, resistance to $1,25(OH)_2D$ could arise at any of several points in this process. Indeed, genetic defects in the response system for androgenic steroids (17) and for glucocorticoids (18) have resulted from lesions at several analogous loci.

Recently, several laboratories have described putative receptors for $1,25(OH)_2D$ in cultured skin fibroblasts (19, 20). Skin fibroblasts cultured from $1,25(OH)_2D$-resistant members of two kindreds were shown to have similar defective nuclear uptake of $[^3H]1,25(OH)_2D_3$ (21). While clinical features distinguished affected members in these latter two kindreds, the nuclear uptake assay did not uncover evidence of heterogeneity in the associated cellular defects. We have now extended our studies on whole-cell nuclear uptake of $1,25(OH)_2D$ in cultured fibroblasts to include four additional affected patients representing four unrelated kindreds. In addition, we have analyzed directly the binding of $[^3H]1,25(OH)_2D_3$ in soluble (300 mM KCl) extracts of cultured fibroblasts from members of all six kindreds. The combined clinical and biochemical data suggest that each of these six kindreds expresses a mutation at a different locus in the effector system for $1,25(OH)_2D$.

METHODS

Patients. Patients showed typical features of resistance to $1,25(OH)_2D$ including early onset rickets with adequate vitamin D intake, hypocalcemia, secondary hyperparathyroidism, and normal or high serum concentrations of $1,25(OH)_2D$. Detailed evaluation of one or more affected members from each kindred has been documented as follows: kindred 1 (6, 20), kindred 2 (7, 20), kindred 3 (10), kindred 4,[2] kindred 5 (9)[2], kindred 6 (15). Distinctive clinical and biochemical features are summarized in Table I.

Materials. Collagenase (type 1, *Clostridium histolyticum*) was obtained from Sigma Chemical Co., St. Louis, MO, fetal calf serum (screened for virus and mycoplasma) from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY, and $1,25(OH)_2D_3$ from Hoffman LaRoche, Nutley, NJ (gift of M. Uskokovic). Aprotinin was from FBA Pharmaceuticals Inc., New York. Ultrapure sucrose (RNAase- and DNAase-free) was from Bethesda Research Laboratories, Rockville, MD, and Bio-Gel HTP hydroxylapatite was from Bio-Rad Laboratories, Richmond, CA. $1,25[23,24(n)^3H](OH)_2D_3$ was from Amersham Corp., Arlington Heights, IL (102 Ci/mmol or from New England Nuclear, Boston, MA (160 Ci/mmol). $[^{14}C]$Ovalbumin was obtained from New England Nuclear.

Cells and cell culture. Fibroblasts were established from skin biopsy specimens (4 mm disposable biopsy punch) (20). Skin biopsies from patients were obtained from arm, thigh, or mons pubis; biopsies from normal controls came from similar sites or from foreskin in one neonate. The biopsy specimen was finely minced and placed in a petri dish (60 mm diam) containing 5 ml of Medium A (improved Eagle's Minimal Essential Medium, 10% fetal calf serum, 10^{-7} M insulin, 0.584 g/liter glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) supplemented with 2 mg/ml collagenase. Specimens were dispersed, transferred to culture flasks (25 cm^2), and incubated for 24 h. After 24 h, the medium was replaced with fresh Medium A without collagenase, and specimens were cultured. On reaching confluency, cells were harvested with 0.05% trypsin-0.02% EDTA in phosphate-buffered saline and transferred to progressively larger flasks. Studies with fibroblasts were performed in passages 4–14. MCF-7 human breast cancer cells (provided by M. E. Lippman) were cultured similarly.

Nuclear uptake of $[^3H]1,25(OH)_2D_3$. Cells were grown to confluency in 150-cm^2 flasks. Then, at 48 and 24 h before harvesting, the growth medium was replaced by Medium B (Medium A without fetal calf serum). Cells were harvested as above and resuspended at a density of 2 to $4 \times (10)^6$/ml in Medium C (Medium B with Tricine-HCl 25 mM, pH 7.4). 0.4 ml of this suspension was added to 0.1 ml of Medium C with $[^3H]1,25(OH)_2D_3$ to yield five or six final concentrations between 0.05 and 5 nM. Low affinity uptake was measured using similar samples containing 1 μM $1,25(OH)_2D_3$. Cells were incubated for 45 min at 37°C, collected by centrifugation, washed with phosphate-buffered saline, and suspended by agitating in ice-cold lysing buffer (0.25 M sucrose, 20 mM Tris pH 7.85, 1.1 mM magnesium chloride, and 0.5% Triton X-100) for 1–2 min. Nuclei were collected by centrifuging the lysed samples at room temperature for 3 min at 1,000 *g*. After discarding the supernate and repeating this

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[2] Balsan, S. Personal communication.

TABLE I
Clinical and Biochemical Data for Patients with Resistance to $1,25(OH)_2D$[*]

	Kindred					
Feature	1	2	3	4	5	6
Parental consanguinity	−	+	+	+	+	−
Affected siblings[‡]	+	+	+	+	+	−
Total alopecia	−	+	+	+	+	+
Serum phosphorus[§]	lo	lo	hi	lo	lo	lo
Serum $24,25(OH)2D$[§]	nl[‖]	?	lo	nl	nl	nl
Eucalcemia with high dose of $25(OH)D_3$, $1\alpha(OH)D_3$, or $1,25(OH)_2D_3$	+	+	−	−	+	?

[*] Affected members of each kindred exhibited early onset rickets with low calcium, high parathyroid hormone, and normal to very high $1,25(OH)_2D$ concentrations in serum.

[‡] In kindreds 1 and 2 both affected siblings were evaluated in detail, and the features were consistent within each kindred. In kindreds 3, 4, and 5 the affected siblings had died before evaluation of the studied patient.

[§] Numerical data are not cited because of differences in normal ranges related to assay methods, patient age, and patient sex.

[‖] Values in the "normal" range may be inappropriate, resulting from defective receptor-mediated induction of 25(OH)D 24-hydroxylase by high circulating levels of $1,25(OH)_2D$.

procedure once, radioactivity associated with the nuclei was determined (20).

Binding of $[^3H]1,25(OH)_2D_3$ to soluble extracts from cells. All procedures were performed with solutions maintained at 0–4°C. Cells were grown and harvested as above, washed in phosphate-buffered saline, and suspended in buffer A (10 mM Tris-HCl pH 7.4, 300 mM KCl, 10 mM sodium molybdate, 1.5 mM EDTA, 1.0 mM dithiothreitol, and aprotinin; 500 Kallikrein Inactivator Units/ml) at a cell concentration of $3\text{–}6 \times (10)^6$/ml.

We included 300 mM KCl and 10 mM sodium molybdate in the buffer because of their stabilizing effect on activity of the receptor(22). The suspension was treated with three 5-s pulses from a sonicator (Heat Systems-Ultra Sonic Inc W-375, Plainview, NY) with microtip set at a gain of 4 on a 50% duty cycle. The sonicate was centrifuged at 100,000 *g* for 60 min. The supernate was saved and will be referred to as "cytosol".[3] Freshly prepared "cytosol" was incubated with varying concentrations of $[^3H]1,25(OH)_2D_3$ (0.05–5 nM) in 12 × 75 polypropylene test tubes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) in a final volume of 0.2 ml for 15 h. Low affinity binding was assessed by coincubation with 1 μM $1,25(OH)_2D_3$. For nuclear uptake and cytosol binding experiments, $[^3H]1,25(OH)_2D_3$ was added in absolute ethanol to yield a final ethanol fraction of <0.02 for nuclear uptake and <0.05 for cytosol binding in all incubations. In preliminary experiments we confirmed that high affinity binding reached apparent equilibrium within 3 h with saturating concentrations of radioligand but required 15 h to reach a plateau at the lowest concentrations of radioligand evaluated (24). Bound and unbound radioligand were separated with hydroxylapatite (25). In brief, hydroxylapatite was prepared in buffer A, washed repeatedly, and stored overnight at 0–4°C. A 50% slurry was prepared by adding a volume of buffer A equal to the settled volume of the resin. Aliquots (0.4 ml) of the slurry were added to cytosol at the end of incubation. Samples were agitated at 5-min intervals for 15 min. Samples were then centrifuged and pellets were washed three times by suspension and sedimentation through buffer A containing 0.5% Triton X-100. $[^3H]1,25(OH)D_3$ was extracted from the washed pellets with 2 ml absolute ethanol at 0–4°C for 15 min. Aliquots (1 ml) of supernate were assayed for radioactivity in 6.5 ml Aquasol.

Sucrose density gradient centrifugation of nuclear extracts. Cells were harvested and incubated with $[^3H]$-$1,25(OH)_2D_3$ ($0.9\text{–}1.5 \times [10]^{-9}$M) with or without 1 μM $1,25(OH)_2D_3$ as indicated above. All subsequent procedures were performed with solutions maintained at 0–4°C. Cells were then washed twice with phosphate-buffered saline, suspended in buffer A without KCl, and homogenized with a tightly fitting Dounce homogenizer. After centrifugation at 1,000 *g* for 10 min, the crude nuclear pellet was suspended in 0.5 ml buffer A and sonicated as described above. To remove unbound $[^3H]1,25(OH)_2D_3$ without diluting extracts, pellets were prepared by centrifuging 0.5 ml of dextran-coated charcoal suspension (0.25%[wt/wt] Norit A, 0.0024% [wt/wt] dextran T-70 in 0.01 M Tris-HCl, pH 7.4) at 1,000 *g* for 10 min. Then 0.5 ml of the nuclear extract was added, mixed, and incubated for 10 min. Suspensions were centrifuged 10 min at 1,000 *g* at 4°C, and 0.35 ml of supernate with $[^{14}C]$ovalbumin was layered on a 5–20% sucrose gradient (total volume 4.3 ml) in buffer A. This was centrifuged 17 h at 0°C at 250,000 *g* using a Beckman L5-65 centrifuge and an SW 60 rotor. Six drop fractions were collected from the bottom of the gradient, added to 0.5 ml H_2O and 5 ml Aquasol, and assayed for radioactivity.

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[3] Our methods do not localize receptors to the cytosolic or nuclear compartment of the cell. Extraction with 300 mM KCl probably solubilizes receptors from both locations (23).

Sucrose density gradient centrifugation of cytosol:[3] Cytosol was incubated for 3 h at 0–4°C in a final volume of 0.5 ml of buffer A with [^{3}H]1,25(OH)$_2$D$_3$ (0.9–1.5 nM) with or without 1 μM 1,25(OH)$_2$D$_3$. Unbound radioligand was removed as described above for nuclear extracts, and sedimentation velocity of radioligand in sucrose gradients was analyzed as above.

Cells in suspension were counted with a hemocytometer. Protein was determined by the method of Lowry et al. (26). High affinity binding was analyzed by the method of Scatchard (27). The criterion for saturable binding was a regression analysis consistent with a single class of binding sites (i.e. the slope differed from zero at the 95% confidence level). When the slope of the regression did not differ significantly from zero, affinity and capacity of the process were considered unmeasurable. A 95% confidence interval was used for all statistical comparisons.

RESULTS

Nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ by dispersed fibroblasts. As indicated previously (20), high affinity nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ is consistently demonstrable in fibroblasts cultured from normal skin (Table II). Similar capacity (9,100±1,100 nuclear uptake sites per cell) and affinity (0.4±0.1 nM) (mean±1 SE from 10 experiments) were obtained with MCF-7 cells from a human breast cancer (28).

In cells from patient 3 the capacity and affinity of nuclear uptake were normal. In cells from the remaining seven patients nuclear uptake was defective; in six it was unmeasurable, while in one (patient 4) the capacity was diminished without change in affinity (Table II).

TABLE II
Saturable Nuclear Uptake of [^{3}H]1,25(OH)$_2$D$_3$ by Dispersed Fibroblasts Cultured from Skin

Donor	Capacity of nuclear uptake	Affinity of nuclear uptake
	sites per cell	*nM*
Normal (8)*	10,300±1,700	0.5±0.1
Patient 1a (3)	UM‡	UM
Patient 1b (3)	UM	UM
Patient 2a (2)	UM	UM
Patient 2b (2)	UM	UM
Patient 3 (3)	4,600±900	0.3±0.1
Patient 4 (2)	800 (500, 1100)	0.5 (0.2, 0.8)
Patient 5 (2)	UM	UM
Patient 6 (3)	UM	UM
Misc.§ (5)	8,900±1,900	1.0±0.4

* Number in parentheses indicates number of tested cell lines established from normals and miscellaneous or number of separate analyses performed for cells from each patient. Data are mean±1 SE or mean and range where number equals two.

‡ UM, unmeasurable.

§ The group of miscellaneous donors includes three parents and one aunt of children with resistance to 1,25(OH)$_2$D and one female with familial x-linked hypophosphatemia.

Capacity and affinity of nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ were normal with fibroblasts from three parents and an aunt of children with resistance to 1,25(OH)$_2$D (Table II).

Sedimentation velocity of nucleus-associated radioligand. Nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ was detectable with dispersed fibroblasts of patients 3 and 4. In patient 3 (Fig. 1) and patient 4 (not shown) the nucleus-associated radioligand exhibited a sedimentation velocity indistinguishable from normal.

Binding of [^{3}H]1,25(OH)$_2$D$_3$ with cytosol. Binding of [^{3}H]1,25(OH)$_2$D$_3$ with cytosol from normal fibroblasts exhibited high affinity and low capacity (Fig. 2). The average binding capacity of 35 fmol/mg protein in normal cells (Table III) corresponds to 8,900 cytoplasmic sites per cell. With cultured MCF-7 cells (28), we observed similar binding capacity (54±6

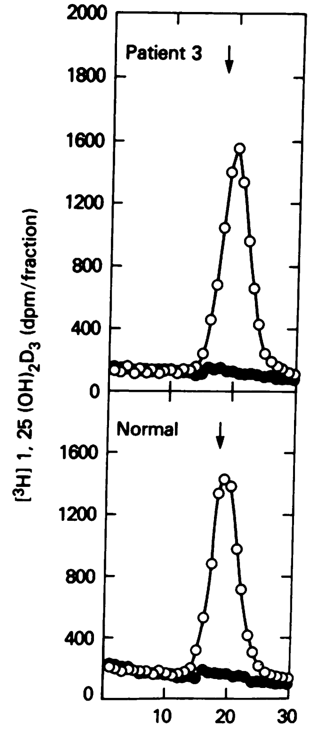

FIGURE 1 Sedimentation of nucleus-associated radioligand from fibroblasts in sucrose gradients for a normal and patient 3. During cellular incubation with radioligand, the concentration of [^{3}H]1,25(OH)$_2$D$_3$ was 1.3 nM. Arrow marks position of [^{14}C]ovalbumin (3.7S). Solid symbols represent binding obtained during coincubation with 1 μM 1,25(OH)$_2$D$_3$. Radioactivity is expressed per milligram nuclear extract protein applied to the sucrose gradient. The top of the gradient is on the right.

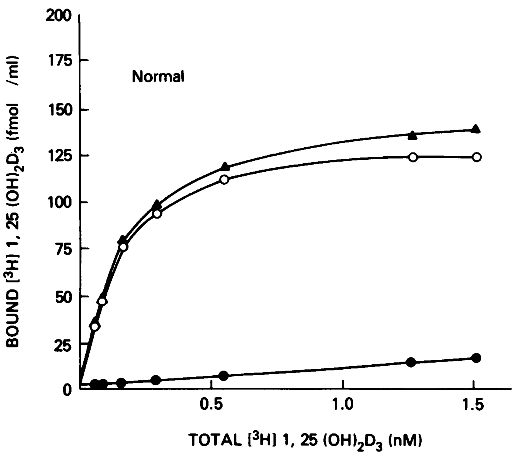

FIGURE 2 Dose-dependency for binding of [^{3}H]1,25(OH)$_2$D$_3$ with cytosol of fibroblasts cultured from normal skin. ▲, Total binding; ○, high affinity binding; ●, low affinity binding. Cytosol protein concentration was 4.1 mg/ml.

fmol/mg protein) and binding affinity (0.19±0.04 nM) (mean±1 SE from six experiments). In cells from patient 3 capacity and affinity of cytosol (Table III) binding were normal (Table III and Fig. 3). In cells from affected members of kindreds 1 and 2 there was nearly normal affinity and capacity of cytosol binding. In cells from patient 4 the binding capacity was <10% normal while affinity was normal. Saturable binding was not detectable with cytosol of cells from patients 5 and 6.

TABLE III

Saturable Binding of [^{3}H]1,25(OH)$_2$D$_3$ with Cytosol of Fibroblasts Cultured from Skin

Donor	Capacity of cytosol binding	Affinity of cytosol binding
	fmol/mg protein	*nM*
Normal (5)[*]	35±2	0.13±0.02
Patient 1a (2)	19 (22, 16)	0.17 (0.20, 0.15)
Patient 1b (2)	18 (20, 16)	0.38 (0.26, 0.50)
Patient 2b (1)	28	0.04
Patient 3 (2)	46 (57, 35)	0.11 (0.11, 0.10)
Patient 4 (2)	4.2 (4.4, 4.0)	0.15 (0.20, 0.09)
Patient 5 (2)	UM[‡]	UM
Patient 6 (2)	UM	UM
Misc.[§] (3)	43±4	0.09±0.01

[*] Number in parentheses indicates number of tested cell lines established from normals and miscellaneous or number of separate analyses performed for cells from each patient. Data are mean±1 SE or mean and range where number equals two.

[‡] UM, unmeasurable.

[§] The group of miscellaneous donors includes two parents of children with resistance to 1,25(OH)$_2$D and one female with familial X-linked hypophosphatemia.

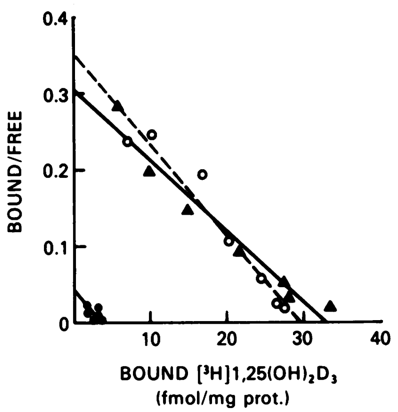

FIGURE 3 Scatchard plots of high affinity binding of [^{3}H]1,25(OH)$_2$D$_3$ with cytosol of fibroblasts cultured from skin of (○, dashed line) normal (replot of data from Fig. 2) (capacity 30 fmol/mg protein, affinity 0.08 nM, $r = -0.97$), (▲, solid line) patient 3 (capacity 33 fmol/mg protein, affinity 0.11 nM, $r = -0.97$), and (●, solid line) patient 4 (capacity 4.0 fmol/mg protein, affinity 0.09 nM, $r = -0.96$).

Capacity and affinity of binding of [^{3}H]1,25(OH)$_2$D$_3$ were normal with cytosol from fibroblasts of two parents with children resistant to 1,25(OH)$_2$D (Table III).

Sedimentation velocity of cytosol-associated radioligand. Sucrose gradient analysis of radioligand after incubation with cytosol[3] extract revealed a single peak at ~3.7S from normal fibroblasts (Fig. 4) and from MCF-7 cells (not shown).

Sedimentation velocities obtained with cytosol from fibroblasts of patients 3 and 4 were indistinguishable from normal (Fig. 4). With cytosol from patient 4 the area of the saturable peak at 3.7S was ~10% of normal. In similar manner the sedimentation velocity of cytosol-associated radioligand was normal for patients 1b and 2b (not shown). Saturable binding was not detectable in the density gradient of the cytosol from fibroblasts of patients 5 and 6 (patient 5 not shown).

DISCUSSION

Previous studies have shown that dispersed fibroblasts cultured from normal skin exhibit nuclear uptake of [^{3}H]1,25(OH)$_2$D$_3$ with characteristics suggestive of mediation by typical hormonal receptors (19, 20). Neither donor age nor cell passage number have major affect on receptor-mediated nuclear uptake of 1,25(OH)$_2$D$_3$ in cultured skin fibroblasts (20, 21). Among six kindreds with presumably hereditary resistance to 1,25(OH)$_2$D, we have observed three patterns with the whole-cell nuclear uptake assay: normal capacity with normal affinity (kindred 3), decreased capacity with normal affinity (kindred 4), or unmeasurable capacity and affinity (kindreds 1, 2, 5, and 6).

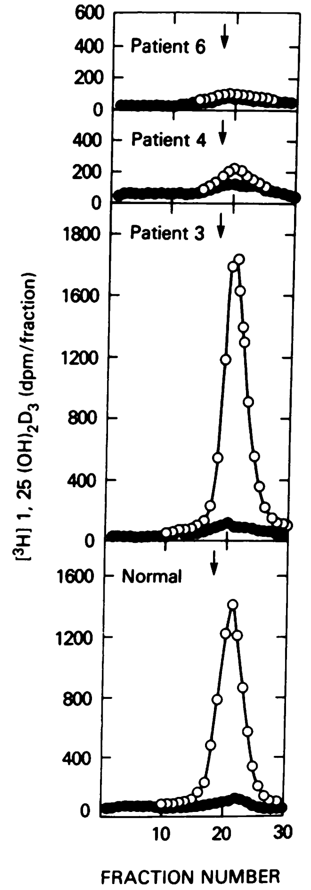

FIGURE 4 Sedimentation of radioligand in sucrose gradient after incubation [at 1.5 nM $(^3H)1,25(OH)_2D_3$] with cytosol of fibroblasts cultured from skin of normal, patient 3, patient 4, and patient 6. Arrow marks position of $[^{14}C]$ovalbumin (3.7S). Solid symbols represent binding obtained during coincubation with 1 μM $1,25(OH)_2D_3$. Radioactivity is corrected to a cytosol sample mass of 1 mg protein applied to the sucrose gradient.

Our present study shows that cytosol of fibroblasts cultured from normal skin binds $[^3H]1,25(OH)_2D_3$ with capacity and affinity similar to those in MCF-7 cancer cells (28) and in cells cultured from several organs known to be targets for $1,25(OH)_2D_3$ (bone [29] and kidney [30]). The similar $1,25(OH)_2D_3$-capacity observed for nuclear uptake and for cytosol binding suggests that the cytosol binding assay is an index of the same processes as the nuclear uptake assay, and that a limiting factor in high affinity nuclear uptake of $1,25(OH)_2D$ is the number of intracellular receptors for $1,25(OH)_2D$. The correlations between data from the nuclear uptake and cytosol binding assays in kindreds 3–6 represent independent evidence that the cytosol binding assay provides a direct analysis of one or more components in the nuclear uptake process.

The two assays that we have employed (whole-cell nuclear uptake and cytosol binding of $[^3H]1,25(OH)_2D_3$) provide complementary information. Whole-cell nuclear uptake reflects a more distal aspect of the effector system for $1,25(OH)_2D$; it is thus more useful for screening of cells and for assessment of certain events distal to binding in cytosol. Affected members from five of six kindreds exhibited deficient whole-cell nuclear uptake. Analysis of cytosol binding provides additional insight into certain lesions associated with deficient nuclear uptake. In our study, deficient nuclear uptake has been associated with three different patterns in the cytosol binding assay; unmeasurable nuclear uptake was associated with normal (kindreds 1 and 2) or with unmeasurable (kindreds 5 and 6) cytosol binding while 90% decrease in nuclear uptake capacity was associated with 90% decrease in cytosol binding capacity (kindred 4).

Sucrose density gradient analysis of nucleus-associated or of cytosol-associated radioligand has not provided major additional insight into the nature of defects but has shown that the radioligand taken up in nucleus or bound to cytosol with high affinity migrates with the same sedimentation velocity whether derived from patient cells or normal cells.

Cultured skin fibroblasts do not reproduce all features of cells that are targets for $1,25(OH)_2D_3$ in vivo. However, interesting speculations can be derived from the correlations between fibroblast-derived and clinical data. In fibroblasts from patients 5 and 6, both saturable nuclear uptake and cytosol binding of $[^3H]1,25(OH)_2D_3$ were unmeasurable. Whereas the data obtained in vitro are compatible with absence of receptors for $1,25(OH)_2D$ in target tissues, the data obtained in vivo make this possibility unlikely for patient 5. While taking $25(OH)D_3$, patient 5 exhibited $1,25(OH)_2D$ levels in the range of 2,800 pg/ml (~70 times the normal mean), and during this time hypocalcemia remitted (9). Receptors may be present in target and skin cells of patient 5 but were not detected in vitro. Receptors that are normal in quantity but severely defective in apparent affinity for $1,25(OH)_2D$ could account for the findings in cultured fibroblasts from patient 5.

Patient 6 may exhibit the consequences of absent or nonfunctional cytosol receptors for $1,25(OH)_2D$. However, since this patient did not take calciferol analogs at sufficient dosage or for sufficient time to document absent clinical response, we cannot prove this possibility at present. Undetectable cytosol binding of $[^3H]1,25(OH)_2D_3$ has been previously reported in two kindreds with resistance to $1,25(OH)_2D$ (31, 32).

In cultured fibroblasts from patient 4, capacity for nuclear uptake of $1,25(OH)_2D$ was ~10% of normal. This was apparently the direct result of a 90% deficiency of cytosol receptors with normal sedimentation velocity and normal apparent affinity. During 1 yr of therapy with $25(OH)D_3$ in patient 4, serum concentrations of $1,25(OH)_2D$ averaged 2,000 pg/ml (~50 times the normal mean) without any change in degree of hypocalcemia.[2] The data suggest that a 90% deficiency of receptors for $1,25(OH)_2D$ in target tissues may impose a limit on the in vivo calcemic affect. For many hormone systems, there is a large excess of unused or "spare" receptors. By inference there would seem to be few spare receptors for $1,25(OH)_2D$. This is consistent with our observation herein that the binding capacity of cytosol receptors is similar to the maximal capacity for nuclear uptake of $1,25(OH)_2D$ in normal fibroblasts. Lack of spare receptors is also consistent with recent observations in chick intestinal mucosa (33) in which there is a close correlation between $1,25(OH)_2D$ occupancy of chromatin-associated sites and biologic response, 10–15% occupancy of sites under normal conditions, and 70% occupancy at $1,25(OH)_2D$ levels that result in maximal biologic response (as measured by cellular levels of immunoreactive intestinal vitamin-D-dependent calcium-binding protein).

Nuclear uptake of hormone was unmeasurable with fibroblasts from affected members of kindreds 1 and 2. However, saturable binding of hormone was detected in fibroblast cytosol from affected members of these two kindreds. The constellation of normal or nearly normal hormone binding in cytosol and defective nuclear uptake is analogous to observations one of us has recently made with cultured skin fibroblasts from a kindred with resistance to androgens.[4] There was a mild increase in binding affinity with cytosol from an affected member of kindred 2, and there was a mild decrease in capacity and affinity of binding with cytosol from both affected members of kindred 1. These changes (modest and of uncertain significance) were not proportional to the unmeasurability of high affinity radioligand uptake in nuclei of whole cells, suggesting that the receptor may be less stable under conditions (such as temperature of 37°C) of the nuclear uptake assay than in the cytosol assay (performed at 0–4°C) or that the receptor may be deficient in capability to mediate localization (of receptor or of hormone-receptor complex) into the nucleus under certain conditions. Since these patients, like patient 5, exhibited a calcemic response to calciferols (Table I), nuclear activation by receptors is likely under certain conditions in target tissues. Though fibroblast interaction with $[^3H]1,25(OH)_2D_3$ was similar in kindreds 1 and 2, clinical features clearly distinguish affected members of the two kindreds. Both affected members of kindred 2 exhibit total alopecia that had begun in the first months of life, while skin appendages are normal in both affected members of kindred 1.

In fibroblasts from patient 3 all evaluations of interaction with $[^3H]1,25(OH)_2D_3$ have been normal thus far. Two explanations can be considered. Firstly, the defect in target tissues for $1,25(OH)_2D$ may be distal to the interaction of receptor-bound $1,25(OH)_2D$ with nuclear chromatin. Such "receptor-positive" mutations have been documented in other hormone systems (17). Alternatively, the defect of $1,25(OH)_2D$ action in patient 3 may be proximal to chromatin activation but may not be expressed with assay methods we have used to date. Patient 3 showed the unusual feature of achieving marked improvement in hypocalcemia while being treated with $24,25(OH)_2D_3$ (10). This had been administered because circulating levels of $24,25(OH)_2D$ were undetectable (<0.4 ng/ml vs. normal of 1.5 to 2.5 ng/ml). If the underlying defect in this kindred is a deficiency of a modulator (such as $24,25(OH)_2D$) of target tissues for $1,25(OH)_2D$, then the secondary defect in the $1,25(OH)_2D$ effector system might not be expressed in cultured skin fibroblasts. Normal whole-cell nuclear uptake has been reported for one other patient with resistance to $1,25(OH)_2D$ (34).

Fibroblasts cultured from affected members of two kindred pairs (kindreds 5 and 6; kindreds 1 and 2) exhibited similar abnormal patterns in their interactions with $1,25(OH)_2D_3$; for each kindred pair, there were clinical features that distinguished affected members of one family from affected members of the other (Table I). Thus the data obtained in vivo (Table I) and in vitro (Tables II and III) indicate that resistance to $1,25(OH)_2D_3$ in these six kindreds reflects five or perhaps six distinct mutations in the genes that determine the effector system for $1,25(OH)_2D$. Our analyses do not establish whether the distinct molecular defects are localized to the receptor molecule(s) or to processes closely associated with them.

The recognition that the molecular defects in kindreds with resistance to $1,25(OH)_2D$ are heterogeneous has provided potential explanations for some of the clinical diversity among these kindreds. Certain features, such as the frequently associated total alopecia, remain largely unexplained. While we have attempted to analyze clinical data as a bioassay for function of $1,25(OH)_2D$ receptors in vivo in target tissues, it will be important to analyze bioresponses to $1,25(OH)_2D$ with patients' tissues in vitro (33, 34). The demonstration of abnormal interaction with $[^3H]$-$1,25(OH)_2D_3$ in skin fibroblasts cultured from members of five of six kindreds with resistance to $1,25(OH)_2D_3$ in vivo indicates that this interaction is

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[4] Eil, C. Familial incomplete male pseudohermaphroditism associated with impaired nuclear androgen retention. Studies in cultured skin fibroblasts. In press.

regulated by some of the same genes that regulate analogous interactions in target tissues for $1,25(OH)_2D$.

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