

## **1,25(OH)<sub>2</sub>D<sub>3</sub> production by T lymphocytes and alveolar macrophages recovered by lavage from normocalcemic patients with tuberculosis.**

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### **Research Article**

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# 1,25(OH)<sub>2</sub>D<sub>3</sub> Production by T Lymphocytes and Alveolar Macrophages Recovered by Lavage from Normocalcemic Patients with Tuberculosis

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## Abstract

To compare extra-renal 1,25(OH)<sub>2</sub>D<sub>3</sub> production in different types of granulomatous disease, and to identify the cell types responsible, we have evaluated the conversion of 25(OH)D<sub>3</sub> in 1,25(OH)<sub>2</sub>D<sub>3</sub> by uncultured cells recovered by bronchoalveolar lavage and blood mononuclear cells from normocalcemic patients with sarcoidosis and tuberculosis. 1,25(OH)<sub>2</sub>D<sub>3</sub> was produced both by lavage cells (12/12 tuberculosis patients, 2/6 sarcoidosis patients) and blood mononuclear cells (3/5 tuberculosis patients, 0/3 sarcoidosis patients) from patients but not controls, but significantly greater amounts were produced by lavage cells from tuberculosis patients than those of sarcoidosis patients ( $P < 0.001$ ). 1,25(OH)<sub>2</sub>D<sub>3</sub> production by lavage cells from tuberculosis patients correlated with the number of CD8<sup>+</sup> T lymphocytes present but not other cell types. T lymphocytes appeared to be an important source of 1,25(OH)<sub>2</sub>D<sub>3</sub> production, since purified T lymphocytes from all patients with tuberculosis produced 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 1,25(OH)<sub>2</sub>D<sub>3</sub> production by these cells correlated closely with that produced by unseparated lavage cells. Because 1,25(OH)<sub>2</sub>D<sub>3</sub> can improve the capacity of macrophages to kill mycobacteria, our results support the conclusion that macrophage-lymphocyte interactions, mediated at least in part by 1,25(OH)<sub>2</sub>D<sub>3</sub>, may be an important component of a successful antituberculous immune response. (*J. Clin. Invest.* 1990. 85:1588–1593.) vitamin D metabolism • tuberculosis • sarcoidosis • T lymphocytes • alveolar macrophages

## Introduction

Recent studies have demonstrated that patients with granulomatous diseases, including sarcoidosis and tuberculosis, can produce 1,25(OH)<sub>2</sub>D<sub>3</sub> at sites of disease activity (1). This spontaneous production of 1,25(OH)<sub>2</sub>D<sub>3</sub> by granulomatous tissues may play an important role in the regulation of granulomatous reactions by influencing the maturation and activity of immune and inflammatory cells (1, 2). Despite the potential importance of 1,25(OH)<sub>2</sub>D<sub>3</sub> production at sites of granulomatous reactions, several relevant questions remain unanswered:

First, 1,25(OH)<sub>2</sub>D<sub>3</sub> production in different types of granulomatous disease has never been compared, and therefore it is

unknown whether the presence of this metabolite merely reflects the existence of a granulomatous response, or whether certain granulomatous reactions are associated with greater 1,25(OH)<sub>2</sub>D<sub>3</sub> production.

Secondly, it is unclear, what cell types spontaneously produce 1,25(OH)<sub>2</sub>D<sub>3</sub>. Fresh tissue (3, 4) or unseparated cells (5) from sites of granulomatous reactions can spontaneously transform 25(OH)D<sub>3</sub> into 1,25(OH)<sub>2</sub>D<sub>3</sub>. It is reasonable to expect that purified subpopulations of these cells would also retain this capacity, but freshly isolated alveolar macrophages from patients with sarcoidosis produce little or no 1,25(OH)<sub>2</sub>D<sub>3</sub>, even though alveolar macrophages from patients with granulomatous diseases do produce 1,25(OH)<sub>2</sub>D<sub>3</sub> when cultured in vitro for 4–7 d (5–9). In this context, the possibility that other cell types, such as T lymphocytes, can produce 1,25(OH)<sub>2</sub>D<sub>3</sub> has not been yet evaluated.

To study these questions, we have evaluated the metabolism of 25(OH)D<sub>3</sub> by fresh cells recovered by bronchoalveolar lavage from controls and normocalcemic patients with tuberculosis and sarcoidosis. The goals of this study were to compare 1,25(OH)<sub>2</sub>D<sub>3</sub> production in different types of granulomatous disease and to identify the cell types responsible for 1,25(OH)<sub>2</sub>D<sub>3</sub> production.

## Methods

### Study populations

**Patients with pulmonary tuberculosis.** The diagnosis of pulmonary tuberculosis was established on the basis of cultures of sputum or gastric aspirates positive for *Mycobacterium tuberculosis* ( $n = 11$ ) and/or the presence of epithelioid granulomata with caseous necrosis on histological study of biopsy specimens ( $n = 4$ ). The 12 patients had a mean age of  $34 \pm 7$  yr. All patients had a normal level of serum calcium ( $2.34 \pm 0.10$  mmol/liter) and normal 24-h urinary calcium excretion ( $0.04 \pm 0.03$  mmol/kg per d; normal range  $< 0.11$  mmol/kg per d) at the time of evaluation. Patients were studied before receiving any antituberculous therapy.

**Patients with pulmonary sarcoidosis.** The diagnosis of pulmonary sarcoidosis was established according to previously described criteria (10). The six patients had a mean age of  $37 \pm 10$  yr. Chest radiographic types were as follows: type I ( $n = 2$ ), type II ( $n = 2$ ), and type III ( $n = 2$ ). The patients had normal serum calcium levels ( $2.37 \pm 0.15$  mmol/liter) and normal urinary calcium excretion ( $0.08 \pm 0.03$  mmol/kg per d) at the time of evaluation. One patient had previously received corticosteroid therapy, which had been discontinued  $> 3$  mo before evaluation.

**Control subjects.** Three normal volunteers and two patients undergoing bronchoscopic evaluation for nonparenchymal pulmonary disorders served as controls. These individuals had an average age of  $28 \pm 8$  yr.

### Bronchoalveolar lavage

Informed consent was obtained from all patients and control subjects. Bronchoalveolar lavage was performed as previously described (10). In

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all patients, a radiologically abnormal lung segment was lavaged when present, and the right middle lobe or lingula lavaged when the radiograph was normal.

Total and differential cell counts and the evaluation of the expression of CD4 and CD8 surface antigens on T lymphocytes were performed as previously described (10).

### Separation of cell populations

Mononuclear cells were isolated from peripheral blood by centrifugation on Lympho-paque (Nyegaard Co., Oslo, Norway), and the cells were resuspended in 20 ml Hanks' balanced salt medium with 0.08% EDTA and centrifuged (100 g for 15 min) two times to remove platelets.

T lymphocytes were isolated from concentrated fresh lavage cells by passage through nylon wool columns using previously described techniques (11). To isolate alveolar macrophages, concentrated lavage cells were suspended in complete medium containing 10% human AB serum and transferred to bacteriologic petri dishes that had previously been incubated with human AB serum for 18 h at 4°C and subsequently washed two times with phosphate buffered saline (12). After a 60-min incubation at 37°C, nonadherent cells were removed and adherent cells were washed twice with complete medium and incubated for 30 min at 4°C in 10 ml Hanks' balanced salt solution containing 0.2% EDTA. Cells which detached spontaneously or after gentle pipetting were recovered and washed in complete medium before use.

The viability of fresh cells recovered by lavage stained with acridine orange and ethidium bromide (13) was: controls, 86±2% (mean±SD); tuberculosis, 81±14%; and sarcoidosis, 90±6%. The viability and purity of lavage T lymphocytes were respectively: controls, 99±1% and 93±5%; tuberculosis, 96±5% and 96±3%; and sarcoidosis, 98±1% and 95±5%. The viability and purity of pulmonary macrophages from tuberculosis patients after separation by adherence were respectively 98±1% and 97±4%.

### Metabolism of 25(OH)D<sub>3</sub> by cells in vitro

To evaluate the capacity of cells to metabolize 25(OH)D<sub>3</sub> in vitro,  $1 \times 10^5$  viable cells were preincubated for 15 min (37°C, 95% air/5% CO<sub>2</sub>) in a 2-cm diameter glass vial containing 1 ml of serum free Dulbecco's modified Eagle's medium containing 1.8 mM Ca<sup>2+</sup> and 0.9 mM phosphate (Boehringer Mannheim GmbH, Penzberg, FRG). 10 µl of ethanol was then added which contained either: (a) 50 nCi [26,27-<sup>3</sup>H]25(OH)D<sub>3</sub> (Radiochemical Centre, Amersham, UK; final concentration  $2.5 \times 10^{-9}$  M and final 20 Ci/mmol sp act); or (b) 1 µg unlabeled 25(OH)D<sub>3</sub> (Roussel-Uclaf Laboratories, Paris, France) with or without 50 nCi [<sup>3</sup>H]25(OH)D<sub>3</sub> (final concentration  $1 \times 10^{-6}$  M and final specific activity 20 mCi/mmol). Incubation was continued for 150 min, after which 2 ml HPLC grade methanol and 2 ml chloroform were added to each sample, the vials agitated, the chloroform phase removed and dried under N<sub>2</sub>, and the residue redissolved in chromatographic solvent (see below). Before chromatography, 100 ng unlabeled synthetic 1,25(OH)<sub>2</sub>D<sub>3</sub> (Hoffman-La Roche, Inc., Nutley, NJ) was added to extracts from cells incubated in the presence of  $2.5 \times 10^{-9}$  M [<sup>3</sup>H]25(OH)D<sub>3</sub>, and 10 nCi synthetic [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub> was added to those incubated with  $1 \times 10^{-6}$  M unlabeled 25(OH)D<sub>3</sub>.

To quantify 1,25(OH)<sub>2</sub>D<sub>3</sub> production, samples were chromatographed using a *n*-hexane:isopropanol solvent system, and the metabolites comigrating with 1,25(OH)<sub>2</sub>D<sub>3</sub> were rechromatographed using a methylene-chloride:isopropanol solvent system as previously described (5). Chromatography of samples on two successive systems was obligatory to isolate 1,25(OH)<sub>2</sub>D<sub>3</sub>, since large amounts of (5E)-19-nor-10-oxo-25(OH)D<sub>3</sub> were produced by the cells and comigrated with 1,25(OH)<sub>2</sub>D<sub>3</sub> in the first, but not the second chromatographic system (data not shown).

For incubations performed in the presence of  $2.5 \times 10^{-9}$  M [<sup>3</sup>H]-25(OH)D<sub>3</sub>, the rate of conversion of [<sup>3</sup>H]25(OH)D<sub>3</sub> into [<sup>3</sup>H]-1,25(OH)<sub>2</sub>D<sub>3</sub> was determined by calculating the percentage of total radioactivity with an appropriate elution profile after the two successive chromatographies. Results are expressed as fmol/10<sup>5</sup> cells per 150

min, based on the assumption that the specific activity of the product was the same as that of the substrate (see below). Thus, conversion of 1% of substrate into [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub> corresponds to the production of 33 fmol. All incubations and the subsequent purification of metabolite were performed in duplicate, and each value reported in the study represents the mean of duplicate determinations. The interassay coefficient of variation of the duplicate determinations of 1,25(OH)<sub>2</sub>D<sub>3</sub> was 25±4%.

In preliminary experiments, 1,25(OH)<sub>2</sub>D<sub>3</sub> production by lavage cells was demonstrated to be linear over the 150-min period studied. Furthermore, < 15% of [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub> added to lavage cell incubations was consumed over the incubation period. When medium without cells (*n* = 4) was incubated in the presence of  $2.5 \times 10^{-9}$  M [<sup>3</sup>H]25(OH)D<sub>3</sub>, 0–0.065% isotope eluted with 1,25(OH)<sub>2</sub>D<sub>3</sub>. This background value has not been subtracted from the values reported for incubations containing cells.

To measure the specific activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> produced in incubations containing  $1 \times 10^{-6}$  M [<sup>3</sup>H]25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub> was purified as described above by sequential chromatography using the two different solvent systems and the absorbance at 254 nm and radioactivity of the isolated metabolite were determined. Ultraviolet (UV) absorption spectra of the purified metabolite were obtained using a HPLC system fitted with a photodiode array detector (model 990; Millipore/Continental Water Systems, St. Quentin en Yvelinnes, France).

### Binding of 25(OH)D<sub>3</sub> metabolites to 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors

To characterize the ability of 25(OH)D<sub>3</sub> metabolites to bind to 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors, metabolites produced in incubations containing  $1 \times 10^{-6}$  M 25(OH)D<sub>3</sub> were purified by successive chromatographies, and tested for their ability to compete with synthetic [<sup>3</sup>H]-1,25(OH)<sub>2</sub>D<sub>3</sub> for binding to 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors present in chick intestinal cytosol as previously described (14). For samples incubated in the presence of [<sup>3</sup>H]25(OH)D<sub>3</sub>, measurement of the quantity of metabolite present was based on radioactivity, assuming that the specific activity was the same as that of the 25(OH)D<sub>3</sub> substrate. For samples incubated in the presence of unlabeled 25(OH)D<sub>3</sub>, measurement of the quantity of metabolite present was based on its absorbance at 254 nm. Each sample was tested in duplicate at three different dilutions. A standard curve was performed in parallel using known amounts of synthetic 1,25(OH)<sub>2</sub>D<sub>3</sub>. The displacement of binding produced by the standards and experimental samples were each fitted to sigmoid curves and compared as previously described (15) using the Ligand program provided by these authors.

### Statistical methods

Results are expressed as mean±SD. Comparison of the production of 25(OH)D<sub>3</sub> metabolites by the three study groups was performed by the Kruskal-Wallis one-way analysis of ranks procedure, and comparisons between subgroups of patients within a given diagnostic category were performed using the Mann-Whitney test. Correlations between the production of 25(OH)D<sub>3</sub> metabolites and other metric variables were evaluated by linear regression. In each case, a *P* < 0.05 was considered significant.

## Results

**1,25(OH)<sub>2</sub>D<sub>3</sub> production by lavage cells and blood mononuclear cells.** Fresh lavage cells from patients with tuberculosis incubated in the presence of  $2.5 \times 10^{-9}$  M [<sup>3</sup>H]25(OH)D<sub>3</sub> produced a metabolite which coeluted with [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub> in two successive chromatographic systems. Further studies supported the conclusion that this metabolite was, in fact, 1,25(OH)<sub>2</sub>D<sub>3</sub>. When lavage cells from these patients were incubated in the presence of  $10^{-6}$  M 25(OH)D<sub>3</sub> and extracts chromatographed successively on the two HPLC systems, a

metabolite coeluting with [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  could also be detected in all cases by monitoring absorbance, and this metabolite had the same capacity as synthetic 1,25(OH) $_2\text{D}_3$  to compete with [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  for binding to 1,25(OH) $_2\text{D}_3$  receptors and its UV spectrum had a maximum at 267 nm and a minimum at 228 nm, values identical to those observed for synthetic 1,25(OH) $_2\text{D}_3$  (data not shown).

Fresh lavage cells from tuberculosis patients produced 1,25(OH) $_2\text{D}_3$  in all cases, although the amounts of total 25(OH) $\text{D}_3$  converted into 1,25(OH) $_2\text{D}_3$  varied over a 10-fold range (Table I). Peripheral blood mononuclear cells from three of the five patients with tuberculosis tested also produced detectable amounts of 1,25(OH) $_2\text{D}_3$ , but the production of this metabolite by blood mononuclear cells was always less than that produced by the same number of lavage cells from the same individual ( $P < 0.05$  by paired analysis). 1,25(OH) $_2\text{D}_3$  production by fresh lavage cells was correlated with the proportion of CD8 $^+$  T lymphocytes present in the cell suspension (Fig. 1;  $r = 0.67$ ,  $P < 0.005$ ), but not with the proportion of CD4 $^+$  T lymphocytes, total lymphocytes or alveolar macrophages present (data not shown).

The conversion of [ $^3\text{H}$ ]25(OH) $\text{D}_3$  into [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  by lavage cells from patients with sarcoidosis was observed in two of six cases, but only small amounts were present (Table I). Thus, the production of 1,25(OH) $_2\text{D}_3$  by lavage cells from patients with sarcoidosis was significantly less than that of cells from patients with tuberculosis ( $P < 0.001$ ). No conversion of [ $^3\text{H}$ ]25(OH) $\text{D}_3$  into [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  was detected by blood mononuclear cells from the three sarcoid patients tested. Similarly, lavage cells and blood mononuclear cells from controls did not produce detectable amounts of 1,25(OH) $_2\text{D}_3$ .

**Identification of cell types recovered by lavage from patients with tuberculosis capable of converting 25(OH) $\text{D}_3$  into 1,25(OH) $_2\text{D}_3$ .** Lymphocytes and alveolar macrophages were the predominant cell types recovered by lavage from patients with tuberculosis, and in 7/12 individuals, these two cell types accounted for  $> 80\%$  of recovered cells. To determine which of these cell types could produce 1,25(OH) $_2\text{D}_3$ , the two populations were separated and studied individually.

**Lymphocytes.** Lavage T lymphocytes from all eight patients with tuberculosis tested were able to convert [ $^3\text{H}$ ]25(OH) $\text{D}_3$  into [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  (Fig. 2). In contrast, lavage T

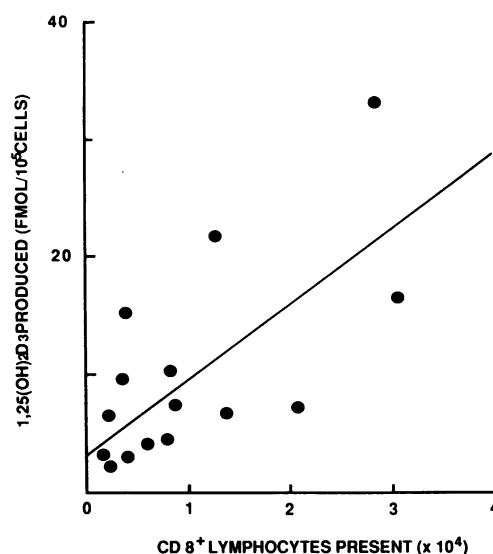


Figure 1. Correlation between 1,25(OH) $_2\text{D}_3$  production by lavage cells from patients with tuberculosis and the number of CD8 $^+$  lymphocytes present. The solid line was determined by linear regression ( $r = 0.67$ ,  $P < 0.005$ ).

lymphocytes from patients with sarcoidosis or normals did not produce detectable amounts of 1,25(OH) $_2\text{D}_3$ .

Additional experiments confirmed the identification of this metabolite as 1,25(OH) $_2\text{D}_3$ . First, purified T lymphocytes from the five tuberculosis patients incubated in the presence of unlabeled  $1 \times 10^{-6}$  M 25(OH) $\text{D}_3$  produced a metabolite detected by UV absorbance at 254 nm which coeluted with synthetic [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  in the two sequential HPLC chromatographic systems. Second, when T lymphocytes from patients were incubated in the presence of  $1 \times 10^{-6}$  M [ $^3\text{H}$ ]25(OH) $\text{D}_3$  ( $20 \pm 2$  mCi/mmol sp act), the specific activity of the metabolite was similar ( $19 \pm 2$  mCi/mmol sp act;  $n = 5$ ). Third, when the metabolite isolated from nine individual patients was tested in the receptor-binding assay, its ability to compete with the binding of [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  to its receptor was similar to that of synthetic 1,25(OH) $_2\text{D}_3$  (Fig. 3). Finally, when the metabolite with chromatographic properties of

Table I. Production of [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  by Blood Mononuclear Cells and Lavage Cells from Control Subjects and Patients with Tuberculosis and Sarcoidosis\*

Cell type	[ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$ production		
	Controls	Tuberculosis	Sarcoidosis
	Fmol/10 $^5$ cells per 150 min		
Blood mononuclear cells	$<0.2$ ( $n = 3$ )	$2.9 \pm 2.6^{\S}$ ( $n = 5$ )	$<1.1$ ( $n = 3$ )
Unseparated lavage cells	$<0.2$ ( $n = 5$ )	$10.1 \pm 8.7^{\parallel}$ ( $n = 12$ )	$2.1 \pm 1.2$ ( $n = 6$ )

\* Results are given as mean  $\pm$  SD.

$^{\dagger}$ ,  $P < 0.05$  comparing blood mononuclear cells and lavage cells;

$^{\S}$ ,  $P < 0.01$  or  $^{\parallel}$ ,  $P < 0.001$  comparing tuberculosis patients with control subjects and sarcoidosis patients.

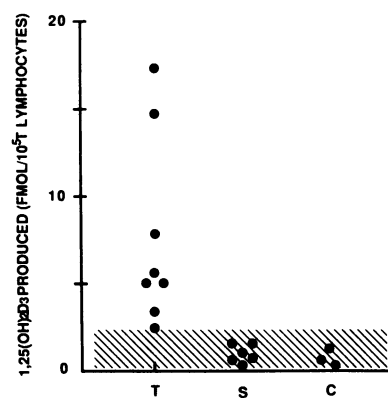


Figure 2. Production of [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  by lavage T lymphocytes. T lymphocytes were purified from cells recovered by bronchoalveolar lavage from normals (C), patients with tuberculosis (T), and patients with sarcoidosis (S).  $1 \times 10^5$  cells were incubated in the presence of  $2.5 \times 10^{-9}$  M [ $^3\text{H}$ ]25(OH) $\text{D}_3$ , and the amount of 1,25(OH) $_2\text{D}_3$  produced was determined. The shaded areas represent the apparent production of 1,25(OH) $_2\text{D}_3$  in incubations performed without added cells.

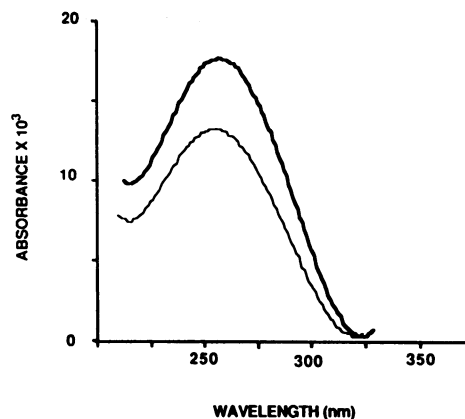
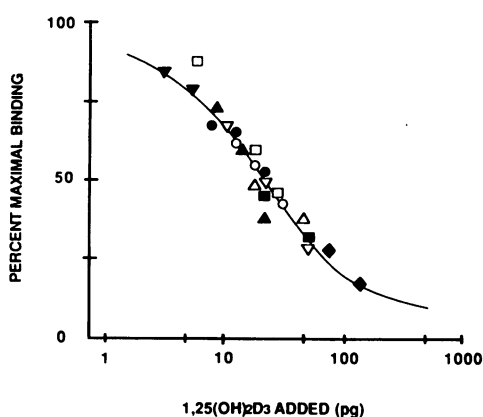


Figure 3. Characterization of  $1,25(\text{OH})_2\text{D}_3$  produced by T lymphocytes. Lavage T lymphocytes from patients with tuberculosis were incubated in the presence of  $1 \times 10^{-6}$  M  $25(\text{OH})\text{D}_3$  and the metabolite comigrating with  $1,25(\text{OH})_2\text{D}_3$  in two consecutive chromatographic systems was isolated. The concentration was determined as described in Methods. (Left) Ability of this metabolite to displace the binding of  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  to its receptor

was compared with that of known amounts of synthetic  $1,25(\text{OH})_2\text{D}_3$  (solid line). Each symbol type represents data from a single individual. (Right) The absorption spectra of this metabolite (heavy solid line) and that of synthetic  $1,25(\text{OH})_2\text{D}_3$  (light solid line).

$1,25(\text{OH})_2\text{D}_3$  was isolated from five different patients, pooled, and rechromatographed using the methylene-chloride:isopropanol solvent system, this metabolite eluted as a sharp single peak with an elution volume identical to that of synthetic  $1,25(\text{OH})_2\text{D}_3$  (data not shown). It had the same capacity as synthetic  $1,25(\text{OH})_2\text{D}_3$  to compete with  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  for binding to receptors present in chick intestinal cytosol (data not shown), as well as a UV spectrum identical to that of synthetic  $1,25(\text{OH})_2\text{D}_3$  (Fig. 3). The only known vitamin D metabolite with these characteristics is  $1,25(\text{OH})_2\text{D}_3$  (16–18).

In addition, a variety of evidence indicated that the  $1,25(\text{OH})_2\text{D}_3$  produced did not result from  $1,25(\text{OH})_2\text{D}_3$  produced by other cell types (e.g., alveolar macrophages) contaminating the lymphocyte preparations. First,  $1,25(\text{OH})_2\text{D}_3$  production by T lymphocytes correlated with the number of  $\text{CD}8^+$  lymphocytes, not the number of alveolar macrophages, present in the original cell population. Second, the production of  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  by purified T lymphocytes correlated very closely with the production of  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  by unseparated fresh lavage cells from the same individual (Fig. 4,  $r$

$= 0.95$ ,  $P < 0.001$ ). Third, the T lymphocytes used for these experiments had a purity of  $> 95\%$ , and therefore the incubations could have contained at most  $5 \times 10^3$  other cell types, whereas incubations containing  $10^4$  alveolar macrophages or fresh lavage cells did not produce detectable amounts of  $1,25(\text{OH})_2\text{D}_3$  (data not shown). Fourth, alveolar macrophages from one tuberculosis patient did not produce detectable amounts of  $1,25(\text{OH})_2\text{D}_3$ , although  $1,25(\text{OH})_2\text{D}_3$  production by unseparated cells from the same individual could be detected (see below).

**Alveolar macrophages.** When purified alveolar macrophages (purity  $97 \pm 4\%$ ) from patients with tuberculosis (all of whose unseparated lavage cells produced  $1,25(\text{OH})_2\text{D}_3$ ) were incubated in the presence of  $2.5 \times 10^{-9}$  M  $[^3\text{H}]-25(\text{OH})\text{D}_3$ , production of  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  was detectable in two cases, but not with cells from the third individual tested ( $1,25(\text{OH})_2\text{D}_3$  production; 9.3, 4.0, and 0 fmol/ $10^5$  cells per 150 min). The metabolite produced by alveolar macrophages from the two individuals that coeluted with synthetic  $1,25(\text{OH})_2\text{D}_3$  was confirmed to have the properties expected for  $1,25(\text{OH})_2\text{D}_3$  by the spectroscopic and receptor-binding methods described earlier (data not shown). The production of  $1,25(\text{OH})_2\text{D}_3$  was directly related to the number of alveolar macrophages included in the incubation between  $10^4$  and  $10^5$  cells/incubation, but no conversion of  $[^3\text{H}]-25(\text{OH})\text{D}_3$  into  $[^3\text{H}]-1,25(\text{OH})_2\text{D}_3$  could be detected in incubations containing  $< 10^4$  macrophages (data not shown).

## Discussion

The spontaneous production of  $1,25(\text{OH})_2\text{D}_3$  by fresh cells or tissue obtained from sites of granulomatous reactions in a small number of patients with sarcoidosis or tuberculosis has previously been reported, all of whom had obvious abnormalities in calcium metabolism (3–5, 7). Our study extends these observations in several ways: (a) It suggests that  $1,25(\text{OH})_2\text{D}_3$  production by immune and inflammatory cells is occurring in most patients with tuberculosis, and is not limited to the small proportion of patients with systemic abnormalities in calcium metabolism (5). (b) It suggests that the production of  $1,25(\text{OH})_2\text{D}_3$  is regulated differently in different granulomatous diseases, for production of  $1,25(\text{OH})_2\text{D}_3$  by lavage cells was detectable in all 12 patients with tuberculosis tested,

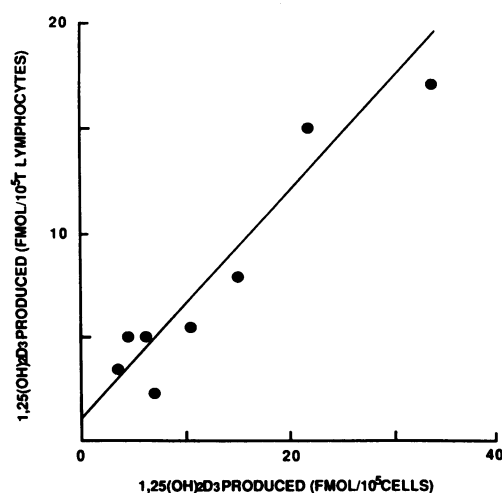


Figure 4. Correlation between the production of  $1,25(\text{OH})_2\text{D}_3$  by fresh lavage cells from patients with tuberculosis and purified lavage T lymphocytes from the same individuals. The solid line was determined by linear regression ( $r = 0.95$ ,  $P < 0.001$ ).

whereas  $1,25(\text{OH})_2\text{D}_3$  production was detectable in only two of the six patients with sarcoidosis and for both individuals only small amounts were produced. (c) This study demonstrates that production of  $1,25(\text{OH})_2\text{D}_3$  is not restricted to sites of granulomatous lesions, because blood mononuclear cells from some patients with tuberculosis were capable of producing detectable amounts of  $1,25(\text{OH})_2\text{D}_3$ .

The results presented here also define the cell types recovered by lavage that can contribute to the production of  $1,25(\text{OH})_2\text{D}_3$ . Our findings indicate that T lymphocytes recovered by lavage from patients with tuberculosis spontaneously produce  $1,25(\text{OH})_2\text{D}_3$ . The production of  $1,25(\text{OH})_2\text{D}_3$  could not be explained by the nonspecific activation of the lymphocytes during the purification procedure, for none of the lavage T lymphocytes from patients with sarcoidosis and normals produced detectable  $1,25(\text{OH})_2\text{D}_3$ . In addition, a variety of evidence indicated that the  $1,25(\text{OH})_2\text{D}_3$  produced did not result from  $1,25(\text{OH})_2\text{D}_3$  produced by other cell types (e.g., alveolar macrophages) contaminating the lymphocyte preparations. It is noteworthy that the close correlation observed between  $1,25(\text{OH})_2\text{D}_3$  production by fresh lavage cells and purified lavage T lymphocytes from the same individual suggests that T lymphocytes are an important source of  $1,25(\text{OH})_2\text{D}_3$  produced by lavage cells.

The production of  $1,25(\text{OH})_2\text{D}_3$  by normal T lymphocytes has not been previously detected, although extrarenal production of  $1,25(\text{OH})_2\text{D}_3$  has been suggested to occur in patients with adult T cell leukemia/lymphoma (19), and HTLV1-transformed cord blood T lymphocytes have been reported to produce  $1,25(\text{OH})_2\text{D}_3$  (20). It is noteworthy that  $1,25(\text{OH})_2\text{D}_3$  production by lavage cells was correlated with the number of  $\text{CD8}^+$  T lymphocytes present, but not with the number of  $\text{CD4}^+$  T lymphocytes, consistent with the possibility that  $\text{CD8}^+$  lymphocytes are the major source of this metabolite. Other explanations are possible for the observed correlation however, such as the possibility that  $\text{CD8}^+$  cells produce cytokines which stimulate  $1\alpha$ -hydroxylase activity in other cells, or that  $1,25(\text{OH})_2\text{D}_3$  favors the expansion of  $\text{CD8}^+$  T lymphocytes (1, 2, 21, 22). Even if  $\text{CD8}^+$  lymphocytes are directly involved in  $1,25(\text{OH})_2\text{D}_3$  production, they must be activated to do so, since the proportion of  $\text{CD8}^+$  T lymphocytes recovered by lavage from patients with sarcoidosis was not significantly different from that of patients with tuberculosis.

These studies also demonstrate for the first time that freshly purified alveolar macrophages from some tuberculosis patients can spontaneously produce  $1,25(\text{OH})_2\text{D}_3$ . In contrast, unfractionated lavage cells from patients with sarcoidosis (containing a majority of alveolar macrophages in each case) produced little or no  $1,25(\text{OH})_2\text{D}_3$ , consistent with the previous observation that fresh alveolar macrophages from sarcoidosis patients produce little or no  $1,25(\text{OH})_2\text{D}_3$  (7). Degradation of  $1,25(\text{OH})_2\text{D}_3$  by the cells cannot explain these results, because little apparent degradation of preformed  $1,25(\text{OH})_2\text{D}_3$  was observed over the period of incubation. Because changes in "maturation" or "differentiation" of alveolar macrophages by in vitro culture increases their capacity to produce this metabolite (22), it is unclear whether the low  $1,25(\text{OH})_2\text{D}_3$  production by freshly isolated sarcoid alveolar macrophages reflects the fact that they are often relatively immature (23), or reflects a basic difference in the production of  $1,25(\text{OH})_2\text{D}_3$  in tuberculosis and sarcoidosis.

In conclusion, this study indicates that  $1,25(\text{OH})_2\text{D}_3$  pro-

duction occurs commonly in tuberculosis, and that T lymphocytes, possibly  $\text{CD8}^+$  T lymphocytes, are an important source of this mediator. Because  $1,25(\text{OH})_2\text{D}_3$  can improve the capacity of macrophages to kill mycobacteria (24–26), our results support the conclusion that macrophage-lymphocyte interactions, mediated at least in part by  $1,25(\text{OH})_2\text{D}_3$ , may be an important component of a successful antituberculous immune response.

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## References

1. Reichel, H., H. P. Koeffler, and A. W. Norman. 1989. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.* 320:980–991.
2. Rigby, W. F. C. 1988. The immunobiology of vitamin D. *Immunol. Today.* 9:54–58.
3. Mason, R. S., T. Frankel, Y. L. Chan, D. Lissner, and S. Posen. 1984. Vitamin D conversion by sarcoid lymph node homogenate. *Ann. Intern. Med.* 100:59–61.
4. Clavel, S., M. Garabedian, C. Tau, and J. Orgiazzi. 1987. Extrarenal synthesis of calcitriol in sarcoidosis. *Presse Med.* 16:107–110.
5. Cadranet, J., A. J. Hance, B. Milleron, F. Paillard, G. Akoun, and M. Garabedian. 1988. Vitamin D metabolism in tuberculosis: production of  $1,25(\text{OH})_2\text{D}_3$  by cells recovered by bronchoalveolar lavage and the role of this metabolite in calcium homeostasis. *Am. Rev. Respir. Dis.* 138:984–989.
6. Adams, J. S., O. P. Sharma, M. A. Gacad, and F. R. Singer. 1983. Metabolism of 25-hydroxyvitamin  $\text{D}_3$  by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* 72:1856–1860.
7. Adams, J. S., and M. A. Gacad. 1985. Characterization of 1-hydroxylation of vitamin  $\text{D}_3$  sterols by cultured alveolar macrophages from patients with sarcoidosis. *J. Exp. Med.* 161:755–765.
8. Adams, J. S., F. R. Singer, M. A. Gacad, O. P. Sharma, M. J. Hayes, P. Vouras, and M. Frholic. 1985. Isolation and structural identification of  $1,25$ -dihydroxyvitamin  $\text{D}_3$  produced by cultured alveolar macrophages in sarcoidosis. *J. Clin. Endocrinol. & Metab.* 60:960–966.
9. Reichel, H., H. P. Koeffler, R. Barbers, and A. W. Norman. 1987. Regulation of  $1,25$ -dihydroxyvitamin  $\text{D}_3$  production by cultured alveolar macrophages from normal human donors and from patients with pulmonary sarcoidosis. *J. Clin. Endocrinol. & Metab.* 65:1201–1209.
10. Lecossier, D., D. Valeyre, A. Loiseau, J. P. Battesti, P. Soler, and A. Hance. 1988. T-lymphocytes recovered by bronchoalveolar lavage from normal subjects and patients with sarcoidosis are refractory to proliferative signals. *Am. Rev. Respir. Dis.* 137:592–599.
11. Julius, M. H., E. Simpson, and L. A. Herzenberg. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645–653.
12. Kumagai, K., K. Itoh, S. Hinuma, and S. Tada. 1979. Pretreatment of plastic petri dishes with fetal calf serum. A simple method for macrophage isolation. *J. Immunol. Methods.* 29:17–25.
13. Parks, D. R., V. M. Bryan, V. R. Oi, and L. A. Herzenberg. 1979. Antigen specific identification and cloning of hybridomas with a fluorescence activated cell sorter. *Proc. Natl. Acad. Sci. USA.* 76:1962–1966.
14. Shepard, R. M., R. L. Horts, A. J. Hamstra, and H. F. Deluca.

1979. Determination of vitamin D and its metabolites in plasma from normal and anephric man. *Biochem. J.* 182:55-69.
15. De Lean, A., P. J. Munson, and D. Rodbard. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose response curves. *Am. J. Physiol.* 235:E97-102.
16. Cohen, M. S., and T. K. Gray. 1984. Phagocytic cells metabolize 25-hydroxyvitamin D<sub>3</sub> in vitro. *Proc. Natl. Acad. Sci. USA.* 80:7538-7541.
17. Mayer, E., J. E. Bishop, N. Onhuma, and A. W. Norman. 1983. Biological assessment of the vitamin D metabolites 1,25-dihydroxy-24-oxo-vitamin D<sub>3</sub> and 1,23,25-trihydroxy-24-oxo-vitamin D<sub>3</sub>. *Arch. Biochem. Biophys.* 224:671-676.
18. Napoli, J. L., J. L. Sommerfeld, and B. C. Pramanik. 1983. 19-nor-10-ketovitamin D derivatives. *Biochemistry.* 22:3636-3640.
19. Breslau, N. A., J. L. McGuire, J. E. Zerwekh, E. P. Frenkel, and C. Y. C. Pak. 1984. Hypercalcemia associated with increased serum calcitriol levels in three patients with lymphoma. *Ann. Intern. Med.* 100:1-7.
20. Fetchik, D. A., D. R. Bertolini, P. S. Sarin, S. T. Weintraub, G. R. Mundy, and J. F. Dunn. 1986. Production of 1,25-dihydroxyvitamin D<sub>3</sub> by human T cell lymphotropic virus-I-transformed lymphocytes. *J. Clin. Invest.* 78:592-596.
21. Koeffler, H. P., H. Reichel, J. E. Bishop, and A. W. Norman. 1985.  $\gamma$ -Interferon stimulates production of 1,25-dihydroxyvitamin D<sub>3</sub> by normal human macrophages. *Biochem. Biophys. Res. Commun.* 127:596-603.
22. Adams, J. S., R. L. Modlin, M. M. Diz, and P. F. Barnes. 1989. Potentiation of the macrophage 25-hydroxyvitamin D-1-hydroxylation reaction by human tuberculosis pleural effusion fluid. *J. Clin. Endocrinol. & Metab.* 69:457-460.
23. Hance, A. J., S. Douches, R. J. Winchester, V. J. Ferrans, and R. G. Crystal. 1985. Characterization of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. *J. Immunol.* 134:284-292.
24. Rook, G. A. W., J. Taverne, C. Leveton, and J. Steele. 1987. The role of gamma-interferon, vitamin D<sub>3</sub> metabolites and tumor necrosis factor in the pathogenesis of tuberculosis. *Immunology.* 62:229-234.
25. Rook, G. A. W. 1988. The role of vitamin D in tuberculosis. *Am. Rev. Respir. Dis.* 138:768-770.
26. Rook, G. A. W., J. Steele, L. Fraher, S. Barker, R. Karmali, J. O'Riordan, and J. Stanford. 1986. Vitamin D<sub>3</sub>, gamma interferon, and control of proliferation of *Mycobacterium tuberculosis* by human monocytes. *Immunology.* 57:159-163.