ready demonstrated that the flow cytometric assay is suitable for the detection of estrogen receptor–positive cells of which there may be less than 5 percent in such samples. Likewise, solid breast tumors with considerable contamination by normal cells and low expression of estrogen receptor as determined by radioreceptor assay (for example, ≤ 5 fmole per milligram of protein) have been successfully identified by flow cytometry (*15*). The reproducibilities of estrogen receptor analysis by flow cytometry and radioreceptor assay compare favorably with a coefficient of variation ranging from 10 to 15 percent.

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10. The concentration of FE required to achieve 50 percent competition varied with the source of cytosols from 5 to 20 n*M* probably as a result of the difference in endogenous estrogen level of the cytosols.
11. Esterase activity is present in cytosols [R. G. Smith, M. D'Istria, N. T. Van, *Biochemistry* **20**, 5557 (1981)]. The fact that esterase activity was not destroyed by mild heat treatment was demonstrated by the observation that heat-treated cytosols (45°C for 30 minutes) still hydrolyzed fluorescein diacetate into fluorescein with marked increase in fluorescence at 520 n*M* (N. T. Van, unpublished result).
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Vitamin D_3–Resistant Fibroblasts Have Immunoassayable 1,25-Dihydroxyvitamin D_3 Receptors

Abstract. *Cultured fibroblasts obtained from patients with tissue resistance to 1,25-dihydroxyvitamin D_3 (vitamin D_3–dependent rickets, type II) contain normal, low, or undetectable concentrations of this hormone's receptor protein as measured by a ligand-binding assay. Extracts from these cells were evaluated for receptors by immunoassay with a recently developed monoclonal antibody to the chick receptor. The results show that a protein sedimenting at 3.7S and recognizable by the antibody exists in comparable concentrations in cells from both normal and resistant patients, irrespective of the hormone-binding abnormalities of the cells. This implies that deficiencies in hormone binding associated with inherited tissue resistance to 1,25-dihydroxyvitamin D_3 probably arise from structural variations in the receptor molecule and not from defective receptor synthesis.*

Vitamin D–dependent rickets, type II (VDDR II), is a rare, heritable syndrome clinically characterized by hypocalcemia, secondary hyperparathyroidism, and rickets, all of which persist despite high circulating levels of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (*1–5*). This constellation of features results from peripheral target organ resistance to 1,25$(OH)_2D_3$ comparable to analogous clinical disorders for the glucocorticoid and androgen hormones (*6–9*). Cultured skin fibroblasts from patients with VDDR II have been used to evaluate the underlying defects associated with this malady (*10, 11*). These studies, coupled with accumulating clinical insights, have shown that this disease is heterogeneous and arises almost exclusively from defects in either the receptor's interaction with 1,25$(OH)_2D_3$ or its nuclear site of action (*10–12*).

One particularly abundant phenotype that has been identified in the fibroblasts of a number of different kindreds appears to be a receptor-lacking variant, as deduced by the absence of hormone binding activity. This has led to the possibility that the syndrome is caused by an inherited deletion of the gene for the 1,25$(OH)_2D_3$ receptor. We have now evaluated this possibility by using a radioligand immunoassay (RLIA) to examine these fibroblasts for 1,25$(OH)_2D_3$ receptors (*13*). Our results indicate the presence of normal amounts of material cross-reacting with the receptor antibody in these cells. This suggests that tissue resistance associated with phenotypes in which hormone binding is lacking (HB^-) may be caused by mutations in the binding domain of the receptor protein and is rarely, if ever, the result of genetic deletion.

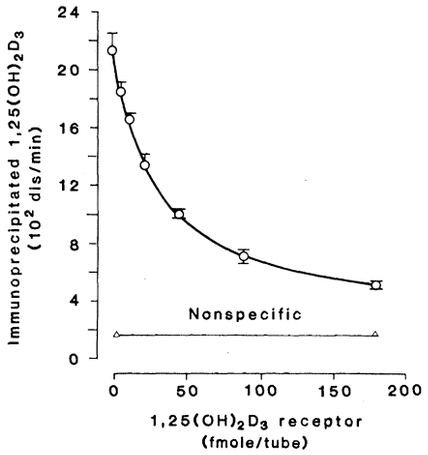

Fig. 1. Standard curve for RLIA. Each point (○) represents the average of triplicate reactions ± the standard error. Reactions were carried out as described. Nonspecific binding (△) was determined in the absence of 9A7γ antibody.

Human fibroblasts were obtained by skin biopsy from normal subjects or from patients, most of whom have been previously described (*1–5, 10, 11*). These cells, as well as the human breast cell carcinoma (MCF-7) and the mouse fibroblast (3T6) lines (American Type Culture Collection, Rockville, Maryland) were maintained in monolayer culture (*8–11, 14*). Cells were harvested at confluence, washed extensively, and disrupted by sonication in KETD buffer (10 m*M* tris-HCl, *p*H 7.4; 1 m*M* EDTA; 0.3*M* KCl, and 5 m*M* dithiothreitol). Cellular extracts used for the 1,25$(OH)_2D_3$–binding assay (*14*) or the immunoassay were obtained after ultracentrifugation.

The 1,25$(OH)_2D_3$ receptors employed in the competition immunoassay were obtained from similarly prepared extracts of chick intestinal mucosa homogenized in KETD buffer. Identical solutions were incubated with either 1,25$(OH)_2[^3H]D_3$ (4 n*M*, 148 Ci/mmole) or 1,25$(OH)_2D_3$ (4 n*M*) to form 1,25$(OH)_2D_3$–receptor complexes. The immunoassay standard curve was created with the 1,25$(OH)_2[^3H]D_3$–receptor complex (80 p*M*), monoclonal antibody (*15–17*), and increasing concentrations of the 1,25$(OH)_2D_3$–receptor complex ranging from 0 to 800 p*M* (*13*) (Fig. 1). Cell or tissue extracts examined by immunoas-

say contained 0.05 to 0.5 mg of protein per sample and 0 to 160 p*M* receptor-related competing material. All immunoassay incubations were carried out in triplicate at 4°C for 16 hours in KETD buffer containing 1 percent fetal bovine serum. Antibody-bound receptor was recovered by immunoprecipitation (*17*), and the $1,25(OH)_2[^3H]D_3$ was then extracted with acetone and quantified by liquid scintillation counting. The monoclonal antibody (9A7γ), which was isolated as described (*16, 17*), has similar equilibrium dissociation constants for chick ($1.8 \times 10^{11} M$) (*17*) and human ($1.4 \times 10^{-11} M$) (*13*) receptors and reacts identically with occupied and unoccupied receptors (*13*). Approximately 40 percent of added $1,25(OH)_2[^3H]D_3$–receptor complex routinely binds to the antibody in the absence of competition under the standard immunoassay conditions. The assay is sensitive to 4 to 5 fmole of competing receptor per tube with intra- and interassay variations of 7 and 12 percent, respectively. This assay can detect and quantify chick intestinal receptors from cytosols directly as well as after sedimentation on sucrose gradients or after chromatography on DNA-cellulose (*13*).

We applied this radioligand immunoassay specifically to the detection and quantitation of $1,25(OH)_2D_3$ receptors in extracts of fibroblasts from patients with VDDR II. The assay of cell extracts from normal fibroblasts, MCF-7 cells, and the fibroblasts from eight different VDDR II patients revealed the presence of cross-reactive material in concentrations at least three times that of the assay background (as determined by minor competition observed in rat liver extracts), which was a linear function of the protein content of the sample assayed. The immunoassay data are summarized in Table 1, where they are contrasted with the hormone-binding values of the fibroblast cytosols, as well as with their qualitative nuclear transfer capabilities (*10, 11*). The immunologic values obtained for normal fibroblasts, MCF-7 cells, and VDDR II–variant fibroblasts (patients 1A, 2B, and 7), all of which show "normal" hormone-binding properties, reveal a narrow range of immunoreactive values which are higher, but generally reflect the level of receptor measured by the ligand-binding assay. In cells in which hormone-binding activity is low (patient 4) or undetectable (patients 5, 6, 8, and 9), the immunoassay values remain comparable to the values obtained in phenotypes in which hormone binding occurs (HB^+). This suggests that a receptor-like molecule is present when quantified via an epitopic site, rather than on the basis of a functional property.

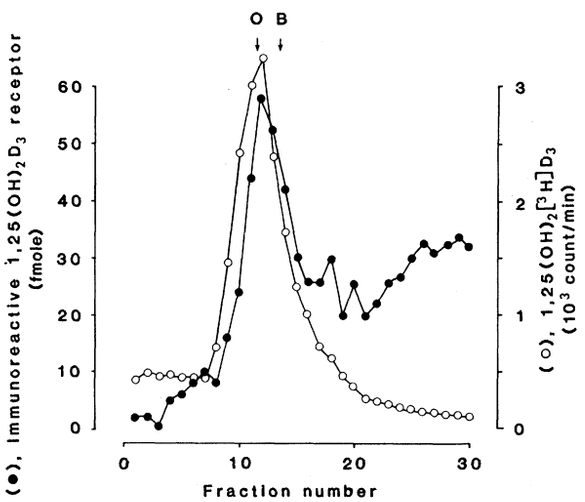

Fig. 2. Sedimentation analysis of putative $1,25(OH)_2D_3$ receptor on 10 to 30 percent sucrose gradients. Cell extracts of human fibroblasts (patient 6) (400 μl; 1.8 mg of protein) and $1,25(OH)_2[^3H]D_3$–labeled cell extracts of mouse 3T6 fibroblasts (400 μl; 2.0 mg of protein) were fractionated (*14*). Fractions were analyzed for receptor either (○) directly by radioactivity (3T6) or (●) indirectly by RLIA (human fibroblasts). Sedimentation markers are (O) ovalbumin, 3.7*S*, and (B) bovine serum albumin, 4.4*S*. The increase in baseline competition by RLIA is due to sucrose interference.

The cell extracts from one individual with an HB^- phenotype (patient 6) were further characterized by immunoassay after sedimentation through hypertonic sucrose gradients (*16*). The sedimentation coefficient of 3.7*S* obtained for this molecule and its comigration with authentic labeled $1,25(OH)_2D_3$ receptor derived from mouse 3T6 fibroblasts [characterized more completely in (*14*)] are both consistent with the hypothesis that the cross-reactive material we have observed is the vitamin D–receptor molecule.

Inspection of Table 1 indicates that the HB^- phenotype is the most common cellular phenotype characteristic of fibroblasts from this series of kindreds with VDDR II. Taken together with our current immunologic data showing that these cell types are in fact receptor-positive (R^+), this constitutes a demonstration of biologically unresponsive HB^-, R^+ phenotypes in VDDR II. Further biochemical heterogeneity within the receptor is also suggested by our data. Cells from patient 4 show low hormone-binding capacity contrasted with normal receptor concentration as judged by immunoassay; this suggests potential molecular instability of hormone binding in vitro or other unknown factors. While cells from patient 6 show lower immunoassayable receptor than was found in the others, we do not know whether this is a reflection of the assay or of an additional variant phenotype. Clinical observations indicate that treatment with pharmacologic levels of $1,25(OH)_2D_3$ or other active vitamin D_3 analogs can elicit restoration of calcium

Table 1. Measurement of $1,25(OH)_2D_3$ receptors by RLIA in patients with type II vitamin D–dependent rickets. Data on $1,25(OH)_2D_3$ binding, the calcemic response of the patient to vitamin D metabolites, and nuclear uptake of $1,25(OH)_2D_3$ are, in part, from (*11*). Abbreviations: N.D., not detected; N.A., not applicable. Values for cytosolic vitamin D receptors are expressed as femtomoles per milligram of protein; the values obtained by RLIA represent the average ± standard deviation of three to six estimations.

Source of cell line	Cytosolic receptor (fmole/mg)		Calcemic response of patient to D metabolites	Nuclear uptake of $1,25(OH)_2D_3$
	$1,25(OH)_2D_3$ binding	RLIA		
Normal 1 (*11*)	35 ± 2	64.8 ± 27	+	+
Normal 2 (*11*)	51	58.9 ± 1	+	+
Patient 2B (*2*)	28	69.3 ± 6	+	−
Patient 1A (*1*)	19	57.7 ± 19	+	−
Patient 7	48	71.6 ± 19	−	+
Patient 4 (*3*)	4.2	48.4 ± 21	−	+
Patient 6 (*5*)	N.D.	23.4 ± 4.5	−	−
Patient 8	N.D.	48.6 ± 14	−	−
Patient 9	N.D.	52.9 ± 13	−	−
Patient 5 (*3*)	N.D.	40.9 ± 5	+	−
MCF-7 (human breast carcinoma)	54 ± 6	58.6 ± 7	N.A.	+
Liver (rat)	N.D.	9	N.A.	N.A.

balance in certain patients (*3*). Thus, patient 5, who was responsive to this treatment, appears to retain a minor physicochemical change in receptor that lowers the receptor's affinity for the hormone without altering the capacity of the receptor to activate genes. In contrast, patients 6, 8, and 9 are unresponsive to treatment, and we predict that in their cases the receptors contain defects in the hormone-binding sites that lead to severely impaired function and consequent tissue resistance to $1,25(OH)_2D_3$. These newly clarified receptor defects must then be added to those previously characterized in VDDR II (*10, 11*), including nuclear transfer deficiencies (patients 1A and 2B) and post-receptor blocks, the latter evidenced here by lack of responsiveness to $1,25(OH)_2D_3$ (patient 7) in spite of apparently normal hormone-binding affinity and nuclear uptake capacity (Table 1).

The HB^- phenotype in VDDR II may have arisen genetically from receptor gene mutation or deletion, molecular abnormalities due to cellular synthesis or processing, or lack of important but unknown environmental principles. Our data rule out several mechanisms, including gene deletion, although they do not specify which of the alternative possibilities might be responsible for tissue resistance to $1,25(OH)_2D_3$. Similar findings will perhaps be observed in comparable tissue-resistant disease states for other steroid hormones (*6–9*). Our observations imply that the clinical manifestations of $1,25(OH)_2D_3$ resistance in VDDR II are rarely, if ever, due to the absence of receptor, but are probably due to internal changes in the molecule that prevents normal function. These aberrations might include molecular instability, lack of conformational changes inducible by $1,25(OH)_2D_3$, modifications in hormone affinity, or reduced capacity to bind to nuclear sites or DNA. Each of these possibilities requires further investigation.

The existence of biologically inactive receptors for $1,25(OH)_2D_3$ and the concomitant tissue resistance to the hormone seen in VDDR II lend strong support for the major $1,25(OH)_2D_3$–mediating role of this protein in normal cells and tissues and for the genomic mechanism of this sterol's action. As a consequence, cellular resistance to $1,25(OH)_2D_3$ leads to clinical symptoms similar to those of vitamin D deficiency, such as hypocalcemia, secondary hyperparathyroidism, and rickets. Presumably these symptoms are due to the lack of effect of $1,25(OH)_2D_3$ in traditional target tissues such as intestine, kidney, and bone, which are primary regulators of calcium homeostasis (*12*). Direct studies in VDDR II–derived fibroblasts have similarly shown these cells to be deficient in other putative receptor-mediated responses such as growth inhibition (*18*) and 25-hydroxyvitamin D_3-24-hydroxylase induction (*19–21*). Taken cumulatively, the present study defines more precisely the nature of the biochemical defects associated with VDDR II and provides further support for the concept that functional receptors are a requirement for the normal mediation of biological activity of $1,25(OH)_2D_3$.

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The Structural Gene for Tetanus Neurotoxin Is on a Plasmid

Abstract. *A pool of synthetic oligonucleotides was prepared based on the amino terminal amino acid sequence of tetanus toxin. This probe hybridized to plasmid DNA isolated from three toxigenic strains of* Clostridium tetani *but not to plasmid DNA from a nontoxigenic strain. These results show that the structural gene for the toxin is on the plasmid. The pCL1 plasmid from one of the toxigenic strains spontaneously deleted 22 kilobase pairs of DNA to form pCL2. Strains harboring this deleted plasmid are nontoxigenic. However, the probe mixture hybridized to pCL2, indicating that the DNA encoding the amino terminus of the toxin had not been deleted. Restriction endonuclease cleavage maps of pCL1 and pCL2 were constructed and indicate the approximate location and orientation of the structural gene for tetanus toxin.*

The symptoms of tetanus were linked to the production of a thermolabile toxin by *Clostridium tetani* in the late 19th century (*1*). Subsequently, much information has been compiled about the size, structure, and biological activities of this powerful neurotoxin, although the exact molecular action of the toxin has not been defined (*2*). The toxin is produced initially as a single polypeptide chain with a molecular weight of approximately 150,000. This chain is cleaved into two subunits with molecular weights of approximately 100,000 and 50,000 by the action of endogeneous proteases (*3*). The genetic mechanism controlling toxin production by *C. tetani* has not been investigated extensively. The genetic factor controlling the production of tetanus toxin is thought to be associated with an extrachromosomal element (*4*). Toxigenic strains of diverse origin have been found to contain a single large plasmid (*5, 6*). A number of nontoxigenic derivatives were isolated from these strains, and in each case the loss of toxigenicity correlated with the loss of the plasmid. However, the precise role of plasmids in toxin production was not elucidated. Plasmids may contain sequences coding for the toxin, or they may code for proteins involved with regulation of a