

CHARACTERIZATION OF 1 α -HYDROXYLATION OF VITAMIN D₃ STEROLS BY CULTURED ALVEOLAR MACROPHAGES FROM PATIENTS WITH SARCOIDOSIS

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The hypercalcemia of sarcoidosis is thought to result from the extrarenal overproduction of an active vitamin D sterol (1, 2). We reported (3, 4) that cultured pulmonary alveolar macrophages (PAM)¹ from patients with sarcoidosis are capable of metabolizing 25-hydroxyvitamin D₃ (25-OH-D₃) to 1 α ,25-dihydroxyvitamin D₃ [1,25-(OH)₂-D₃], suggesting that 1,25-(OH)₂-D₃, the naturally occurring active metabolite of vitamin D₃, is a hypercalcemia-causing factor in sarcoidosis. However, the characteristics of this 25-OH-D₃-converting activity of sarcoid PAM in vitro are poorly understood. It does appear that the 1 α -hydroxylation process is specific for cells derived from patients with sarcoidosis: 1,25-(OH)₂-D₃ is not synthesized by PAM from patients with other types of pulmonary disease (4). In addition, the specific activity of the cellular converting reaction is greatest in PAM derived from patients with diffuse, infiltrative pulmonary disease and clinical evidence of abnormal calcium metabolism (3, 4). The aim of the current report is to describe the kinetics of 1,25-(OH)₂-D₃ production by these cells in vitro and to delineate factors that may be important regulators of the conversion reaction.

Materials and Methods

Vitamin D₃ Sterols. [³H-1 α ,2 α]vitamin D₃ (sp act, 25 Ci/mmol); [³H-26,27-methyl]25-hydroxyvitamin D₃ ([³H]25-OH-D₃) (161 Ci/mmol); and [³H-26,27-methyl]24(R),25-dihydroxyvitamin D₃ ([³H]24,25-(OH)₂-D₃) (158 Ci/mmol) were obtained from Amersham Corp., Arlington Heights, IL. Authentic 25-OH-D₃ was a gift from Dr. John Babcock (Upjohn Co., Kalamazoo, MI). Authentic, crystalline standards of 1,25-(OH)₂-D₃, 24,25-(OH)₂-D₃, 1 α -hydroxyvitamin D₃ (1 α -OH-D₃), 25,26-dihydroxyvitamin D₃ [25,26-(OH)₂-D₃], and 1,24(R),25-trihydroxyvitamin D₃ [1,24,25-(OH)₃-D₃] were generously provided by Dr. Milan Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). All labeled and unlabeled

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¹*Abbreviations used in this paper:* FCS, fetal calf serum; HPLC, high performance liquid chromatography; IFN, interferon; 1,24,25-(OH)₃-D₃, 1,24(R),25-trihydroxyvitamin D₃; 1,25-(OH)₂-D₃, 1 α ,25-dihydroxyvitamin D₃; 1 α -OH-D₃, 1 α -hydroxyvitamin D₃; 24,25-(OH)₂-D₃, 24(R), 25-dihydroxyvitamin D₃; 25-OH-D₃, 25-hydroxyvitamin D₃; 25,26-(OH)₂-D₃, 25,26-dihydroxyvitamin D₃; PAM, pulmonary alveolar macrophage.

TABLE I
Clinical Data on Patients With Sarcoidosis

Patient	Age	Sex	Serum calcium	Urinary calcium excretion (mg Ca/100 ml GF)*	Serum 1,25-(OH) ₂ -D	Serum angiotensin-converting enzyme activity
	yrs		mg/dl		pg/ml	IU/l
1	55	F	14.4	1.34	76	59
2	33	F	10.0	0.31	59	81
3	25	F	9.6	0.11	40	31
4	26	F	12.2	***-‡	63	59
5	31	M	14.5	0.92	68	92
Normal range:			8.5-10.5	<0.16	30-65	10-35

* GF, glomerular filtrate.

‡ Obtained during furosemide therapy.

sterols were purified on silica Sep-Pak cartridges (Waters Associates, Milford, MA) before use. Dexamethasone sodium phosphate was obtained from Merck Sharp & Dohme Div., Merck & Co., Inc., West Point, PA. Preparations of human immune (gamma) interferon (IFN- γ) and human alpha interferon (IFN- α) were purchased from Interferon Sciences, New Brunswick, NJ.

Macrophage Cultures. Primary monolayer cultures of PAM were established by standard technique (5) from the alveolar lavage fluid of five patients with diffuse pulmonary sarcoidosis (biopsy proven). Clinical laboratory data on patients at the time of lavage are shown in Table I. The cells were plated and maintained in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (KC Biological, Inc., Lenexa, KA), 10 μ g/ml insulin, 4 nM glutamine, 50 U/ml penicillin, and 50 mg/ml streptomycin at 37°C in an atmosphere of 95% air/5% CO₂. The medium was replaced with fresh medium every 3rd d. After 3 d in culture, >98% of the adherent cell population was esterase positive (6).

Metabolism Experiments. After 5 d in primary culture, the capacity of confluent PAM monolayers from each patient to metabolize 5 nM [³H]25-OH-D₃ to [³H]1,25-(OH)₂-D₃ was established. Thereafter, metabolism experiments were performed in cultures (0.25-1.0 \times 10⁶ cells/well) ranging in age from 7 to 17 d, by previously described techniques (3). Briefly, cultures were preincubated for 16 h in serum-free medium with or without vitamin D₃ sterols (solubilized in 0.2% ethanol), dexamethasone, or IFN. This medium was replaced with BGJ_B medium (Gibco Laboratories) containing radiolabeled sterol and incubated for 3 h in the incubator: accumulation of generated [³H]1,25-(OH)₂-D₃ was linear over 5 h (Fig. 1). All subsequent experiments were incubated for 3 h. A known amount (150 ng) of unlabeled sterol was then added to the incubation medium, the medium was collected, the monolayer was harvested by scraping, and the medium and cells were extracted in methanol/methylene chloride. The lipid extracts were chromatographed on Sep-Pak cartridges (7) to obtain the vitamin D metabolite fraction of interest. This fraction was then subjected to a three-step purification procedure on normal-phase high performance liquid chromatography (HPLC) over a 3.9 mm \times 30 cm μ -Porasil column (DuPont Instruments, Wilmington, DE). The first chromatographic purification was performed in *n*-hexane/methanol/isopropanol (92:4:4) followed by HPLC in methylene chloride/methanol (98:2) and methylene chloride/isopropanol (94:6). The fractions containing the authentic standard of interest were retained, pooled, and subjected to scintillation counting to determine the yield of metabolite.

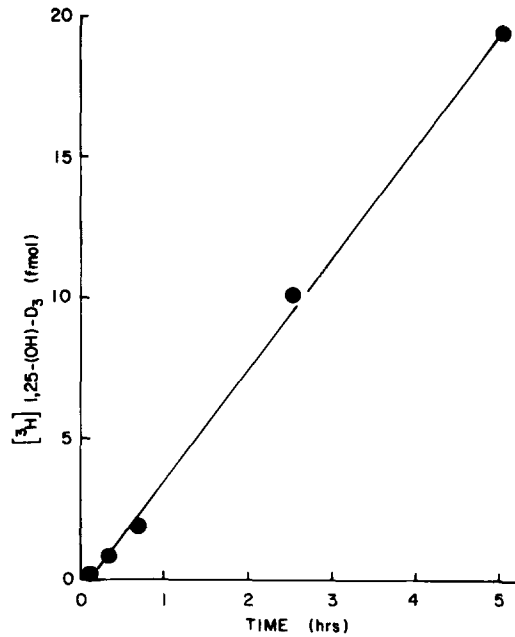


FIGURE 1. Synthesis of [³H]1,25-(OH)₂-D₃ from 5 nM [³H]25-OH-D₃ by cultured PAM from a patient with sarcoidosis over time. Each point is the mean of duplicate determinations.

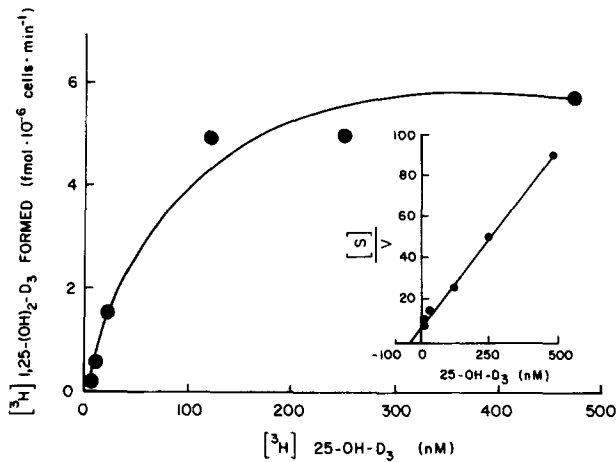


FIGURE 2. Kinetic analysis of [³H]1,25-(OH)₂-D₃ synthesis from [³H]25-OH-D₃ by cultured human PAM from a representative patient (patient 1, Table II) with sarcoidosis. Established monolayer cultures of PAM containing 1.5×10^5 cells per culture were exposed to increasing concentrations of [³H]25-OH-D₃ for 3 h. A single-reciprocal plot of the data (insert) yielded a K_m (x intercept) and velocity (V_{max} , 1/y intercept) of 53 nM and $6.3 \text{ fmol} \cdot 10^{-6} \text{ cells} \cdot \text{min}^{-1}$, respectively. Each point is the mean of duplicate determinations.

Results

Fig. 2 depicts the synthesis of [³H]1,25-(OH)₂-D₃ by PAM monolayer cultures derived from a representative patient with sarcoidosis and abnormal calcium metabolism, during incubation with increasing amounts of [³H]25-OH-D₃. The

TABLE II
Kinetic Analysis of [^3H]1,25-(OH) $_2$ -D $_3$ Synthesis by Cultured PAM
from Patients With Sarcoidosis

Patient	Substrate Range	V_{\max} (fmol · 10 $^{-6}$ cells · min $^{-1}$)	K_m
	<i>nM</i>		<i>nM</i>
1	3–150	5.9	52
	1–500	6.3	53
2	3–180	11.0	70
3	5–320	1.2	76
4	10–300	9.1	109
5	10–300	40.6	210

rate of [^3H]1,25-(OH) $_2$ -D $_3$ synthesis was dependent on the concentration of substrate, [^3H]25-OH-D $_3$ (Fig. 2). A single-reciprocal plot of these data (insert, Fig. 2) demonstrated the K_m for [^3H]25-OH-D $_3$ (concentration resulting in half-maximal formation rate of [^3H]1,25-(OH) $_2$ -D $_3$) to be 53 nM. The cumulative kinetic data for PAM derived from all five patients with sarcoidosis is summarized in Table II. Cells from all patients produced a labeled metabolite that cochromatographed with authentic 1,25-(OH) $_2$ -D $_3$. The yield of metabolite was greater in PAM cultures derived from the four patients with a clinical abnormality in calcium homeostasis; it resulted primarily from an increase in the velocity (V_{\max}) of the conversion reaction. The K_m for [^3H]25-OH-D $_3$ varied only fourfold between PAM from five different patients, whereas the V_{\max} varied nearly 40-fold. Two examinations, 5 d apart, of the kinetics of [^3H]1,25-(OH) $_2$ -D $_3$ production by PAM from the same host (patient 1, Table II) yielded comparable results. Interestingly, no radiolabeled product with the chromatographic mobility of 24,25-(OH) $_2$ -D $_3$ was observed even in cell extracts from cultures exposed to concentrations of substrate [^3H]25-OH-D $_3$ as high as 500 nM.

A study of the substrate specificity of the PAM 1 α -hydroxylation reaction was made in cultures from patient 2 using [^3H]25-OH-D $_3$, [^3H]24,25-(OH) $_2$ -D $_3$, and [^3H]vitamin D $_3$ as substrates (Fig. 3). The presence of an additional hydroxyl function at C24 in the side chain did not hinder hydroxylation at C1 in the A ring of the molecule. The yield of [^3H]1,24,25-(OH) $_3$ -D $_3$ from [^3H]24,25-(OH) $_2$ -D $_3$ was not significantly different from the amount of [^3H]1,25-(OH) $_2$ -D $_3$ made from [^3H]25-OH-D $_3$. However, absence of the C25 hydroxyl group had a profound inhibitory effect on the generation of 1 α -hydroxylated product. The specific activity of the PAM conversion reaction of [^3H]vitamin D $_3$ to [^3H]1 α -OH-D $_3$ was only 15% of that observed with the side chain-substituted substrates.

In an attempt to discern factors that influence 1,25-(OH) $_2$ -D $_3$ synthesis by sarcoid macrophages *in vivo*, we investigated *in vitro* the effects of preexposure of PAM to 1,25-(OH) $_2$ -D $_3$ or to glucocorticoid, dexamethasone, or human IFN, known stimulators of macrophage activity. Fig. 4 shows the effects of preincubation of PAM monolayers from patient 5 with increasing amounts of radioinert 1,25-(OH) $_2$ -D $_3$ on the synthesis of [^3H]1,25-(OH) $_2$ -D $_3$. Exposure to 0.75 and 7.5 nM 1,25-(OH) $_2$ -D $_3$ had little effect on [^3H]1,25-(OH) $_2$ -D $_3$ production. Preincu-

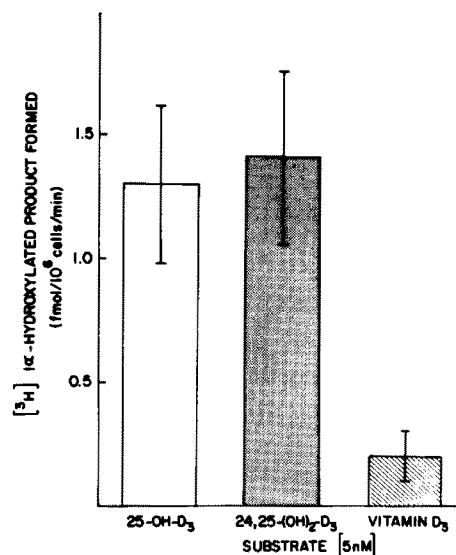


FIGURE 3. 1α -Hydroxylated metabolite formation by cultured, human PAM from a patient with sarcoidosis and altered calcium metabolism (patient 2, Table II) after incubation with 5 nM [^3H]25-OH-D₃, [^3H]24,25-(OH)₂-D₃, and [^3H]vitamin D₃. The yield of metabolite was determined by the amount of radiolabeled product that cochromatographed with authentic 1,25-(OH)₂-D₃, 1,24,25-(OH)₃-D₃, and 1α -OH-D₃, respectively. Each value is the mean \pm SD of triplicate determinations.

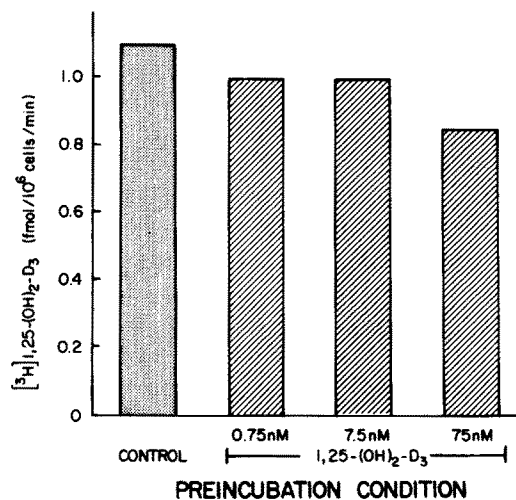


FIGURE 4. Effects of preincubation with radioinert 1,25-(OH)₂-D₃ on [^3H]1,25-(OH)₂-D₃ synthesis in cultured human PAM from a hypercalcemic host (patient 5 in Table I) with sarcoidosis. PAM were preincubated for 18 h in culture medium containing 0.5% FCS with or without radioinert 1,25-(OH)₂-D₃. For determination of [^3H]1,25-(OH)₂-D₃ from 5 nM [^3H] 25-OH-D₃, the medium was removed and replaced with serum-free BGJ_B medium. Each value is the mean of duplicate determinations.

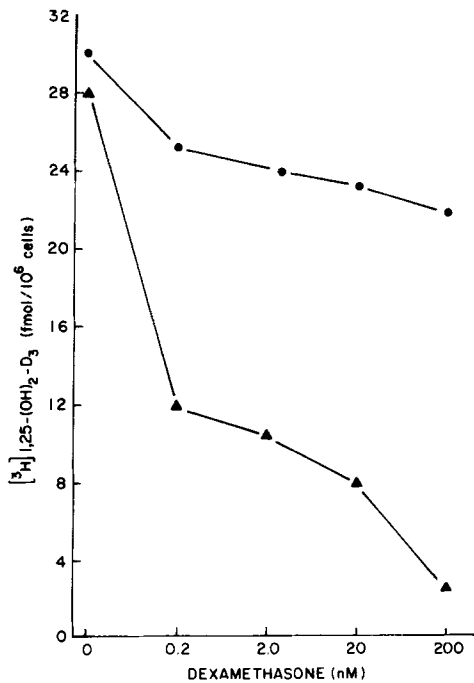


FIGURE 5. Effect of dexamethasone on [³H]1,25-(OH)₂-D₃ synthesis by cultured, human PAM from two patients (patients 1 [▲] and 4 [●]; see Table I) with sarcoidosis. PAM monolayers were preincubated for 18 h in serum-free medium with or without dexamethasone. The medium was removed and replaced with serum-free BG_JB medium for determination of [³H]1,25-(OH)₂-D₃ synthesis from 5 nM [³H]25-OH-D₃. Each point is the mean of triplicate [▲] or duplicate [●] determinations.

bation with 75 nM 1,25-(OH)₂-D₃ reduced [³H]1,25-(OH)₂-D₃ synthesis by only 20%, indicating a relative resistance to product inhibition of the conversion reaction. Preincubation of cells from two subjects (patients 1 and 4) with sarcoidosis and hypercalcemia with increasing amounts of dexamethasone resulted in a dose-dependent inhibition of [³H]1,25-(OH)₂-D₃ synthesis (Fig. 5). Although the half-maximal inhibitory concentration of dexamethasone was the same in cells from both individuals (0.2 nM), the magnitude of the decrease in hormone production was much greater in cells from patient 1. By comparison, IFN- γ stimulated [³H]1,25-(OH)₂-D₃ synthesis by sarcoid PAM. As shown in Fig. 6, preincubation of PAM from three different patients with IFN- γ exerted a dose-dependent stimulatory effect on the conversion of 5 nM [³H]25-OH-D₃ to [³H]1,25-(OH)₂-D₃, in each case. At the lower concentrations of IFN the stimulatory action was specific for IFN- γ . Preincubation of cells with 1,000 μ g/ml IFN- α resulted in only a modest increase (50%) in [³H]1,25-(OH)₂-D₃ synthesis in PAM from the host whose cells had the greatest increase (200%) in hormone production after exposure to IFN- γ . In cells from a second host, IFN- α exerted an inhibitory effect on the PAM [³H]25-OH-D₃ conversion reaction at all concentrations of the lymphokine. A kinetic analysis of the IFN- γ -mediated stimulatory action is depicted in Fig. 7. IFN- γ clearly increased the yield of [³H]1,25-(OH)₂-D₃ at each concentration of substrate [³H]25-OH-D₃. As demonstrated in the

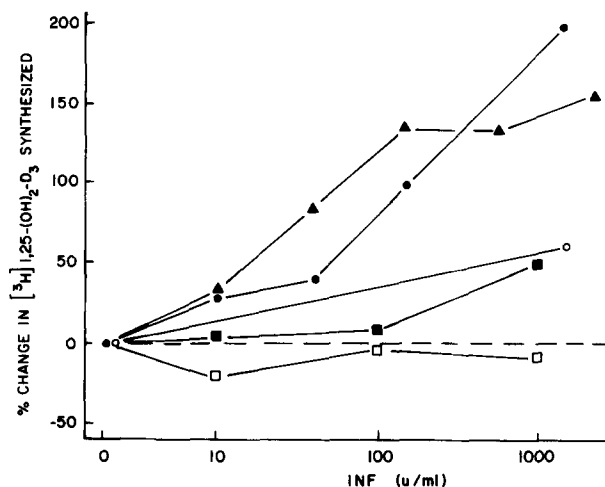


FIGURE 6. Effect of IFN- γ (closed symbols) and IFN- α (open symbols) on [^3H]1,25-(OH) $_2$ -D $_3$ synthesis by cultured, human PAM from three patients (patients 3 [□], 4 [▲], 5 [●]; see Table I) with sarcoidosis. Cell monolayers were preincubated for 18 h in medium containing 1.0% FCS with or without increasing amounts of IFN- γ or IFN- α . The medium was removed and replaced with serum-free BGJ $_B$ medium for determination of [^3H]1,25-OH-D $_3$ synthesis from 5 nM [^3H]25-OH-D $_3$. Each point is the mean of duplicate determinations.

reciprocal plot of the data (insert, Fig. 7), the majority of the increase in [^3H] 1,25-(OH) $_2$ -D $_3$ synthesis was attributable to an increase in the velocity of the conversion reaction. The V_{\max} of 1 α -hydroxylation was 34.5 fmol \cdot 10 $^{-6}$ cells \cdot min $^{-1}$ after preincubation of cells with IFN- γ , and 9.1 fmol \cdot 10 $^{-6}$ cells \cdot min $^{-1}$ in its absence. The K_m for [^3H]25-OH-D $_3$ with or without IFN- γ were similar, 109 and 145 nM, respectively.

Discussion

The conversion of 25-OH-D to 1,25-(OH) $_2$ -D in nonpregnant, mammalian and avian species is a strictly controlled metabolic event catalyzed by a cytochrome P-450 mixed-function oxidase located in the mitochondria of renal epithelial cells (8). The only pathologic human condition in which there is good evidence for the extrarenal synthesis of 1,25-(OH) $_2$ -D is sarcoidosis (9). Previous data from our laboratory (3) suggest that the macrophage, a ubiquitous constituent of the noncaseating granulomata of sarcoidosis, is the synthetic source of 1,25-(OH) $_2$ -D in the disease. In the current report, kinetic analysis of the 25-OH-D $_3$ -1 α -hydroxylation reaction in cultured PAM from patients with sarcoidosis yielded an apparent K_m for 25-OH-D $_3$ of 52–210 nM. This value is in the range previously reported for the 25-OH-D $_3$ -24-hydroxylase in cultured mouse kidney cells (10), dispersed rat renal epithelial cells (11), mouse kidney homogenates (12), and reconstituted mammalian enzyme (13). If the proposed affinities of the renal enzyme and the PAM hydroxylating mechanism *in vitro* are applicable *in vivo*, then one might predict that the sarcoid macrophage competes with the renal 1 α -hydroxylase for its preferred substrate, 25-OH-D $_3$.

Although the PAM 1 α -hydroxylation reaction and the renal 1 α -hydroxylase are similar in their affinity and specificity for 25-hydroxylated substrates, there

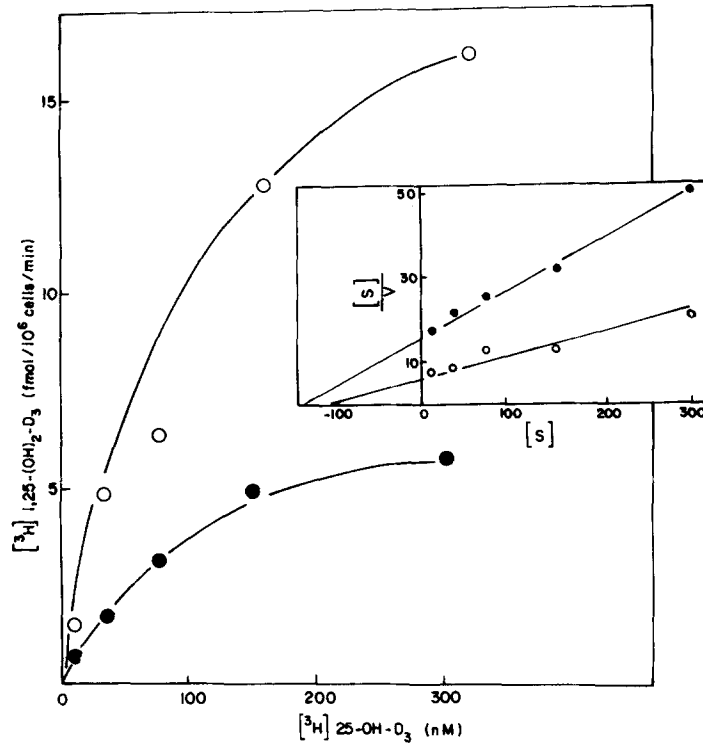


FIGURE 7. Kinetic analysis of $[^3\text{H}]1,25\text{-(OH)}_2\text{-D}_3$ synthesis by cultured, human PAM from a single hypercalcemic patient with sarcoidosis (patient 4, Table II) preincubated in the presence (○) or absence (●) of IFN- γ . PAM monolayers were preincubated for 18 h in medium containing 1.0% FCS with or without 1,000 U/1 IFN- γ . This medium was removed and replaced with serum-free BGJ_B medium for determination of $[^3\text{H}]1,25\text{-(OH)}_2\text{-D}_3$ synthesis in the presence of increasing amounts of $[^3\text{H}]25\text{-OH-D}_3$. Each point is the mean of duplicate determinations. A single-reciprocal plot of the data (insert) disclosed a K_m for 25-OH-D₃ (x intercept) and velocity (v_{\max} , $1/y$ intercept) of 109 nM $[^3\text{H}]25\text{-OH-D}_3$ and 34.5 fmol·10⁻⁶ cells·min⁻¹, respectively, in cells exposed to IFN- γ , and 145 nM $[^3\text{H}]25\text{-OH-D}_3$ and 9.1 fmol·10⁻⁶ cells·min⁻¹ in cells not exposed to IFN- γ .

appear to be some important differences between the two. First, even in the presence of high concentrations of substrate 25-OH-D₃ or after preincubation with high concentrations of 1,25-(OH)₂-D₃, a potent inducer of the renal 25-OH-D₃ 24-hydroxylase (14), the PAM 1 α -hydroxylation process was not accompanied by detectable 24-hydroxylating activity. An accompanying 24-hydroxylase is a characteristic of all the other renal 1 α -hydroxylation systems previously described (10–13, 15, 16). Therefore, unlike the situation in the renal epithelial cell, the sarcoid macrophage does not have a 24-hydroxylase competing for substrate. Furthermore, 1,25-(OH)₂-D₃ synthesized in the sarcoid PAM is not likely to be metabolized to 1,24,25-(OH)₃-D₃, a metabolite that is biologically less potent than 1,25-(OH)₂-D₃ (17, 18). A second discrepancy is the apparent lack of metabolic control in the 1 α -hydroxylation of 25-OH-D in hypercalcemic patients with sarcoidosis. Patients 1 and 5 reported here (Table I) are good examples of this event in vivo. Despite being hypercalcemic, their serum level of 1,25-(OH)₂-D was in the supranormal range, constituting inappropriate genera-

tion of hormone. This clinical observation was born out *in vitro*. Preincubation of cultured PAM from a hypercalcemic host with 1,25-(OH)₂-D₃ reduced the yield of [³H]1,25-(OH)-D₃; however, the degree of suppression [only 20% with 75 nM 1,25-(OH)₂-D₃] was far less than that previously reported for the suppression of the 25-OH-D₃-1 α -hydroxylase either in preparations of renal tissue *in vitro* after pretreatment of vitamin D-deficient animals *in vivo* with 1,25-(OH)₂-D₃ (19) or in cultured kidney cells preincubated in medium containing 10⁻⁸-10⁻⁷ M 1,25-(OH)₂-D₃ (10, 15). These data indicate that the inhibitory effect of 1,25-(OH)₂-D₃ on its own synthesis or, as proposed by Eisman et al. (20), 1,25-(OH)₂-D₃ induction of its own metabolism, may not be fully operative in the PAM 1 α -hydroxylation reaction. The lack of accompanying 24-hydroxylating activity and significant product inhibition of the PAM 25-OH-D₃-1 α -hydroxylation reaction, coupled with the relatively high affinity of the macrophage conversion reaction for 25-OH-D₃, suggests that the sarcoid macrophage has the potential to rival the naturally occurring renal enzyme in its efficiency of 1,25-(OH)₂-D₃ synthesis.

These dissimilar characteristics of the PAM and renal hydroxylating mechanisms suggest that the sarcoid macrophage does not harbor the classic 1 α -hydroxylase (21). Moreover, it is also unlikely that 1,25-(OH)₂-D₃ synthesis in sarcoid PAM occurs via the mitochondrial cytochrome P-450-catalyzed peroxxygenase pathway recently reported by Warner (21). In this NADPH-independent system, both 1,25-(OH)₂-D₃ and 24,25-(OH)₂-D₃ are generated from substrate 25-OH-D₃. No 24,25-(OH)₂-D₃ production was identified in our metabolism experiments, even at high concentrations of 25-OH-D₃. It is possible that the PAM 1 α -hydroxylation reaction is linked to the production of reactive oxygen intermediates. The generation of hydrogen peroxide by activated human monocytes is exquisitely sensitive to glucocorticoids (23) and may explain the steroid-mediated inhibition of 1,25-(OH)₂-D₃ synthesis by sarcoid PAM. Interestingly, the suppressive action *in vitro* correlates with the long-recognized antihypercalcemic action of glucocorticoids *in vivo* in patients with sarcoidosis (24).

In light of the suppressive actions of glucocorticoids on the generation of 1,25-(OH)₂-D₃ by sarcoid PAM, we were curious to know whether IFNs, known stimulators of macrophage action, might enhance sterol synthesis by these cells. Mononuclear inflammatory cells, derived from the alveolar space of patients with sarcoidosis and high intensity alveolitis, are prolific in their synthesis of lymphokines, including interleukin 1, interleukin 2, and IFN- γ (25). As demonstrated in Figs. 6 and 7, IFN- γ accelerates the velocity of the conversion reaction in PAM *in vitro*, increasing the synthetic yield of 1,25-(OH)₂-D₃ by as much as 200%. Whether IFN- γ is an important modulator of macrophage 1,25-(OH)₂-D₃ synthesis *in vivo*, and whether 1,25-(OH)₂-D₃, recently shown (26) to be a specific and potent inhibitor of human T helper/inducer lymphocyte function, is a biologically active lymphokine in the alveolar space in sarcoidosis, are intriguing but yet unanswered questions.

Summary

We investigated the 1 α -hydroxylation of vitamin D₃ sterols by cultured pulmonary alveolar macrophages (PAM) from patients with sarcoidosis with or

without clinically abnormal calcium homeostasis. Like the naturally occurring renal 1α -hydroxylase, the PAM 1α -hydroxylation reaction exhibited a high affinity for 25-hydroxyvitamin D₃ (25-OH-D₃) and a preference for substrates containing a 25-hydroxyl group in the side chain of the sterol. Unlike the renal enzyme, the PAM 1α -hydroxylating mechanism was not accompanied by 24-hydroxylating activity, even after preincubation with 75 nM 1,25-dihydroxyvitamin D₃ [1,25-(OH)₂-D₃] or exposure to high concentrations of substrate (500 nM 25-OH-D₃). The PAM 25-OH-D₃- 1α -hydroxylation reaction was stimulated by gamma interferon and inhibited by exposure to the glucocorticoid dexamethasone. The characteristics of the PAM hydroxylation process in vitro appear to reflect the efficiency of the extrarenal production of 1,25-(OH)₂-D₃ and the therapeutic efficacy of glucocorticoids in patients with sarcoidosis and disordered calcium metabolism.

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