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1,25-Dihydroxyvitamin D_3 Increases Type 1 Interleukin-1 Receptor Expression in a Murine T Cell Line

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Abstract The biologically active metabolite of vitamin D_3, 1,25 $(OH)_2$ D_3, exerts important immunoregulatory effects in addition to being a central mediator of calcium/phosphate metabolism. Utilizing an interleukin 1 responsive murine T cell line and ^{125}I-interleukin 1α, we show that 1,25 $(OH)_2$ D_3 (5,50 nM) enhanced ^{125}I-interleukin 1α binding up to almost 2-fold over control. This 1,25 $(OH)_2$ D_3 effect occurred in a dose-dependent manner and was detectable after 24 h but not before 7 h of culture. Scatchard analysis of ^{125}I-interleukin 1α binding data demonstrated that 1,25 $(OH)_2$ D_3 enhanced interleukin 1 receptor number without a significant change in affinity. The biologically less potent metabolite of vitamin D_3, 25 (OH) D_3, also augmented ^{125}I-interleukin 1α binding but at steroid levels 2–3 log orders greater than 1,25 $(OH)_2$ D_3. This observation, combined with the presence of high-affinity ^{3}H-1,25 $(OH)_2$ D_3 receptors (88 sites/cell, K = 0.45 nM) in cytosolic extracts, strongly suggests that the nuclear vitamin D receptor mediates this steroid's effect on interleukin 1 receptor expression. Based on the capacity of an anti-type 1 interleukin 1 receptor monoclonal antibody (35F5) to block 1,25 $(OH)_2$ D_3-enhanced ^{125}I-interleukin 1α binding, we conclude that this steroid augments type 1 interleukin 1 receptor expression. When combined with interleukin 1, a cytokine that also impacts MD10 interleukin 1 receptor expression, 1,25 $(OH)_2$ D_3 enhanced interleukin 1 receptor expression. Northern blots hybridized with a ^{32}P-type 1 interleukin 1 receptor cDNA probe show that 1,25 $(OH)_2$ D_3 enhanced type 1 interleukin 1 receptor steady state mRNA levels. Functionally, 1,25 $(OH)_2$ D_3 pretreatment augmented the MD10 proliferative response to suboptimal levels of interleukin 1 (< 100 fM interleukin 1α). These findings further support 1,25 $(OH)_2$ D_3's role as an immunoregulatory molecule and provides a possible mechanism by which this steroid could potentiate certain immune activities.

Key words: Northern analysis, vitamin D receptor, metabolite specificity, immune regulation, cellular proliferation, MTT assay

Vitamin D_3 is a steroid hormone with metabolites having the capacity to influence a wide variety of systemic and cellular activities [Haussler, 1986; Reichel et al., 1989] in addition to those pivotal to bone and mineral metabolism. The most potent vitamin D_3 metabolite, 1,25 $(OH)_2$ D_3, exerts its effects primarily through its interaction with the vitamin D receptor (VDR), which is localized in the nucleus. This receptor, a member of the thyroid hormone receptor superfamily, contains both "zinc-finger" DNA binding regions and hormone recognition sites [Baker et al., 1988; McDonnell et al., 1987]. In accordance with its pleiotropic effects, VDRs have been documented in a wide variety of cells and tissues [Haussler, 1986; Reichel et al., 1989].

With the discovery of VDRs in monocytes and activated T [Provvedini et al., 1983; Bhalla et al., 1983; Merke et al., 1984] and B cells [Provvedini et al., 1986], efforts have been focused on the effects of this steroid class on the immune system. In the human, 1,25 $(OH)_2$ D_3 inhibits lectin induced lymphocyte proliferation [Tsoukas et al., 1984; Rigby et al., 1984; Saggese et al., 1989], and production of IL-2 [Tsoukas et al., 1984; Rigby et al., 1987; Matsui et al., 1986; Saggese et al., 1989], interferon gamma [Reichel et al., 1987; Saggese et al., 1989; Matsui et al., 1986], granulocyte-macrophage colony stimulating factor [Tobler et al., 1988], and antibody [Komoriya et al., 1985; Provvedini et al., 1986; Iho et al., 1986; Lemire et al., 1984]. In the mouse, 1,25 $(OH)_2$ D_3 fails to inhibit lectin-induced mitogenesis of splenic T cells but does

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impair antigen and alloantigen T cell stimulation [Bhalla et al., 1984] as well as thymocyte proliferation induced by interleukin 1 (IL-1), IL-2, and/or phytohemagglutinin exposure [Ravid et al., 1984; Koizumi et al., 1985; Muller et al., 1988].

While these reports suggested that 1,25 $(OH)_2$ D_3, like dexamethasone, is immunoinhibitory, we and others have observed that 1,25 $(OH)_2$ D_3 may be immunostimulatory in certain situations. In myelo/monocytic leukemia cell lines, 1,25 $(OH)_2$ D_3 stimulated the expression of class II molecules either alone or in combination with interferon gamma [Bar-Shavit et al., 1983; Morel et al., 1986]. Further, these observations may be associated with the capacity of the sterol to augment gamma interferon receptors as has been documented in the malignant promonocytic cell line HL60 [Zuckerman et al., 1988]. As regards monocytic cytokines, IL-1 production is augmented in isolated, adherent peripheral blood monocytes and the murine macrophage cell line P388D1 subsequent to 1,25 $(OH)_2$ D_3 exposure [Bhalla et al., 1986; Hodler et al., 1985].

While these observations underscored the capacity for 1,25 $(OH)_2$ D_3 to enhance immune activities of monocytes or monocytic leukemic cells, the immunostimulating effects of this steroid on T cells are not as well documented. We have previously reported that proliferation of the type 2 murine T helper (TH2) cell line D10.G4.1 was increased by 1,25 $(OH)_2$ D_3 in the presence of lectin [Lacey et al., 1987]. This phenomenon was associated with an elevation of immunoreactive IL-2 receptors, but not with change in bioassayable growth factor production [Lacey et al., 1987]. In human T cells, 1,25 $(OH)_2$ D_3 enhanced the capacity of interleukin 2 to augment the expression of Tac antigen, the low affinity IL-2 receptor [Tamori et al., 1989]. Haverty et al. have demonstrated the capacity for 1,25 $(OH)_2$ D_3 to affect IL-2 production by a primate T lymphoma cell line in a biphasic manner. By using vitamin D deficient serum, these authors demonstrated that 1,25 $(OH)_2$ D_3 at low, but physiologic concentrations, increased IL-2 production by these cells [Haverty et al., 1987]. At higher concentrations, the steroid inhibited IL-2 production.

While these studies have documented the T cell and monocyte immunostimulating capacity of 1,25 $(OH)_2$ D_3 in certain circumstances, it remains a distinct possibility that the steroid positively impacts other aspects of immune cell function. This report documents 1,25 $(OH)_2$ D_3 effects on the expression of receptors for interleukin 1, a cytokine pivotal to many aspects of the immune response [Dinarello et al., 1989]. These studies utilized the murine T cell line MD10 that were derived from the TH2 cell line D10.G4.1 [Lacey et al., 1987]. MD10 cells express measurable IL-1Rs (interleukin 1 receptors) [Civitelli et al., 1989] and proliferate in response to low IL-1 concentrations (10–50 fM) [Lacey et al., 1987]. We previously reported that IL-1R expression on this T cell line is upregulated by IL-4, a finding that may correlate with the capacity of IL-1 and IL-4 to synergistically promote MD10 proliferation [Lacey et al., 1990]. As will be shown, we have found that 1,25 $(OH)_2$ D_3 upregulates the expression of MD10 IL-1R either alone or in the presence of IL-1. The fact that this vitamin D response was metabolite-dependent, combined with the presence of MD10 VDRs, suggests that this is a "genomic" effect of the vitamin. Based on antibody-blocking studies, 1,25 $(OH)_2$ D_3 appears to increase type 1 IL-1R levels. Furthermore, the steroid increases steady-state levels of type 1 IL-1R mRNA. Functionally, 1,25 $(OH)_2$ D_3 MD10 pretreatment potentiates the proliferative effect of low IL-1 concentrations. These data suggest that type 1 IL-1R upregulation may be one means by which 1,25 $(OH)_2$ D_3 could enhance specific immune responses.

METHODS

Media, Buffers, and Chemicals

The media used for MD10 cell culture (D10 medium) was RPMI 1640 (Hazelton-Dutchland, Denver, PA) supplemented as described [Lacey et al., 1987]. The binding buffer (BB) used in the IL-1R assay was RPMI 1640, HEPES—25 mM, pH-7.2 supplemented with 5% heat-inactivated FCS (HIFCS) [Kilian et al., 1986]. The acid strip buffer was NaCl—130 mM, Na acetate—20 mM, pH 3.0. The buffer used to obtain MD10 cellular extracts for the 1,25 $(OH)_2$ D_3 receptor assays was KCl—0.3 M; Tris—10 mM, pH 7.4; EDTA—1.5 mM; dithiothreitol—1 mM, phenylmethylsufonyl fluoride (PMSF)—1 mM (KTEDP). The hydroxylapatite wash buffer was KTEDP without either KCl or PMSF supplemented with 0.1% Triton-X 100 (ICN, Irvine, CA). The hydroxylapatite (Biorad, Richmond, CA) solution consisted of a slurry of hydroxylapatite:Triton X-100 free hydroxylapatite wash buffer (50:50).

Cytokines, Vitamin D_3 Metabolites, and Antibodies

Human recombinant IL-1α (HrIL-1α) supplied as the carboxy terminal 154 amino acids of the 271 amino acid human IL-1α precursor was kindly provided by Dr. P. Lomedico (Hoffmann-La Roche, Nutley, NJ). Purified murine IL-4 (MpIL-4) was a gift from J. Ohara (University of Colorado School of Medicine, Denver, CO). Rat T cell growth factor (RTCGF) was produced via con A stimulation of rat splenocytes as described [Lacey et al., 1987]. The vitamin D_3 metabolites were the kind gift of Milan Uskokovic (Hoffmann-LaRoche, Nutley, NJ) and were dissolved as concentrated stock solutions in absolute ethanol and stored in the dark at −20°C. The final concentration of ethanol in all experiments was always less than 0.01%, an amount that does not affect cellular viability nor enhance type 1 IL-1R expression. A monoclonal anti-murine type 1 IL-1 receptor antibody (35F5) was kindly provided by R. Chizzonite (Hoffmann-LaRoche, Nutley, NJ). Purified rat IgG was obtained from Sigma (St. Louis, MO).

Cells

MD10 cells were cultured as described [Lacey et al., 1987]. The cells were used a minimum of 10 days after last passage and were separated from culture debris by centrifugation over a Ficoll-Hypaque gradient (d = 1.077) prior to their use in further experiments. The anti-murine IL-4 hybridoma, 11B11 [Ohara et al., 1985] (American Tissue Culture Collection, Rockville, MD), was maintained in D10 media supplemented with 10% HIFCS at cell concentrations between 10^5–10^6 cells/ml.

IL-1 Bioassay

MD10 cells, at least 8 days after last passage, were rinsed once in D10 media after gradient separation, suspended in D10 medium supplemented with 10% HIFCS, and then added to microtitre wells (2×10^4 cells/well, 200 μl volume) containing various conditions. The cultures were incubated for a 72 h period. To assess cell growth, the MTT dye conversion assay [Mosmann, 1983] was employed. The amount of dye converted (as assessed by spectroscopy in an ELISA plate reader (absorbance—570 nm; reference—630 nm)) is directly related to viable cell number [Mosmann, 1983]. Triplicate determinations were made.

^{125}I-HrIL-1α Preparation

HrIL-1α was labeled with Na ^{125}I (ICN, Irvine, CA) utilizing enzymobeads as described [Kilian et al., 1986; Lacey and Erdmann, 1990]. The ligand specific activity ranged from 1,400 to 3,000 cpm/fmol and the biochemical and functional integrity of the preparation were verified by SDS-PAGE analysis and the MD10 bioassay [Lacey et al., 1987].

^{125}I-HrIL-1α Binding Assay

Cell surface IL-1R expression was assessed as described [Lacey and Erdmann, 1990]. Briefly, MD10 cells were rinsed with serum free BB and then suspended in BB at 3–5 × 10^6 cells/ml. In some experiments, the cells were acid stripped by suspending the cell pellet in 1 ml of acid strip buffer for 60 s followed by the rapid addition of serum free BB (neutral pH). This treatment effectively removes receptor-bound IL-1 [Lacey and Erdmann, 1990]. The cells were centrifuged (1,000 rpm, 10 min, 4°C) and then suspended in BB. ^{125}I-HrIL-1α binding was performed in a total volume of 0.5 ml in 2 ml microcentrifuge tubes placed at a 45° angle on a rocking platform (Bellco, Vineland, NJ) at 4°C for 1–2 h. Nonspecific binding was determined in the presence of 20 nM HrIL-1α. After incubation, 3 × 150 μl aliquots were layered over an oil mixture (300 μl, Dow Corning 702 diffusion pump fluid: Apiezon A oil; 2.5:1) in 0.5 ml microcentrifuge tubes and centrifuged at 14,000 rpm (5 min) in a microfuge. The cell pellet containing tips were cut and the pellet associated ligand assessed in a gamma counter. The data are reported as the mean (n = 3) ± SD. Some of the data were subjected to Scatchard analysis [Scatchard, 1949].

1,25 $(OH)_2$ D_3 Receptor Assay

The MD10 1,25 $(OH)_2$ D_3 receptor was assessed utilizing a modification of a hydroxylapatite batch assay method [Wecksler et al., 1979]. MD10 cells were removed from culture, subjected to Ficoll-Hypaque gradient centrifugation, and rinsed three times with cold phosphate-buffered saline. The resulting cell pellet was suspended in KTEDP buffer (7×10^6 cells/100 μl) and sonicated in an ice water bath for 5 min (Heat Systems-Ultrasonics, Farmingdale, NY, model W220, probe #C2, power setting—9). The resultant solution was cleared of debris by centrifugation (SS24 rotor, Sorvall Superspeed centrifuge (Dupont/NEN, Wilmington, DE), 18,000

RPM, 30 min, 4°C). Both the 1,25 $(OH)_2$ D_3 (1 μM) and various concentrations of ^{3}H-1,25 $(OH)_2$ D_3 were diluted in 100% ethanol and constituted, when combined, 10 μl out of a total of 110 μl. Following an 18 h incubation at 4°C, the receptor bound and free ligand were separated by the addition of the hydroxylapatite slurry. The hydroxylapatite was then washed 3 times with hydroxylapatite wash solution followed by ethanol (100%) extraction. To assess the level of ^{3}H-1,25 $(OH)_2$ D_3 bound, scintillation spectroscopy was performed on the dried extracts. The data are reported in cpm (counting efficiency = 50%) and represent triplicate determinations for the total cpm bound and duplicate determinations for the non-specific cpm bound. Some of the data were subjected to Scatchard analysis [Scatchard, 1949].

Type 1 IL-1 Receptor Plasmid and Northern Blots

The insert used to create plasmid P4ZIL-1R1.5 (containing a 500 bp type 1 IL-1R cDNA sequence) was derived from the DNA amplification product of reverse transcribed EL4.6.1 total cell RNA (gift of D. Chaplin, Washington University, St. Louis, MO) as described (Lacey et al., in press). The type 1 IL-1R primers were based on the sequence published for the cDNA [Sims et al., 1988] and are as follows: **5**′-TGGTACAGGGACTCCTGCTCTGGTT corresponding to bases 1,048–1,072 and **3**′-GTCTCCTGACCAGCAAATGACTCCG, which is the reverse complement of bases 1,482–1,506.

For RNA gels, MD10 total cellular RNA was prepared by the method of Chirgwin [Chirgwin et al., 1979], quantified by absorbance spectroscopy (1 A_{260} = 40 μg/ml) and electrophoresed in 1% Seakem agarose (FMC, Rockland, ME) in a buffer composed of MOPS—20 mM (pH-6.8), sodium acetate—5 mM, and EDTA—1 mM and formaldehyde (0.23%) [Chaplin et al., 1983]. The RNA was then electroblotted onto Zeta probe nylon membranes according to manufacturer's instructions (Biorad, Richmond, CA). The blots were prehybridized in cocktail containing 50% formamide, 0.25 M $NaHPO_4$ (pH 7.2), 0.25 M NaCl, 1 mM EDTA, and 2% SDS for 1–2 h at 42°C. The type 1 murine IL-1R probe was generated by labeling the gel-purified DNA amplification product of plasmid P4ZIL-1R1.5 with ^{32}P-αdCTP (3,000 Ci/mmol, ICN, Irvine, CA) by random priming (Random Primed DNA Labeling Kit, Boehringer-Mannheim, Indianapolis, IN). The probe specific activity was estimated at 10^8–10^9 cpm/μg DNA. Hybridization (16–20 h, 42°C) was performed using 10^6 cpm/ml probe in the same buffer used for prehybridization. The membranes were then washed 3 times in 2× SSC, 0.1% SDS at room temperature and then once in 0.2× SSC, 0.1% SDS at 56°C for 1 h. For autoradiography, the blots were exposed to Kodak XAR film at −80°C.

TABLE I. 1,25 $(OH)_2$ D_3 Increases ^{125}I-HrIL-1α Binding by MD10 Cells

Condition[a]	Culture period (h)	^{125}I-HrIL-1α bound[b]
Control	0	1,764 ± 115[c]
Control	24	10,214 ± 821
Ethanol	24	8,282 ± 809
1,25 $(OH)_2$ D_3		
0.5 nM	24	12,542 ± 683
5.0 nM	24	18,798 ± 926[d]
50 nM	24	20,490 ± 879[d]

[a]MD10 cells were isolated from passage conditions and exposed to control, ethanol, or various concentrations of 1,25 $(OH)_2$ D_3 for 24 h. The final ethanol concentration utilized was the same as that present in the 50 nM 1,25 $(OH)_2$ D_3 group and represents less than 0.01%.
[b]^{125}I-HrIL-1α binding was performed as described with 1 nM labeled IL-1 and 20 nM competing unlabeled HrIL-1α.
[c]The results represent the mean (n = 3) ± SD of the net ^{125}I-HrIL-1α bound normalized to cpm/1 × 10^6 cells.
[d]$P < .01$ when compared to 24 h control.

RESULTS

To determine whether MD10 IL-1R expression could be influenced by 1,25 $(OH)_2$ D_3, MD10 cells were exposed to various concentrations of the steroid for 24 h followed by ^{125}I-HrIL-1α binding. As Table I demonstrates, 1,25 $(OH)_2$ D_3 augmented ^{125}I-HrIL-1α binding in a dose-dependent fashion. Both 5.0 and 50 nM 1,25 $(OH)_2$ D_3 increased specific ^{125}I-HrIL-1α binding by approximately 84 and 100%, respectively, when compared to control. At 0.5 nM 1,25 $(OH)_2$ D_3, the IL-1R upregulating effect was much less. The magnitude of IL-1R upregulation induced by 1,25 $(OH)_2$ D_3 was variable when separate experiments were compared and averaged 83% above control for cells treated with 5 nM 1,25 $(OH)_2$ D_3 (n = 6, range: 22–164%). To determine the time required for 1,25 $(OH)_2$ D_3 to augment ^{125}I-HrIL-1α binding, MD10 cells were exposed to the steroid for different time periods followed by ligand binding experiments. As demonstrated in Figure 1, the 1,25 $(OH)_2$ D_3 enhancing effect on ^{125}I-HrIL-1α binding was not observed within the first 7 h of culture. By 24 h,

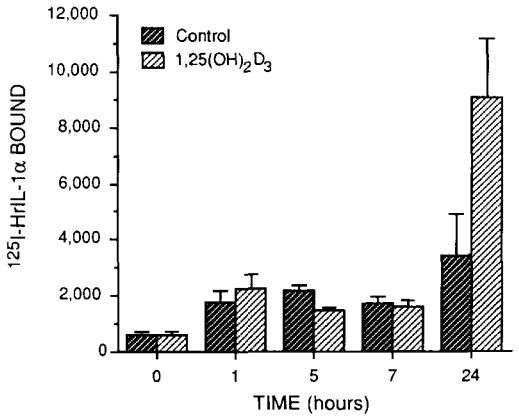

Fig. 1. 1,25 $(OH)_2$ D_3 modulates MD10 ^{125}I-HrIL-1α binding in a time-dependent manner. MD10 cells (5×10^5 cells/ml) were cultured in the presence of 5 nM 1,25 $(OH)_2$ D_3 or ethanol. At various times, paired flasks were harvested and utilized in ^{125}I-HrIL-1α (1 nM) binding experiments as described (see Methods). Data represent the mean (n = 3) and SD of the net ^{125}I-HrIL-1α (cpm) bound/1×10^6 cells.

however, ^{125}I-HrIL-1α binding was increased more than 2-fold when compared to control.

While the above studies documented the capacity for 1,25 $(OH)_2$ D_3 to augment MD10 cell ^{125}I-HrIL-1α binding utilizing IL-1R saturating ligand concentrations, it was unclear whether this observation reflected a change in IL-1R number or affinity. Figure 2 shows the results of equilibrium binding studies utilizing varying ligand concentrations. Consistent with our previously reported findings [Lacey and Erdmann, 1990], MD10 cells expressed saturable cell surface ^{125}I-HrIL-1α binding sites (Fig. 2A). Resolution of the data on Scatchard plots (Fig. 2B) demonstrated that MD10 1,25 $(OH)_2$ D_3 treatment resulted in increased IL-1R expression (B_{maxD_3} = 7,833 receptors/cell, B_{maxCtl} = 5461 receptors/cell) with no significant change in the IL-1R K (K_{D_3} = 255 pM, K_{Ctl} = 218 pM).

The next experiments were performed to determine the mechanism for the IL-1R upregulating effect driven by 1,25 $(OH)_2$ D_3. Two experiments were performed to document the potential role of the VDR in this effect. In the first experiment, we assessed MD10 cellular extracts for the presence of VDRs utilizing a hydroxylapatite-based ^{3}H-1,25 $(OH)_2$ D_3 binding assay [Wecksler and Norman, 1979]. We found that MD10 cellular extracts contained saturable ^{3}H-1,25 $(OH)_2$ D_3 binding sites as shown in Figure 3A. Resolution of the data on Scatchard plots, shown in Figure 3B, demonstrated the presence of a single, high-affinity ^{3}H-1,25 $(OH)_2$ D_3 binding site

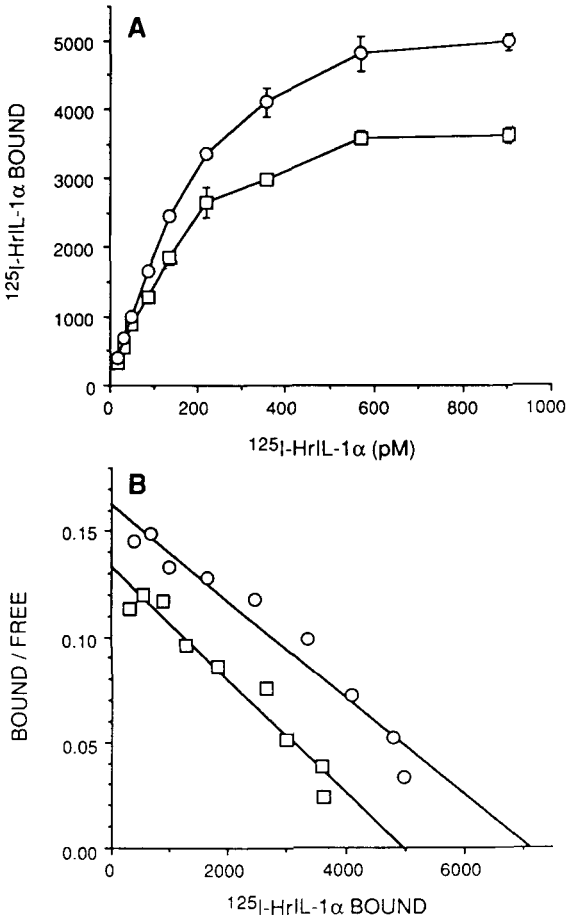

Fig. 2. 1,25 $(OH)_2$ D_3 increases MD10 ^{125}I-HrIL-1α binding through increased surface expression of a saturable, high-affinity site. MD10 cells (5×10^5 cells/ml) were treated with 5 nM 1,25 $(OH)_2$ D_3 or an equivalent ethanol concentration for 24 h. ^{125}I-HrIL-1α binding was performed with various ^{125}I-HrIL-1α concentrations as described (see Methods). **A:** Specific ^{125}I-HrIL-1α (cpm) binding curves for 1,25 $(OH)_2$ D_3 (○) and ethanol (□) treated cells. **B:** Scatchard analysis of the data presented in A.

(Kd = 0.45 nM). The number of VDRs expressed was 88 receptors/cell.

In the second experiment, both 1,25 $(OH)_2$ D_3 and 25 (OH) D_3 were utilized as the MD10 stimuli. If the VDR was mediating the 1,25 $(OH)_2$ D_3 effect, IL-1R upregulation induced by 25 (OH) D_3 would require a 2–3 log order higher concentration of this metabolite than 1,25 $(OH)_2$ D_3. As shown in Figure 4, this was the case. The biologically less potent metabolite 25 (OH) D_3, like 1,25 $(OH)_2$ D_3, increased ^{125}I-HrIL-1α binding. Because this effect required a much greater 25 (OH) D_3 concentration relative to 1,25 $(OH)_2$ D_3, it was likely that the VDR mediated this steroid effect.

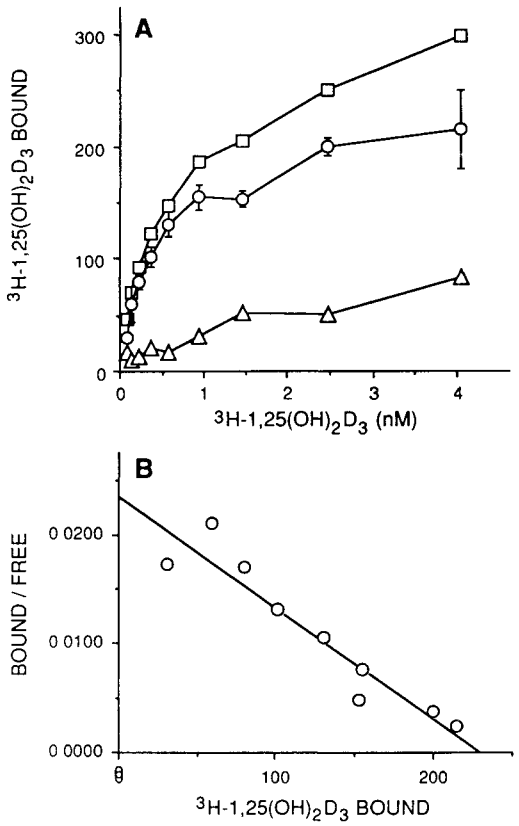

Fig. 3. MD10 cells express high-affinity receptors for 1,25 $(OH)_2$ D_3 MD10 cell extracts were prepared as described from cells freshly isolated from passage conditions The extracts were subsequently employed in the 1,25 $(OH)_2$ D_3R assay (see Methods) **A:** Binding curves of MD10 extracts demonstrating the total (□), nonspecific (△), and net (○) cpm ^{3}H-1,25 $(OH)_2$ D_3 bound **B:** Scatchard analysis of the data in A

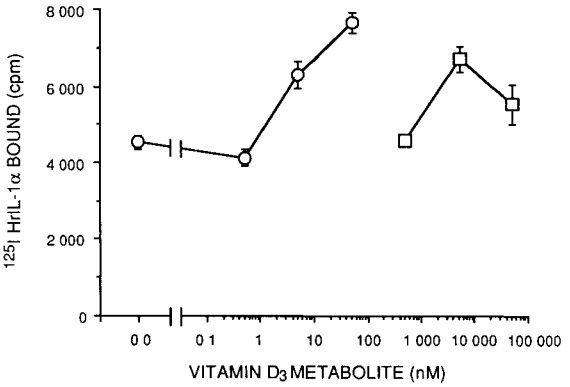

Fig. 4. 1,25 $(OH)_2$ D_3 and 25 (OH) D_3 effects on the MD10 IL-1R MD10 cells (5×10^5 cells/ml) were cultured in the presence of various concentrations of 1,25 $(OH)_2$ D_3 (○), 25 (OH) D_3 (□) or ethanol-containing control media for 24 h ^{125}I-HrIL-1α (1 nM) binding was then performed (see Methods) The data are expressed as the mean (n = 3) ± SD of the specific ^{125}I-HrIL-1α (cpm) bound/1 × 10^6 cells

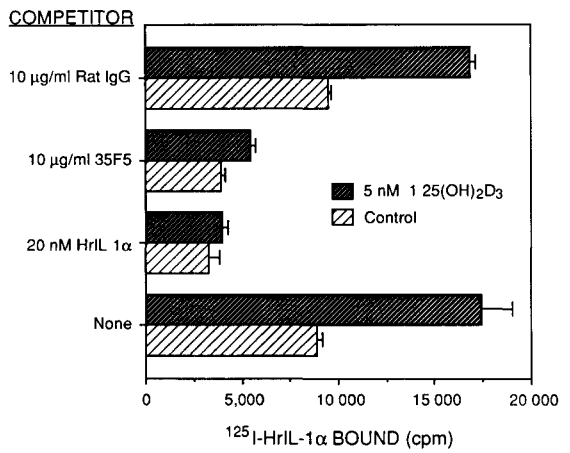

Fig. 5. 1,25 $(OH)_2$ D_3 increases MD10 type 1 IL-1R expression MD10 cells (5×10^5 cells/ml) were exposed to media or media supplemented with 5 nM 1,25 $(OH)_2$ D_3 for 24 h Prior to the addition of ^{125}I-HrIL-1α (1 nM), the cells from each treatment group were exposed to buffer alone or buffer supplemented with 20 nM HrIL-1α, 10 μg/ml 35F5, or 10 μg/ml rat IgG for 1 h at 4°C ^{125}I-HrIL-1α (1 nM) binding was then performed (see Methods) The data are expressed as the mean (n = 3) ± SD of the specific ^{125}I-HrIL-1α (cpm) bound/1 × 10^6 cells

To determine if the 1,25 $(OH)_2$ D_3-increased ^{125}I-HrIL-1α binding was mediated by elevated type 1 IL-1R expression, ^{125}I-HrIL-1α binding experiments were performed utilizing an anti-type 1 IL-1R monoclonal antibody as a blocking agent. As shown in Figure 5, preexposure of MD10 cells to either 20 nM HrIL-1α or 10 μg/ml 35F5 results impaired ^{125}I-HrIL-1α binding to the same extent. In contrast, 10 μg/ml of rat IgG failed to impede ^{125}I-HrIL-1α binding. These results suggested that 1,25 $(OH)_2$ D_3 enhanced ^{125}I-HrIL-1α binding by increasing type 1 IL-1R levels at the cell surface.

Previous studies from this laboratory have documented that IL-4 induces MD10 IL-1R up-regulation [Lacey and Erdmann, 1990]. These observations raised the possibility that 1,25 $(OH)_2$ D_3 enhanced the production of IL-4, which in turn could have stimulated IL-1R expression in an autocrine manner. To address this possibility, MD10 cells were treated with 1,25 $(OH)_2$ D_3 in the presence of anti-murine IL-4 (11B11) hybridoma supernatants. As Table II shows, MD10 cells treated with 1,25 $(OH)_2$ D_3 in the presence of 11B11 containing supernatants (1:20 dilution) still enhanced ^{125}I-HrIL-1α binding whereas the capacity of IL-4 (50 U/ml) to augment ^{125}I-HrIL-1α binding was completely inhibited by the antibody-containing supernatant.

Because IL-1 also influences MD10 IL-R expression, we explored the effects of 1,25 $(OH)_2$

TABLE II. Anti-IL-4 Antibody Effects on 1,25 $(OH)_2$ D_3 and IL-4 Induced MD10 IL-R Upregulation

Condition[a]	11B11	^{125}I-HrIL-1α[b]	T/C[c]
Control	−	10,223 ± 435[d]	1.0
	+	9,552 ± 431	0.93
IL-4	−	16,928 ± 810	1.66
	+	10,018 ± 465	0.98
1,25 $(OH)_2$ D_3	−	14,186 ± 1424	1.39
	+	13,069 ± 1024	1.28

[a]MD10 cells were exposed to media alone, IL-4 (50 U/ml) or 1,25 $(OH)_2$ D_3 (5 nM) in the presence and absence of 11B11 supernatant (1:20 dilution) for 24 h.
[b]^{125}I-HrIL-1α (1 nM) binding was determined as described (see Methods).
[c]T/C ratio determined by cpm condition/cpm control.
[d]Data are expressed as the mean (n = 3) net ^{125}I-HrIL-1α bound ± SD.

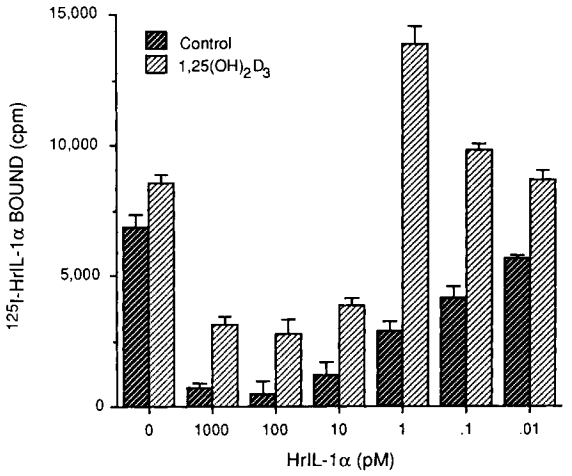

Fig. 6. 1,25 $(OH)_2$ D_3 increases IL-1 treated MD10 ^{125}I-HrIL-1α binding. MD10 cells (5 × 10^5 cells/ml) were treated with various concentrations of HrIL-1α in the presence and absence of 5 nM 1,25 $(OH)_2$ D_3 for 24 h. At the end of this period, the cells were acid-stripped to remove surface bound IL-1 and then utilized in ^{125}I-HrIL-1α (1 nM) binding experiments. The data are expressed as the mean (n = 3) ± SD of the net ^{125}I-HrIL-1α (cpm) bound/1 × 10^6 cells.

D_3 combined with IL-1 on MD10 IL-1R regulation. Figure 6 shows the results of MD10 ^{125}I-HrIL-1α binding where the cells had been exposed for 24 h to a range of IL-1 (0.01–1,000 pM HrIL-1α) in the presence and absence of 5 nM 1,25 $(OH)_2$ D_3. In the absence of steroid, high IL-1 levels markedly downregulate IL-1R expression. However, this marked downregulatory effect was significantly antagonized by the coaddition of 5 nM 1,25 $(OH)_2$ D_3. The effect of the steroid in the presence of low IL-1 levels was even more interesting. When 1 pM HrIL-1α and

TABLE III. 1,25 $(OH)_2$ D_3 Reverses IL-1 Induced MD10 IL-1R Downregulation

Condition[a]	24 h addition[b]	^{125}I-HrIL-1α bound[c]
Control (0)	—	4,992 ± 406[d]
Control (24)	—	12,839 ± 518
HrIL-1α (24)	—	606 ± 241
HrIL-1α (48)	—	Not detectable
HrIL-1α (48)	1,25 $(OH)_2$ D_3	1,700 ± 306

[a]MD10 cells were treated with 100 pM HrIL-1α or media alone for the hours indicated in parentheses.
[b]5 nM 1,25 $(OH)_2$ D_3 was added to one of two paired flasks.
[c]^{125}I-HrIL-1α binding (1 nM) was performed after acid-stripping the cells as described (see Methods).
[d]The data are reported as the mean (n = 3) ± SD of the net cpm ^{125}I-HrIL-1α bound/1 × 10^6 cells.

5 nM 1,25 $(OH)_2$ D_3 were combined in MD10 cell treatments, the level of IL-1R expression was greater than that observed when either agent was used alone. To assess the capacity of 1,25 $(OH)_2$ D_3 to reverse the IL-1R-downregulatory effects of IL-1, MD10 cells were treated with 100 pM HrIL-1α for 24 h then 5 nM 1,25 $(OH)_2$ D_3 was added for an additional 24 h. As Table III shows, a 24 h 100 pM IL-1 treatment diminished MD10 ^{125}I-HrIL-1α binding to approximately 5% of that exhibited by cells treated with media alone. After an additional 24 h, the level of ^{125}I-HrIL-1α binding exhibited by IL-1 treated cells, not steroid exposed, became undetectable. However, the 24 h IL-1 treated MD10 cells that were subsequently 1,25 $(OH)_2$ D_3-treated, showed a marked increase in ^{125}I-HrIL-1α binding at 48 h. The level of ^{125}I-HrIL-1α binding observed in this group exceeded that exhibited by MD10 cells treated with IL-1 alone for either 24 or 48 h. These results indicate that the steroid possesses the capacity, in MD10 cells, to both antagonize and reverse IL-1-induced IL-1 receptor downregulation.

The time delay between 1,25 $(OH)_2$ D_3 and increased ^{125}I-HrIL-1α binding together with the probable involvement of the VDR, as noted in Figures 1 and 4, indicated that this 1,25 $(OH)_2$ D_3 effect likely involved changes in gene expression. To explore this possibility, we examined the level of type 1 IL-1R mRNA on Northern blots prepared with total cell RNA from MD10 cells treated for 24 h with the steroid in the presence and absence of high and low levels of IL-1. As shown in Figure 7, MD10 cells, exposed to media alone, expressed detectable levels of type 1 IL-1R message. Whereas 1 pM HrIL-1α treatment had little impact on type 1 IL-1R

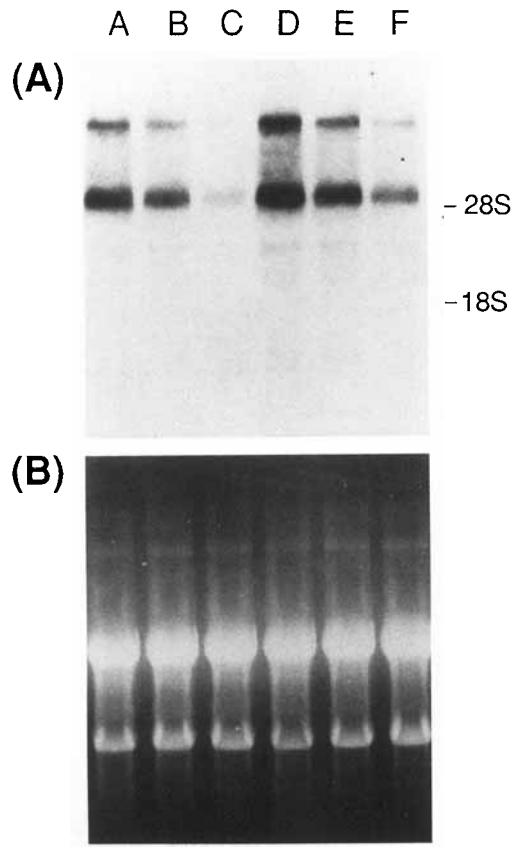

Fig. 7. 1,25 $(OH)_2$ D_3 increases MD10 steady-state type 1 IL-1R mRNA levels. MD10 cells (5×10^5 cells/ml) were treated with media alone or media containing various combinations of HrIL-1α (1, 100 pM) or 5 nM 1,25 $(OH)_2$ D_3 for 24 h. Total cell RNA was purified, electrophoresed (20 μg/lane), electroblotted onto nylon, hybridized with a ^{32}P-type 1 IL-1R probe, and autoradiographs prepared as described (see Methods). The lanes are as follows: **A,** control; **B,** 1 pM HrIL-1α; **C,** 100 pM HrIL-1α; **D,** 1,25 $(OH)_2$ D_3; **E,** 1,25 $(OH)_2$ D_3 + 1 pM HrIL-1α; **F,** 1,25 $(OH)_2$ D_3 + 100 pM HrIL-1α. A and B are photographs of the autoradiograph and ethidium bromide-stained gel, respectively.

mRNA levels, 100 pM HrIL-1α treatment led to a significant decline in the amount of type 1 IL-1R mRNA. Importantly, 1,25 $(OH)_2$ D_3 increased type 1 IL-1R mRNA levels both alone and in combination with either amount of IL-1. In particular, note the effect of the steroid on type 1 IL-1R mRNA levels in cells treated with 100 pM IL-1. Similar to its effects on ^{125}I-HrIL-1α binding by MD10 cells treated with high IL-1 concentrations, 1,25 $(OH)_2$ D_3 antagonized the downregulation of MD10 type 1 IL-1R mRNA induced by this cytokine treatment.

While these effects of 1,25 $(OH)_2$ D_3 on the type 1 IL-1R were provocative, it was not clear whether this IL-1R upregulation was functionally relevant. To address this issue, we examined the impact of the steroid on IL-1-driven MD10 proliferation. For these studies, we pretreated the cells for 24 h with 5 nM 1,25 $(OH)_2$ D_3 to upregulate the type 1 IL-1R. Following this treatment, we rinsed the cells, exposed them to low levels of HrIL-1α for 72 h, and then assessed cellular proliferation using the MTT assay. As shown in Figure 8A, 1,25 $(OH)_2$ D_3 pretreatment enhanced cellular proliferation at very low IL-1 levels with an optimal enhancing effect observed at approximately 30 fM HrIL-1α. At this cytokine level, cellular proliferation was increased by approximately 60%. To determine whether the IL-1-induced proliferative effect on 1,25 $(OH)_2$ D_3-treated MD10 cells was mediated by the type 1 IL-1 receptor, we explored the impact of 35F5 on IL-1-induced cellular proliferation. As shown in Figure 8B, cells exposed to 35F5 fail to proliferate in response to IL-1 concentrations (<100 fM) where 1,25 $(OH)_2$ D_3 treatment results in the greatest proliferation enhancing effects (Fig. 8A).

DISCUSSION

While originally viewed as the steroid hormone principally involved in calcium/phosphate homeostasis, the range of 1,25 $(OH)_2$ D_3 effects and the types of cells influenced by this steroid lie well beyond those typically associated with bone and mineral metabolism [Haussler, 1986; Reichel et al., 1989]. In this report, we document the capacity for 1,25 $(OH)_2$ D_3 to alter the expression of T cell receptors for the inflammatory cytokine IL-1. Utilizing a T cell derived from a murine TH2 cell clone that expresses readily detectable IL-1R, we have shown that IL-1R expression is increased by 1,25 $(OH)_2$ D_3 in a dose- (Table I), time- (Fig. 1), and metabolite- (Fig. 4) dependent manner. Scatchard analysis of the ^{125}I-HrIL-1α binding data show that the major change in ^{125}I-HrIL-1α binding can be explained through an increase in site number and not a significant change in site affinity (Fig. 2). The capacity for 35F5 to block ^{125}I-HrIL-1α binding (Fig. 5) indicates that the steroid increases the expression of the type 1 IL-R. The mechanism involved is likely mediated through the VDR based on the metabolite specificity (Fig. 4) of the response and the documented presence of measurable 1,25 $(OH)_2$ D_3R (Fig. 3). The capacity for the steroid to augment steady-state type 1 IL-1R mRNA levels (Fig. 7) suggests that either increased message stability and/or

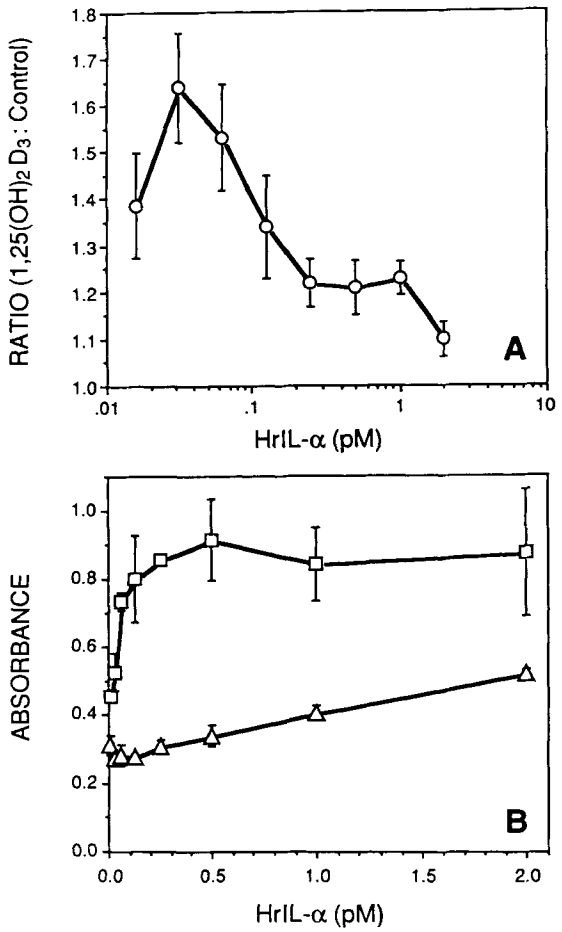

Fig. 8. 1,25 $(OH)_2$ D_3 pretreatment increases IL-1-induced MD10 proliferation through the type 1 IL-1 receptor. **A:** MD10 cells (5×10^5 cells/ml) were pretreated with media alone or media containing 5 nM 1,25 $(OH)_2$ D_3 for 24 h. The cells were rinsed and then exposed to various amounts of HrIL-1α as indicated. Following 72 h the MTT assay was performed as described. The ratio of the absorbances (570 nm with reference of 630 nm) of the 1,25 $(OH)_2$ D_3 treated:control groups were determined for each of three separate experiments and the mean and SD of the pooled data are displayed. **B:** MD10 cells (5×10^5 cells/ml) were pretreated with media containing 5 nM 1,25 $(OH)_2$ D_3 for 24 h, rinsed and then employed in the IL-1 bioassay. Prior to their exposure to various amounts of HrIL-1α, the cells were exposed to either 35F5 (Δ, 10 μg/ml) or the same amount of rat IgG (□) for 30 min. Following a 72 h incubation, cellular proliferation was assessed using the MTT assay as described. The results are reported as the mean (n = 3) ± SD.

increased type 1 IL-1R gene transcription plays a role in this effect.

It is unlikely that IL-4 is a cytokine intermediary required for 1,25 $(OH)_2$ D_3-induced IL-1R upregulation for two reasons. First, 1,25 $(OH)_2$ D_3 can enhance IL-1R expression in MD10 cells despite the presence of anti-IL-4 monoclonal antibodies (Table II). This experiment does not exclude the possibility that 1,25 $(OH)_2$ D_3 induced the production of IL-4 that subsequently operated within a compartment not accessible to the antibody. However, the observed failure of the cells to proliferate in response to 1,25 $(OH)_2$ D_3 is inconsistent with that possibility because IL-4 stimulates MD10 proliferation [Lacey et al., 1987].

Two different cell surface receptors for IL-1 have been identified [Horuk et al., 1987; Chizzonite et al., 1989; Bomstyk et al., 1989] and cloned [Sims et al., 1988; McMahan et al., 1991]. While it was originally thought that the type 1 IL-1R was restricted to T cells and mesenchymal cells and the type 2 IL-1R restricted to monocytes and B cells, use of the type II IL-1R cDNA as a probe in Northern blots indicate that such a restricted distribution of these receptor subtypes is unlikely [McMahan et al., 1991]. Evidence presented in this report suggests that, in MD10 cells, the type 1 IL-1R predominates (Fig. 5) and is upregulated by 1,25 $(OH)_2$ D_3. These results in no way exclude the possibility that the steroid may also influence the expression of the type 2 IL-R in other cells.

A potential physiologic role for 1,25 $(OH)_2$ D_3 as an immunoenhancer is suggested by its capacity to increase IL-1-driven proliferation of MD10 cells (Fig. 8A). Steroid-enhanced MD10 proliferation became apparent at suboptimal IL-1 levels that are far below the IL-1R Kd. Reasoning that engagement of only a small number of IL-1 receptors can trigger maximal cellular responses [Dower et al., 1986], we hypothesize that the modest increase in IL-1R content induced by 1,25 $(OH)_2$ D_3 would only become manifest, biologically, in conditions where ligand/receptor complexes were at limiting numbers. In MD10 cells, that circumstance occurs at IL-1 levels at or below approximately 100 fM. Consistent with this prospect, the maximal 1,25 $(OH)_2$ D_3 effect was observed at ligand concentrations just below this amount (30 fM, Fig. 8A). At higher IL-1 concentrations, this potentiation was not observed, which was likely due to the presence of excess ligand/receptor complexes. Furthermore, the capacity for 35F5 to block IL-1-driven proliferation (Fig. 8B) suggests that it is the type 1 IL-1 receptor that mediates IL-1 driven MD10 proliferation.

In addition to 1,25 $(OH)_2$ D_3, a number of substances enhance IL-1R expression, including peptides, glucocorticoids, and prostanoids. In

mesenchymal cells, platelet derived growth factor [Bonin and Singh, 1988], fibroblast growth factor [Chandrasekhar and Harvey, 1989], and prostaglandin E_2 [Akahoshi et al., 1988b] have been found to enhance IL-1R expression by mechanisms involving protein synthesis. In peripheral blood lymphocytes, glucocorticoids have the capacity to elevate IL-1R levels [Akahoshi et al., 1988a]. Concanavalin A, in the presence of accessory cells, can enhance IL-1R expression in freshly isolated T cells [Shirakawa et al., 1987]. As mentioned above, we have recently reported the capacity of IL-4 to augment IL-1R levels in this T cell line confirming the results of Savage et al. [Savage et al., 1989]. Whether or not the 1,25 $(OH)_2$ D_3 effect on the IL-1R is specific for only this T cell, for T cells in general, or for that matter, both B and T lymphocytes is unknown. Further, we have recently documented that this steroid also increases type 1 IL-1R expression in MC3T3-E1 cells (Lacey et al., in press), a mesenchymal cell line that expresses an osteoblastic phenotype, raising the possibility that this steroid may enhance the catabolic activities induced by the cytokine [Dinarello and Savage, 1989] in connective tissue cells.

While 1,25 $(OH)_2$ D_3 is an immunosuppressive agent as regards IL-2-dependent immune responses, cytokines other than IL-2 are important in certain types of immune activities. For instance, the presence of at least two types of T helper cell clones [Mosmann et al., 1986] in the mouse and the restricted lymphokine secretion pattern exhibited by these two T cell classes has suggested to some that, in vivo, these different T cell types represent one mechanism by which the immune system could tailor itself to meet certain challenges [Mosmann et al., 1989]. Agents with inhibitory effects on IL-2 or interferon gamma production, such as 1,25 $(OH)_2$ D_3, may exert different influences on T cell types whose products do not include either of these cytokines. Because IL-2 is neither produced by TH2 cells nor necessary for their proliferation, it is possible that 1,25 $(OH)_2$ D_3 could enhance the activities of this T cell subset. Reasoning that MD10 cells were derived from a TH2 clone that was stimulated by 1,25 $(OH)_2$ D_3 [Lacey et al , 1987], this scenario is possible and needs to be further investigated. In any event, the capacity of 1,25 $(OH)_2$ D_3 to augment type 1 IL-1R in this T cell lends further support to the concept that this steroid possesses the capacity to both inhibit *and* augment immune cell activity.

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