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METABOLISM OF 25-HYDROXYVITAMIN D_3 TO 24,25-DIHYDROXYVITAMIN D_3 BY BLOOD DERIVED MACROPHAGES FROM A PATIENT WITH ALVEOLAR RHABDOMYOSARCOMA DURING SHORT-TERM CULTURE AND 1α,25-DIHYDROXYVITAMIN D_3 AFTER LONG-TERM CULTURE

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Summary—We have examined the ability of blood-derived monocytes and macrophages isolated from a patient with alveolar rhabdomyosarcoma and hypercalcaemia, to form 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) or 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) from 25-hydroxyvitamin D_3 ($25(OH)D_3$). Adherent monocyte-macrophage cells incubated with $25(OH)D_3$ over the initial 2 days in culture synthesized 1.9 pmol $24,25(OH)_2D_3$/h/incubation (representing 0.63 pmol/h/10^6 cells), whereas macrophages synthesized 1.03 and 1.15 pmol $1\alpha,25(OH)_2D_3$/h/incubation after 1 and 4 weeks in culture respectively. In a further experiment synthesis of $1\alpha,25(OH)_2D_3$ by long-term cultured macrophages fell from 2.25 to 0.04 pmol/h/incubation following exposure to 10 nM $1\alpha,25(OH)_2D_3$ for 7 days, whereas $24,25(OH)_2D_3$ synthesis was induced (0.46 pmol/h/incubation). The vitamin D_3 metabolites were identified by co-chromatography with authentic $24,25(OH)_2D_3$ or $1\alpha,25(OH)_2D_3$ in three different high-performance liquid chromatography systems. Serum $1\alpha,25(OH)_2D_3$ in the patient was markedly suppressed at 5 pg/ml (normal 20–50 pg/ml) indicating that raised $1\alpha,25(OH)_2D_3$ was not the cause of the hypercalcaemia, but rather, that raised calcium may have suppressed renal $1\alpha,25(OH)_2D_3$ synthesis. Administration of APD (3-amino-1-hydroxypropylidine-1,1-bisphosphonate) corrected the hypercalcaemia in the patient suggesting that increased bone resorption was responsible for the raised calcium.

The results of this study show for the first time that immature blood derived monocyte-macrophage cells can synthesize $24,25(OH)_2D_3$ before they mature into macrophages able to synthesize $1\alpha,25(OH)_2D_3$.

INTRODUCTION

Normal metabolism of vitamin D_3 involves sequential hydroxylation of the parent secosterol to 25-hydroxyvitamin D_3 ($25(OH)D_3$) in the liver and then to 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) which has limited biological activity, or to the active hormonal form 1α,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) in the kidney. The renal synthesis of $1\alpha,25(OH)_2D_3$ is primarily stimulated by parathyroid hormone (PTH), synthesis of which is increased by hypocalcaemia, whereas synthesis of $24,25(OH)_2D_3$ can be increased by $1\alpha,25(OH)_2D_3$ [1, 2]. $1\alpha,25(OH)_2D_3$ appears to act by first binding to a specific intracellular receptor protein, forming a complex that then induces a series of genomic interactions which results in activation or repression of gene transcription. By these mechanisms $1\alpha,25(OH)_2D_3$ stimulates intestinal calcium and phosphate absorption; increases osteoblast mediated bone mineralization and indirectly increases osteoclast mediated bone resorption; decreases synthesis of PTH; decreases renal $25(OH)D_3$-1α-hydroxylase activity and increases $25(OH)D_3$-24-hydroxylase activity in renal cells, keratinocytes and fibroblasts. It also induces differentiation and inhibits proliferation of keratinocytes, fibroblasts, monocytes and lymphocytes, and inhibits secretion of interleukin 2 by T-lymphocytes and immunoglobulins by B-lymphocytes [2].

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Macrophages from pateints with sarcoidosis [3, 4], peritonitis [5], arthritis [6] or tuberculosis [7] can also synthesize $1\alpha,25(OH)_2D_3$ *in vitro*. Similarly $25(OH)D_3$-1α-hydroxylase activity is expressed by normal macrophages *in vitro* following activation with gamma-interferon (γ-INF) [4, 8, 9], bacterial lipopolysaccharide (LPS) [4, 10] or tumour necrosis factor-alpha (TNF-α) [11]. Conversely, $25(OH)D_3$-24-hydroxylase activity is only occasionally seen in activated macrophages [4, 9]. In patients with sarcoidosis [3], tuberculosis [7], Hodgkin's lymphoma [12] or malignant lymphoma [13] hypercalcemia has been described which was related to raised serum $1\alpha,25(OH)_2D_3$. In the light of these observations we investigated the metabolism of $25(OH)D_3$ by blood derived monocyte-macrophage cells from a patient with hypercalcaemia (serum calcium 3.8 mM) who was later diagnosed as having alveolar rhabdomyosarcoma with bone marrow involvement.

EXPERIMENTAL

Blood samples (20 ml) were taken from the patient before and 5 days after administration of 60 mg 3-amino-1-hydroxypropylidine-1,1-bisphosphonate (APD) given as a single 6 h infusion to control the hypercalcaemia. These blood samples were initially centrifuged at 500 *g* for 20 min to collect plasma for vitamin D metabolite measurements [14]. The cells were then resuspended in 20 ml of serum-free RPMI-1640 medium containing 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomysin (Flow Labs Ltd) and layered onto Ficoll lymphocyte separating media (LSM; Flow Labs Ltd), which was centrifuged at 500 *g* for 20 min to separate the lower density lymphocytes and monocytes from high density granulocytes and erythrocytes. The low density cells from the pre-APD sample were washed using serum-free RPMI-1640 medium, resuspended in a further 8 ml of medium to give 5×10^6 cells/ml and plated as 2 ml aliquots into 4 tissue culture wells. Two aliquots were incubated at 37°C in a humidified atmosphere of 95% air 5% CO_2 for 30 min and then the adherent monocyte-macrophages (approx. 3×10^6) and non-adherent cells (approx. 7×10^6 monocytes and lymphocytes) were separated and incubated with $[^3H]25(OH)D_3$ for 48 h. The adherent cells in the second pair of aliquots were maintained in culture in 10 mls of medium (changed every 3–4 days) containing 10% new born calf serum (Gibco BRL) for 1 and 4 weeks, after which they were resuspended in 2 ml of incubation medium and incubated with $[^3H]25(OH)D_3$ for 24 h. The cells incubated after 1 week in culture were fixed for morphological examination whereas the 4 week samples was kept in culture for another 2 weeks. Fresh $1\alpha,25(OH)_2D_3$ (10 nM, donated by Dr M. R. Uskocovic) was then included in the medium each day and a series of 24 h incubations carried out on days 1, 2, 3 and 7 to examine the effect of $1\alpha,25(OH)_2D_3$ on the ability of the macrophages to metabolize $[^3H]25(OH)D_3$. Incubations were also attempted with cells from the blood sample taken 5 days after APD treatment, however, these cells did not thrive in culture.

The assays of $[^3H]25(OH)D_3$ metabolism were carried out by incubating cells in 2 ml of serum-free RPMI-1640 medium supplemented with 1.5 mg/ml bovine serum albumin (Immuno Ltd). 50,000 dpm $[^3H]25(OH)D_3$ (6.5 TBq/mmol, Amersham International) and 0.125 nmol $25(OH)D_3$ (25 ng/ml, Roussel–Uclaf) at 37°C in a humidified atmosphere of 95% air 5% CO_2. Vitamin D_3 metabolites were extracted from each incubation by mixing the medium with 3 ml of chloroform and 2 ml of methanol, which for some incubations was also used to rinse and fix the cells. The extracts were centrifuged at 500 *g* for 20 min at 4°C and the lower chloroform layer removed, dried under a stream of nitrogen gas and resuspended in 1 ml of ethanol for storage at −20°C. Half of each extract was initially analysed by normal phase high-performance liquid chromatography (HPLC) using a Zorbax–Sil column (4.6 mm × 25 cm; DuPont Company) developed with a mobile phase of *n*-hexane, propan-2-ol and methanol (110:4:4, by vol) at 2 ml/min. Further aliquots were then analysed by a second normal-phase HPLC system developed with dichloro-methane and methanol (97:2.5, v/v) and by a reverse-phase HPLC system using a Zorbax–ODS column (4.6 mm × 25 cm; DuPont) developed with methanol and water (85:15, v/v) at 2 ml/min. Timed eluent fractions were collected and the radioactivity associated with the substrate, $24,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ estimated by liquid scintillation counting. Standard $25(OH)D_3$, $24,25(OH)_2D_3$ (donated by Dr M. R. Uskocovic) and $1\alpha,25(OH)_2D_3$ and their tritiated forms were also chromatographed and the retention times of these markers determined by u.v. absorption at 265 nm and liquid scintillation counting respectively. Results are expressed as pmol synthesized/h/incubation

because the non-adherent cells were heterogenous and the adherent macrophages were difficult to trypsinize for accurate cell counting. However, approximate cell numbers were estimated using a haemocytometer.

Cell morphology was determined by expression of non-specific esterase activity (NSE) [15] and by Jenner–Giemsa staining using methanol-fixed cytocentrifuge preparations made with approximately 40,000 cells per slide or using adherent cells fixed in tissue culture wells.

Serum calcium concentrations were estimated by atomic absorption flame spectrophotometry.

RESULTS

Monocyte-macrophage cells that had been separated from erythrocytes and granulocytes using lymphocyte separating medium and from lymphocytes by adherence in tissue wells, synthesized 1.9 pmol [^{3}H]24,25(OH)$_2$$D_3$/h/incubation (representing 0.63 pmol/h/10^6 cells) from [^{3}H]25(OH)D_3 over the first 48 h in culture. The

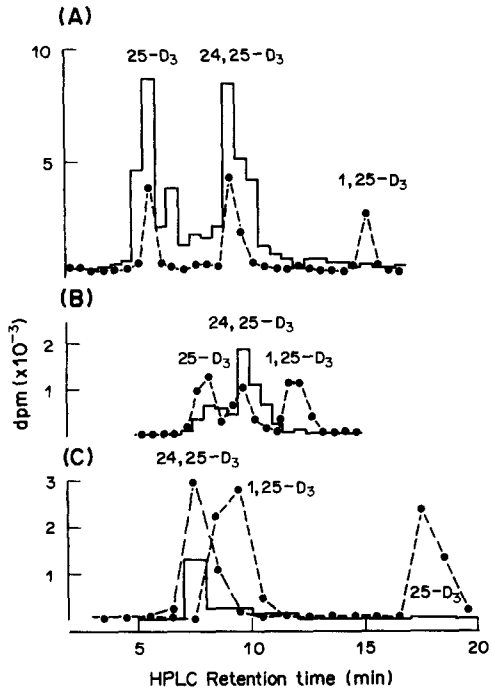

Fig. 1. Analysis of [^{3}H]24,25(OH)$_2$$D_3$ synthesized by adherent monocyte-macrophage cells following a 48 h incubation period using 3 different HPLC systems. (A) Zorbax-SIL column developed with hexane, propan-2-ol, methanol (110:4:4) at 2 ml/min. (B) Zorbax–SIL column developed with dichloromethane and methanol (97:2.5) at 2 ml/min. (C) Reverse-phase Zorbax–ODS column developed with methanol and water (85:15) at 2 ml/min. Dashed traces represent the chromatography of standard tritium labelled metabolites.

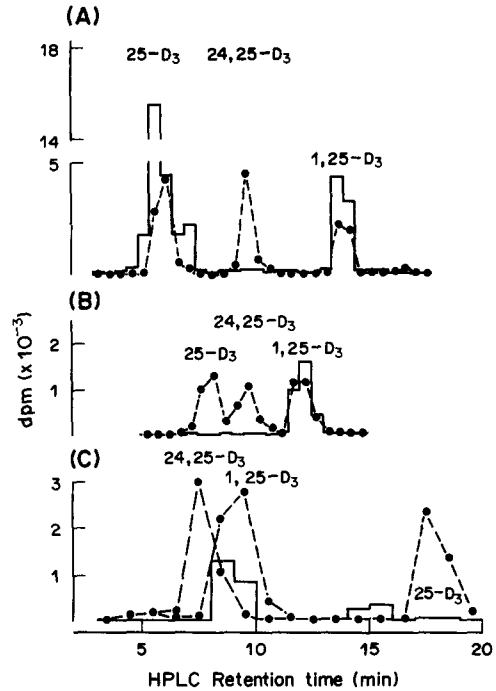

Fig. 2. Analysis of [^{3}H]1α,25(OH)$_2$$D_3$ synthesized by macrophages following a 24 h incubation period after 1 week in culture, using 3 different HPLC systems. (A) Zorbax-SIL column developed with hexane, propan-2-ol, methanol (110:4:4) at 2 ml/min. (B) Zorbax–SIL column developed with dichloromethane and methanol (97:2.5) at 2 ml/min. (C) Reverse-phase Zorbax–ODS column developed with methanol and water (85:15) at 2 ml/min. Dashed traces represent the chromatography of standard tritium labelled metabolites.

morphology of these adherent monocyte-macrophages was confirmed by Jenner–Giemsa staining and by expression of NSE activity. The identity of the [^{3}H]24,25(OH)$_2$$D_3$ synthesized was confirmed by co-chromatography with authentic 24,25(OH)$_2$$D_3$ in 3 different HPLC systems (Fig. 1). The parallel incubations carried out with non-adherent cells (monocytes and lymphocytes) synthesized much less [^{3}H]24, 25(OH)$_2$$D_3$ (0.92 pmol/h/incubation) representing 0.13 pmol/h/10^6 cells. After 1 week in culture the adherent cells which had developed the morphology of more mature macrophages synthesized 1.03 pmol [^{3}H]1α,25(OH)$_2$$D_3$/h/incubation from the substrate. Again the identity of the product was confirmed by co-chromatography with the authentic metabolite in three HPLC systems (Fig. 2).

The ability of the macrophages (some of which were multinucleated) to synthesize [^{3}H]1α, 25(OH)$_2$$D_3$ (1.15 pmol/h/incubation) was confirmed in cells cultured for 4 weeks. A further experiment using these macrophages after 6 weeks culture, in which the cells were incubated

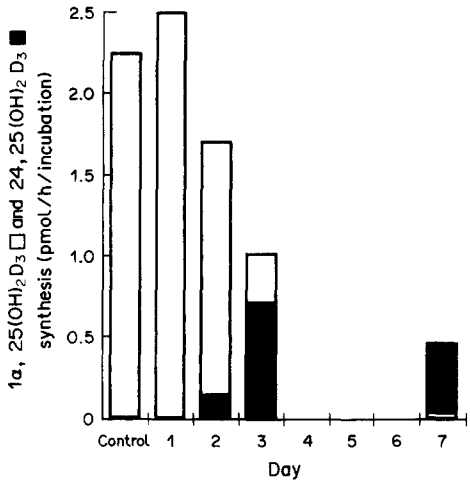

Fig. 3. Synthesis of 1α,25-dihydroxyvitamin D_3 (□) and 24,25-dihydroxyvitamin D_3 (■) by macrophages after 6 weeks in culture and following exposure to 10 nM 1α,25-dihydroxyvitamin D_3 over a further 7 days.

with [^{3}H]25(OH)D_3 in the presence of 10 nM 1α,25$(OH)_2D_3$ in a series of 24 h incubations carried out after 1, 2, 3 and 7 days, demonstrated that this high concentration of 1α,25$(OH)_2D_3$ reduced [^{3}H]1α,25$(OH)_2D_3$ synthesis from 2.25 to 0.04 pmol/h/incubation and induced [^{3}H]24, 25$(OH)_2D_3$ synthesis (0.46 pmol/h/incubation) (Fig. 3). Although previous studies have demonstrated that 1α,25$(OH)_2D_3$ can accelerate multinucleation of normal human blood monocytes [16], many of the cells in this study were already multinucleated after 6 weeks and exposure to 1α,25$(OH)_2D_3$ did not appear to further increase the proportion of such cells.

Hypercalcaemia in the patient was treated by administration of APD which reduced the serum calcium level from 3.8 to 2.4 mM after 3 days, which is within the normal range (2.2–2.6 mM). Measurement of serum vitamin D_3 metabolites demonstrated that 25(OH)D_3 levels were 20.9 ng/ml before and 21.7 ng/ml after treatment which were within the normal range (10–50 ng/ml). However, serum 1α,25$(OH)_2D_3$ levels were 5.1 pg/ml before and 4.6 pg/ml after APD treatment which were markedly below the normal range (20–50 pg/ml), indicating that the hypercalcaemia did not result from excess 1α,25$(OH)_2D_3$ synthesis. Cells isolated from blood after 5 days treatment with APD did not metabolise [^{3}H]25(OH)D_3 nor did they thrive in culture.

DISCUSSION

These results demonstrate that blood derived monocytes and macrophages from a patient with alveolar rhabdomyosarcoma were able to synthesize 24,25$(OH)_2D_3$ in short-term primary culture and 1α,25$(OH)_2D_3$ after 1, 4 and 6 weeks in culture. These cells were separated by standard density gradient centrifugation methods and by adherence in cell culture, and were characterized by well established histological methods. The labelled vitamin D_3 metabolites produced were also identified unequivocally.

This study represents the first observation of 24,25$(OH)_2D_3$ synthesis by monocyte-macrophage cells following only short-term culture. However, a previous study reported that in 3 out of 10 cultures of normal or sarcoid-activated macrophages, limited 24,25$(OH)_2D_3$ synthesis was induced by 1–100 nM 1α,25$(OH)_2D_3$ after 2–4 days in culture [4]. In another study γ-INF was used to stimulate normal macrophages to synthesize 1α,25$(OH)_2D_3$, but after several days 24, 25$(OH)_2D_3$ was the major product [9]. This change in 25(OH)D_3 metabolism was believed to be induced by an intracellular action of 1α,25$(OH)_2D_3$ on the two hydroxylases or the genes that regulate them.

Several previous studies have demonstrated that disease activated human macrophages expressed only 25(OH)D_3-1α-hydroxylase activity after either 24 h short-term culture [5, 6] or 2–7 day long-term culture [3–7]. Similarly, cultured blood-, alveolar- or bone marrow-derived normal macrophages, which synthesized small amounts of 1α,25$(OH)_2D_3$, significantly increased synthesis following exposure to γ-INF [4, 8, 9], LPS [4, 10] or TNF-α [11], synthesis which was partially inhibited by 1α,25$(OH)_2D_3$ [4]. In this respect only the macrophages maintained in long-term culture in this study behaved in a similar manner to normal or disease activated macrophages and synthesized 1α,25$(OH)_2D_3$. Furthermore, it took several days for a high concentration of 1,25$(OH)_2D_3$ to inhibit 25(OH)D_3-1α-hydroxylase activity and induce 25(OH)D_3-24-hydroxylase activity.

Alveolar rhabdomyosarcomas are tumours of soft tissues of viscera which are often difficult to diagnose and may metastasise to the breasts, ovaries or bone marrow or present with the characteristics of leukaemia [17, 18]. Hypercalcaemia is seen in a small proportion of these patients and usually occurs in association with bone metastases [19]. The patient in the present case had bone involvement, was hypercalcaemic and had low serum levels of 1α,25$(OH)_2D_3$ although the concentration of the precursor

25(OH)D_3 was normal. Thus the hypercalcaemia was not caused by raised 1α,25(OH)$_2D_3$; indeed, it is more likely that renal synthesis of 1α,25(OH)$_2D_3$ was suppressed by the raised calcium. However, serum 1α,25(OH)$_2D_3$ levels did not increase following correction of the hypercalcaemia, suggesting other mechanisms for reduced synthesis. The hypercalcaemia itself was controlled with APD which inhibits osteoclastic bone resorption [20–22] indicating that the raised calcium arose from this source. It is possible that the increased bone resorption was caused by parathyroid hormone-related peptide(s) (PTHrP) which are secreted in a humoral fashion by many tumors and are known to induce bone resorption [23], although this has not yet been demonstrated for alveolar rhabdomyosarcomas. The APD also appeared to reduce the ability of blood derived cells to thrive in culture indicating that the APD may have affected the development of monocyte precursors.

Overall, the data suggest that monocytes or immature macrophages can express 25(OH)D_3-24-hydroxylase activity *in vitro* before they differentiate into cells that express 25(OH)D_3-1α-hydroxylase activity. The significance of the initial ability of the cells to synthesize 24,25(OH)$_2D_3$ is not clear since the biological function of this metabolite is unknown. However, expression of this enzyme often correlates with expression of receptors for 1α,25(OH)$_2D_3$ [2], although this was likely it was not tested. In the human promyelomonocytic leukaemia cell-line (HL60) which does express receptors, 1–100 nM 1α,25(OH)$_2D_3$ induces expression of the 25(OH)D_3-24-hydroxylase [24], which may form 1α,24,25(OH)$_3D_3$ and represent the initial step in the metabolic inactivation of 1α,25(OH)$_2D_3$ to calcitroic acid [25]. In the present case 1α,25(OH)$_2D_3$ is unlikely to be the primary stimulus for expression of 25(OH)D_3-24-hydroxylase by the monocyte-macrophage cells since the patient had serum 1α,25(OH)$_2D_3$ levels of 4–5 pg/ml (0.01 nM) which are probably too low to induce this activity. However, the ability of the cells to synthesize 24,25(OH)$_2D_3$ must have been related in some way to the rhabdomyosarcoma or to the hypercalcaemia, since macrophages from patients with other disorders like arthritis [6] or a history of peritonitis [5] did not synthesize this metabolite after short-term primary culture. The induction of 25(OH)D_3-1α-hydroxylase activity following culture was probably a function of increased maturity and activation of the macrophages which were characterised by increased size, multinucleation and expression of non-specific esterase activity. Although 1α,25(OH)$_2D_3$ inhibited its own synthesis and induced that of 24,25(OH)$_2D_3$, this effect was observed with a high concentration of 1,25(OH)$_2D_3$ (10 nM) and was relatively slow in onset.

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REFERENCES

1. Haussler M. R. and McCain T. A.: Basic and clinical concepts related to vitamin D metabolism and action. *New Engl. J. Med.* **297** (1977) 974–983.
2. Reichel H., Koeffler P. H. and Norman A. W.: The role of the vitamin D endocrine system in health and disease. *New Engl. J. Med.* **320** (1989) 980–991.
3. Adams J. S., Sharma O. P., Gacad M. A. and Singer F. R.: Metabolism of 25-hydroxyvitamin D_3 by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* **72** (1983) 1856–1860.
4. Reichel H., Koeffler H. P., Barbers R. and Norman A. W.: Regulation of 1,25-dihydroxyvitamin D_3 production by cultured alveolar macrophages from normal human donors and from patients with pulmonary sarcoidosis. *J. Clin. Endocr. Metab.* **65** (1987) 1201–1209.
5. Hayes M. E., O'Donoghue D. J. O., Ballardie F. W. and Mawer E. B.: Peritonitis induces the synthesis of 1α,25-dihydroxyvitamin D_3 in macrophages from CAPD patients. *FEBS Lett.* **220** (1987) 307–310.
6. Hayes M. E., Denton J., Freemont A. J. and Mawer E. B.: Synthesis of the active metabolite of vitamin D, 1,25(OH)$_2D_3$, by synovial fluid macrophages in arthritic disease. *Ann. Rheum. Dis.* **48** (1989) 723–9.
7. Cadranel J., Hance A. J., Milleron B., Paillard F., Akoun G. M. and Garabedian M.: Vitamin D metabolism in tuberculosis: Production of 1,25(OH)$_2D_3$ by cells recovered by bronchoalveolar lavage and the role of this metabolite in calcium homeostasis. *Am. Rev. Respir. Dis.* **138** (1988) 984–9.
8. Koeffler H. P., Reichel H., Bishop J. E. and Norman A. W.: Gamma-interferon stimulates production of 1,25-dihydroxyvitamin D_3 by normal human macrophages. *Biochem. Biophys. Res. Commun.* **127** (1985) 596–603.
9. Reichel H., Koeffler H. P. and Norman A. W.: Synthesis *in vitro* of 1α,25-dihydroxyvitamin D_3 and 24,25-dihydroxyvitamin D_3 by interferon-gamma-stimulated normal human bone marrow and alveolar macrophages. *J. Biol. Chem.* **262** (1987) 10931–10937.
10. Reichel H., Koeffler P., Bishop J. E. and Norman A. W.: 25-hydroxyvitamin D_3 metabolism by lipopolysaccharide-stimulated normal human macrophages. *J. Clin. Endocr. Metab.* **64** (1987) 1–9.
11. Pryke A. M., Duggan C., White C. P., Posen S. and Mason R. S.: Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. *J. Cell. Physiol.* **142** (1990) 652–656.

12. Davies M., Mawer E. B., Hayes M. E. and Lumb G. A.: Abnormal vitamin D metabolism in Hodgkin's lymphoma. *Lancet* **i** (1985) 1186–1188.
13. Rosenthal N., Insogna K. L., Godsall J. W., Smaldone L., Waldron J. A. and Stewart A. F.: Elevations in circulating 1,25-Dihydroxyvitamin D_3 in three patients with lymphoma-associated hypercalcaemia. *J. Clin. Endocr. Metab.* **60** (1985) 29–33.
14. Mawer E. B., Hann J. T., Berry J. L. and Davies M.: Vitamin D metabolism in patients intoxicated with ergocalciferol. *Clin. Sci.* **68** (1985) 135–41.
15. Freemont A. J. and Davies J. S.: Acid esterase activity in lymphocytes and other cells: a comparison of six alpha-napthyl based substrates. *Med. Lab. Sci.* **39** (1982) 405–407.
16. Provvedini D. M., Deftos L. J. and Manolagas S. C.: 1,25-Dihydroxyvitamin D_3 promotes *in vitro* morphologic and enzymatic changes in normal human monocytes consistent with their differentiation into macrophages. *Bone* **7** (1986) 23–28.
17. Young R. H. and Scully R. E.: Alveolar rhabdomyosarcoma metastatic to the ovary. A report of two cases and a discussion of the differential diagnosis of small cell malignant tumors of the ovary. *Cancer* **64** (1989) 899–904.
18. Nunez C., Abboud S. L., Lemon N. C. and Kemp J. A.: Ovarian rhabdomyosarcoma presenting as leukaemia. *Cancer* **52** (1983) 297–300.
19. Leblanc A., Caillaud J. M., Hartmann O., Kalifa C., Flamant F., Patte C., Tournade M. F. and Lemerle J.: Hypercalcaemia preferentially occurs in unusual forms of childhood non-hodgkin's lymphoma, rhabdomyosarcoma, and Wilms' tumor: a study of 11 cases. *Cancer* **54** (1984) 2132–2136.
20. Mundy G. R., Wilkinson R., Heath D. A.: Comparative study of available medical therapy for hypercalcaemia of malignancy. *Am. J. Med.* **74** (1983) 421–432.
21. Sleeboom H. P., Bijvoet O. L. M., van Oosterom A. T., Gleed J. H. and O'Riordan J. L. H.: Comparison of intravenous (3-amino-1-hydroxypropylidene)-1-bisphosphonate and volume repletion in tumour-induced hypercalcaemia. *Lancet* **ii** (1983) 239–243.
22. Lowik C. W., Boonekamp P. M., van de Pluym G., van de Wee-Pals L., Bloys van Treslong-de Groot H. and Bijvoet O. L.: Bisphosphonates can reduce osteoclast bone resorption by two different mechanisms. *Adv. Exp. Med Biol.* **208** (1986) 275–281.
23. Mundy G. R.: Hypercalcaemia of malignancy revisited. *J. Clin. Invest.* **82** (1986) 1–6.
24. Hayes M. E., Bayley D. and Mawer E. B.: Metabolism of 25-hydroxyvitamin D_3 to 24,25-dihydroxyvitamin D_3 and its sensitivity to ketoconazole in 1α,25-dihydroxyvitamin D_3-differentiated HL60 cells. *J. Molec. Endocr.* **3** (1989) 199–205.
25. Reddy G. S. and Tserng K-Y: Calcitroic acid, end product of renal metabolism of 1,25-dihydroxyvitamin D_3 through C-24 oxidation pathway. *Biochemistry* **28** (1989) 1763–1769.