# Differential metabolism of 25-hydroxyvitamin D₃ by cultured synovial fluid macrophages and fibroblast-like cells from patients with arthritis

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## Abstract
Differential metabolism of 25-hydroxyvitamin D₃ (25(OH)D₃) has been shown for macrophages and fibroblast-like cells (possibly synoviocytes) cultured for two to 50 days after isolation from the synovial fluid of 12 patients with various forms of inflammatory arthritis. Macrophages synthesised the active metabolite of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the synthesis of which was increased by bacterial lipopolysaccharide, a known macrophage activating factor. In contrast, fibroblast-like cells formed 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), synthesis of which was stimulated by 1,25(OH)₂D₃ and inhibited by lipopolysaccharide. The synthesis of 1,25(OH)₂D₃ and 24,25(OH)₂D₃ by macrophages and fibroblast-like cells respectively was inhibited by ketoconazole, indicating that both hydroxylases are dependent on cytochrome P-450.

Mean (SEM) synovial fluid and serum 25(OH)D₃ concentrations were 16·7 (1·7) and 22·2 (2·6) ng/ml and those of 1,25(OH)₂D₃ were 29·4 (4·8) and 43·3 (4·0) pg/ml respectively. In most cases concentrations were lower in synovial fluid than in paired serum samples, but in two patients 1,25(OH)₂D₃ concentrations were greater in synovial fluid than in serum, suggesting local synthesis within the affected joints.

Vitamin D₃ is hydroxylated by cytochrome P-450 dependent enzymes to 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver and then to 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) or 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) in the kidney. Both 25(OH)D₃ and 24,25(OH)₂D₃ have limited biological effect on calcium homeostasis, whereas 1,25(OH)₂D₃ promotes intestinal calcium absorption, stimulates osteoblast mediated bone growth and mineralisation, increases osteoclastic bone resorption, inhibits renal 25(OH)D₃-1α-hydroxylase activity, and increases renal 25(OH)D₃-24-hydroxylase activity.[1][2] All these actions seem to be induced after binding of 1,25(OH)₂D₃ to specific intracellular receptors and formation of a hormone-receptor complex that selectively activates or suppresses the transcription of various genes.

Studies on the extrarenal metabolism of 25(OH)D₃ have shown that 1,25(OH)₂D₃ is also synthesised in vitro by normal human macrophages activated with γ interferon,[3] bacterial lipopolysaccharides,[4] or tumour necrosis factor α,[5] and by alveolar macrophages from patients with granulomatous conditions, such as sarcoidosis.[6] In sarcoidosis this synthesis appears to be dependent on substrate and is not homeostatically controlled, in contrast with the well regulated renal synthesis of 1,25(OH)₂D₃ by parathyroid hormone, calcium, and 1,25(OH)₂D₃.[1][2] Our own studies have shown that macrophages, activated by peritonitis, from the peritoneal cavity of patients with renal failure receiving continuous ambulatory peritoneal dialysis[7] and macrophages cultured from synovial fluid of patients with arthritis also synthesise 1,25(OH)₂D₃.[8]

Receptor binding studies on samples from patients with rheumatoid arthritis have shown that peripheral blood lymphocytes and fibroblasts derived from the synovial tissue express specific receptors for 1,25(OH)₂D₃[9][10] but the role of the metabolite in these cells is not known.

Studies on the synthesis of 24,25(OH)₂D₃ in cultured cells have shown that this metabolite is occasionally formed by macrophages[11][12] and is almost invariably synthesised by fibroblasts.[13–15] In both cell types 24,25(OH)₂D₃ synthesis also seems to be increased by 1,25(OH)₂D₃.

As colonies of fibroblast-like cells may form in long term cultures of macrophages derived from synovial fluid we carried out this study to establish whether these two cell types express differential metabolism of 25(OH)D₃. We also measured 25(OH)D₃ and 1,25(OH)₂D₃ concentrations in paired serum and synovial fluid samples from the patients from whom cells were obtained.

## Patients and methods
Samples of synovial fluid were obtained from 12 patients with knee joint effusions attending the rheumatology clinics of two local hospitals. Patients were assigned to well recognised diagnostic groups on the basis of clinical criteria and synovial fluid total and differential white cell counts (see table), supplemented by appropriate immunological, radiological, and crystallographic investigations. Only two patients with symmetrical polyarthritis or oliogoarthropathies could not be assigned to a specific diagnostic group and were therefore classified as nonspecific inflammatory arthritis.

Cells were harvested from synovial fluid (10–70 ml) by centrifugation at 2000 g for 15 minutes followed by resuspension in RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol/l glutamine, and 50 μg/ml streptomycin (Flow Laboratories) to give about 10×10⁶ cells/ml. Cells were then plated at a density of 5×10⁶ cells per well (6–24 wells per

## Summary of patient data

| Sample | Diagnosis | Sex | Age | Synovial fluid white cell count (×10⁹/l) | White cell differential ratios (%) | | | | 25(OH)D₃ (ng/ml) | | 1,25(OH)₂D₃ (pg/ml) | | Principal cell type present in culture | Day of culture on which cells were incubated (inc) with [³H]25(OH)D₃ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Poly* | Lymph* | Macro* | Synov* | Serum | SF* | Serum | SF | | |
| A | Reactive arthritis (Reiter's) | M | 46 | 3·12 | 37 | 23 | 39 | <1 | 29·9 | 24·9 | 30·0 | 16·0 | Macrophages, Fibroblasts | 15 (24 h inc, fig 1a) 15 (24 h inc, fig 1b) |
| B | Rheumatoid arthritis+ osteoarthritis | F | 26 | 4·20 | 13 | 77 | 9 | <1 | 18·8 | 11·1 | 49·0 | 15·0 | Mixed | 14 (5 h inc, figs 2, 4) |
| C | Non-specific inflammatory arthritis | F | 70 | 3·30 | 46 | 39 | 13 | 2 | 16·4 | 13·7 | 30·0 | 21·0 | Macrophages | 7 (5 h inc, fig 2) |
| D | Rheumatoid arthritis | M | 70 | 25·48 | 82 | 16 | 1 | <1 | 20·5 | 15·5 | 51·2 | 11·5 | Macrophages, Fibroblasts | 13 (6 h inc, figs 2, 8) 50 (6 h inc, fig 7) |
| E | Rheumatoid arthritis | F | 78 | 10·04 | 75 | 23 | 2 | 0 | 4·1 | 3·9 | 37·0 | 24·0 | Mixed | 6 (5 h inc, figs 2, 4) |
| F | Rheumatoid arthritis | F | 24 | 10·40 | 92 | 7 | 1 | 0 | 24·9 | 19·5 | 66·0 | 43·0 | Mixed Mixed | 7 (6 h inc, fig 6) 16 (6 h inc, fig 2) |
| G | Hypertrophic osteoarthritis | F | 68 | <0·10 | — | — | — | — | 27·8 | 17·8 | 62·0 | 27·0 | Mixed, Fibroblasts | 14 (6 h inc, fig 2) 24 (5 h inc, fig 5) |
| H | Rheumatoid arthritis | F | 73 | 2·08 | 86 | 13 | 1 | 0 | 27·4 | 19·7 | 20·0 | 17·0 | Macrophages, | 11 (6 h inc, fig 2) |
| I | Rheumatoid arthritis | F | 63 | 25·62 | 94 | 3 | 3 | 0 | 14·9 | 11·2 | 42·0 | 23·0 | Macrophages | 17 (5 h inc, fig 2) |
| J | Non-specific inflammatory arthritis | M | 56 | 12·42 | 6 | 80 | 11 | 3 | — | — | — | — | Fibroblasts | 19 (6 h inc, fig 5) |
| K | Juvenile chronic arthritis | F | 21 | 42·40 | 98 | 1 | 1 | 0 | 35·1 | 24·9 | 37·0 | 38·0 | Macrophages | 2 (6 h inc, fig 6) |
| L | Reactive arthritis (Reiter's, Still's) | M | 17 | | | | | | 24·1 | | 48·0 | | Macrophages, | 6/14 (6 h inc, fig 3) |
| | (left knee) | | | 24·50 | 61 | 32 | 7 | 0 | | 17·7 | | 61·0 | | |
| | (right knee) | | | 25·80 | 75 | 18 | 7 | 0 | | 18·5 | | 57·0 | | |

*Poly=polymorph; lymph=lymphocyte; macro=macrophage; synov=synoviocyte; SF=synovial fluid.

sample) in six-well multidishes (Nunc) and cultured overnight in an atmosphere of 95% air/ 5% $CO_2$ at 37°C. Next day non-adherent polymorphs, lymphocytes, and monocytes were decanted, leaving adherent macrophages and colony forming synoviocyte/fibroblast-like cells, which were cultured in 10 ml of fresh RPMI-1640 medium. Cells were used for experiments after two to 50 days when it was apparent which cell types were present. The ability of macrophages and fibroblast-like cells to metabolise 25(OH)D₃ was examined first and then the effects of culturing cells with lipopolysaccharide (0·5–100 µg/ml) for three to 11 days or with 1,25(OH)₂D₃ ($10^{-11}$–$10^{-7}$ mol/l) for three or seven days on the synthesis of 24,25(OH)₂D₃ or 1,25(OH)₂D₃ were tested. In addition, we tested the effects of adding ketoconazole ($10^{-9}$–$10^{-6}$ mol/l), a cytochrome $P$-450 enzyme inhibitor (Sigma), to the assays of 25(OH)D₃-hydroxylase activity.

The assays of 25(OH)D₃ metabolism were carried out in 2 ml of serum free RPMI-1640 incubation medium containing 1·5 mg/ml of bovine serum albumin (BSA; Immuno Diagnostika), 50 000 dpm [³H]25(OH)D₃ (6·5 TBq/mmol; Amersham International), 0·125 nmol 25(OH)D₃, (25 ng/ml; Roussel UCLAF) for five, six, or 24 hours (see table) in an atmosphere of 95% air/5% $CO_2$ at 37°C. Incubations were carried out in triplicate and were stopped by mixing the medium with 3 ml of chloroform and 2 ml of methanol, which had been used to rinse and fix the cells. The extracts were centrifuged at 500 $g$ for 20 minutes at 4°C and the lower chloroform layer containing vitamin D₃ metabolites removed, dried under a stream of nitrogen gas, resuspended in 1 ml of ethanol and stored at −20°C. Each chloroform extract was analysed on a manual injection normal phase high performance liquid chromatography (HPLC) system developed with

a mobile phase of n-hexane: propan-2-ol: methanol, 110:6:4 by volume at 2 ml/min using a model 6000A HPLC solvent delivery pump (Waters Associates) and a Zorbax-Sil HPLC column (25 cm×4·6 mm; Du Pont). For each analysis eluent fractions were collected by a model 202 fraction collector (Gilson) and the radioactivity associated with the substrates 24,25(OH)₂D₃ and 1,25(OH)₂D₃ estimated by a 1217 Rackbeta liquid scintillation counter (LKB Wallac). Standard 25(OH)D₃, 24,25 (OH)₂D₃, and 1,25(OH)₂D₃ and their tritiated forms were also chromatographed, and the retention times of these markers determined by ultraviolet absorption at 265 nm by a model 440 absorbance detector (Waters Associates) with an AR 25 linear chart recorder (Pye Unicam) or by liquid scintillation counting respectively.

For each experiment the cell numbers were determined with a haemocytometer after trypsinisation of parallel cultures, and the mean of four determinations from each well was used to normalise 25(OH)D₃-24-hydroxylase and 25(OH)₃-1α-hydroxylase activity for cell number. Cytocentrifuge preparations were also prepared for identification of cell lineage, which was determined by their morphology in Giemsa stained preparations and their reaction for non-specific esterase using α-naphthyl propionate as substrate.[16]

Paired 3 ml serum and synovial fluid samples were also analysed for 25(OH)D₃ and 1,25(OH)₂D₃ as previously described in detail.[17] Briefly, samples for assay were mixed with 3 ml of acetonitrile, applied to C18 silica Sep-paks (Waters Associates), and vitamin D metabolites were eluted in acetonitrile. Further separation of the metabolites was achieved by normal phase HPLC developed with hexane: isopropanol:methanol, 110:6:4 by volume at 2 ml/min using an automatic sample injector (Waters Associates), a programmable fraction

collector (LKB Superac), and a model 745 data module (Waters Associates). The $25(OH)D_3$ fraction was reapplied to the automated HPLC system, developed with hexane:isopropanol, 98:2 by volume at 2 ml/min and the concentration determined from the height of the ultraviolet absorption peak recorded at 265 nm after calibration with a $25(OH)D_3$ standard (detection limit 2 ng/ml). The fraction containing $1,25(OH)_2D_3$ was assayed by radioimmunoassay with a detection limit of 3 pg/ml using an antiserum 02282.

Statistical analysis was carried out by Student's $t$ test and, where appropriate, $p<0.05$ or $p<0.01$ has been indicated as * or ** respectively.

## Results

The table gives details of the samples from 12 patients with arthritis (six with rheumatoid arthritis; two with reactive arthritis; one with juvenile chronic arthritis; two with non-specific inflammatory arthritis; two with osteoarthritis—one secondary to rheumatoid arthritis). Mean (SEM) concentrations of vitamin $D_3$ metabolites in serum and synovial fluid respectively were 22·2 (2·6) and 16·7 (1·7) ng/ml for $25(OH)D_3$ and 43·3 (4·0) and 29·4 (4·8) pg/ml ($p<0.05$) for $1,25(OH)_2D_3$. In all patients synovial fluid $25(OH)D_3$ was lower than in paired serum samples and only one serum sample had a low value ($<5$ ng/ml). In two patients (one with rheumatoid arthritis, one with hypertrophic osteoarthritis) serum $1,25(OH)_2D_3$ was above the normal reference range (18–56 pg/ml, mean 37 (2SD)) and in two patients (one with juvenile chronic arthritis, one with reactive arthritis) synovial fluid $1,25(OH)_2D_3$ concentrations were higher than in serum. The table also lists details of the cell types cultured from each synovial fluid sample and the number of days after isolation that incubations with $[^3H]25(OH)D_3$ were carried out.

The macrophage cultures were characterised by non-proliferating cells which initially had the morphology of mononucleated monocytes and immature macrophages but after several days in culture had the appearance of larger more mature macrophages, many of which were multinucleated (three to 25 nuclei). These cells also stained for non-specific esterase, an enzyme characteristically expressed by monocytes and macrophages.[16] In contrast, proliferating fibroblast-like cells could not readily be identified in early cultures; however, after a few days, rapidly growing colonies of these cells could be seen. It is most likely that the fibroblast-like cells developed from synoviocytes, which were identified in only five (A,B,C,D, and J) of the original 12 samples, four of which developed fibroblast colonies. Fibroblast-like cells also developed in samples F and G, of which sample G was from an osteoarthritic patient with a synovial fluid white cell count too low for a routine differential cell count to be carried out. However, osteoarthritic synovial fluid usually contains significant numbers of synoviocytes in addition to macrophages.
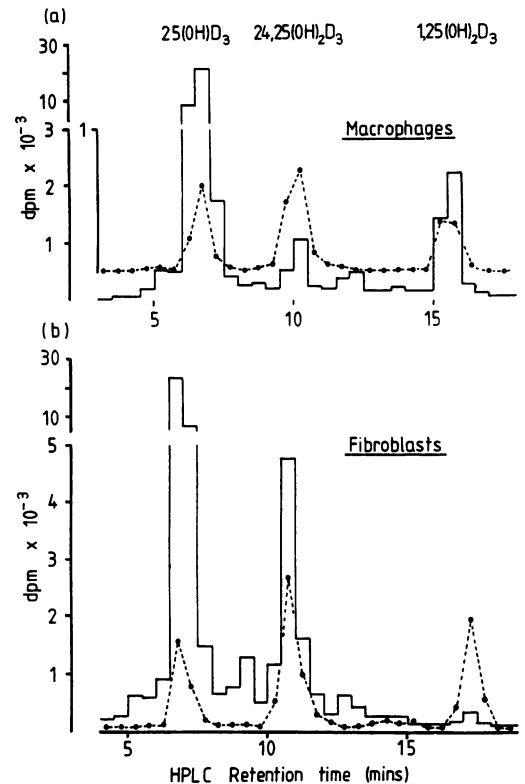
Figure 1a shows the HPLC analyses of



Figure 1  Metabolism of $[^3H]25(OH)D_3$ in a 24 hour in vitro incubation system by (a) 15 day cultures of macrophages derived from synovial fluid and (b) by fibroblasts from a patient with reactive arthritis (sample A). Bars represent the amount of tritiated material in eluent fractions obtained by high performance liquid chromatography (HPLC) of cell extracts on a Zorbax-Sil column developed with n-hexane:propan-2-ol:methanol (110:6:4). The dotted traces represent the HPLC of standard tritiated $25(OH)D_3$, $24,25(OH)_2D_3$, and $1,25(OH)_2D_3$, which were used as markers.

$[^3H]25(OH)D_3$ metabolism by cells cultured for 15 days after isolation from the synovial fluid of a patient with reactive arthritis (Reiter's disease; sample A). These cells were predominantly macrophages (many multinucleated) which formed $1,25(OH)_2D_3$, though some fibroblast-like cells were also present, accounting for some synthesis of $24,25(OH)_2D_3$. Figure 1b shows the synthesis of $24,25(OH)_2D_3$ by a 15 day culture of fibroblast-like cell colonies from the same sample. These cultures contained different cell types probably because the macrophages had been seeded from clumps of aggregated cells, whereas the fibroblast-like cells had developed from a suspension of single cells.

Figure 2 shows that exposure to lipopolysaccharide (0·5–100 µg/ml) for three to four days increased $1,25(OH)_2D_3$ synthesis in five cultures of macrophages (samples C, D, E, H, I) and three macrophage cultures that also contained significant numbers of fibroblast-like cells (samples B, F, G). For all samples, except sample E, this increase was significant ($p<0.01$). An experiment carried out using fibroblast-free macrophage cultures from both knees of a patient with reactive arthritis (sample L) showed that lipopolysaccharide (20 µg/ml; days 3–14) maintained high levels of $1,25(OH)_2D_3$ synthesis compared with cells cultured in the absence of lipopolysaccharide for 14 days (fig 3).

Figure 4 compares the effect of $1,25(OH)_2D_3$ $(10^{-10}-10^{-7}$ mol/l) or lipopolysaccharide (20 µg/ml) included in the culture medium for three days on $1,25(OH)_2D_3$ synthesis by synovial fluid cells from two patients with rheumatoid arthritis (one with secondary osteoarthritis). In cells from sample B (mixed culture) increasing concentrations of $1,25(OH)_2D_3$ in the medium decreased the formation of $1,25(OH)_2D_3$
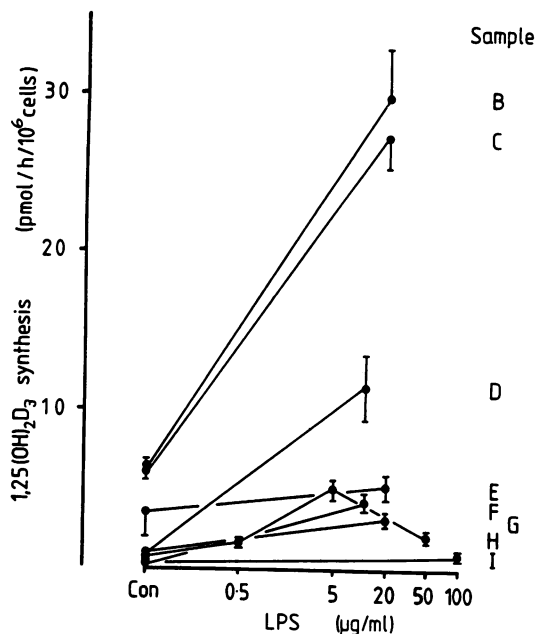


*Figure 2   Effect of bacterial lipopolysaccharide (LPS) after three or four days on the synthesis of $1,25(OH)_2D_3$ by macrophages which had been cultured for six to 17 days after isolation from the synovial fluid of eight patients with various forms of arthritis. Values are the means (SEM) of three determinations (see table for further details). For each sample, except sample E, synthesis was significantly increased compared with controls ($p<0.01$). Con=control.*
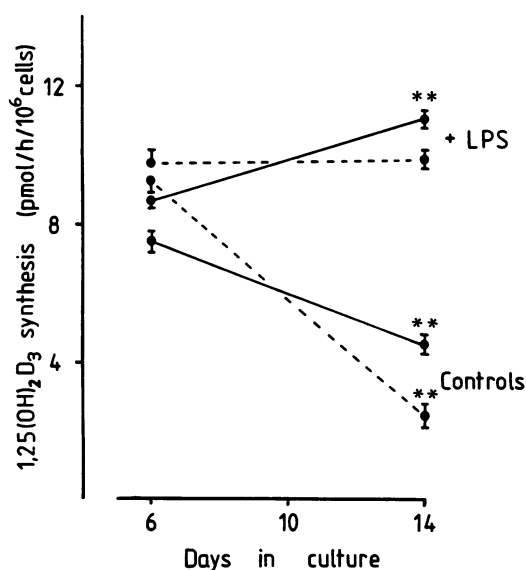


*Figure 4   Effect of bacterial lipopolysaccharide (LPS) (●---●) or $1,25(OH)_2D_3$ (●———●), incubated with macrophages for three days, on the synthesis of $1,25(OH)_2D_3$ after 12 days in culture following isolation from the synovial fluid of two patients with rheumatoid arthritis. Values are the means (SEM) of three determinations ($*p<0.05$; $**p<0.01$); see table for further details. Con=control.*

compared with control cells cultured in the absence of the hormone ($p<0.05$), where lipopolysaccharide greatly increased $1,25(OH)_2D_3$ synthesis ($p<0.01$). In macrophages (fibroblast-free) cultured from sample E neither $1,25(OH)_2D_3$ nor lipopolysaccharide altered $1,25(OH)_2D_3$ synthesis; in addition, neither factor induced $24,25(OH)_2D_3$ synthesis. In contrast, $1,25(OH)_2D_3$ was a potent stimulator of $24,25(OH)_2D_3$ synthesis by fibroblast-like cells in three samples which included sample B (mixed culture) and samples J and G, which were macrophage-free (fig 5). In two of these



*Figure 3   Synthesis of $1,25(OH)_2D_3$ by synovial fluid macrophages cultured for six and 14 days after isolation from the left (●———●) and right (●---●) knees of a patient with reactive arthritis (sample L). Cells were cultured with and without (controls) bacterial lipopolysaccharide (LPS) from day 3. Values are the means (SEM) of three determinations ($** p<0.01$).*
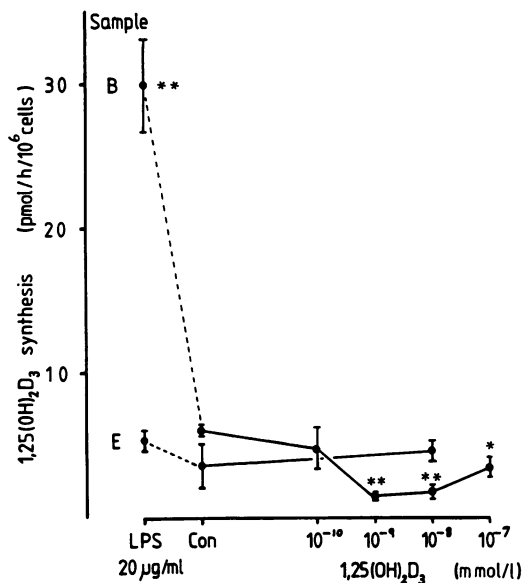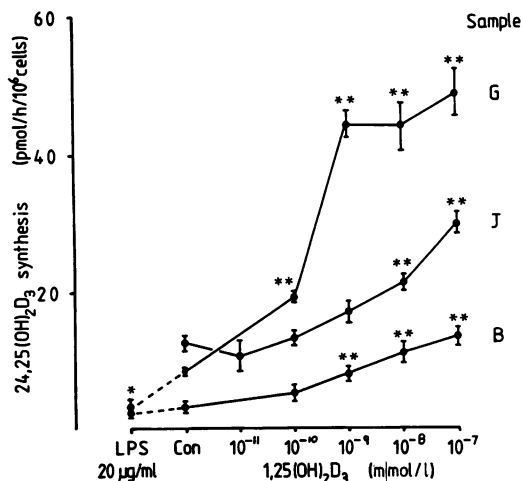


*Figure 5   Effect of bacterial lipopolysaccharide (LPS) after three days (●---●) or $1,25(OH)_2D_3$ (●———●) after three or seven days on the synthesis of $24,25(OH)_2D_3$ by fibroblast-like cells which had been cultured for 14–24 days after isolation from the synovial fluid of three patients with various forms of arthritis. Values are the means (SEM) of three determinations ($*p<0.05$; $**p<0.01$); see table for further details. Con=control.*
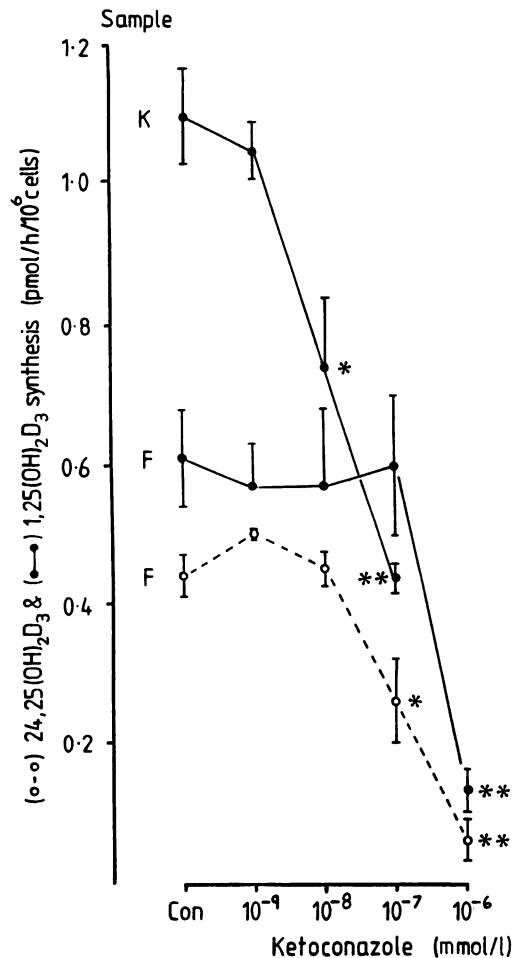
Figure 6   Effect of ketoconazole present during the six hour incubation with $25(OH)D_3$ on $1,25(OH)_2D_3$ (●————●) or $24,25(OH)_2D_3$ (○———○) synthesis by macrophages and fibroblast-like cells, respectively, cultured for two and seven days after isolation from the synovial fluid of two patients with arthritis. Values are the means (SEM) for three determinations (*p<0·05; **p<0·01); see table for further details. Con=control.



Figure 7   Effect of ketoconazole present during the six hour incubation with $25(OH)D_3$ on $24,25(OH)_2D_3$ synthesis by fibroblast-like cells which had been maintained in culture for 50 days and treated with $1,25(OH)_2D_3$ from day 47 after isolation from the synovial fluid of a patient with rheumatoid arthritis (sample D). Values are the means (SEM) of three determinations (**p<0·01). Con=control.



Figure 8   Effect of ketoconazole present during the six hour incubation with $25(OH)D_3$ on $1,25(OH)_2D_3$ synthesis by macrophages that had been cultured for 13 days and treated with bacterial lipopolysaccharide (LPS) from day 8 after isolation from the synovial fluid of a patient with rheumatoid arthritis (sample D). Values are means (SEM) of three determinations (*p<0·05; **p<0·01). Con=control.

samples (B and G) lipopolysaccharide reduced $24,25(OH)_2D_3$ synthesis.

Figure 6 shows the inhibition of $1,25(OH)_2D_3$ synthesis by macrophages (sample K) and of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ synthesis by a mixed culture (sample F) by the drug ketoconazole, a cytochrome $P$-450 enzyme inhibitor. Similarly, figs 7 and 8 show ketoconazole inhibition of $1,25(OH)_2D_3$ potentiated $24,25(OH)_2D_3$ synthesis by fibroblasts and lipopolysaccharide induced synthesis of $1,25(OH)_2D_3$ by macrophages (sample D) respectively.

## Discussion

We examined the metabolism of $25(OH)D_3$ by cultured macrophages and fibroblast-like cells and confirmed our original observation that synovial fluid derived macrophages synthesise the active vitamin D metabolite, $1,25(OH)_2D_3$, in vitro.[8] In addition, we showed that $1,25(OH)_2D_3$ is formed from physiological concentrations (25 ng/ml) of the substrate. We also showed for the first time that synovial fluid derived synoviocyte/fibroblast-like cells can synthesise $24,25(OH)_2D_3$, though previous
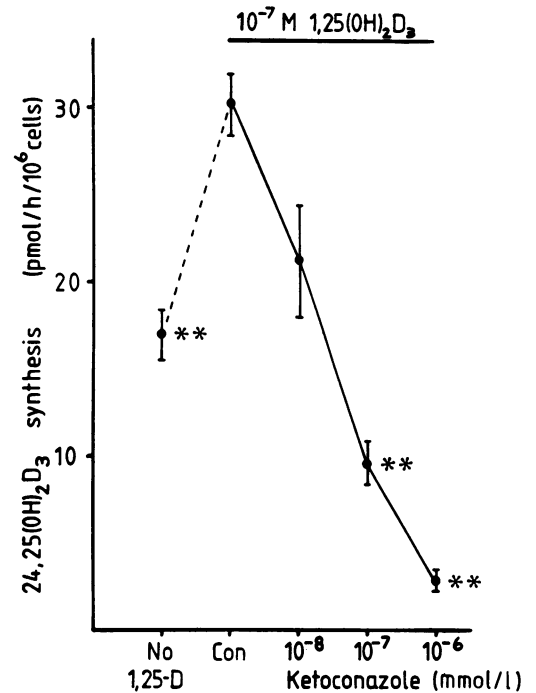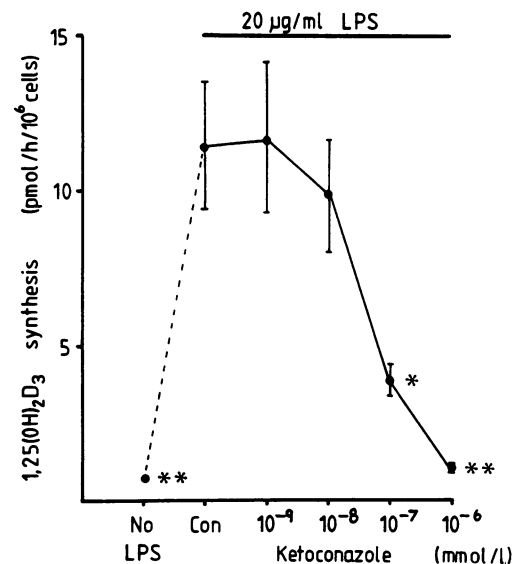
studies in vitro have shown synthesis of $24,25(OH)_2D_3$ by articular cartilage and by cultured chondrocytes.[18] We showed that both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ are formed in mixed cultures of synovial fluid cells containing macrophages and fibroblast-like cells, with the predominant metabolite relating to the cell type present. Cultures containing only fibroblast-like

cells, however, did not synthesise $1,25(OH)_2D_3$, and cultures containing only macrophages did not synthesise $24,25(OH)_2D_3$. In contrast, other studies have shown that macrophages from a patient with alveolar rhabdomyosarcoma[11] and macrophages derived from normal human blood and bone marrow can synthesise $24,25(OH)_2D_3$.[12] In the latter study, however, the cells were cultured with γ-interferon and initially formed $1,25(OH)_2D_3$ before synthesising $24,25(OH)_2D_3$ after prolonged exposure.[12] Previous studies have also shown that lipopolysaccharide stimulates macrophages to form $1,25(OH)_2D_3$[4] and that $1,25(OH)_2D_3$ stimulates fibroblasts to form $24,25(OH)_2D_3$,[13-15] but synovial fluid cells have not previously been investigated. We have now shown similar effects for lipopolysaccharide and $1,25(OH)_2D_3$ on $25(OH)D_3$ metabolism in synovial fluid macrophages and fibroblast-like cells respectively. In our study it was also apparent that lipopolysaccharide reduced fibroblast $24,25 (OH)_2D_3$ synthesis, but the effect of $1,25(OH)_2D_3$ on its own synthesis by macrophages was less clear as synthesis was inhibited in one sample and unchanged in another. We also showed that synthesis of both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ was inhibited in a dose dependent manner by ketoconazole, cytochrome $P$-450 inhibitor,[19][20] indicating that the $25(OH)D_3$-24-hydroxylase and the $25(OH)D_3$-1α-hydroxylase are both enzymes dependent on cytochrome $P$-450.

The assays of $25(OH)D_3$ concentrations were lower in all synovial fluid samples than in paired serum samples, with mean (SEM) values of 16·7 (1·7) and 22·2 (2·6) ng/ml respectively. This observation has previously been reported in other studies,[21][22] one of which also showed that both $24,25(OH)_2D_3$ and the vitamin D binding protein were present in synovial fluid at about half serum concentrations.[21] The lower concentration of vitamin D binding protein in synovial fluid is the most likely cause of the lower $25(OH)D_3$ concentrations in synovial fluid than in serum. $1,25(OH)_2D_3$ concentrations were also lower in most synovial fluid samples, with mean (SEM) values of 29·4 (4·8) and 43·3 (4·0) pg/ml ($p<0·05$) for synovial fluid and serum respectively. The exceptions were one patient with juvenile chronic arthritis, in whom synovial fluid and serum concentrations were similar, and a patient with Reiter's disease, in whom the synovial fluid of both left and right knees had greater $1,25(OH)_2D_3$ concentrations than in serum. Reiter's disease is a reactive arthritis, previously shown to be the most likely form to have synovial fluid macrophages capable of forming $1,25(OH)_2D_3$,[8] and thus the raised concentrations of $1,25(OH)_2D_3$ were probably synthesised locally by activated macrophages present in the synovial fluid. In two other patients (one with rheumatoid arthritis, one with hypertrophic osteoarthritis) serum but not synovial fluid $1,25(OH)_2D_3$ concentrations were above the normal reference range (18–56 pg/ml). In these patients the high serum values were probably not related to the ability of cells within synovial fluid to form $1,25(OH)_2D_3$ but to the more mature activated macrophages

likely to be found within synovial tissue itself. Overall, the determinants of serum and synovial fluid $1,25(OH)_2D_3$ concentrations may be severalfold and include: the synovial fluid vitamin D binding protein concentration, the numbers and activity of monocytes and macrophages entering synovial fluid and the joint tissue, the rate of synovial fluid turnover and equilibration of locally synthesised $1,25(OH)_2D_3$ with serum, and the effect of drugs used in the treatment of arthritis on the metabolism of $25(OH)D_3$.

Further evidence that extrarenal synthesis of $1,25(OH)_2D_3$ may occur in patients with rheumatoid arthritis is provided by a study in which the serum $1,25(OH)_2D_3$ response to a challenge dose of $25(OH)D_3$ was significantly greater in patients than in healthy controls.[22] In the latter study two patients also had initial serum $1,25(OH)_2D_3$ concentrations above normal and two patients had synovial fluid $1,25(OH)_2D_3$ concentrations greater than those in paired serum samples. In the few studies published to date, however, there is no evidence that patients with rheumatoid arthritis as a group have higher than normal circulating concentrations of $1,25(OH)_2D_3$ as a result of net renal and extrarenal synthesis.[22][23] This may in part result from feedback regulation of the renal $1,25(OH)_2D_3$ synthesis by that formed outside the kidney. Synthesis of $1,25(OH)_2D_3$ by macrophages, which is poorly regulated and seems to depend on the substrate concentration, may only result in raised serum concentrations when the $25(OH)D_3$ concentrations are abnormally high or the mass of tissue affected is large, as in some cases of sarcoidosis[24] and malignant disease.[25]

The presence of specific receptors for $1,25(OH)_2D_3$ in normal human monocytes and activated lymphocytes[26][27] and in peripheral blood lymphocytes[9] and synovial tissue derived fibroblasts[10] from patients with rheumatoid arthritis is consistent with an immunological role for $1,25(OH)_2D_3$ in joint disease. Many of the reported receptor mediated effects of $1,25(OH)_2D_3$ may be relevant to arthritis; for example, this metabolite seems to inhibit proliferation of both B and T activated lymphocytes and to reduce interleukin 2 production by activated T lymphocytes in vitro.[28][29] $1,25(OH)_2D_3$ has also been shown to promote differentiation of monocytes into macrophages and the fusion of macrophages to multinucleated giant cells with bone resorbing activity.[30][31] This is in addition to the known ability of $1,25(OH)_2D_3$ to inhibit collagen synthesis by osteoblasts and to promote bone resorption.[32] The latter may be achieved by direct action on the differentiation of preosteoclasts or by indirect action of $1,25(OH)_2D_3$ on osteoblasts to induce bone resorption by mature multinucleated osteoclasts, which unlike osteoblasts do not seem to have receptors for $1,25(OH)_2D_3$.[33] Thus within the synovial fluid and synovium $1,25(OH)_2D_3$ may have complex paracrine and immunoregulatory functions which may be highly relevant to the joint disease and to the localised periarticular osteoporosis associated with arthritis.[34][35] Control of its synthesis and

elucidation of its action in the arthritic joint clearly warrant further study.

1 Haussler M R, McCain T A. Basic and clinical concepts related to vitamin D metabolism and action. *N Engl J Med* 1977; 297: 974–83.

2 Reichel H, Koeffler H P, Norman A W. The role of the vitamin D endocrine system in health and disease. *N Engl J Med* 1989; 320: 980–91.

3 Koeffler H P, Reichel H, Bishop J E, Norman A W. Gamma-interferon stimulates production of 25-dihydroxyvitamin D$_3$ by normal human macrophages. *Biochem Biophys Res Commun* 1985; 127: 596–603.

4 Reichel H, Koeffler H P, Bishop J E, Norman A W. 25-Hydroxy vitamin D$_3$ metabolism by lipopolysaccharide-stimulated normal human macrophages. *J Clin Endocrinol Metab* 1987; 64: 1–9.

5 Pryke A M, Duggan C, White C P, Posen S, Mason R S. Tumor necrosis factor-alpha induces vitamin D-1-hydroxylase activity in normal human alveolar macrophages. *J Cell Physiol* 1990; 142: 652–6.

6 Adams J S, Sharma O P, Gacad M A, Singer F R. Metabolism of 25-dihydroxyvitamin D$_3$ by cultured pulmonary alveolar macrophages in sarcoidosis. *J Clin Invest* 1983; 72: 1856–60.

7 Hayes M E, O'Donoghue D J O, Ballardie F W, Mawer E B. Peritonitis induces the synthesis of 1α, 25-dihydroxyvitamin D$_3$ in macrophages from CAPD patients. *FEBS Lett* 1987; 220: 307–10.

8 Hayes M E, Denton J, Freemont A J, Mawer E B. Synthesis of the active metabolite of vitamin D, 1,25(OH)$_2$D$_3$, by synovial fluid macrophages in arthritic disease. *Ann Rheum Dis* 1989; 48: 723–9.

9 Manolagas S C, Werntz D A, Tsoukas C D, Provvedini D M, Vaughan J H. 1,25-Dihydroxyvitamin D$_3$ receptors in lymphocytes from patients with rheumatoid arthritis. *J Lab Clin Med* 1986; 108: 596–600.

10 Sambrook P N, Eisman J A, Breit S. 1,25-Dihydroxyvitamin D$_3$ receptors in synovial cells in rheumatoid arthritis [abstract]. *Aust N Z J Med* 1987; 17: 120.

11 Hayes M E, Bayley D, Drayson M, et al. 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. *Journal of Steroid Biochemistry and Molecular Biology*. 1991; 38: 301–6.

12 Reichel H, Koeffler P H, Norman A W. Synthesis in vitro of 1,25-dihydroxyvitamin D$_3$ and 24,25-dihydroxyvitamin D$_3$ by interferon-gamma-stimulated normal human bone marrow and alveolar macrophages. *J Biol Chem* 1987; 262: 10931–7.

13 Gamblin G T, Liberman U A, Eil C, Donns Jr D, Degrange D A, Marx S J. Vitamin D dependent rickets type II-defective induction of 25-hydroxyvitamin D-24-hydroxylase by 1,25-dihydroxyvitamin D$_3$ in cultured skin fibroblasts. *J Clin Invest* 1985; 75: 954–60.

14 Cancela L, Rebut-Bonneton C. Regulation of 24-hydroxylase activity in mouse skin fibroblasts by cholecalciferol derivatives, triamcinolone acetonide and a calcium modulating agent, nicardipine. *J Steroid Biochem* 1987; 28: 479–84.

15 Barsony J, McKoy W, DeGrange D A, Lieberman U A, and Marx S J. Selective expression of a normal action of the 1,25-dihydroxyvitamin D$_3$ receptor in human skin fibro-
blasts with hereditary severe defects in multiple actions of that receptor. *J Clin Invest* 1989; 83: 2093–101.

16 Freemont A J, Davies J S. Acid esterase activity in lymphocytes and other cells: a comparison of six alpha-naphthyl based substrates. *Med Lab Sci* 1982; 39: 405–7.

17 Mawer E B, Hann J T, Berry J L, Davies M. Vitamin D metabolism in patients intoxicated with ergocalciferol. *Clin Sci* 1985; 68: 135–41.

18 Garabedian M, Leiberherr M, N'Guyen T M, Corvol M T, Bailly Du Bois M, Balsan S. The in vitro production and activity of 24,25-dihydroxycholecalciferol in cartilage and calvarium. *Clin Orthop* 1978; 135: 241–8.

19 Loose D S, Kan P B, Hirst M A, Marcus R A, Feldman D. Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P-450 dependent enzymes. *J Clin Invest* 1983; 71: 1495–9.

20 Henry H. Effect of ketoconazole and miconazole on 25-dihydroxyvitamin D$_3$ metabolism by cultured chick kidney cells. *J Steroid Biochem* 1984; 23: 991–4.

21 Fairney A, Starffen A M, May C, Seifert M H. Vitamin D metabolites in synovial fluid. *Ann Rheum Dis* 1987; 46: 370–4.

22 Mawer E B, Hayes M E, Still P E, et al. Evidence for non-renal synthesis of 1,25-dihydroxyvitamin D in patients with inflammatory arthritis. *J Bone Mineral Res* 1991; 6: 733–9.

23 Bird H A, Wright V, Hennes U, Theiss E. Comparison of serum 1,25-dihydroxycholecalciferol concentrations in rheumatoid arthritis and osteoarthritis. *Ann Rheum Dis* 1982; 41: 257–8.

24 Sandler L M, Winearls C G, Fraher L J, Clemens T L, Smith R, O'Riordan J L H. Studies of the hypercalcaemia of sarcoidosis: effect of steroids and exogenous vitamin D$_3$ on circulating concentrations of 1,25-dihydroxy vitamin D$_3$. *Q J Med* 1984; 210: 165–80.

25 Davies M, Mawer E B, Hayes M E, Lumb G A. Abnormal vitamin D metabolism in Hodgkin's lymphoma. *Lancet* 1985; i: 1186–8.

26 Provvedini D M, Tsoukas C D, Deftos L J, Manolagas S C. 1,25-dihydroxyvitamin D$_3$ receptors in human leukocytes. *Science* 1983; 221: 1181–3.

27 Bhalla A K, Amento E P, Clemens T L, Holick M F, Krane S M. Specific high-affinity receptors for 1,25-dihydroxyvitamin D$_3$ in human peripheral blood mononuclear cells: presence in monocytes and induction in T lymphocytes following activation. *J Clin Endocrinol Metab* 1983; 57: 1308–10.

28 Shiozawa S, Shiozawa K, Tanaka Y, Fujita T. 1α, 25-Dihydroxyvitamin D$_3$ inhibits proliferative response of T- and B-lymphocytes in serum-free culture. *Int J Immunopharmacol* 1987; 9: 719–23.

29 Tsoukas C D, Provvedini D M, Manolagas S C. 1,25-Dihydroxyvitamin D$_3$: a novel immunoregulatory hormone. *Science* 1984; 224: 1438–40.

30 Abe E, Miyaura C, Tanaka H, et al. 1α,25-Dihydroxyvitamin D$_3$ promotes fusion of mouse alveolar macrophages both by direct mechanisms and by spleen cell-mediated indirect mechanisms. *Proc Natl Acad Sci USA* 1983; 80: 5583–7.

31 Bar-Shavit Z, Teitlebaum S L, Reitsma P, et al. Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D$_3$. *Proc Natl Acad Sci USA* 1983; 80: 5907–11.

32 Raisz L G, Kream B E, Smith M A, Simmons H A. Comparison of the effects of vitamin D metabolites on collagen synthesis and resorption of fetal rat bone in organ culture. *Calcif Tissue Int* 1980; 32: 135–8.

33 Narbaitz R, Stumpf W E, Sar M, Huang S, DeLuca H F. Autoradiographic localisation of target cells for 1,25-dihydroxyvitamin D$_3$ in bones from fetal rat. *Calcif Tissue Int* 1983; 35: 177–82.

34 Sambrook P N, Reeve J. Bone disease in rheumatoid arthritis. *Clin Sci* 1988; 74: 225–30.

35 Sambrook P N, Shawe D, Hesp R, et al. Rapid periarticular bone loss in rheumatoid arthritis: possible promotion by normal circulating concentrations of parathyroid hormone or calcitriol (1,25-dihydroxyvitamin D$_3$). *Arthritis Rheum* 1990; 33: 615–22.