

Metabolism of 25-Hydroxyvitamin D₃ by Cultured Pulmonary Alveolar Macrophages in Sarcoidosis

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ABSTRACT Metabolism of [³H]25-hydroxyvitamin D₃(25-OH-D₃) was studied in primary cultures of pulmonary alveolar macrophages (PAM) from seven patients with sarcoidosis and two patients with idiopathic pulmonary fibrosis. Production of a [³H]1,25-dihydroxyvitamin D₃ (1,25-[OH]₂-D₃)-like metabolite of [³H]25-OH-D₃ was detected in lipid extracts of cells from five patients with sarcoidosis. Synthesis of this compound in vitro was limited to viable PAM and was greatest in cells derived from a patient with hypercalcemia and an elevated serum concentration of 1,25-dihydroxyvitamin D. The tritiated PAM metabolite coeluted with authentic 1,25-(OH)₂-D₃ in three different solvent systems on straight-phase high performance liquid chromatography (HPLC) and demonstrated binding to extracted receptor for 1,25-(OH)₂-D₃, which was identical to that of commercially available [³H]1,25-(OH)₂-D₃ of comparable specific activity. Incubation of PAM with high concentrations of 25-OH-D₃ resulted in production of an unlabeled metabolite that co-chromatographed with the ³H-PAM metabolite on HPLC and that was bound with high affinity by both the specific receptor for 1,25-(OH)₂-D₃ and antiserum to 1,25-(OH)₂-D₃.

INTRODUCTION

Disordered calcium metabolism is a frequently encountered clinical problem in sarcoidosis. The reported incidence of hypercalcemia in the disease may range as high as 20% (1), while the incidence of hy-

percalciuria, a major source of morbidity in sarcoidosis, may be twice as great (2). The proposed etiology for this phenomenon is extrarenal overproduction of the active metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25-[OH]₂-D)¹ (3, 4). A possible cellular source for 1,25-(OH)₂-D or a related sterol is the macrophage, a ubiquitous constituent of the noncaseating granulomata of sarcoidosis. In this report, we demonstrate production in vitro of a 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂-D₃)-like metabolite of [³H]25-hydroxyvitamin D₃ (25-OH-D₃) by primary cultures of pulmonary alveolar macrophages (PAM) harvested from patients with active sarcoidosis.

METHODS

Patients. Bronchoalveolar lavage was performed in seven patients with biopsy-proven sarcoidosis and two patients with idiopathic pulmonary fibrosis. Five patients with sarcoidosis had roentgenographic evidence of diffuse infiltrative pulmonary disease with or without hilar lymphadenopathy (stage II or III), while two patients demonstrated only hilar lymph node involvement (stage I). Lavage was performed on two separate occasions in one patient (Patient 1, Table I) with active pulmonary sarcoidosis; it was done initially when his serum concentration of calcium was 15.1 mg/dl (normal, 8.5–10.5 mg/dl) and his serum 1,25-(OH)₂-D level (5) was 94 pg/ml (normal, 30–70 pg/ml) and 4 mo hence, after self-initiated withdrawal from glucocorticoid therapy, when he had a serum calcium concentration of 9.7

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¹ Abbreviations used in this paper: HPLC, high performance liquid chromatography; 25-OH-D₃, 25-hydroxyvitamin D₃; 1,25-(OH)₂-D, 1,25-dihydroxyvitamin D; 1,25-(OH)₂-D₃, 1,25-dihydroxyvitamin D₃; IEC, intestinal epithelial cells; PAM, pulmonary alveolar macrophages.

mg/dl. Another subject (Patient 2, Table I) had a normal serum concentration of calcium (9.8 mg/dl) and 1,25-(OH)₂-D (51 pg/ml) but was hypercalciuric (0.31 mg calcium/100 ml glomerular filtrate, normal < 0.16) at the time of lavage. None of the other patients from whom lavage specimens were obtained had past or current evidence of either hypercalcemia or hypercalciuria. No patients were receiving glucocorticoid treatment.

Cell cultures. Primary monolayer cultures of PAM were established by standard techniques (6) from 40 to 60 ml of bronchoalveolar lavage fluid. Harvested cells were plated in Costar plasticware (Costar, Cambridge, MA) at an approximate density of 2×10^5 cells/cm² in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 4 mM glutamine, 10 µg/ml insulin, 50 U/ml penicillin, and 50 µg/ml streptomycin (all from Gibco Laboratories, Grand Island, NY) and maintained in an atmosphere of 5% CO₂:95% air at 37°C. Nonadherent cells were decanted in the culture medium 12–24 h after plating the cells. Thereafter, the culture medium was changed twice weekly. Nonviable macrophages (ascertained by failure to exclude trypan blue) were harvested from the culture medium and retained for use as controls in subsequent experiments.

[³H]25-OH-D₃ metabolism. After 7–12 d in primary culture, the homogeneity of the cell populations was confirmed by histochemical staining for nonspecific esterase activity by using α-naphthyl acetate as substrate (7), and the culture medium was decanted and replaced with serum-free BGJ_b medium. After a 36-h preincubation period, this medium was replaced with serum-free BGJ_b medium containing 5 nM [26,27-methyl-³H]25-OH-D₃ (Amersham Corp., Arlington Heights, IL) solubilized in 0.1% absolute ethanol. [³H]25-OH-D₃ was purified on a silica Sep-Pak cartridge (Waters Associates, Milford, MA) before use (8). Cultures were incubated for 3 h at 37°C in [³H]25-OH-D₃-containing medium. At the conclusion of 3-h incubation period, the medium was aspirated and the cell monolayer harvested by gentle scraping. The lipid was extracted from the cells and the incubation medium in methanol-methylene chloride, and the resultant extracts were chromatographed on silica Sep-Pak cartridges. The bulk of the radioactivity in the form of unmetabolized [³H]25-OH-D₃ was eluted in 15 ml of 25% ethylacetate in *n*-hexane. More polar material was eluted in 15 ml of 100% ethylacetate. The latter fraction was evaporated under nitrogen and resolubilized for straight-phase high performance liquid chromatography (HPLC) over a 3.9 mm × 30 cm µPorasil column (Waters Associates) in a mobile phase of 92% *n*-hexane:4% methanol:4% isopropanol eluting at 2.0 ml/min. Each sample was co-chromatographed with crystalline standards of 25-OH-D₃, 24(R),25-(OH)₂-D₃, 25,26-(OH)₂-D₃, and 1,25-(OH)₂-D₃; and the radioactivity coeluting with unlabeled standards was determined. Co-chromatography of labeled product with 1,25-(OH)₂-D₃ was confirmed in each case by straight-phase HPLC in a different solvent system of 98% methylene chloride:2% methanol (see Fig. 1 C). The quantity of labeled metabolite co-chromatographing with 1,25-(OH)₂-D₃ was expressed in terms of femtomoles per 10⁶ cells per minute⁻¹ (specific activity). Parallel incubations of cultured rat intestinal epithelial cells (IEC) (9), nonviable macrophages, and culture medium alone with [³H]1,25-(OH)₂-D₃ served as controls *in vitro*.

25-OH-D₃ metabolism. Primary cultures of PAM from Patient 1b (Table I) containing $\sim 10 \times 10^6$ cells were preincubated in serum-free BGJ_b medium for 12 h and then exposed to 200 nM 25-OH-D₃ for 12 h. The incubation medium was collected and the lipid was extracted, chromatographed on silica Sep-Paks, and stored in ethanol at -70°C under ni-

trogen. Serum-containing medium was replaced for 48 h, and the above procedure was repeated. After six such cyclic incubation periods, the six extracts were pooled, spiked with tracer amounts (2 µCi) of the 1,25-(OH)₂-D₃-like ³H-PAM metabolite, and chromatographed successively on straight-phase HPLC in 92% *n*-hexane:4% methanol:4% isopropanol, 98% methylene chloride:2% methanol, and 95% methylene chloride:5% isopropanol. The unlabeled PAM metabolite that was co-chromatographing with the ³H-PAM metabolite was quantitated by its UV absorbance at 254 nm in a Waters 440 in-line detector.

Metabolite binding. The binding of the labeled and unlabeled PAM metabolite to the chick intestinal cytosol receptor for 1,25-(OH)₂-D₃ and to an antibody generated against 1,25-(OH)₂-D₃ was compared with that of commercially available [³H]1,25-(OH)₂-D₃ and authentic 1,25-(OH)₂-D₃, respectively. High affinity receptor for 1,25-(OH)₂-D₃ was extracted in TKM buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 5 mM dithiothreitol, 300 mM KCl, and 10 mM sodium molybdate) from a high-speed centrifugate (100,000 *g* for 1 h) of rachitic chick duodenal mucosa (10). 1-ml aliquots of the resultant cytosol preparation (diluted 1:40 with TKM buffer) were added to sterols solubilized in 20 µl of ethanol and incubated for 1 h at 23°C. The 1,25-(OH)₂-D₃ antiserum, that was raised in sheep immunized with 1,25-dihydroxy-25-hemisuccinate vitamin D₃, was kindly provided by Dr. Tom Clemens. The radioimmunoassay, which used serial dilutions of the PAM metabolite as unlabeled ligand, was performed by a modification of the method of Clemens et al. (11). In both assay systems, bound radioactivity was separated from unbound label by dextran-coated charcoal and quantitated by scintillation counting.

RESULTS

Table I shows the results of the [³H]25-OH-D₃ metabolism experiments in PAM cultures from the five patients with sarcoidosis in which 1,25-(OH)₂-D₃-co-chromatographing material was identified. In the initial experiment, [³H]25-OH-D₃ metabolism was examined in primary PAM cultures from the index case (Patient 1) with sarcoidosis-associated hypercalcemia. In the lipid extracts of both macrophages and their incubation medium, there was a homogeneous peak of radiolabeled material that co-chromatographed with authentic 1,25-(OH)₂-D₃ on HPLC in 92% *n*-hexane:4% methanol:4% isopropanol; 11.1 and 3.7% (total 14.8%) of the lipid-extractable label in the cells and incubation medium, respectively, co-chromatographed with 1,25-(OH)₂-D₃. Readmission of the index case to the hospital with an exacerbation of his pulmonary disease provided an opportunity to reevaluate the metabolic capacity of PAM from this patient when he was not overtly hypercalcemic. As was demonstrated in the initial experiments with cells from this host, [³H]25-OH-D₃ was metabolized to a more polar product which co-chromatographed with 1,25-(OH)₂-D₃ on HPLC. However, the yield of metabolite at the same substrate concentration was reduced 75% when compared with that of PAM from the initial harvest. To verify the elution position of the 1,25-(OH)₂-D₃-like metabolite, a portion

TABLE I
Metabolism of [³H]25-OH-D₃ by Cultured Alveolar Macrophages from Patients with Sarcoidosis to a ³H-Sterol That Co-chromatographs with 1,25-(OH)₂-D₃

Patients	n*	Specific activity of [³ H]25-OH-D ₃	Enzyme specific activity
		(Ci/mmol)	(fmol per 10 ⁶ cells per min ⁻¹)
1†a‡	1	30	8.6
b	3	158	2.2±0.2
2‡	3	158	1.6±0.1
3	2	158	0.43
4	3	158	0.47±0.32
5	4	158	0.29±0.01

* n is the number of replicate incubations for that particular sample. Where n is three or more, data are expressed as the mean±SD. † Cultures from patient 1 were established and examined on two separate occasions, initially (a) when he was hypercalcemic and subsequently (b) when he was not. The use of [³H]25-OH-D₃ of higher specific activity in subsequent incubations of cells from the index case and other patients was elected in order to maximize the yield of high specific activity product from replicate cultures.

‡ Presence of hypercalcemia at the time of study.

§ Presence of hypercalciuria at the time of study.

of the PAM extract was co-chromatographed in two additional solvent systems on straight-phase HPLC (Fig. 1). In all three solvent systems evaluated, there was co-chromatography of the tritiated PAM metabolite with standard 1,25-(OH)₂-D₃.

After incubation with [³H]25-OH-D₃, 1,25-(OH)₂-D₃-co-chromatographing sterol was detected in PAM cultures from four additional patients with sarcoidosis; all of these patients had stage II or III pulmonary disease (Table I). With the exception of cells from Patient 2, who was hypercalciuric at the time of the study, the specific activity of the PAM-derived enzyme was at least an order of magnitude below that of cells from the hypercalcemic patient. Labeled metabolite with the chromatographic mobility of 1,25-(OH)₂-D₃ was not identified in extracts of PAM from either of the two patients with stage I pulmonary sarcoidosis or the two patients with nonsarcoidosis-related pulmonary disease. In addition to being specific for cells from a host with sarcoidosis, production of the [³H]1,25-(OH)₂-D₃-like metabolite was also specific for viable PAM. Incubation of nonviable PAM (n = 6) and IEC cell cultures (n = 6) with [³H]25-OH-D₃ failed to result in production of material that co-chromatographed on HPLC with 1,25-(OH)₂-D₃. However, IEC cells did synthesize a substantial amount of a periodate-sensitive (12), radiolabeled product (5.2±2.1% [mean±SD] of the total lipid-extractable tritium) that co-chromatographed

with 24(R),25-(OH)₂-D₃. Lipid extracts of medium alone contained no product more polar than the starting material, [³H]25-OH-D₃.

In an attempt to functionally characterize the tritiated metabolite, the PAM-derived material was employed as radiolabeled ligand for binding to the high-affinity intracellular binding protein (receptor) for 1,25-(OH)₂-D₃ (Fig. 2). Displacement of the labeled PAM metabolite and commercially available [³H]1,25-(OH)₂-D₃ from extracted receptor by increasing the concentrations of unlabeled 1,25-(OH)₂-D₃ was identical. To generate unlabeled metabolite for ligand binding studies, we took advantage of the fact that approximately 25% of the metabolite synthesized by monolayer PAM cultures reappears in the incubation medium. Repetitive incubation of cells from a single host (Patient 1b, Table I) with 200 nM 25-OH-D₃ yielded a detectable quantity of unlabeled metabolite that co-chromatographed

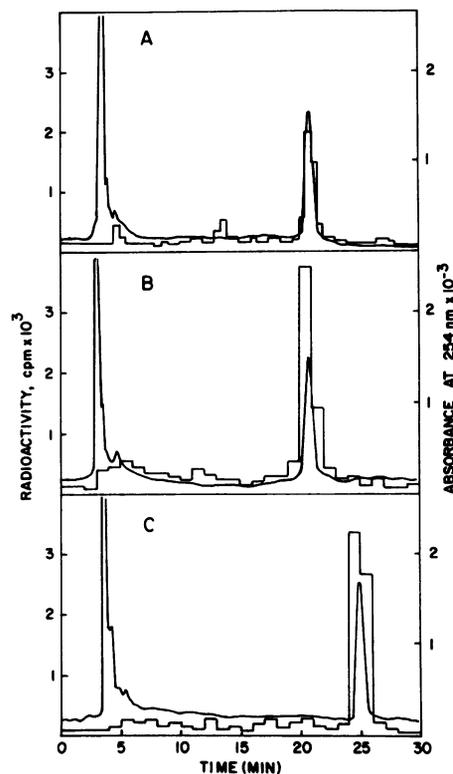


FIGURE 1 Straight-phase HPLC of a portion of the 100% ethylacetate fraction from Sep-Pak preparative chromatography of a lipid extract of sarcoid PAM. Chromatography of the sample and a crystalline standard of 1,25-(OH)₂-D₃ was performed in three different solvent systems: 92% n-hexane:4% methanol:4% isopropanol at 2.0 ml/min (A), 95% methylene chloride:5% isopropanol at 1.5 ml/min (B), and 98% methylene chloride:2% methanol at 1.5 ml/min (C).

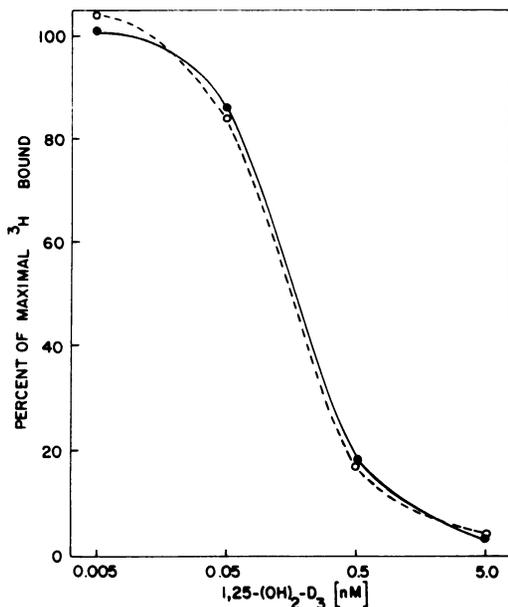


FIGURE 2 Displacement of [26,27-*methyl*- ^3H]1,25-(OH) $_2$ -D $_3$ (●) and ^3H -PAM metabolite (○) from receptor for 1,25-(OH) $_2$ -D $_3$ by unlabeled 1,25-(OH) $_2$ -D $_3$. The specific activity of [^3H]1,25-(OH) $_2$ -D $_3$ and ^3H -PAM metabolite (derived and purified from cultures of Patient 1b [Table I]) was 161 and 158 Ci/mmol, respectively.

graphed on HPLC with both [^3H]1,25-(OH) $_2$ -D $_3$ and the ^3H -PAM metabolite in the three solvent systems used (Fig. 1). Assuming the metabolite possessed the same UV absorption spectrum as its precursor, 25-OH-D $_3$, the HPLC-purified compound was equivalent to 60 pmol of 1,25-(OH) $_2$ -D $_3$. As shown in Fig. 3, the displacement of [^3H]1,25-(OH) $_2$ -D $_3$ from both the receptor for 1,25-(OH) $_2$ -D $_3$ and antibody to the hormone was identical for equimolar concentrations of the PAM metabolite and authentic 1,25-(OH) $_2$ -D $_3$.

DISCUSSION

It is now recognized that the macrophage is capable of synthesizing a variety of compounds with proven or potential bioactivity. These products include a number of peptides, nucleotide metabolites, reactive oxygen metabolites, arachidonic acid derivatives (13-16), and potent androgenic steroids (17). In the present report, we demonstrate that cultured PAM from patients with active pulmonary sarcoidosis metabolize a sterol hormone precursor, 25-OH-D $_3$, to a 1,25-(OH) $_2$ -D $_3$ -like metabolite. Production of this compound *in vitro* was observed only in cells from patients with diffuse pulmonary sarcoidosis (stage II or III) and was greatest in PAM derived from patients with either hypercalcemia

or hypercalciuria. Coelution of the PAM metabolite and authentic 1,25-(OH) $_2$ -D $_3$ was documented in three different mobile phases on straight-phase HPLC (Fig. 1). Co-chromatography of PAM metabolite and 1,25-(OH) $_2$ -D $_3$ in a mobile phase of 98% methylene chloride:2% methanol (Fig. 1 C) was of particular interest. According to a recent report by Gray et al. (18), this solvent system successfully separated 1,25-(OH) $_2$ -D $_3$ from a [^3H]25-OH-D $_3$ metabolite produced by rodent peritoneal macrophages. Therefore, the metabolite synthesized by rodent macrophages and that of human PAM origin are probably different.

Repetitive incubation of PAM from a sarcoid patient (Patient 1b, Table I) with high concentrations of unlabeled 25-OH-D $_3$ resulted in production of metabolite that co-chromatographed on HPLC with [^3H]1,25-(OH) $_2$ -D $_3$ as well as the ^3H -PAM metabolite. In addition, the unlabeled PAM metabolite from this host was bound

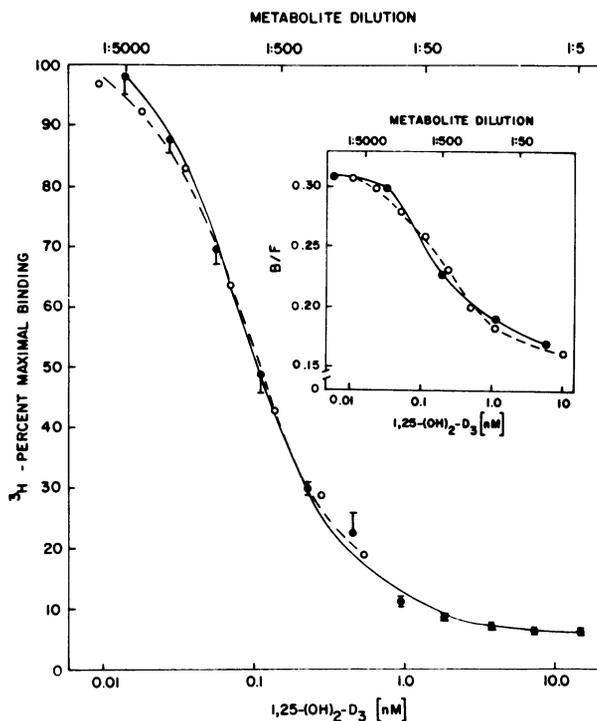


FIGURE 3 An analysis of competitive binding by using authentic 1,25-(OH) $_2$ -D $_3$ (○) and serial dilutions of the 1,25-(OH) $_2$ -D $_3$ -co-chromatographing macrophage metabolite (●) as unlabeled ligands, [26,27-*methyl*- ^3H]1,25-(OH) $_2$ -D $_3$ as labeled ligand, and either an extract of the chick intestinal receptor for 1,25-(OH) $_2$ -D $_3$ or 1,25-(OH) $_2$ -D $_3$ -specific antibody (inset) as the binding protein. There were 60 pmol of sterol in the original undiluted sample of the macrophage metabolite. Each point represents either the mean \pm SD result for triplicate samples or the mean of duplicate determinations.

with high affinity to both specific receptor for 1,25-(OH)₂-D₃ and antibody to 1,25-(OH)₂-D₃. While these data strongly suggest that the macrophage may be the synthetic source of "serum-assayable" 1,25-(OH)₂-D in sarcoidosis, they do not exclude the possibility that the PAM-derived metabolite is chromatographically and functionally similar but structurally different from 1,25-(OH)₂-D₃. Absolute identity of the macrophage metabolite must await structural identification of the compound.

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