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Suppressive effect of 1,25-dihydroxyvitamin D_3 and its analogues EB 1089 and KH 1060 on T lymphocyte proliferation in active ulcerative colitis

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Abstract

This study examined the effect exerted by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] and two vitamin D analogues, EB 1089 and KH 1060, on the proliferation of T lymphocytes obtained from ulcerative colitis (UC) patients and healthy controls. The proliferative response of T lymphocytes to phytohaemagglutinin treatment was first analyzed on days three, five, and seven of culture. Cell proliferation was significantly lower in UC patients than that observed in healthy controls. The highest proliferation value, in either controls or patients, was registered on day five of culture. On day seven, a decrease in proliferation occurred, less evident in patients with respect to controls, whereas on day three, controls and patients showed the same proliferation value. The response of T lymphocytes of either healthy controls or UC patients to 1,25$(OH)_2D_3$, EB 1089, or KH 1060 was then investigated, treating the cells for three, five, and seven days with 10 nM vitamin D derivatives. In the presence of these compounds, cell proliferation was significantly inhibited in both groups, but on day seven, the inhibition of lymphocyte proliferation was remarkable in controls, whereas in patients it was similar to that registered on day five. The highest inhibition values were always obtained in the presence of KH 1060, and the time dependence was continuous in controls, but in the presence of EB 1089 only in patients. T lymphocytes prepared from healthy controls and UC patients were then cultured for five days in the presence of vitamin D derivatives at three different concentrations (0.1, 1, and 10 nM). In the two groups, a dose-dependent inhibition was registered in the presence of 1,25$(OH)_2D_3$ or EB 1089, while the inhibition of proliferation exerted by KH 1060 was not dose-dependent. The results obtained suggest an option for the use of the two non-hypercalcemic vitamin D analogues in the therapy of UC patients, perhaps in association with other immunosuppressive drugs.

Keywords: 1,25-Dihydroxyvitamin D_3; Vitamin D analogues; T lymphocyte proliferation; Active ulcerative colitis

1. Introduction

It has been widely demonstrated that the hormonal form of vitamin D_3, 1,25$(OH)_2D_3$, is a regulator of mineral absorption and also influences many other cell functions. The discovery of receptors for vitamin D (VDR) in cell lines different from the classic lines has prompted revaluation of the biological role of vitamin D [1,2]. 1,25$(OH)_2D_3$ has been demonstrated to induce differentiation of both human and murine myeloid leukemia cell lines [3,4] and of myelocytic precursors from human bone marrow [5–8]. In particular, a role for 1,25$(OH)_2D_3$ as a regulator of immune cell differentiation and proliferation has been proposed [6]. In the cells of the myeloid lineage, VDR can be further enhanced by 1,25$(OH)_2D_3$ treatment with concomitant differentiation phenotype [9,10]. In contrast to human monocytes, resting peripheral T and B lymphocytes do not present 1,25$(OH)_2D_3$ receptors, since VDR expression is lost once the cells leave the thymus and enter the circulation as T or B cells. However, it has been shown that VDR expression in these cells is re-induced upon *in vitro* activation by mitogenic lectins such as PHA and concanavalin A [11–13]. It has been demonstrated that 1,25$(OH)_2D_3$ is a potent inhib-

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Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; IBD, inflammatory bowel disease; IFN-γ, interferon-gamma; IL-2, interleukin 2; IL-4, interleukin 4; FBS, fetal bovine serum; PBMC, human peripheral blood mononuclear cells; PHA, phytohaemagglutinin; T_H, T helper lymphocytes; T_S, T suppressor lymphocytes; UC, ulcerative colitis; and VDR, vitamin D receptor.

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itor of T cell proliferation [14,15]. $1,25(OH)_2D_3$ blocks the transition of cells from early G_1 phase (G_{1a}) to late G1 (G_{1b}), but does not affect movement from G_0 to G_{1a} or from G_{1b} to S phase [16]. The preferential target for $1,25(OH)_2D_3$ appears to be T_H cells, and the established anti-IL-2 effect of $1,25(OH)_2D_3$ suggests that this hormone could play a role in directing T_H cell responses. Initial studies proposed the suppression of T_H cell activity by $1,25(OH)_2D_3$ [17], while further research has shown that $1,25(OH)_2D_3$ inhibits IL-2 production and IFN-γ secretion by T cells [18,19]. Moreover, a recent study reports that the total T lymphocyte population contains VDR, whose levels are increased when activated and treated with $1,25(OH)_2D_3$, while B lymphocytes do not contain a detectable amount of VDR, suggesting that B lymphocytes are not likely to be directly regulated by the hormone [20].

Since the clinical use of $1,25(OH)_2D_3$ is severely limited by its remarkable effects on intestinal calcium absorption and the risk of associated side effects, intensive research activity has been directed at finding new vitamin D analogues which are active in promoting cellular differentiation and cell growth inhibition, but with reduced calcemic activity and a consequently more favorable therapeutic profile. Among these analogues, EB 1089, at low doses, induces a significant inhibition in cell proliferation and tumor growth in the absence of a rise in serum calcium, thereby suggesting a possible therapeutic application for this compound as antitumor agent [21]. Moreover, this analogue induces apoptosis of breast cancer cells [22] and inhibits, *in vitro*, cellular proliferation of human colon cancer cells [23]. Recently, a study on the administration of EB 1089 to patients with advanced breast and colorectal cancer was reported for the first time [24]. KH 1060, another vitamin D derivative which belongs to a family of 20-epi-vitamin D_3 analogues, has been observed to be more potent *in vitro* than $1,25(OH)_2D_3$ as an inhibitor of clonal growth of leukemic cells [25,26] and also exerts a considerable inhibitory effect on breast cancer cell lines [27]. Moreover, it has been reported that the combination of KH 1060 and 9-*cis*-retinoic acid synergistically and irreversibly inhibits the clonal proliferation of HL-60 cells, inducing both differentiation and apoptosis [28].

Ulcerative colitis (UC) and Crohn's disease, known as inflammatory bowel disease (IBD), are characterized by chronic inflammation of the intestinal mucosa. In particular, UC, which affects the mucosa of the colon and rectum, is characterized by remission and flare-up and is likely associated with an alteration of the immune system [29]. T lymphocytes play an important role in the pathogeny of IBD, and T cell regulation may be of central importance in the inflammatory process. It has been reported that activated T cells expressing IL-2 receptors are increased in lamina propria from patients with IBD [30], and Hearing *et al*. [31] demonstrated that patients with severe UC who fail to respond to steroid treatment have steroid-resistant T lymphocytes. Perez-Machado *et al*. [32] observed that reduced proliferative responses to IL-4 may be involved in UC, even if these authors demonstrated that there were no significant differences in the percentage of T cell subpopulations in UC patients and healthy controls. Increased levels of soluble mediators of inflammation as well as of the cell immune system (macrophages, neutrophils, and lymphocytes) have been found in the intestinal mucosa and submucosa of IBD patients [33,34].

Since $1,25(OH)_2D_3$ is known to be a modulator of immunocompetent cell activity, we investigated the role of $1,25(OH)_2D_3$ and of two non-hypercalcemic vitamin D analogues, EB 1089 and KH 1060, in active UC patients. In particular, the object of the present study was to investigate the effect of $1,25(OH)_2D_3$ and its analogues on the proliferation of T lymphocytes obtained from UC patients, in comparison with healthy controls, in order to verify whether $1,25(OH)_2D_3$ or its analogues could exert an immunosuppressive effect on the proliferation of T lymphocytes of UC patients and whether the possible inhibitory response could be dose-dependent. The results obtained suggest the possible employment of the vitamin D analogues, in particular KH 1060, in therapy as well as that of other immunosuppressive agents.

2. Materials and methods

2.1. Patients

Ten patients (six men and four women with a mean age of 37.1 years; range 27–54) with active ulcerative colitis (UC patients) and seven age- and sex-matched healthy controls were studied. The diagnosis of UC was previously established on the basis of clinical symptoms and on endoscopic and histologic demonstration. All patients entering this study showed mild-to-moderate active disease and did not take steroids or other immunosuppressive drugs.

2.2. Reagents

RPMI-1640 medium HEPES modification, PBS, heat-inactivated FBS, L-glutamine, antibiotics, PHA, and Whatman glass microfiber filter were obtained from Sigma. Ficoll–Paque Research Grade was purchased from Amersham Pharmacia Biotech. Uni-Sorb columns were provided by Novamed Ltd. $1,25(OH)_2D_3$, EB 1089, and KH 1060 stock solutions in 4 mM isopropanol were a gift from Leo Pharmaceutical Products. Stock solutions were stored at $-20°$, protected from light, and freshly diluted in culture medium before each experiment. [^{3}H]Thymidine was purchased from New England Nuclear. All other chemicals were the highest grade available from E. Merck.

2.3. T lymphocyte isolation

PBMC were obtained by gradient density (1.077) centrifugation (30 min at 400 $\times$ *g*) of heparinized venous

blood diluted 1:2 with PBS on Ficoll–Paque. About 95% of mononuclear cells at the interface, containing PBMC, were collected and washed twice with PBS. PBMC were further fractionated by the nylon wool column separation technique. Uni-Sorb columns and ready-to-use polypropylene tubes packed with specially treated nylon wool were used. Briefly, PBMC, suspended in 1.5 mL RPMI-1640 medium supplemented with 10% heat-inactivated (56° for 30 min) FBS, were injected into the column. After incubation of the column at 37° for 60 min, the non-adherent cell population, T lymphocyte-enriched, was collected by washing the column twice with 10 mL medium to completely remove non-adherent T cells. This method is reported to yield excellent B and T preparations (> 90% purity). Lymphocyte viability was determined by the trypan blue exclusion test.

2.4. Culture conditions and proliferation studies

The nylon wool non-adherent cell population (T lymphocyte-enriched) was cultured in RPMI-1640 supplemented with 25 mM Hepes, 10%, (v/v) heat-inactivated FBS, 60 mg/L (100 U/mL) of penicillin, 100 mg/L of streptomycin, and 0.29 g/L of L-glutamine. One milliliter, containing 6×10^4 cells, was dispensed in 24-well microtiter plates, to which 10 μg/mL of PHA and 1,25(OH)$_2$D$_3$ or EB 1089 or KH 1060 (0.1–10 nM final concentration) were added. Control assays treated with PHA and vehicle (isopropanol) alone were performed. Cultures were incubated at 37° in humidified atmosphere containing 5% CO_2 for three, five, and seven days. Eighteen hours before the end of incubation, 1 μCi of [^{3}H]thymidine was added to each well. The cells were then harvested on Whatman glass microfiber filter, scintillation liquid was added, and the radioactivity was measured in a liquid scintillation counter (Beckman). All cultures were run in triplicate. Cell viability was always examined by trypan blue exclusion.

2.5. Statistical analysis

Differences between means were evaluated by Student's *t*-test and were considered significant at $P < 0.05$.

3. Results

3.1. Proliferative response by mitogen T lymphocytes in active ulcerative colitis

The proliferative response of PHA-activated T lymphocytes from UC patients and healthy controls was first studied, and the data are reported in Fig. 1. Lymphocyte proliferation was analyzed on days 3, 5, and 7 of culture in the presence of 10 μg/mL of PHA, which, under our experimental conditions, was the optimum mitogenic dose. On day 3, patients and controls showed the same relatively low proliferation value. Proliferation then rapidly increased,

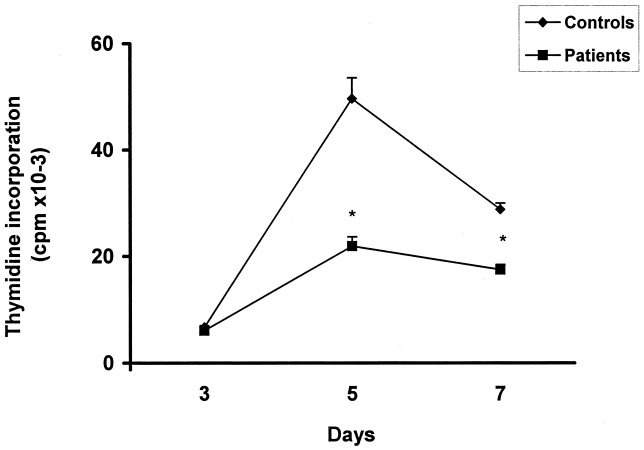

Fig. 1. Proliferative response of T lymphocytes from UC patients and healthy controls to PHA (10 μg/mL) stimulation for three, five, and seven days. Each value represents the mean ± SD of 6–8 separate experiments, each one performed in triplicate. Results are expressed as counts per minute (cpm $\times 10^{-3}$). Statistical differences between the two groups (*$P < 0.01$) were observed on days five and seven of culture.

reaching its highest value, in both controls and patients, on day five of culture. On day 7, the proliferation decreased, but this decrease was less evident in patients in comparison with controls. However, the proliferative response to PHA of purified T lymphocytes from UC patients, on days 5 and 7, was significantly lower than that observed in healthy controls. In particular, the day 5 and day 7 values of [^{3}H]thymidine incorporation into T lymphocytes from patients were approx. 56% and 40%, respectively, lower than those registered in healthy controls.

3.2. Effect of 1,25(OH)$_2$D$_3$, EB 1089, and KH 1060 on T lymphocyte proliferation

The response of T lymphocytes from either healthy controls or UC patients to 1,25(OH)$_2$D$_3$, EB 1089, and KH 1060 treatment was investigated by incubating the cells for 3, 5, and 7 days with 10 nM vitamin D derivatives. The data obtained, expressed as cpm $\times 10^{-3}$, are reported in Fig. 2A and B. In the presence of 1,25(OH)$_2$D$_3$, cell proliferation was significantly inhibited in both groups at all the times studied. The inhibitory effect increased on day 5 both in controls and in patients, reaching about 34% and 37%, respectively. The inhibition in proliferation induced by 1,25(OH)$_2$D$_3$ on days 3 and 5 was similar in controls and in UC patients. On day 7, a different behavior of T cell response was observed: a remarkable inhibition of T lymphocyte proliferation was evident in controls (about 79%), whereas the inhibitory effect in UC patients was similar to that registered on day 5, i.e. approx. 41%. A similar trend was observed when T lymphocytes were treated with 10 nM EB 1089, even if on day 3 of incubation, T lymphocytes prepared from UC patients were more sensitive to the treatment with the vitamin D analogue in comparison with

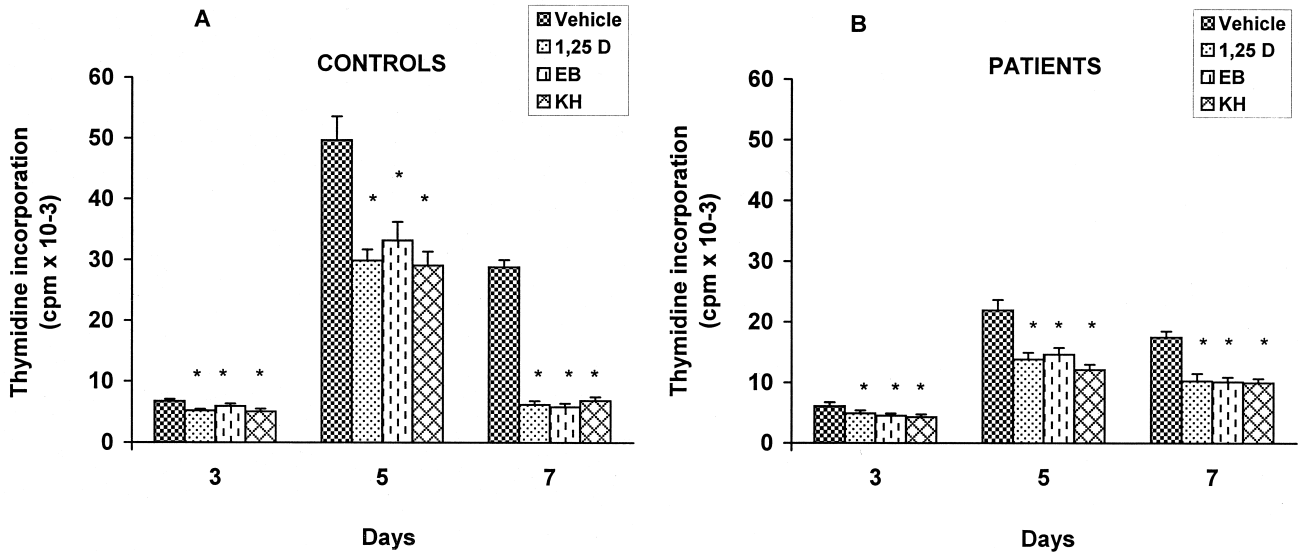

Fig. 2. Effect of 10 nM $1,25(OH)_2D_3$, EB 1089, and KH 1060 on PHA-activated T lymphocyte proliferation from healthy controls (a) and UC patients (b) after treatment for three, five, and seven days. [^{3}H]Thymidine incorporation is expressed as cpm $\times$ 10^{-3}. Each value represents the mean $\pm$ SD of 5–8 separate experiments, each one performed in triplicate. *$P < 0.01$ (in comparison with the vehicle treatment).

healthy controls (25.5% inhibition versus 11.5%). Here again, the inhibition in cell proliferation, increased on day 5, was similar for controls and UC patients (32% and 33%, respectively). On the contrary, on day 7, a further and remarkable inhibitory effect on T lymphocyte growth was observed in controls (80% inhibition), while in UC patients, the decrease in proliferation was not so accentuated (42%) but was still significant in comparison with that registered on day 5. The treatment of T lymphocytes with 10 nM KH 1060 produced an inhibition profile very similar to that obtained in the presence of the other two vitamin D derivatives even if, on day 5, KH 1060 was a more potent inhibitor of T lymphocyte proliferation than $1,25(OH)_2D_3$ and EB 1089 both in controls and in UC patients (41.5 and 45% inhibition, respectively). On day 7, the inhibitory effect on proliferation showed a different trend in controls and patients, but similar to that obtained with $1,25(OH)_2D_3$ and EB 1089. Here too, in controls, an inhibition value of about 76% was registered, while in UC patients it was 43%. These results, taken together, demonstrate that the inhibitory effect exerted by $1,25(OH)_2D_3$ and its analogues on the proliferation of T lymphocytes, prepared from healthy controls, was time-dependent, while in UC patients the time dependence was present on days 3 and 5, when $1,25(OH)_2D_3$ and KH 1060 were used, and was continuous in the presence of EB 1089 only.

3.3. Effect of different doses of $1,25(OH)_2D_3$, EB 1089, and KH 1060 on T lymphocyte proliferation

In Fig. 3, the data obtained when T lymphocytes from healthy controls and UC patients were cultured for 5 days in the presence of $1,25(OH)_2D_3$, EB 1089, and KH 1060 at three different concentrations (0.1, 1, and 10 nM) are reported. Day 5 of proliferation was chosen, since the maximal mitogenic response was detected on this day (see Fig. 1). The treatment of T lymphocytes with vitamin D derivatives yielded very similar results in controls and UC patients. Different behavior was shown by $1,25(OH)_2D_3$, EB 1089, and KH 1060. Indeed, in the presence of 0.1 nM $1,25(OH)_2D_3$ or EB 1089, a lack of inhibition was registered, while a significant dose-dependent inhibition of T cell proliferation was observed at 1 and 10 nM concentrations,

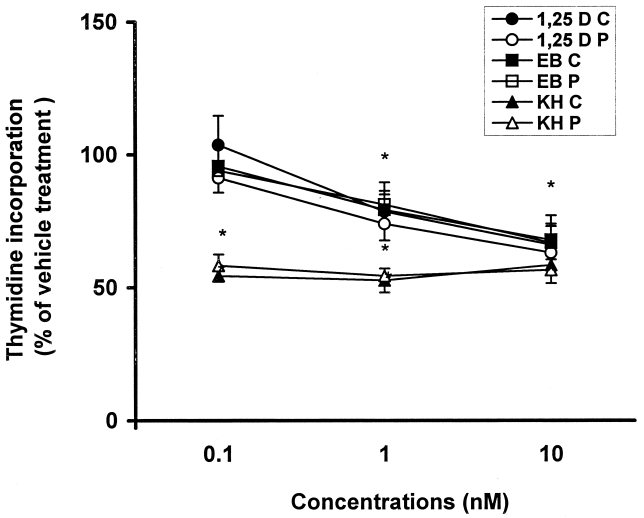

Fig. 3. Dose dependence of $1,25(OH)_2D_3$, EB 1089, and KH 1060, at three different concentrations (0.1, 1, and 10 nM), on PHA-activated T lymphocyte proliferation from healthy controls and UC patients after treatment for five days. Results are expressed as the percentage of the value of [^{3}H]thymidine incorporation determined in cultures treated with vehicle alone. Each value represents the mean $\pm$ SD of 6–8 separate experiments, each one performed in triplicate. *$P < 0.01$ (in comparison with the vehicle treatment).

the highest inhibition occurring at 10 nM. On the other hand, the inhibition of proliferation exerted by KH 1060 was not dose-dependent under our experimental conditions, being in fact remarkable at 0.1 nM concentration (ranging from 42% to 48% in controls and from 42% to 44% in UC patients, taking into account all the doses used). KH 1060 therefore proved to be a more potent inhibitor of T lymphocyte proliferation than $1,25(OH)_2D_3$ and EB 1089 in both healthy controls and UC patients.

4. Discussion

Previous studies have demonstrated that $1,25(OH)_2D_3$ is a potent inhibitor of T lymphocyte proliferation and IL-2 production in normal PBMC [16,35,36]. Rigby *et al*. [16] reported that $1,25(OH)_2D_3$, while blocking the transition from early (G_{1a}) phase cell cycle to late (G_{1b}), has no effect on IL-2 receptor expression, an early G_1 event, but markedly inhibits transferrin receptor, a late G_1 event. In particular, it has been demonstrated that T_H lymphocytes are the specific cellular target for the immunoinhibitory effect of $1,25(OH)_2D_3$ [17], while the hormone does not exert any effect on T_S and B cells. Recently, Veldman *et al*. [20] reported that the highest concentrations of VDR are found in CD8 lymphocytes, although significant amounts are also present in CD4 lymphocytes. Furthermore, B lymphocytes do not contain detectable amounts of VDR, suggesting that CD8 lymphocytes may be a major site of $1,25(OH)_2D_3$ action.

Our results on the proliferative response of T lymphocytes from UC patients in comparison with healthy controls, on day five, are in accordance with those reported by Manzano *et al*. [37], thereby confirming the defective proliferative response in these patients. However, we demonstrated a different proliferative behavior on days three and seven. On day three, Manzano and co-workers found a significant difference in T lymphocyte proliferation between patients and controls, while we registered a similar value in the two groups. On day seven of our study, the decrease in proliferation of T lymphocytes prepared from UC patients was not so marked as that of controls, while Manzano *et al*. [37] did not find any difference when comparing the two groups. The discrepancy between our data and those reported by these authors could be due to a different cellular density, which may influence PHA-induced proliferation, or to the utilization of a different method for T lymphocyte purification. We carried out our studies on a T lymphocyte-enriched population, purified by nylon wool column and therefore containing about 1–4% monocytes, as reported in the literature [38,39], to avoid the possibility that the proliferative answer to the compounds used could be influenced by a variable number of monocytes. However, monocytes were present at a concentration high enough to induce lymphocyte proliferation [40] and the immunosuppressive effect of $1,25(OH)_2D_3$. Our results, which indicate an inhibitory effect of $1,25(OH)_2D_3$ on T lymphocyte proliferation, are in accordance with those reported in the literature, but in our case, we found a lower inhibition at 10 nM hormone in comparison with the value of 56% reported by Lemire *et al*. [17] for the action of $1,25(OH)_2D_3$ on [^{3}H]thymidine incorporation into T_H cells. This discrepancy could be due to the fact that, for the proliferation studies, these authors utilized a higher number of cells than we did, and only T_H cells. On the contrary, we used a population of T lymphocytes without separating the subsets T_H and T_S.

The inhibitory effect exerted by $1,25(OH)_2D_3$ and EB 1089 on the proliferation of T lymphocytes, obtained from UC patients and healthy controls, on day five, was dose-dependent, and the inhibitory effect similarly increased in both healthy controls and UC patients. KH 1060 proved to be the most powerful inhibitor, showing the highest inhibition value at the lowest dose, i.e. 0.1 nM, without dose dependence. It is likely that the dose dependence could be highlighted by treating T lymphocytes with KH 1060 at a dosage lower than 0.1 nM. Indeed, when lower concentrations of KH 1060 were tested, i.e. 0.01, 0.001, and 0.0001 nM, an inhibition in cell proliferation was registered that was always significant in comparison with the vehicle-treated controls, being approx. 20% with the lowest dose. It must be pointed out that, at these same concentrations, $1,25(OH)_2D_3$ was ineffective (data not shown). However, we chose to use the same concentrations for all the vitamin D derivatives in order to compare their effects on cell proliferation. The inhibitory effect exerted by EB 1089 on the proliferation of T lymphocytes is, to our knowledge, the first report on this subject. The time-dependent inhibitory effect on T lymphocyte proliferation was present in controls, while in UC patients, the difference in proliferation between days five and seven was significant, but not remarkable, with EB 1089 treatment only.

An important difference in the behavior of T lymphocyte proliferation on day seven therefore exists between healthy controls and UC patients. The fact that $1,25(OH)_2D_3$ and its analogues exerted virtually the same inhibition on the proliferation of T lymphocytes of UC patients, on days five and seven, may be due either to genetic factors or acquired ones, either as a consequence of T lymphocyte activation during the disease [41] or of the lack of activation of apoptotic processes absent in the UC patients, but present in the controls. This last hypothesis needs to be investigated, particularly in the light of a recent report by Perez-Machado et al. [32]. These authors reported that, in spite of the fact that the percentage of cells in growth phase $S + G_2M$ at two and three days of PHA stimulation was significantly decreased in UC patients, the percentage of CD4+ and CD8+ cells in UC patients showing apoptosis was not significantly different than in the control group, but it must be remarked that these authors did not carry out experiments on day five or seven.

In conclusion, the two non-hypercalcemic vitamin D analogues tested by us *in vitro* could be used in the therapy

of UC patients, perhaps in association with other immunosuppressive drugs working with different mechanisms, as has been proposed for the therapy of several forms of cancer. In particular, KH 1060 could be administered at a dosage lower than that used in our experiments, due to its high inhibitory activity on T cell proliferation. This approach could be theoretically subjected to criticism: in UC patients, peripheral T lymphocyte proliferation is basically impaired [37], contrary to what happens in the colon wall, where T cells actively proliferate (in response to IL-2) and where they show a response model type T_H2 [42,43]. In fact, an evident contradiction exists between the proliferative response in the peripheral blood and in afflicted sites; the reasons for such behavior are still unknown. In any event, all drugs that show clinical activity on UC patients *in vivo* are characterized by some effects on the proliferative activity of the T cells, in the sense of inhibition. In our opinion, the most interesting speculation arising from the present research is that the effect of $1,25(OH)_2D_3$ and its analogues on T lymphocyte proliferation seems to be highly specific and highly reproducible, even if further studies are required to verify the action of the vitamin D derivatives on T lymphocyte subsets, on T lymphocytes obtained from inflamed tissue, and on possible apoptotic phenomena.

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