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Vitamin D metabolism by alveolar immune cells in tuberculosis: correlation with calcium metabolism and clinical manifestations

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Vitamin D metabolism by alveolar immune cells in tuberculosis: correlation with calcium metabolism and clinical manifestations. J.L. Cadranel, M. Garabédian, B. Milleron, H. Guillozzo, D. Valeyre, F. Paillard, G. Akoun, A.J. Hance. ©ERS Journals Ltd 1994.

ABSTRACT: The aim of this study was to investigate the relationship between the pulmonary vitamin D metabolism in tuberculosis and the calcium metabolism abnormalities and other clinical characteristics of the disease.

The metabolism of 25-hydroxyvitamin D_3 (25(OH)D_3) by alveolar immune cells recovered by bronchoalveolar lavage (BAL) was evaluated in parallel to the results of calcium metabolism, 25(OH) D and 1,25 dihydroxyvitamin D (1,25(OH)$_2$D) plasma levels and other clinical parameters obtained in 14 tuberculosis patients.

Whilst predominant metabolites produced by lavage cells in patients and controls were 5(E) - and 5(Z) -19-nor-10-oxo-25(OH)D_3, 1,25(OH)$_2D_3$ was produced by cells from all tuberculosis patients but not by cells from controls. Calcium metabolism abnormalities were observed in only some patients, but the production of 1,25 (OH)$_2D_3$ by lavage cells was found to correlate both with 1,25(OH)$_2$D levels (r=0.67) and post-load urinary calcium excretion (r=0.59). 1,25(OH)$_2D_3$ production by lavage cells was increased in patients of black origin, and those presenting with hilar adenopathy without pulmonary infiltrates, and was correlated with the number of lymphocytes recovered by lavage (r=0.87).

We conclude that 1,25(OH)$_2D_3$ production by alveolar immune cells makes a major contribution to the abnormalities in calcium metabolism seen in tuberculosis patients, and may be partly dependent on the clinical characteristics evaluated here.

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Patients with tuberculosis can produce 1,25 dihydroxyvitamin D_3 (1,25(OH)$_2D_3$) and other vitamin D metabolites at sites of disease activity [1–3]. The spontaneous production of vitamin D metabolites by granulomatous tissues could have both unfavourable and favourable clinical consequences [4].

The production of 1,25(OH)$_2D_3$ or other vitamin D metabolites could have deleterious effects, mediated through an endocrine pathway. Hypercalcaemia with increased levels of plasma 1,25(OH)$_2$D has been observed in some tuberculosis patients, including several anephric patients [1, 5–8]. It has also been suggested that the production of 1,25(OH)$_2D_3$ by granulomatous tissue could contribute to the cachexia and fever seen in patients with tuberculosis [4, 9].

On the other hand, several lines of evidence suggest that 1,25(OH)$_2D_3$, operating through an autocrine/paracrine pathway, may be beneficial. Indeed, it has been demonstrated that cells obtained from sites of granulomatous reactions both convert 25 hydroxyvitamin D_3 (25(OH)D_3) into 1,25(OH)$_2D_3$ and express 1,25(OH)$_2D_3$ receptors [2, 3, 10]. Furthermore, *in vitro* studies indicate that 1,25(OH)$_2D_3$ may play an important role in the regulation of granulomatous reactions [11], and can improve the ability of alveolar macrophages to inhibit the growth of mycobacteria [12–15].

Recent studies suggest that extrarenal production of 1,25(OH)$_2D_3$ occurs in the majority of patients with tuberculosis [2, 3], but no information is available comparing the production of vitamin D metabolites and the clinical presentation of these patients. Similarly, although few patients with tuberculosis have obvious abnormalities in calcium metabolism, the degree to which extrarenal 1,25(OH)$_2D_3$ production correlates with more subtle abnormalities in calcium homeostasis has not been evaluated. The purpose of this study was, therefore, to identify and quantify the metabolites of 25(OH)D_3 produced by fresh cells recovered by bronchoalveolar lavage (BAL) from patients with tuberculosis and control subjects, and to correlate the production of these metabolites with the clinical characteristics of the tuberculosis patients, including both the existence of abnormalities in calcium metabolism and the type of inflammatory lesions present.

Methods

Study populations

Patients with pulmonary tuberculosis. Pulmonary tuberculosis was diagnosed on the basis of positive cultures of sputum or gastric aspirates (n=13) and/or the presence of epithelioid granulomata with caseous necrosis on histological study of biopsy specimens (n=4). The 14 patients (10 men and 4 women) had a mean±SD age of 34±7 yrs. Eight were of black ethnic origin, and six were Caucasians. None of them was human immunodeficiency virus (HIV) positive. Five of the subjects were smokers (24±12 cigarettes·day^{-1}). Tuberculin skin tests were strongly positive in all patients. Chest X-ray showed localized infiltrates with or without cavitation in 10 patients, and hilar adenopathy but without parenchymal infiltrates in the remaining four. Patients were studied prior to receiving any antituberculous therapy.

Control subjects. Three normal volunteers and two patients undergoing bronchoscopic evaluation for non-parenchymal pulmonary disorders served as controls. These individuals (2 men and 3 women) had an average age of 28±8 yrs. Two were cigarette smokers (3–20 cigarettes·day^{-1}).

Evaluation of calcium metabolism

Serum and urinary calcium concentrations were determined by atomic absorption photometry (Model 303, Perkin-Elmer, Norwalk, CT, USA), and serum calcium concentrations were corrected for proteinaemia, as described by ALSEVER and GOTLIN [16]. Serum and urinary phosphate were determined by a colorimetric method (Technicon, Rax T). Cyclic adenosine monophosphate (cAMP) was measured using a commercial radiocompetition assay (Amersham, Les Ulis, France). Immunoreactive parathyroid hormone (PTH) was measured by an immunoradiometric assay using an antiserum directed against both 1–34 and 39–84 peptides (Incstar Corp., Stillwater, MN, USA). Plasma 25(OH)D and 1,25(OH)$_2$D were measured as described previously [1].

Calcium absorption tests were performed as described previously [17]. Briefly, after 3 days, during which the patient had a diet containing less than 400 mg of calcium·day^{-1}, blood and urine samples were drawn at 9.30 a.m. The patient was then given a standard breakfast and absorbed 1,500 mg of calcium. Blood and urine samples were collected again at 1.30 p.m.

Normal ranges for the results of calcium metabolism evaluation were obtained from a group of 27 healthy volunteers. They were 20 men and 7 women, with a mean age of 42±10 yrs.

Bronchoalveolar lavage

Informed consent was obtained from all patients and control subjects. BAL was performed using 4 or 5 aliquots (50 ml each) of sterile saline [18]. A radiologically abnormal lung segment was lavaged when present, and the right middle lobe or lingula when the radiograph was normal.

Culture of lavage fluid was systematically performed and always remained negative for bacteria. Total and differential cell counts and the evaluation of the expression of CD4 and CD8 surface antigens on T-lymphocytes were performed as described previously [18]. The viability of cells recovered by lavage and stained with acridine orange and ethidium bromide was: controls 86±2%; and tuberculosis patients, 81±14%. The number and types of cells recovered by lavage from patients and control subjects are summarized in table 1.

Table 1. – Numbers and types of cells recovered by bronchoalveolar lavage from patients with tuberculosis and from control subjects

Cell type	Controls (n=5)	Tuberculosis (n=14)
Total cells cells·ml^{-1}	234.3±227.6	353.0±175.5
Macrophages %	86.7±9.8	50.1±29.4
Lymphocytes %	11.9±8.3	31.8±23.1
Neutrophils %	0.8±0.8	16.4±27.0
Eosinophils %	0.4±0.4	1.0±2.3
Mast cells %	0.2±0.2	0.7±2.0
CD4+ lymphocytes		
Cells·ml^{-1}	5.9±7.4	55.2±50.1
Percentage total cells	6.0±4.3	20.9±18.4
CD8+ lymphocytes		
Cells·ml^{-1}	3.9±3.7	26.0±16.0
Percentage total cells	4.0±1.0	10.5±7.4

Metabolism of 25(OH)D$_3$ by cells in vitro

Techniques used to quantify and characterize the production of vitamin D metabolites by lavage cells have been described previously [1, 3]. Briefly, lavage cells were washed twice using Dulbecco's modified Eagle medium (Boerhinger Mannheim GmbH, Penzberg, Germany) and 1×10^{6} cells were incubated for 150 min in the presence of [^{3}H]25(OH)D$_3$ (final concentration either 2.5×10^{-9} M or 1×10^{-6} M), and vitamin D metabolites were extracted and purified by sequential passage on a high performance liquid chromatography (HPLC) system using two different solvent systems (n-hexane: isopropanol and methylene chloride:isopropanol). The rate of conversion of [^{3}H]25(OH)D$_3$ into [^{3}H]1,25 (OH)$_2$D$_3$ and [^{3}H](5E)- or (5Z)-19-nor-10-oxo-25(OH) D$_3$ was determined by calculating the percentage of total radioactivity with an appropriate elution profile after purification by HPLC. Results are expressed as fmoles·10^{-5} cells per 150 min, based on the assumption that the specific activity of the product was the same as that of the substrate. All incubations and the subsequent purification of metabolites were performed in duplicate, and each value reported in the study represents the mean of duplicate determinations.

The interassay coefficient of variation of duplicate determinations was: 1,25$(OH)_2D_3$ 25±4%; (5Z)-19-nor-10-oxo-25$(OH)D_3$ 16±6%; and (5Z)-19-nor-10-oxo-25 $(OH)D_3$ 17±5%. The intra-assay coefficient of variation of two determinations of the same sample was 5±3%; 3±2%; and 4±2% for the three metabolites evaluated, respectively. Methods used for determining the specific activity of metabolites, their UV spectra, and their capacity to bind 1,25$(OH)_2D_3$ receptors have been described previously [1, 3].

Statistical methods

Results are expressed as mean±SD. Comparison of the production of metabolites between two groups was performed by the Kruskal-Wallis one-way analysis of ranks procedure. Correlations between the production of 25$(OH)D_3$ metabolites and other metric variables were evaluated by linear regression. In each case, a $p<0.05$ was considered significant.

Results

Characterization of 25(OH)D_3 metabolites synthesized by cells in vitro

Cells recovered by BAL from normals and patients with tuberculosis were able to metabolize 25$(OH)D_3$ into more polar metabolites. When extracts containing metabolites of $[^3H]$25$(OH)D_3$ were separated by HPLC using the n-hexane:isopropanol solvent system, two predominant peaks of radioactivity were observed, which eluted after the $[^3H]$25$(OH)D_3$ substrate. A first peak (region "1" in fig. 1a) eluted slightly ahead of synthetic 1,25$(OH)_2D_3$, and was composed almost entirely of (5Z)-19-nor-10-oxo-25$(OH)D_3$ (see below). A second peak coeluted with unlabelled synthetic 1,25$(OH)_2D_3$ (region "2" in fig. 1a).

When the metabolites coeluting with synthetic 1,25 $(OH)_2D_3$ (region "2" in fig. 1a) were pooled, concentrated and rechromatographed, using the methylene chloride:isopropanol solvent system, four peaks of radioactivity could be resolved (fig. 1b): a small peak eluting immediately after the solvent front (elution volume 0.30 relative to synthetic 1,25$(OH)_2D_3$), which was not further analysed; two closely adjacent peaks (elution volumes 0.54 and 0.69, respectively, relative to synthetic 1,25$(OH)_2D_3$) corresponding to the (5Z)- and (5E)-isomers of 19-nor-10-oxo-25$(OH)D_3$ (referred to hereafter as 5Z and 5E) (see below); and, in some samples, a sharp well-defined peak which coeluted with synthetic 1,25$(OH)_2D_3$.

Evidence supporting the conclusion that the metabolite which coeluted with 1,25$(OH)_2D_3$ in two successive HPLC chromatographic steps was 1,25$(OH)_2D_3$ has been published previously [1, 3]. Additional findings supported the idea that the metabolites, the chromatographic properties of which were similar to 5Z and 5E were, in

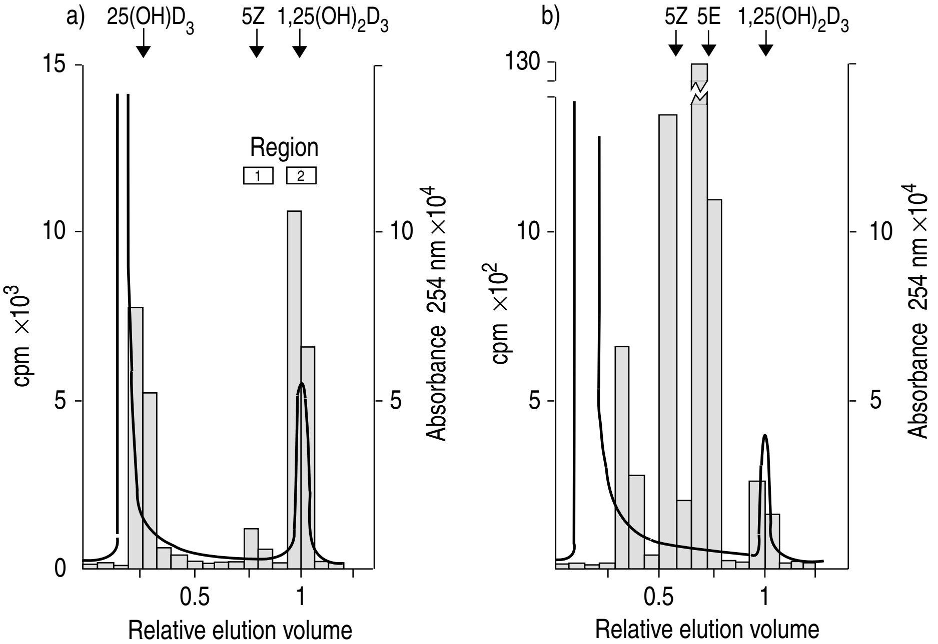

Fig. 1. – Separation of 25 hydroxyvitamin D_3 (25$(OH)D_3$) metabolites by high performance liquid chromatography (HPLC). a) 1×10^5 fresh lavage cells from a patient with tuberculosis were incubated in the presence of 2.5×10^{-9} M $[^3H]$25$(OH)D_3$. The $[^3H]$25$(OH)D_3$ metabolites were extracted, unlabelled synthetic 1,25 dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) was added and the sample chromatographed, using a straight phase HPLC system equilibrated with n-hexane:isopropanol solvent system as described in [3]. Fractions were collected each minute, and aliquots were counted to measure radioactivity. b) Fractions identified as region "2" in (a) were pooled, concentrated, and rechromatographed using the methylene chloride:isopropanol system [3]. Fractions were collected, and aliquots were counted to measure radioactivity. Absorbance at 254 nm (solid line) was monitored continuously. Elution volumes are expressed relative to that of 1,25$(OH)_2D_3$. Elution volume of synthetic 25$(OH)D_3$, (5Z)- and (5E)-isomers and 1,25$(OH)_2D_3$ are shown at the top of the figure. cpm: counts per minute. Note difference in cpm values between a) ($\times10^3$) and b) ($\times10^2$).

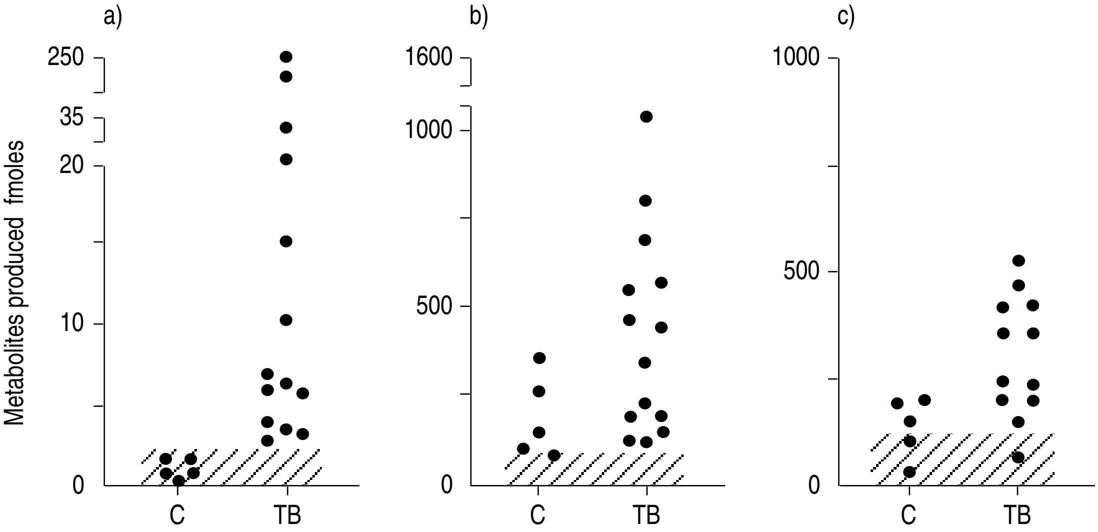

Fig. 2. – Metabolism of 25 hydroxyvitamin D_3 ($25(OH)D_3$) by lavage cells from controls (C) and patients with tuberculosis (TB). 1×10^5 fresh lavage cells were incubated in the presence of $[^3H]25(OH)D_3$ (see methods). The $[^3H]25(OH)D_3$ metabolites were purified by high performance liquid chromatography (HPLC), and the amount of metabolites with chromatographic properties expected for: a) 1,25 dihydroxyvitamin D_3 $1,25(OH)_2D_3$; b) (5E)-isomer; and c) (5Z)-isomers was determined. The shaded area corresponds to the range of values obtained from four experiments performed as above, but in the absence of cells.

fact, these metabolites. Firstly, the putative 5Z, isolated using the n-hexane:isopropanol solvent system, eluted mostly as a single peak at the position expected for 5Z when rechromatographed using the methylene chloride:isopropanol solvent system. In addition, a minor fraction eluted at the position expected for 5E, as would be expected from the isomerization of 5Z into 5E [19, 20]. Similarly, when the putative 5E, purified by sequential chromatography using the two solvent systems, was rechromatographed using the methylene chloride:isopropanol solvent system, some radioactivity which initially had an elution volume relative to 1,25 $(OH)_2D_3$ of 0.69, now had an elution volume of 0.54. This phenomenon would be expected for the isomerization of 5E into 5Z [19, 20]. Furthermore, when cells were cultured in the presence of 10^{-6} M $25(OH)D_3$ and these metabolites were purified by HPLC, the products had a UV spectrum similar to that of synthetic 5Z and 5E, and did not compete with the binding of synthetic $[^3H]1,25(OH)_2D_3$ in the receptor binding assay [19, 20].

Metabolism of $[^3H]25(OH)D_3$ by lavage cells

When fresh lavage cells from patients with tuberculosis were incubated in the presence of 2.5×10^{-9} M $[^3H]25(OH)D_3$, detectable amounts of $[^3H]$ 5Z and 5E were produced in all cases (fig. 2b and c). $[^3H]1,25(OH)_2D_3$ was also produced by lavage from all tuberculosis patients tested (fig. 2a). In contrast, lavage cells from controls did not produce detectable amounts of 1,25 $(OH)_2D_3$. Interestingly, lavage cells from two patients produced considerably more $1,25(OH)_2D_3$ than the remaining 12 patients (fig. 2a); these two patients had overt hypercalcaemia and elevated plasma $1,25(OH)_2D$ levels (see below). The amount of $1,25(OH)_2D_3$ produced by lavage cells was 10–100 fold smaller than the amount of 19-nor-10-oxo-$25(OH)D_3$ produced by these cells. No correlation was observed between $1,25(OH)_2D_3$ production by lavage cells and that of 5Z- or 5E-isomers. Lavage cells from controls also produced considerable amounts of 5Z and 5E. The production of 5Z, but not of 5E, by lavage cells from controls was slightly lower than that of lavage cells from patients with tuberculosis ($p<0.05$).

Relationship between the conversion of $25(OH)D_3$ into $1,25(OH)_2D_3$ by lavage cells and the characteristics of the patients

Calcium and phosphorus metabolism. Two patients had hypercalcaemia and increased post-load urinary calcium excretion, associated with elevated plasma levels of $1,25(OH)_2D_3$, normal serum concentrations of phosphorus (table 2, patients No. 1 and 2) and low serum PTH concentrations (data not shown). Five other patients had normocalcaemia but increased post-load urinary calcium excretion (patients Nos. 4 and 9–12); two of these had elevated $1,25(OH)_2D$ plasma levels, whereas three had a normal value. The remaining seven patients had normal calcium metabolism and plasma $1,25(OH)_2D$ levels (patients Nos 3, 5–8, 13, 14). Although, plasma 1,25 $(OH)_2D$ levels were normal (n=10) or elevated (n=4), plasma $25(OH)D$ concentrations were at or below the lower limit of normal (tuberculosis patients 3.9±2.0 ng·ml^{-1}; controls 16±7 ng·ml^{-1}; $p<0.01$). Finally, serum PTH concentrations (50.4±23.6 pg·ml^{-1}; normal range <80 pg·ml^{-1}), nephrogenic cAMP (1.5±0.4 nmoles·dl^{-1} glomerular filtrate; normal range <3 nmoles·dl^{-1}), and tubular reabsorption of phosphate (93±3%, normal range >82%) were normal in all patients.

There was a correlation between post-load urinary calcium excretion and both $1,25(OH)_2D$ plasma levels

Table 2. – Calcium and phosphorus metabolism in patients with tuberculosis

Pt No.	Plasma $1,25(OH)_2D$ $pg \cdot ml^{-1}$	Plasma 25(OH)D $ng \cdot ml^{-1}$	Serum calcium $mmoles \cdot l^{-1}$	Serum phosphate $mmoles \cdot l^{-1}$	Urinary calcium ($mmole \cdot mmole^{-1}$ creatinine) Fasting	Post-load	$1,25(OH)_2D_3$ production (lavage cells)*
1	110	4	3.50	1.30	0.20	2.5	230
2	108	6	2.95	1.05	0.17	1.80	250
3	10	5	2.32	1.30	0.10	0.22	33.0
4	106	4	2.43	1.39	0.43	2.74	21.5
5	27	1	2.52	1.19	0.23	0.66	14.9
6	46	5	2.40	0.91	0.18	0.70	10.2
7	51	7	2.37	0.92	0.28	0.62	7.2
8	37	2	2.22	0.98	ND	ND	6.6
9	67	6	2.45	1.45	0.15	1.09	6.3
10	29	2	2.34	0.83	0.24	0.81	6.3
11	35	6	2.35	1.05	0.29	1.11	4.3
12	24	2	2.30	1.09	0.22	1.29	3.6
13	31	3	2.26	1.18	0.20	0.65	3.3
14	49	1	2.18	1.07	0.02	0.27	3.0
Normal range	(20–60)	(6–30)	(2.10–2.55)	(0.89–1.21)	<(0.30)	<(0.77)	

*: results are expressed as fmol $1,25(OH)_2D_3$ produced by 10^5 fresh lavage cells incubated in the presence of 2.5×10^{-9} M $25(OH)D_3$ for 150 min. ND: not determined; Pt: patient; $1,25(OH)_2D$: 1,25 dihydroxyvitamin D; 25(OH)D: 25 hydroxyvitamin D.

($r=0.84$; $p=0.0001$) (fig. 3a) and $1,25(OH)_2D_3$ production by lavage cells ($r=0.59$; $p=0.035$). Furthermore, a correlation was also found between $1,25(OH)_2D$ plasma levels and $1,25(OH)_2D_3$ production by lavage cells ($r=0.67$; $p=0.008$) (fig. 3b). The quantity of 1,25 $(OH)_2D_3$ produced by lavage cells from hypercalcaemic patients was 20 fold higher than that of non-hypercalcaemic patients (240±10 *vs* 10.1±8.7 fmol; $p=0.03$).

Clinical features. $1,25(OH)_2D$ plasma level and $1,25(OH)_2D_3$ production by lavage cells were correlated with the proportion of lymphocytes present ($r=0.46$; $p=0.03$; and $r=0.87$; $p=0.0001$, respectively) (fig. 3c). No other correlations between $1,25(OH)_2D_3$ production by lavage cells and the proportion of other inflammatory cells (macrophages, neutrophils, eosinophils and mastocytes) and/or CD4 and CD8 lymphocyte subsets were found. Both patients with hypercalcaemia and very high 1,25 $(OH)_2D_3$ production had extensive cavitary disease and a high proportion of lymphocytes recovered by lavage (>40%). Other patients whose lavage cells produced more modest amounts of $1,25(OH)_2D_3$ had disease of similar form and severity and equivalent numbers of lymphocytes were recovered by lavage (fig. 3c).

Of the other clinical variables evaluated, two were identified which were associated with an increased production of $1,25(OH)_2D_3$ by fresh lavage cells. Firstly, lavage cells from black patients with tuberculosis produced more $1,25(OH)_2D_3$ than did those from Caucasian patients ($p=0.03$). Secondly, cells from patients with hilar adenopathy but no parenchymal abnormality produced more $1,25(OH)_2D_3$ than did patients with other forms of tuberculosis ($p=0.05$). These two clinical variables were not, however, independent. Indeed, 4 out of 8 black patients presented with hilar adenopathy only, whereas this presentation was not seen in the six

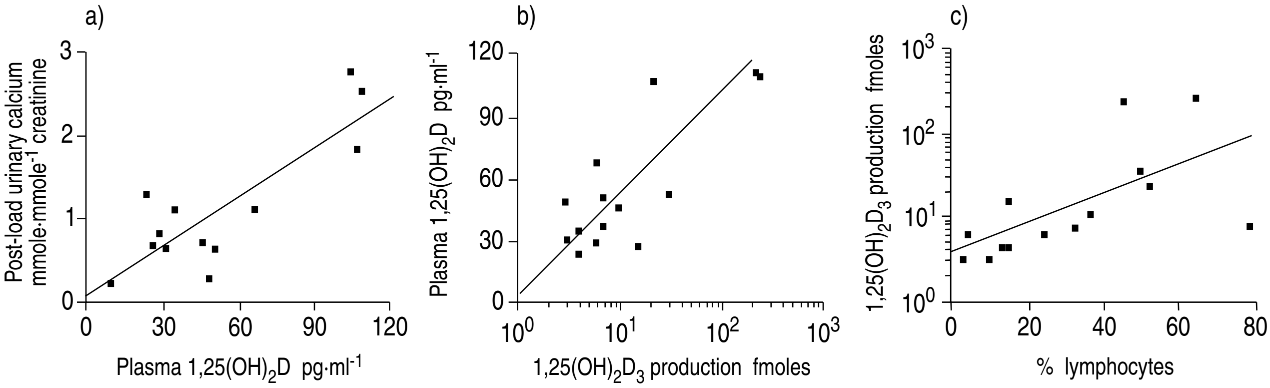

Fig. 3. – Correlations between: a) post-load urinary calcium excretion and plasma 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) level; b) plasma $1,25(OH)_2D$ level and 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) production by lavage cells; c) $1,25(OH)_2D_3$ production by alveolar cells and the percentage of lymphocytes recovered in bronchoalveolar lavage. Note the log scale in (b) and (c).

Caucasians evaluated ($p=0.04$). In addition, more lymphocytes were recovered by lavage from black patients than from Caucasians 42.5±24.5% (n=8) and 15.2±15.8% (n=6) of total lavage cells, respectively ($p=0.01$).

Relationship between 5Z- or 5E-isomer production by lavage cells and clinical characteristics. No correlations were observed between the production of 5E- and 5Z-isomers by lavage cells from patients with tuberculosis and the indices of calcium and phosphorus metabolism evaluated in this study, or the clinical features of the patients (data not shown).

Discussion

In this study we have characterized the metabolites of $25(OH)D_3$ produced by immune and inflammatory cells, obtained by bronchoalveolar lavage, from patients with tuberculosis and controls, and compared the production of these metabolites with clinical characteristics of the patients. Our results suggest that: 1) spontaneous production of $1,25(OH)_2D_3$ by these alveolar cells occurs commonly in tuberculosis, but represents a minor proportion of the total vitamin D metabolites produced; 2) increases in circulating $1,25(OH)_2D$ are likely to make a major contribution to the abnormalities of calcium metabolism observed in tuberculosis patients, but do not entirely explain these abnormalities; and 3) the production of $1,25(OH)_2D_3$ by lavage cells is quite variable, and depends, in part, on the clinical characteristics of the tuberculosis patients.

$25(OH)D_3$ metabolites produced by lavage cells

The major metabolites of $25(OH)D_3$ produced by fresh lavage cells both from controls and patients with tuberculosis had chromatographic and spectroscopic characteristics previously described for the (5Z)- and (5E)-isomers of 19-nor-10-oxo-$25(OH)D_3$ [19–22]. Considerable overlap was observed in the production of these metabolites by cells from controls and patients, and only the production of the 5Z-isomer by lavage cells was slightly different when comparing the two study groups. The production of 19-nor-10-oxo-$25(OH)D_3$ by a variety of epithelial and haematopoietic cells has been described previously [19, 21, 22]. However, the physiological role of this metabolite, particularly in the control of $1\alpha,25(OH)D_3$ hydroxylase activity, if any, has not been established [19, 21, 22].

In contrast to controls, immune and inflammatory cells from tuberculosis patients were also capable of synthesizing $1,25(OH)_2D_3$. The production of $1,25(OH)_2D_3$ at sites of granulomatous reactions has previously been reported in sarcoidosis and tuberculosis [1–3, 23–25]. However, the characteristics of this production are quite different in these two diseases. In sarcoidosis, the $1,25(OH)_2D_3$ production has been found in 7 day cultured alveolar macrophages obtained only from patients who had known calcium metabolism abnormalities [25]. It may also be observed after *in vitro* activation by various mediators, *i.e.* lipopolysaccharides and gamma-interferon, in macrophages from patients without such calcium metabolism abnormalities [23, 25]. By contrast, in tuberculosis, $1,25(OH)_2D_3$ production has been found in fresh as well as in cultured alveolar cells; even if macrophages may also contribute sometimes to this production, lymphocytes are the predominant source of vitamin D metabolism in lung of tuberculosis patients [1, 3].

$1,25(OH)_2D_3$ production and calcium metabolism

In this study, significant correlations were observed between serum calcium and post-load urinary calcium excretion and both the plasma levels of $1,25(OH)_2D$ and spontaneous production of $1,25(OH)_2D_3$ by lavage cells. Similar findings have been reported previously for patients with sarcoidosis [17, 25, 26]. These results are entirely consistent with the idea that $1,25(OH)_2D_3$ produced by immune and inflammatory cells, acting in an endocrine fashion, contributes to the abnormalities of calcium metabolism observed in patients with tuberculosis.

Although "normal" $1,25(OH)_2D$ plasma levels in hypercalciuria patients represent an inappropriate elevation of the circulating levels of this hormone, it should be emphasized that some of the findings in our study suggest that increased circulating levels of $1,25(OH)_2D$ are probably not the only determinant of abnormal calcium metabolism in these patients. In particular, clearly abnormal post-load urinary calcium excretion was observed in seven patients, whereas plasma $1,25(OH)_2D$ was elevated in only four of these individuals. In the remaining three patients, plasma $1,25(OH)_2D$ levels were, in fact, near the middle of the normal range. In this context, patients with sarcoidosis and tuberculosis have previously been described in whom abnormalities in calcium metabolism did not correlate closely with plasma $1,25(OH)_2D$ [1, 26, 27]. The local production of $1,25(OH)_2D_3$ in tissues responding to this hormone (*e.g.*, gut, bone or kidney) or the production of other mediators (tumour necrosis factor-α (TNF-α), prostaglandin E_2 (PGE_2), interleukin-1 (IL-1), *etc.*) by immune and inflammatory cells [28–30], which independently modify calcium metabolism [31], are two possible explanations for the dissociation between plasma $1,25(OH)_2D$ levels and post-load calcium excretion observed in this and previous studies.

It is also noteworthy that lavage cells from all patients produced $1,25(OH)_2D_3$, whereas only a minority of patients had elevated plasma $1,25(OH)_2D$. Furthermore, although a significant correlation was observed between $1,25(OH)_2D_3$ production by lavage cells and plasma $1,25(OH)_2D$, the correlation was not strong ($r=0.67$). The low plasma $25(OH)D$ concentrations observed in our patients could, in theory, reduce the $1,25(OH)_2D_3$ production by these individuals *in vivo* (due to a lack of substrate), thereby explaining the weak correlation between *in vivo* and *in vitro* findings. Several findings argue against this possibility. Firstly, no correlation was observed between plasma 25(OH)D levels and 1,25(OH)$_2$D levels. Furthermore, no difference has been observed in prior studies comparing 25(OH)D levels in patients with granulomatous diseases who did and did not have elevated 1,25(OH)$_2$D plasma levels and hypercalcaemia [17, 26]. The ability of 1,25(OH)$_2$D$_3$ production by immune and inflammatory cells to increase plasma 1,25(OH)$_2$D probably depends on a number of factors, including: 1) the rate of 1,25(OH)$_2$D$_3$ synthesis by immune cells; 2) the extent to which extrarenal 1,25 (OH)$_2$D$_3$ production occurs in multiple sites, such as production by circulating cells [3]; 3) the magnitude of compensatory changes in renal 1,25(OH)$_2$D$_3$ production; and 4) the degree to which 1,25(OH)$_2$D$_3$ is retained at sites of immune reactions by cells expressing 1,25(OH)$_2$D$_3$ receptors [10]. In view of this complexity, it is not surprising that *in vitro* production of 1,25 (OH)$_2$D$_3$ did not correlate closely with plasma 1,25 (OH)$_2$D levels.

1,25(OH)$_2$D$_3$ production in tuberculosis and clinical features

In this study, three factors were identified which were associated with increased production of 1,25(OH)$_2$D$_3$ by lavage cells, the proportion of T-lymphocytes among cells recovered by lavage, black ethnic origin, and presentation of tuberculosis as isolated adenopathy. The strong correlation found between 1,25(OH)$_2$D$_3$ production by lavage cells and the proportion of T-lymphocytes is in accordance with the prior observation that T-lymphocytes are an important source of extrarenal 1,25(OH)$_2$D$_3$ production in tuberculosis [3]. By contrast, in the present study, we did not find, as previously shown [3], a correlation between the 1,25(OH)$_2$D$_3$ production by alveolar cells and the proportion of CD8-lymphocytes present in the cell incubation. However, such a strong correlation can be observed when the two hypercalcaemic patients, with their predominant CD4-lymphocytic alveolitis, are excluded in the statistical analysis (data not shown). It is possible that 1,25(OH)$_2$D$_3$ production during tuberculosis normally results from the CD8-lymphocytes activation [3], whilst hypercalcaemia may be related to the stimulation of 1α-hydroxylase activity in macrophages by cytokines produced by CD4-lymphocytes (gamma-interferon, IL-2), such as in sarcoidosis [24].

The difference in 1,25(OH)$_2$D$_3$ production observed when comparing patients of different ethnic origin or patients with different clinical presentations may also simply reflect differences in the intensity of the lymphocytic alveolitis found in these two groups. Indeed, in this study, lymphocytes were increased to a greater extent in lavage from black patients compared to that observed in Caucasians. Furthermore, as observed here, tuberculosis presenting with hilar adenopathy is more common for black patients than Caucasians, and is associated with an intense lymphocytic alveolitis [32, 33].

It is clear, however, that the proportion of lymphocytes among cells recovered by lavage was not the sole determinant of 1,25(OH)$_2$D$_3$ production. Lavage cells from the two patients with hypercalcaemia produced extremely large amounts of 1,25(OH)$_2$D$_3$ *in vitro*. The proportion of lymphocytes in these cases was elevated (>40%), but lavage cells from several other patients which contained equivalent proportions of lymphocytes produced only modest amounts of 1,25(OH)$_2$D$_3$. Similarly, both patients who produced large quantities of 1,25(OH)$_2$D$_3$ had extensive bilateral pulmonary abnormalities with cavitation, but lavage cells from other patients with equally extensive disease did not produce equivalent amounts of 1,25(OH)$_2$D$_3$. Thus, further studies are necessary to define the mechanism responsible for induction of 1,25(OH)$_2$D$_3$ production by immune cells in tuberculosis. Once such factors (gamma-interferon, IL-2, TNF-α *etc.*) are better characterized, it will be of interest to evaluate whether these processes occur to a different extent in tuberculosis patients with different clinical characteristics, and to determine whether or not activation of 1,25(OH)$_2$D$_3$ synthesis might improve the capacity of the immune system to kill mycobacteria, as has been suggested by *in vitro* studies [11–15].

We conclude that the production of 1,25(OH)$_2$D$_3$ by immune alveolar cells makes a major contribution to the abnormalities in calcium metabolism seen in these patients, but that such abnormalities can be present in patients with normal circulating 1,25(OH)$_2$D levels. Considerable variation in 1,25(OH)$_2$D$_3$ production is observed in these patients, which can only be partly explained by difference in the clinical characteristics evaluated here.

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