Regulation of Lymphokine Production and Human T Lymphocyte Activation by 1,25-Dihydroxyvitamin D3

Specific Inhibition at the Level of Messenger RNA

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Abstract

The steroid hormone, $1\alpha,25$-dihydroxyvitamin D_3 (calcitriol), has been shown to inhibit T cell proliferation, primarily through inhibition of interleukin 2 (IL-2) production. In these experiments, we show that calcitriol also markedly inhibited production of the lymphokine, gamma interferon (IFN-γ), by activated human T lymphocytes. Regulation of both IL-2 and IFN-γ production as well as transferrin receptor (TfR) expression by calcitriol was apparent at the messenger RNA (mRNA) level as determined by Northern blotting. The decrease in IL-2 and IFN-γ mRNA that occurred with calcitriol treatment was coordinate and not apparent up to 12 h after phytohemagglutinin stimulation, whereas decreased accumulation of TfR mRNA was not present before 24–36 h. Furthermore, the effects of calcitriol on IL-2, IFN-γ, and TfR mRNA accumulation were specific; actin mRNA accumulation was comparable between control and treated cells. These data indicate that calcitriol regulated proteins associated with T cell activation at the transcriptional level and that these effects were mediated in a specific, coordinate fashion.

Introduction

Recent studies have suggested that $1\alpha,25$-dihydroxyvitamin D_3 (calcitriol) can modulate lymphocyte growth and function (1–3). Awareness of this immunoregulatory activity of calcitriol was prompted by studies of vitamin D metabolism as well as the demonstration of specific receptors for vitamin D in monocytes and activated lymphocytes (4, 5). Human pulmonary alveolar macrophages from patients suffering from sarcoidosis have been shown capable of 1α-hydroxylation, which converts 25-hydroxyvitamin D_3 to calcitriol by a metabolic process distinct in its regulation from classical renal metabolism of vitamin D (6). Local production of calcitriol by macrophages may be sufficient to cause systemic effects even in the absence of renal production of calcitriol (7, 8). Moreover, normal human monocytes and macrophages can be induced by gamma interferon (IFN-γ)[1] or lipopolysaccharide (LPS) to exhibit 1α-hydroxylase activity (9). These findings, coupled with the discovery of vitamin D receptors in monocytes and activated, but not resting, T and B cells suggested that calcitriol may be a macrophage-derived product with immunomodulatory activity. Studies examining this activity have revealed that calcitriol is a potent inhibitor of T cell proliferation and immunoglobulin production (1–3). The antiproliferative action of calcitriol specifically blocks transition of T cells from early G_1 (G_{1A}) to late G_1 (G_{1B}) as well as their acquisition of transferrin receptors (TfR), and appears to be primarily mediated through inhibition of interleukin 2 (IL-2) production (1, 10). The ability of vitamin D metabolites to mediate this activity correlates with the affinity with which they are bound by vitamin D receptors (1–3). In the studies described in this report, we present evidence that calcitriol has an additional profound effect on immune reactivity by blocking IFN-γ production by lectin-activated T cells. Furthermore, we have determined that the induction of IL-2 and IFN-γ production as well as TfR expression that accompanies T cell activation is specifically inhibited by calcitriol at the level of mRNA.

Methods

Cell preparation and culture. Peripheral blood mononuclear cells (PBMC) from normal donors were isolated by Ficoll–Hypaque density centrifugation, cultured at 2×10^6/ml in RPMI 1640 medium that was supplemented with 8% fetal calf serum (FCS) (both Hazelton Research Products, Denver, PA), and stimulated with 1 μg/ml phytohemagglutinin (PHA) (Wellcome Reagent Ltd., Beckenham, England) in the absence or presence of calcitriol (the generous gift of Dr. Milan Uskokovic, Hoffman-LaRoche Inc., Nutley, NJ) or 25-hydroxyvitamin D_3 (Upjohn Co., Kalamazoo, MI). Control cultures contained equivalent concentrations of ethanol that never exceeded 0.01%. In RNA isolation experiments, PBMC obtained by cytophoresis of normal volunteers were isolated and partially depleted of monocytes by rotation in polypropylene tubes at 8 rpm for 1 h at 4°C to induce monocyte clumping. This cell suspension was then layered on ice cold FCS and clumped monocytes separated from lymphocytes by sedimentation for 20 min at 4°C. The lymphocytes obtained were 70–80% T3 positive as determined by flow cytometry. Cells were cultured at 4×10^6/ml in RPMI 1640 with 8% FCS in the absence or presence of 10 nM calcitriol and stimulated with 1 μg/ml PHA alone or in combination with 10 ng/ml phorbol myristic acetate (PMA) (Sigma Chemical Co., St. Louis, MO).

Assay of IFN-γ production. Supernatants were harvested and clarified through centrifugation at 10,000 g and were frozen at -20°C until shortly before assay. Supernatants were analyzed for the level of IFN-γ by solid phase radioimmunoassay (RIA) (Centocor Corp., Malvern, PA) that utilized two murine monoclonal antibodies to different epitopes of biologically active IFN-γ. Antibody coupled to polystyrene beads was incubated with culture supernatant, subsequently washed, and a second ^{125}I-labeled antibody was added to a different non–cross-reacting epitope of IFN-γ. After washing, binding of the second antibody to beads was quantified by using a gamma counter. Levels of IFN-γ were then expressed as reference units based on the values obtained with a standard that was provided by the National Institutes of Health.

Measurement of IL-2, IFN-γ, TfR, and actin mRNA levels. Total RNA was extracted by a guanidinium isothiocyanate method with cesium chloride gradient centrifugation (11). Poly(A)-RNA was selected by

1. *Abbreviations used in this paper:* IFN-γ, gamma interferon; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SSC, saline plus 0.015 M sodium citrate, pH 7.0; TfR, transferrin receptors.

oligo(dT)–cellulose chromatography, then size fractionated by formaldehyde–agarose gel electrophoresis, transferred to a Biotrans nylon membrane (ICN Radiochemicals, Div. ICN Biomedicals Inc., Irvine, CA) in 20× saline plus 0.015 M sodium citrate, pH 7.0 (SSC), and baked under vacuum at 80°C for 2 h. Filters were prehybridized overnight at 42°C in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and salmon sperm DNA (200 μg/ml). Hybridizations were performed at 42°C for 48 h in prehybridization mix that contained 10% dextran sulfate and 1×10^6 cpm of ^{32}P-labeled cDNA probes for IL-2 (12), IFN-γ, or human actin (13) that had been nick-translated with 800 Ci/mmol [^{32}P]dCTP (Amersham Corp., Arlington Heights, IL) to a specific activity of 1–2×10^8 dpm/μg DNA. Filters were washed twice with 2× SSC that contained 0.02% sodium pyrophosphate and 0.5% Sarkosyl at 20°C, then washed four times with 0.1× SSC that contained 0.01% sodium pyrophosphate and 0.5% Sarkosyl at 50°C. Blots were dried, and filters exposed at −70°C to Kodak XAR film using one intensifying screen. Sizes of mRNAs were estimated from the position of 28S (4.8 kilobase [kb]) and 18S (2 kb) ribosomal RNA (rRNA) bands that were present in the poly(A)-RNA preparation. Similar data were observed in two other experiments. For Northern blot analysis of the kinetics of calcitriol effect, RNA was extracted at various time intervals (6–48 h after PHA stimulation) from PBMC of three or four different donors. Total cellular RNA was then pooled and poly(A)-RNA was isolated as described above. Northern blotting and hybridization with ^{32}P-labeled IL-2 complementary DNA (cDNA) were performed as described above. After hybridization, blots were stripped of ^{32}P-labeled probe by two incubations at 85–90°C for 20-min each in 0.1× SSC that contained 0.01% sodium pyrophosphate and 0.05% Sarkosyl. Blots were subsequently stripped and hybridized with ^{32}P-labeled IFN-γ, TfR, and actin cDNA probes.

Results

Effect of vitamin D compounds on IFN-γ production. The lymphokine IFN-γ is produced by activated T cells in response to mitogen or antigen and has a broad range of immunoregulatory activity, including macrophage activation (14). Perhaps more relevant has been the demonstration that IFN-γ can regulate macrophage production of calcitriol (6, 9). Moreover, agents that inhibit the production of IL-2 (glucocorticoids, cyclosporin A) (15, 16) have been shown to also inhibit production of IFN-γ (17, 18). We therefore examined the effect of calcitriol on IFN-γ production by PBMC that were stimulated with PHA for 72 h (Table I). Calcitriol blocked IFN-γ production in a concentration-dependent fashion. Half-maximal suppression of immunoreactive IFN-γ production was observed at calcitriol concentrations of 1 nM, with maximal effect apparent at 10 nM calcitriol. The observed dose–response curve is similar to that reported with inhibition of IL-2 production (1, 10). As found in previous work, 25-hydroxyvitamin D$_3$, which binds the vitamin D receptors 100-fold less avidly than calcitriol (19), was without detectable activity until much higher concentrations (1 mM). These findings demonstrate the specificity of this activity of calcitriol and suggest that this inhibition is dependent on ligand binding to vitamin D receptors. We have confirmed these results through bioassay by utilizing the inhibition of viral cytopathic effect on human fibroblasts by IFN-γ (data not shown).

Northern blot analysis of the effect of calcitriol on T cell activation. To clarify the mechanism of action of calcitriol, we examined its effect on the level of accumulation of mRNA for IL-2 and IFN-γ at 20 h after PHA stimulation by using Northern blotting (Fig. 1). In RNA from PHA-stimulated cells, we observed a 1.0-kb RNA species that hybridized with the ^{32}P-labeled full length IL-2 cDNA probe, which was consistent with the size of

Table I. Inhibition of IFN-γ Production by Vitamin D Compounds*

Treatment	IFN-γ		
	Donor 1	Donor 2	Donor 3
	U/ml	U/ml	U/ml
Ethanol	888	1,070	1,070
1,25-(OH)$_2$-D$_3$ ($10^{-10}M$)	680	945	1,017
1,25-(OH)$_2$-D$_3$ ($10^{-9}M$)	365	545	722
1,25-(OH)$_2$-D$_3$ ($10^{-8}M$)	205	165	260
1,25-(OH)$_2$-D$_3$ ($10^{-7}M$)	210	165	ND
25-OH-D$_3$ ($10^{-8}M$)	945	1,040	ND
25-OH-D$_3$ ($10^{-7}M$)	936	973	ND
25-OH-D$_3$ ($10^{-6}M$)	175	127	ND

* Inhibition of IFN-γ production by vitamin D compounds. Human PBMC stimulated with PHA were cultured (2 $\times$ 10^6 cells/ml) for 72 h in the absence or presence of varied concentrations of calcitriol (1,25-[OH]$_2$-D$_3$), 25-hydroxyvitamin D$_3$ (25-OH-D$_3$), or ethanol. Supernatants were then assayed for IFN-γ by RIA. Data shown represent the average of duplicate samples with a maximum range of < 10% among replicates.
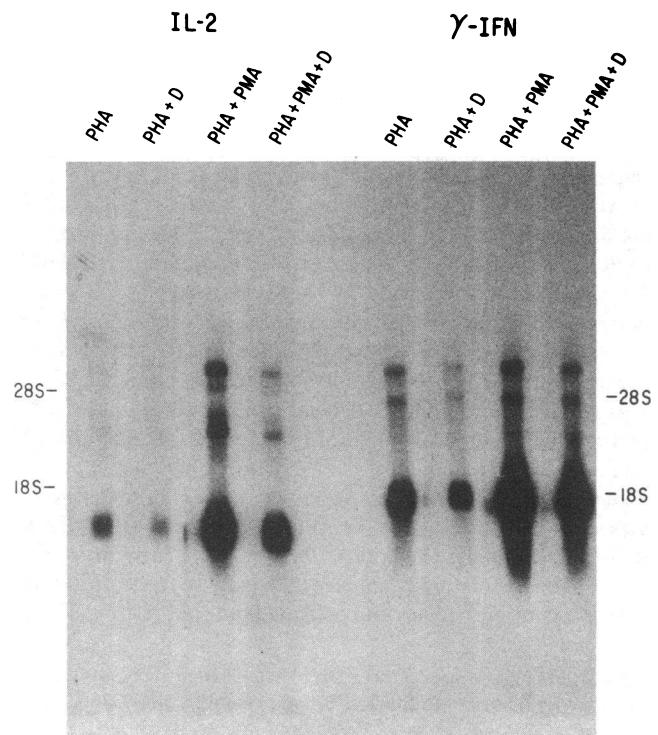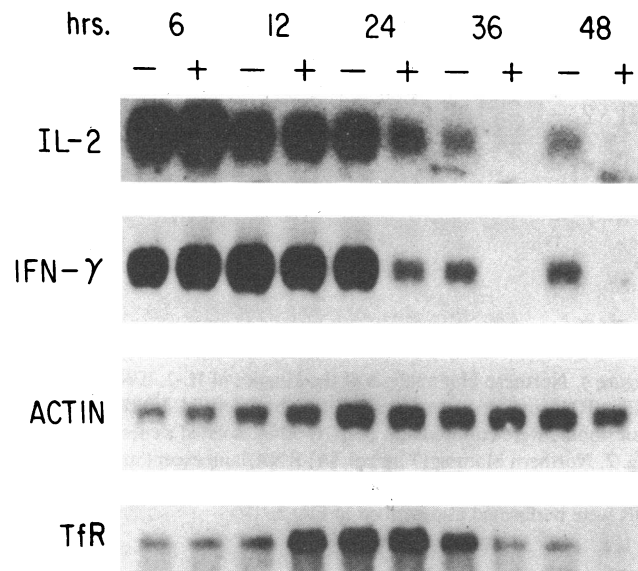

Figure 1. Northern blot analysis of IL-2 and IFN-γ mRNA from PBMC activated in the absence or presence of calcitriol. 10 μg/lane poly(A)-RNA from PBMC that had been cultured at 4 $\times$ 10^6/ml in the absence or presence of 10 nM calcitriol for 20 h after activation with PHA alone or in combination with 10 ng/ml PMA, were size-fractionated and analyzed for the level of IL-2 and IFN-γ mRNA by using Northern blotting. Blots were overexposed to demonstrate larger mRNA species.

IL-2 mRNA previously reported (20, 21). The amount of IL-2 mRNA in poly(A)-RNA from calcitriol-treated PBMC was markedly decreased relative to control. Treatment of cells with PHA and PMA resulted in a significant increase in the level of detectable IL-2 mRNA, as previously shown (22). Nevertheless, calcitriol treatment caused a significant reduction in the level of IL-2 mRNA, which suggests that the addition of PMA to PHA did not overcome the effects of calcitriol. This observation is consistent with our previous finding that PMA addition to PHA does not affect the antiproliferative activity of calcitriol (10). Also, at least two species of larger mRNAs that hybridized with the IL-2 probe were observed in poly(A)-RNA from cells activated with PHA and PBMC, which was consistent with the size of precursor IL-2 mRNAs that have been previously reported (21). The level of expression of each of these larger species of IL-2 mRNA (perhaps representing precursors) was also reduced by calcitriol treatment.

In companion experiments, a similar pattern of reduction in IFN-γ mRNA accumulation by calcitriol was observed (Fig. 1). Northern blotting with a ^{32}P-labeled IFN-γ cDNA and IFN-γ-L8-9 that contained ∼ 75% of the coding sequence of mature IFN-γ revealed a 1.4-kb band in poly(A)-selected RNA from PHA-stimulated cultures that was augmented by the addition of PMA. Under both sets of conditions (PHA without and with PMA), calcitriol treatment of cells reduced the levels of detectable IFN-γ mRNA. In addition, two bands of larger mRNA (∼ 4.4 and 5.2 kb) that hybridized with ^{32}P-labeled IFN-γ cDNA were observed in each experiment, and also apparently increased in response to PMA treatment. Calcitriol treatment reduced the level of expression of these larger mRNA species as well. These data demonstrate that accumulation of both mature and possibly precursor forms of IL-2 and IFN-γ mRNA were reduced by calcitriol treatment.

To determine if IL-2 is necessary for IFN-γ production (23, 24) or if there is coordinate regulation of the expression of these genes (25, 26), we examined the kinetics of the effect of calcitriol on the levels of IL-2 and IFN-γ mRNA in lectin-stimulated cells (Figs. 2 and 3). In this experiment, the level of IL-2 mRNA accumulation was observed to be maximal at 6 h. The level of IL-2 mRNA decreased at 12 h, and slightly increased at 24 h. Calcitriol treatment did not affect the level of accumulation of IL-2 mRNA until 24 h, at which point a progressive reduction in IL-2 mRNA relative to controls was observed. Interestingly, at 6 h, calcitriol slightly but consistently augmented the level of IL-2 mRNA relative to controls, while at 12 h equivalent levels of IL-2 mRNA were observed. Similarly, no significant inhibitory effect of calcitriol on IFN-γ mRNA accumulation was observed before 24 h, after which calcitriol caused a progressive reduction in the levels of IFN-γ mRNA. As found with IL-2 mRNA, calcitriol treatment appeared to effect a slight increase in the level of IFN-γ mRNA relative to controls at 6 h, whereas a slight decrease in IFN-γ mRNA first became apparent at 12 h. In a second experiment (Fig. 4), this biphasic pattern of induction of IL-2 and IFN-γ was more evident, with decreased IL-2 and IFN-γ mRNA accumulation at 12 h relative to that seen at 6 and 24 h. Nevertheless, in each experiment, the inhibition of IL-2 and IFN-γ mRNA accumulation observed with calcitriol treatment was not apparent until 12–24 h.

Induction of TfR gene transcription by T lymphocytes has been shown to follow that of IL-2 and IFN-γ (25), and has been demonstrated to be dependent on IL-2 interacting with its receptor (27). In previous work we have observed that calcitriol
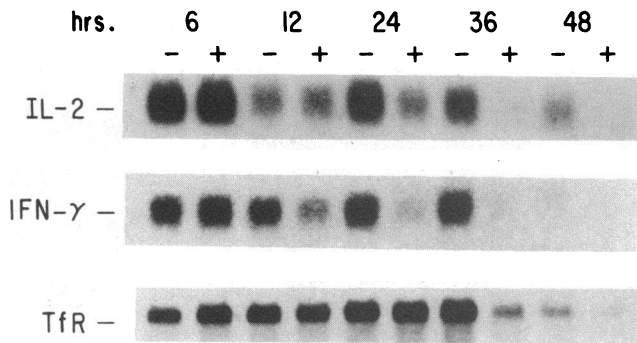

Figure 2. Northern blot analysis of the kinetics of IL-2, IFN-γ, and TfR mRNA induction and its inhibition by calcitriol. PBMC from three donors were cultured in the absence (−) or presence (+) of calcitriol (10 nM), and RNA was extracted at various time intervals (6–48 h after PHA addition). Total cellular RNA for each time point and treatment was pooled and poly(A)-RNA was isolated by oligo(dT)–cellulose chromatography. Northern blotting (5 μg poly[A]-RNA/lane) and hybridization with ^{32}P-labeled IL-2 cDNA were performed as described in Fig. 1. After hybridization and autoradiography with IL-2 cDNA, blots were stripped and subsequently rehybridized with ^{32}P-labeled IFN-γ, TfR, and actin cDNA probes. Blots were overexposed for sake of photography.

inhibited TfR expression on activated T cells, whereas IL-2 receptors were unaffected (10). We therefore examined the effect of calcitriol on the kinetics of TfR mRNA accumulation in activated T cells by using the TfR cDNA pcD-TR1 (Figs. 2–4) (28). We observed that peak TfR mRNA accumulation occurred much later than with IL-2 or IFN-γ (24–36 h after PHA stimulation). No reduction in TfR mRNA accumulation by calcitriol was observed before 24 h, with inhibition becoming marked by
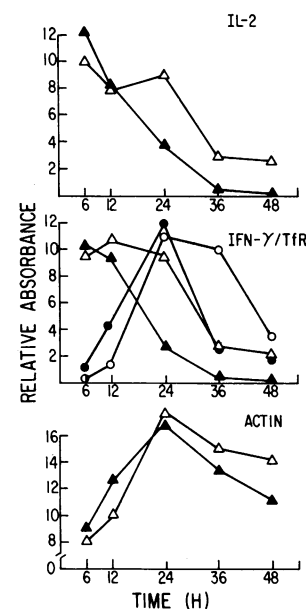
Figure 3. Time course of IL-2, IFN-γ, TfR, and actin mRNA accumulation and its inhibition by calcitriol. Autoradiograms demonstrated in Fig. 2 were quantified by densitometry, and relative absorbance was plotted as a function of time. Decreased exposures of the IL-2 and IFN-γ blots shown in Fig. 2 were utilized for densitometry. Open symbols (○, △) represent values obtained with ethanol controls; closed symbols (●, ▲) represent calcitriol treatment. In the middle panel, IFN-γ and TfR mRNA accumulation are represented by triangles and circles, respectively.

hrs.   6   12   24   36   48
     − + − + − + − + − +

IL-2 −

IFN-γ −

TfR −

Figure 4. Northern blot analysis of the kinetics of IL-2, IFN-γ, and TfR mRNA induction and its inhibition by calcitriol. PBMC from four donors were cultured and poly(A)-RNA isolated as described in Fig. 2. Northern blotting (7 μg poly[A]-RNA/lane except at 48 h where 4 μg/lane was used) and hybridizations with IL-2, IFN-γ, and TfR were performed as described in Fig. 2.

36 h in these two experiments. Interestingly, at earlier times (6–24 h) in each experiment, it appeared that calcitriol augmented the accumulation of TfR mRNA relative to controls. Though similar to observations with IL-2 and IFN-γ mRNA, the increase in TfR mRNA accumulation in calcitriol-treated cells relative to control cells seen at 6–12 h (Figs. 2 and 3) and 24 h (Fig. 4) was more apparent. The slightly different rates at which the effect of calcitriol is noted may be due to donor variation, as different sets of donors were used in each experiment. Finally, the selectivity of this activity of calcitriol was demonstrated by the finding that despite a significant reduction in the level of IL-2, IFN-γ, and TfR mRNAs with calcitriol treatment, comparable levels of actin mRNA were observed throughout (Figs. 2 and 3).

Discussion

In this paper, we examined the immunomodulating activity of calcitriol at both the cellular and molecular level. IL-2 and IFN-γ production have been shown to accompany T cell activation. We have found that calcitriol inhibits IFN-γ production by PHA-stimulated T cells with a concentration-dependence similar to that observed with IL-2 (1–3). Furthermore, we found that calcitriol reduces the level of accumulation of both IL-2 and IFN-γ mRNA 20 h after stimulation. Time course studies of IL-2 and IFN-γ mRNA accumulation after T lymphocyte stimulation are comparable, which suggests coordinate regulation of their expression. Moreover, the time course of the reduction by calcitriol of IL-2 and IFN-γ mRNA levels was similar. These data therefore suggest that the reduction in IFN-γ mRNA accumulation by calcitriol is not due to decreased IL-2 production. Rather, the decreased levels of IL-2 and IFN-γ mRNA expression effected by calcitriol treatment may be mediated through a common pathway. In addition, consistent with our earlier observations on IL-2 production (4), no significant reduction in either IL-2 or IFN-γ mRNA is seen with calcitriol treatment in the first 12 h after lectin stimulation.

These findings demonstrate that calcitriol, like cyclosporin A and glucocorticoids, can specifically regulate the production of both IFN-γ and IL-2 and that these effects are apparent at the level of the mRNAs that specifically encode these proteins. These effects appear to be relatively specific, with both levels of actin mRNA and Tac antigen expression (10) not significantly affected by calcitriol treatment. Whether these changes are due

to decreased RNA transcription, or increased degradation of IL-2 or IFN-γ mRNA, has not been established. Studies of the effect of calcitriol treatment on nuclear transcription and message stability will help address these issues.

In contrast to glucocorticoids (15), resting T cells do not express a receptor for calcitriol unless activated (4, 5). Consistent with this observation, calcitriol did not inhibit the initial increase in cellular RNA that accompanies early T cell activation (G_0–G_{1A} transition) (10). Rather, calcitriol appeared to specifically inhibit G_{1A}–G_{1B} transition and the acquisition of TfR, both of which have been shown to be IL-2-dependent events (27–29). Our findings here illustrate that calcitriol does not significantly reduce the level of IL-2 or IFN-γ mRNAs for at least the first 6–12 h, whereas TfR mRNA accumulation is not inhibited for the first 24 h. These observations are consistent with the possibility that until vitamin D receptors are induced in T cells, calcitriol cannot effect any changes in the level of expression of these products of T cell activation. Alternatively, vitamin D receptors may be induced quite early (< 6 h) in T cell activation, but the process that regulates the level of IL-2, IFN-γ, and TfR mRNA expression may only become sensitive to the inhibitory action of calcitriol after 6–12 h. Either possibility is consistent with our findings that calcitriol blunts, but does not totally abrogate, the production of these lymphokines or the expression of TfR by activated T lymphocytes.

The rapid (6 h) induction of most of IL-2 mRNA by PHA seen in our first experiment is consistent with that previously reported using either the Jurkat cell line or PBMC (21, 30). This is in contrast to the levels of IL-2 mRNA as measured by Xenopus laevis oocyte assay, where peak levels of IL-2 production were found with mRNA that was obtained 20 h after lectin stimulation (26, 31). These different observations may result from different assay techniques: levels of IL-2 mRNA measured by hybridization of a cDNA probe may not accurately reflect the utility of this IL-2 mRNA in a Xenopus oocyte translation system. Another possibility is suggested by our observation that IL-2 mRNA expression appears to initially decline at 12 h in each experiment followed by an increased accumulation at 24 h. It may be that a second peak in IL-2 mRNA expression occurs between 12 and 24 h. Such a finding would be consistent with the observation that T cell activation occurs in an asynchronous fashion (32). Thus, in a heterogeneous population of T cells stimulated with lectin, different T cells might be activated to express IL-2 mRNA at different times. The demonstration that calcitriol inhibits IL-2 and IFN-γ mRNA at 20–24 h could possibly reflect the sensitivity to vitamin D of T cells that are activated later. It is unlikely, however, that differential rates of activation of CD4+ and CD8+ T cells could account for these effects, since we have observed equivalent sensitivity and kinetics of each of these T cell subsets to the antiproliferative activity of calcitriol (33).

Not accounted for by these hypotheses is the slight but consistent and specific increase in mRNA for IL-2 and IFN-γ we observe at 6 h with calcitriol treatment relative to controls. Similar, more dramatic augmentation of mRNA accumulation with calcitriol treatment was observed with TfR in each experiment before any inhibition was observed. Calcitriol may induce this biphasic response directly on the T cell, first stimulating initial T cell activation, followed by a specific inhibition of IL-2, IFN-γ, and TfR mRNA accumulation. Alternatively, this early augmentation of IL-2, IFN-γ, and TfR mRNA accumulation may be mediated through the effect of vitamin D compounds on

accessory cell function. Interleukin 1 (IL-1) production by accessory cells has been shown to be important in T cell activation (34–36). Calcitriol has been found to augment IL-1 production by monocytes (36), perhaps resulting in more rapid, efficient T cell activation. Increases in cytoplasmic-free calcium have been implicated in both initial T cell activation (37, 38) as well as IL-1 production by monocytes (39). Recent work has demonstrated that calcitriol can induce rapid increases in cytoplasmic-free calcium (40), which suggests a mechanism that could account for either of these activities. Since induction of TfR expression on T cells is dependent on an interaction of IL-2 with its receptor (27), the augmentation in TfR mRNA observed with calcitriol at 6 and 12 h may be a consequence of increased IL-2 message and subsequent IL-2 production. Alternatively, calcitriol could directly effect this biphasic response on all the genes involved in T cell activation that are susceptible to its action.

These data demonstrate the specificity of calcitriol in modulating T cell activation and proliferation as well as lymphokine production. The conversion by monocytes and macrophages of 25-hydroxyvitamin D₃ into calcitriol may therefore represent a negative feedback loop on T cell proliferation and IFN-γ production. Once adequate levels of macrophage activation are achieved, local production of calcitriol may regulate T lymphocyte-driven immune reactivity and inflammation. Calcitriol may thus be unique as a steroid hormone produced both by and for cells of the immune system.

Acknowledgments

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