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T Lymphocytes and Their CD4 Subset Are Direct Targets for the Inhibitory Effect of Calcitriol[1]

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We studied the direct effects of the hormone calcitriol on the activation and proliferation of pure T lymphocytes and their subsets. Calcitriol inhibited the proliferation of T lymphocytes stimulated in the absence of monocytes with phytohemagglutinin (PHA) and either a monocytic culture supernatant or a combination of monocyte-derived interleukin 1 and interleukin 6. This inhibition was not influenced by the concentration of the stimulating agents. The minimal effective concentration of calcitriol was 10^{-10} *M*. In contrast, the interleukin 2 (10 U/ml)-driven growth of PHA-stimulated T lymphocytes was not significantly altered by calcitriol at 10^{-8} *M*. The hormone had also no influence on the T lymphocyte proliferation induced by a combination of PHA and the anti-CD28 monoclonal antibody 9.3. Pure T lymphocytes, after incubation for 5 days with PHA and monocytic factors, expressed a high level of transferrin receptors. This phenomenon was strongly suppressed on both CD4 and CD8 subsets when 10^{-8} *M* calcitriol had been present during the culture. Moreover, the proliferation of pure CD4 cells was directly inhibited by calcitriol in similar conditions as for unseparated T lymphocytes. We conclude that T lymphocytes and their CD4 subset are direct targets for the inhibitory effect of calcitriol.

INTRODUCTION

The hormonal form of vitamin D, 1,25-dihydroxyvitamin D_3 or calcitriol, is known to affect human T lymphocyte function *in vitro* (for review see (1, 2)). In cultures of peripheral blood mononuclear cells (PBMC),[2] it inhibits mitogen- and antigen-induced T lymphocyte proliferation and suppresses the production of lymphokines e.g., interleukin 2 (IL-2), interferon-γ and colony-stimulating factor. Calcitriol also decreases the expression of T lymphocyte-membrane activation markers such as transferrin receptor (TfR) and HLA-DR, while the expression of IL-2 receptors is unaffected. Decreased proliferation is therefore thought to result mainly from a reduction in IL-2 production (3–10).

These inhibitory effects of calcitriol are certainly mediated via receptor-dependent mechanisms, as the order of anti-proliferative potency of several vitamin D analogs

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[2] Abbreviations used: CD, cluster of differentiation; IL, interleukin; mAb, monoclonal antibody; MO, monocyte (cell); MO sup, monocytic supernatant; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells, TfR, transferrin receptor.

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on PBMC parallels their receptor-binding affinity (11). Moreover, PBMC from patients with defective receptors are refractory to the anti-proliferative effect of calcitriol (12). T lymphocytes themselves, however, are not necessarily the primary targets for calcitriol. Indeed, accessory cells (e.g., monocytes) are needed for the proliferative response of T lymphocytes to mitogens or antigens (13, 14). Monocytes (MO) have been shown to express considerable levels of calcitriol receptors before stimulation, while T lymphocytes only acquire the receptors progressively after stimulation *in vitro* (15–17).

The inhibitory influence of calcitriol on T lymphocyte activation may therefore be dependent on the suppression of MO accessory function. Several studies have supported this possibility (18–20). Two groups, on the other hand, have claimed that calcitriol exerts its inhibitory effect directly on T lymphocytes (21, 22). Other groups, however, using more purified T lymphocytes, recently reported that the direct anti-proliferative effect of calcitriol is minimal (20) or very moderate (23).

To demonstrate direct effects of calcitriol on T lymphocytes, a culture system should be used in which pure T lymphocytes can be directly activated despite elimination of all accessory cells. MO accessory cell function in T lymphocyte activation with phytohemagglutinin (PHA) can partly be replaced by crude monocytic supernatant (MO sup) (13, 24) or by the combination of the monokines interleukin 1β (IL-1) and interleukin 6 (IL-6) (24, 25, 26). The anti-CD28 monoclonal antibody (mAb) 9.3 can also replace the requirement for MO (27–29). Finally, exogenous IL-2 can induce proliferation of PHA-stimulated T lymphocytes in the absence of monocytic accessory signals (24, 26, 30). We took advantage of these techniques to study the influence of calcitriol on proliferation and TfR expression of pure T lymphocytes and of their subsets.

MATERIALS AND METHODS

Reagents: calcitriol. Preservative-free 1,25-dihydroxyvitamin D_3, a generous gift of Dr. Uskokovic (Hoffmann–LaRoche Inc., Nutley, NJ), was stored at −20°C in glass vials with a concentration of 2×10^{-5} *M* in absolute ethanol.

Monoclonal antibodies: complement-fixing IgM-mAb, used for cytotoxicity. Anti-Leu11b (CD16) and anti-Leu7 (CD57) were purchased from Becton–Dickinson (Mountain View, CA); anti-NKH1A (anti-natural killer cell), anti-MO1 ($CD11_c$), and anti-MO2 (CD14) were purchased from Coulter Co. (Hialeah, FL).

IgG-mAb used for immunomagnetic T lymphocyte separation were anti-Leu3a (CD4) from Becton–Dickinson and anti-FK18 (CD8) from Monosan (Sanbio, Uden, The Netherlands).

FITC- or PE-conjugated mAb. Anti-Leu4 (CD3), anti-Leu M3 (CD14), anti-Leu15 ($CD11_b$), anti-Leu7, anti-HLA-DR, anti-Leu2a (CD8), anti-Leu3a (CD4), and anti-TfR mAb were all purchased from Becton–Dickinson. Fluorescence studies were carried out on a fluorescence-activated cell sorter (FACScan) (Becton–Dickinson).

Ascites fluid containing mAb 9.3 (anti-CD28) was a generous gift from Dr. P. J. Martin (Fred Hutchinson Cancer Research Center, Seattle, WA).

T-lympho-kwik. (One Lambda Inc., Los Angeles, CA) contains mAb (anti-monocyte and anti-B lymphocyte) and a source of lytic complement.

Immuno-magnetic equipment. Dynabeads M450 coated with sheep anti-mouse IgG and a specially designed magnet were purchased from Dynal A. S. (Oslo, Norway).

PHA (purified grade) was purchased from Wellcome (Dartford, UK).

Cytokines. Recombinant IL-2 was obtained from Janssen Chimica (Beerse, Belgium). Natural IL-1β and IL-6 were a gift from Dr. J. Van Damme (Rega Institute, K. U. Leuven, Belgium). They were purified from supernatants of stimulated PBMC as described (31, 32).

Cell-free MO sup was prepared by incubating adherent cells (from 30×10^6 PBMC) in a petri dish overnight at 37°C with 6 ml of RPMI 1640 containing 20% fetal calf serum and indomethacin (1 mg/liter). The supernatant was centrifuged, dialyzed against PBS and sterilized by filtration. The pool of MO sup used was derived from 10 different human donors. The MO sup was devoid of IL-2 (<0.02 U/ml) but contained 150 U/ml IL-1β (as measured by ELISA from Cistron, Pine Brooks, NJ) and 2000 U/ml IL-6 as measured by hybridoma growth activity (31, 32).

Lymphocyte separation techniques. PBMC were obtained from normal adult volunteer blood or buffy coats by centrifugation on Ficoll–Paque (Pharmacia, Uppsala, Sweden). After washing, PBMC were resuspended in RPMI 1640 supplemented with penicillin (50 U/ml), streptomycin (50 mg/liter), L-glutamine (0.29 g/liter) and 10% fetal calf serum. This mixture was the medium for all cultures.

Monocytes were prepared from PBMC in two steps: cold aggregation and adherence to plastic. Ten milliliter fractions of PBMC (5×10^6/ml in complete medium) were incubated on a rolling table at 4°C for 30 min. In these circumstances, MO form visible microaggregates while lymphocytes stay in suspension (33). Keeping the tubes with the aggregates upright in ice for 15 min allowed the MO to sediment in the conical bottom. The nonaggregated lymphocytes were carefully aspirated and used for T lymphocyte purification. The sedimented MO were resuspended in complete medium and further enriched by adherence to plastic culture dishes for 90 min at 37°C. Adherent cells in complete medium were stored in a 5% CO_2 incubator until the next day. Shortly before coculture with T lymphocytes, MO were harvested from the dishes by means of a rubber policeman. The resulting cell population contained 71–96% CD14 (+) cells and only 0.05–7.0% CD3 (+) lymphocytes (as studied by immunofluorescence).

T lymphocytes were purified from the nonaggregated PBMC by complement-dependent lysis of non-T lymphocytes. Thirty million of the nonaggregated cells were treated with 0.8 ml of T-lympho-kwik for 1 hr at 37°C. The resulting enriched T lymphocyte population still contained some MO and "null cells". Therefore they were incubated with additional mAb: 100 ng of anti-Leu11b, 8 ng of anti-Leu7, 100 ng of anti-MO1, 100 ng of anti-MO2, and 100 ng of NKH1A were added per 1×10^6 cells and allowed to react with the cells for 30 min at 4°C. After removing the unbound mAb by washing, 20×10^6 cells were treated again with 0.8 ml of T-lympho-kwik for 1 hr at 37°C. The purified T lymphocytes were then resuspended at 2×10^6/ml in complete medium and stored overnight at 37°C in the CO_2 incubator before use. Purity was assessed by immunofluorescence: 96.7–99.8% of the cells were CD3 (+). Contaminating cells were exclusively null cells: $CD11_b$ and partly Leu7 positive but negative for the monocytic marker CD14 and also negative for HLA-DR. This T lymphocyte population no longer responded to PHA alone or IL-2 alone.

CD4 and CD8 subset separation using Dynabeads (34). Pure human T lymphocytes were incubated with mouse mAb of the IgG class for 30 min at 4°C: for CD8 cell elimination anti-FK18 (25 μl/10^7 cells) and for CD4 cell elimination anti-Leu2a (3 μg/10^7 cells). Before mixing the treated cells with the beads, both were washed to remove unbound antibodies. Treated T lymphocytes were then incubated together with the magnetic beads for 30 min at 4°C. The rosetted cells were isolated from the

free cells by applying a magnet to the outer wall of the test tube. This last step was repeated twice. Only the negatively selected cells were further used. Purified CD4 lymphocytes were >96% CD4 and <1% CD8; purified CD8 lymphocytes were >92% CD8 and <1% CD4 in two experiments. Contaminating cells were null cells.

Culture conditions. Cell cultures for proliferation studies were carried out in five-fold in 96-well culture plates with a flat bottom (Costar, Broadway, Cambridge, MA). Final cell concentration was 0.25×10^6/ml (50×10^3/well). Final PHA concentration was 0.25 μg/ml unless otherwise indicated. Further additions were MO (2.5×10^3/well = 5% of total cell content), crude cell-free MO sup (5, 10, or 25% of well volume), or pure interleukins. IL-2 was used at 2 or 10 U/ml (10 U/ml was the optimal concentration). IL-1β and IL-6 were always used together (IL-1 2 U/ml plus IL-6 20 U/ml). In some experiments anti-CD28 was added as the second signal at concentrations of 10 ng/ml or 100 ng/ml (100 ng/ml was the optimal conc). Calcitriol was used at 10^{-12} to 10^{-8} *M*. All these reagents were always diluted in complete medium.

The microculture plates were incubated at 37°C in a 5% CO_2 incubator for 5 days, unless otherwise indicated. After this culture period, 1 μCi [*methyl*-^{3}H]thymidine, 2 Ci/mmol (Amersham, Buckinghamshire, UK) diluted in RPMI 1640, was added per well. After 8 hr, lymphocytes were harvested on a filter paper (Whatman 934 AH, Maidstone, UK) by a semiautomatic cell-culture harvesting system and the retained radioactivity was measured in a liquid scintillation counter (Packard Model 3380).

Cell cultures for evaluation of TfR expression were carried out similarly with slight modifications. T lymphocytes (0.33×10^6/ml) were incubated with PHA (0.25 μg/ml) alone, or with PHA and either MO sup (25 vol %) or a combination of IL-1 (4 U/ml) and IL-6 (50 U/ml). Calcitriol was added in paired cultures at 10^{-8} *M*. The cultures were incubated in 24-well plates with flat bottoms during 5 days.

Statistical analysis was performed by the Wilcoxon signed rank test, except for the data of Fig. 2, where a paired Student's *t* test was used.

RESULTS

Calcitriol Inhibits Proliferation of Pure T Lymphocytes Stimulated with PHA and a Cell-Free Monocytic Supernatant

In accordance with our previous data (24), the purified T lymphocytes did not proliferate when stimulated with PHA alone: in 21 experiments the [^{3}H]thymidine incorporation after 5 days in unstimulated T lymphocytes was 239 ± 52 cpm (mean ± SEM) and in T lymphocytes stimulated with PHA 0.25 μg/ml 701 ± 25 cpm. Adding back 5% MO to PHA-stimulated T lymphocytes resulted in a [^{3}H]thymidine incorporation of 63,634 ± 10,478 cpm in 10 experiments. The crude MO sup at 10 vol% also partly restored the proliferation: 23,521 ± 6,433 cpm in 7 experiments.

In a first series of experiments we compared the inhibitory effect of calcitriol on T lymphocyte proliferation in the presence of either monocytes, monocytic culture supernatant, or IL-2. We used a dose of 10^{-8} *M* calcitriol because this concentration has been shown to be strongly inhibitory for T lymphocyte proliferation in cultures of PBMC (11). [^{3}H]Thymidine incorporation in these cultures was measured at different time intervals, in order to study the kinetics of the calcitriol effect. As shown in Fig. 1 (top), the presence of calcitriol inhibited the growth of T lymphocytes cocultured with MO. This effect was marginal at Day 2, but inhibition steadily increased thereafter: 21% at Day 3, 50% at Day 4, and 75% at Day 5. When MO were replaced by the MO sup, calcitriol similarly had a progressive but even stronger inhibitory

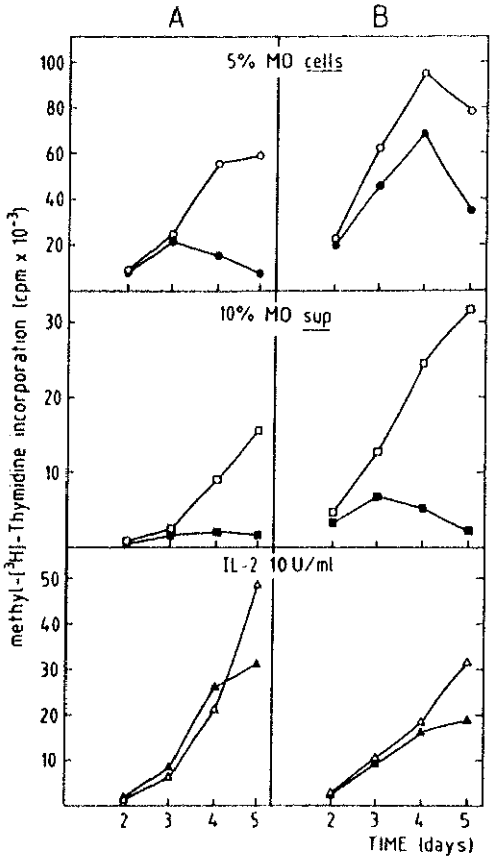

FIG. 1. Kinetic effect of calcitriol on PHA-induced T lymphocyte proliferation. Pure T lymphocytes of two different donors (A and B) were stimulated with PHA (0.25 μg/ml) and 5% MO (top, ○, ●), MO sup 10% (middle, □, ■) or IL-2 10 U/ml (bottom, △, ▲). The cells were cultured for 2, 3, 4, and 5 days before addition of [*methyl*-^{3}H]thymidine. Each open symbol (○, □, △) represents the mean incorporation of five microcultures without added calcitriol; the solid symbols (●, ■, ▲) represent results of cultures with 10^{-8} M calcitriol added at Day 0.

influence on T lymphocyte proliferation: 29% inhibition on Day 2, 43.5% on Day 3, 79% on Day 4, and 91% on Day 5 (Fig. 1, middle). These observations demonstrate that calcitriol directly inhibits T lymphocyte proliferation in a culture system with no added MO. In the same experiments, we also studied the effect of calcitriol on IL-2-driven growth of PHA-stimulated T lymphocytes. As previously shown, PHA directly renders pure T lymphocytes responsive to IL-2 (26, 30). Addition of IL-2 10 U/ml thus resulted in a high [^{3}H]thymidine incorporation in T lymphocytes (36,182 ± 8,729 cpm in 19 experiments). The IL-2-driven proliferation was not influenced by 10^{-8} M calcitriol on Days 2, 3, and 4. There was a moderate inhibition by calcitriol on Day 5 only (Fig. 1, bottom). In all three proliferation systems, [^{3}H]thymidine incorporation decreased after Day 5 and the relative inhibition by calcitriol also diminished (data not shown).

Since T lymphocytes showed an optimal proliferative response and a maximal sensitivity to calcitriol after 5 days, we selected this culture period for all further experiments.

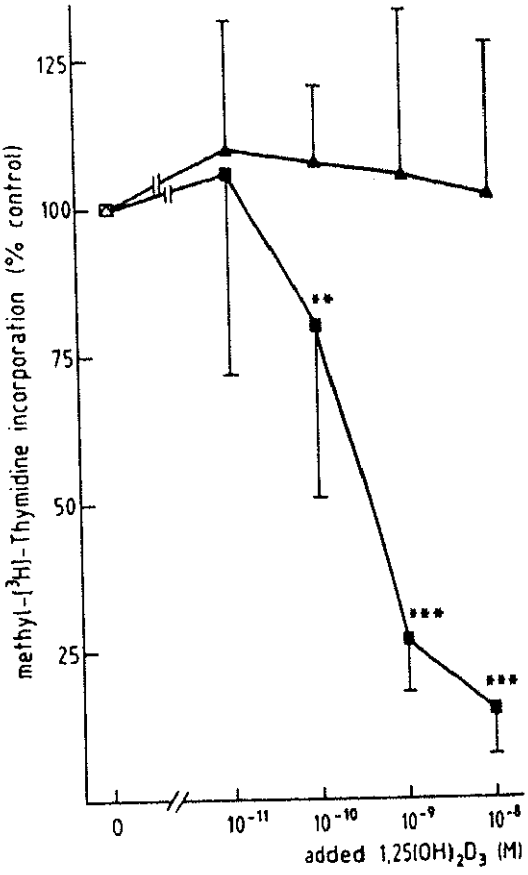

FIG. 2. Dose–response effect of calcitriol on proliferation of T lymphocytes. Pure T lymphocytes from seven different donors were stimulated with PHA 0.25 μg/ml and either MO sup 10% (□, ■) or IL-2 10 U/ml (△, ▲). The lymphocytes were cultured for 5 days before addition of [*methyl*-^{3}H]thymidine. Results of proliferation in the presence of different concentrations of calcitriol are expressed as percentages (mean ± SD) of control cultures (without added calcitriol). Statistical analysis was performed by comparing the effect of calcitriol at each concentration on proliferation in both systems. $^{**}P < 0.01$, $^{***}P < 0.001$.

Dose–Response Effect of Calcitriol on Pure T Lymphocyte Proliferation

In a next series of experiments a dose–response curve for the inhibitory effect of calcitriol was constructed (Fig. 2). The PHA-stimulated proliferation of T lymphocytes in the presence of 10% MO sup was slightly decreased at 10^{-10} *M* calcitriol, but it was very strongly inhibited at the higher concentrations. In parallel experiments, the IL-2 (10 U/ml)-driven growth was not significantly influenced, at any concentration of calcitriol used. The difference of the calcitriol effect in both systems was highly significant from 10^{-10} *M* on.

Effect of the Cytokine Concentration on the Sensitivity of T Lymphocytes to Calcitriol

The use of superoptimal (25%), optimal (10%), or suboptimal (5%) concentrations of the MO sup did not alter the inhibitory effect of 10^{-8} *M* calcitriol: inhibition in all

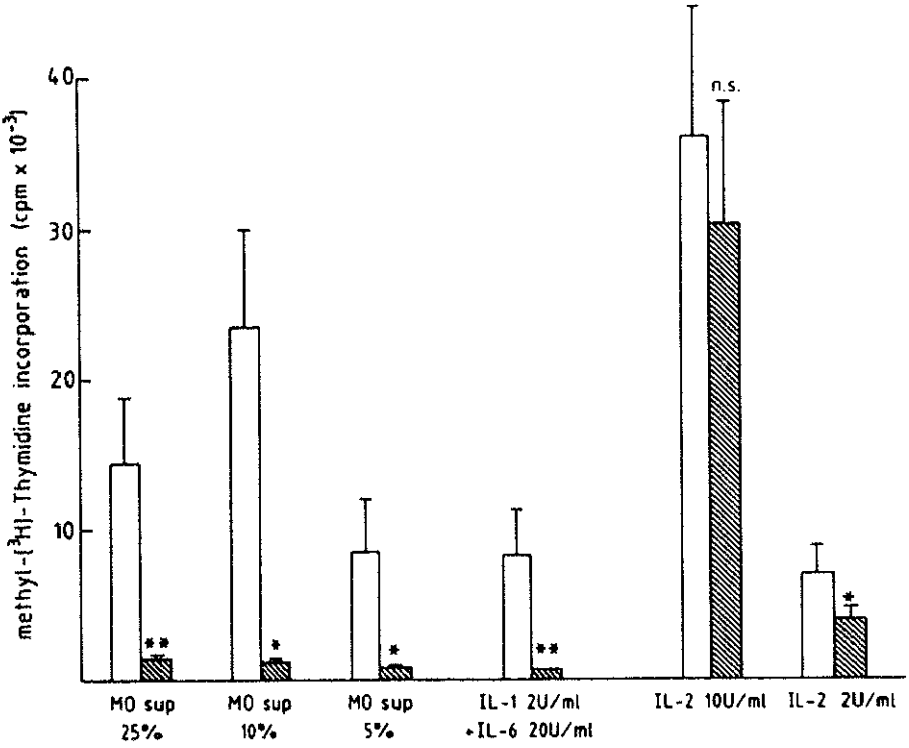

FIG. 3. Effect of the concentration of monocytic factors or IL-2 on the inhibition by calcitriol. Pure T lymphocytes were stimulated with PHA and MO sup 25% ($n = 12$), 10% ($n = 7$), or 5% ($n = 6$); with PHA and IL-1β 2 U/ml + IL-6 20 U/ml ($n = 10$); with PHA and IL-2 10 U/ml ($n = 19$) or IL-2 2 U/ml ($n = 6$). Results are expressed in cpm (mean + SEM) of [*methyl*-^{3}H]thymidine incorporation after 5 days. Open bars represent cultures without added calcitriol, shaded bars represent those with 10^{-8} M calcitriol. $^*P < 0.05$, $^{**}P < 0.01$.

experiments was more than 70% and usually more than 85% (Fig. 3). Moreover, an increase in PHA concentration up to 1.25 μg/ml could not attenuate the effect of calcitriol (data not shown). We next replaced the crude MO sup by a combination of purified monokines (IL-1β 2 U/ml and IL-6 20 U/ml). These factors induced a similar level of [^{3}H]thymidine incorporation in PHA-stimulated T lymphocytes as did 5% MO sup. The inhibitory influence of calcitriol was also very comparable (Fig. 3). Thus in T lymphocyte cultures supplemented with either MO sup or purified monokines, the inhibition by 10^{-8} M calcitriol on T lymphocyte growth was very pronounced and not attenuated by the dose of the accessory signal.

We then examined the inhibition by calcitriol on IL-2-driven T lymphocyte growth at different IL-2 concentrations. Calcitriol did not significantly alter the proliferative response of PHA-stimulated T lymphocytes to an optimal concentration of IL-2 (10 U/ml) in 19 experiments (Fig. 3). Calcitriol produced a moderate but significant inhibition ($P < 0.05$) when a suboptimal IL-2 concentration of 2 U/ml was used (Fig. 3). Inhibition was much less pronounced than in the paired cultures supplemented with MO sup ($P < 0.001$). It has to be noted that in the absence of calcitriol, T lymphocyte proliferation driven by IL-2 2 U/ml was of similar magnitude as that induced by 5% MO sup (Fig. 3). Interleukin 2-driven T lymphocyte growth was thus relatively

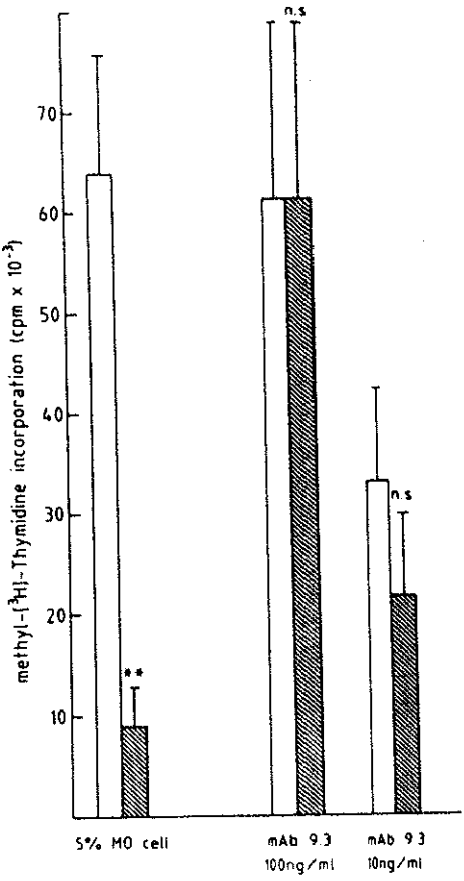

FIG. 4. Effect of calcitriol on T lymphocyte proliferation with MO (cells) or mAb 9.3. Pure T lymphocytes were stimulated with PHA and 5% MO ($n = 11$) or with PHA and the mAb 9.3 at 100 ng/ml ($n = 10$) or at 10 ng/ml ($n = 10$). [*methyl*-^{3}H]Thymidine incorporation (mean ± SEM) after 5 days in cultures without calcitriol (open bars) and with calcitriol 10^{-8} *M* (hatched bars) is represented. $^{**}P < 0.01$.

insensitive to the inhibitory action of calcitriol, unless low concentrations of IL-2 were used.

Effect of Calcitriol on T Lymphocyte Growth in the Presence of mAb 9.3 as the Accessory Signal

The mAb 9.3 (anti-CD28) alone is unable to induce proliferation of pure T lymphocytes but in combination with PHA, it has a strong comitogenic effect and can substitute for monocytes (27–29). The optimal concentration of the mAb (100 ng/ml) combined with PHA induced a proliferation as high as in cultures of T lymphocytes stimulated with PHA + 5% MO (cells) (Fig. 4). Calcitriol produced a pronounced inhibition on the MO-supported growth, while it had on average no effect on the cultures costimulated with mAb 9.3. (100 ng/ml or 10 ng/ml).

Effect of Calcitriol on the Expression of TfR in Pure T Lymphocytes and Their Subsets

Pure T lymphocytes were cultured for 5 days with medium, PHA alone, or PHA in combination with either MO sup (25%) or IL-1β (4 U/ml) and IL-6 (50 U/ml) with

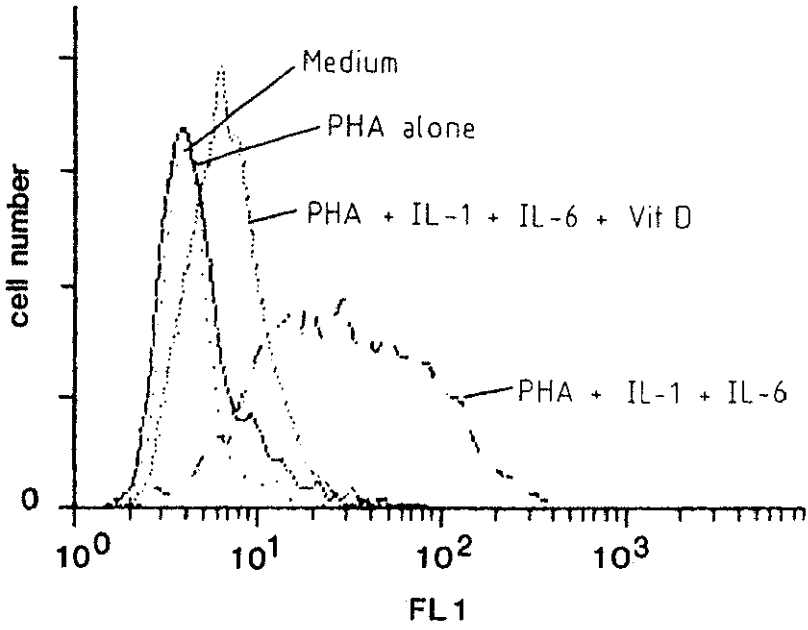

FIG. 5. Effect of calcitriol on TfR expression in CD8 cells. This figure represents the results of one of the experiments summarized in Table 1. TfR expression (FL1) is shown in CD8 (+) cells (selected on FACScan as Leu2a (+) cells). Culture additions are represented in the figure. Vit D stands for calcitriol 10^{-8} *M*.

or without 10^{-8} *M* calcitriol. TfR expression on T cell subsets was then analyzed by means of two-color immunofluorescence with anti-CD8 and anti-TfR mAb.

PHA alone induced no TfR expression. Addition of MO sup or of the combination of IL-1 and IL-6 induced a high percentage of TfR (+) T lymphocytes, both in the CD8 (+) and the CD8 (−) subset. When calcitriol was also present in the culture, the percentage of TfR (+) T lymphocytes was reduced to almost background levels in both subsets (Table 1 and Fig. 5).

Anti-proliferative Effect of Calcitriol on T Lymphocyte Subsets

CD4 and CD8 T lymphocytes were separated from purified T lymphocytes and the effect of calcitriol on their proliferation was studied. Cell separation was performed using an immunomagnetic technique (34). Both subsets were then cultured with PHA and either MO (cells), MO sup, or IL-2 under conditions previously used for T lymphocytes. Representative results of one out of two experiments are shown in Table 2. The CD4 population behaved exactly like the unseparated T lymphocytes: [^{3}H]thymidine incorporation could be induced by combining PHA with MO, MO sup, or IL-2. Inhibition by calcitriol was more pronounced in the cultures with MO or MO sup, than in those with IL-2. The CD8 lymphocytes proliferated in the presence of monocytes and they were inhibited by calcitriol in this system. The IL-2-driven growth of CD8 lymphocytes was not significantly altered by calcitriol. On the other hand, CD8 lymphocytes could not be stimulated by PHA and MO sup: they died during culture.

These experiments thus prove the direct inhibitory influence of calcitriol on the CD4 subset when stimulated with PHA and MO sup.

TABLE I

Effect of Calcitriol on TfR Expression in Pure T Lymphocytes

Culture addition[a]		% TfR-positive cells[b]	
Accessory signal	Calcitriol (10^{-8} M)	CD8(+) subset	CD8(−) subset
—	−	5.9 ± 1.7	3.0 ± 0.5
MO sup	−	64.9 and 62.1	25.0 and 26.8
MO sup	+	3.7 and 3.9	3.9 and 2.7
IL-1 + IL-6	−	69.6 ± 12.7	42.8 ± 13.0
IL-1 + IL-6	+	4.1 ± 1.0	2.5 ± 1.0

[a] Pure T lymphocytes (0.33×10^6/ml) were cultured with PHA 0.25 μg/ml, combined with either MO sup 25% ($n = 2$) or IL-1 4 U/ml + IL-6 50 U/ml ($n = 4$). Calcitriol was added in paired cultures.

[b] Cells were stained on Day 5 with anti-Leu2a-PE and anti-TfR–FITC for analysis on a FACScan. Results are expressed as percentages (mean ± SD) of TfR-positive cells within the Leu2a(+) resp. Leu2a(−) subset.

DISCUSSION

We and others have previously shown that calcitriol inhibits the proliferation of T lymphocytes in PBMC cultures stimulated with mitogens or antigens (1, 2, 11). These effects have been explained by a decreased production of the T cell growth factor IL-2 (3–5, 7), at the level of its mRNA, suggesting a nuclear action of calcitriol directly on T lymphocytes (8, 10). The anti-proliferative effect, however, could also be explained by a MO-dependent action of the hormone, since it was much less pronounced when the percentage of MO within the PBMC was reduced (18, 19). Moreover, pretreatment of MO with calcitriol markedly reduced their accessory signal for PHA-induced IL-2 production by T lymphocytes (19) and for lectin or antigen-induced T lymphocyte proliferation (19, 20, 23, 35). Calcitriol-treated monocytes were reported to produce less lymphocyte-activating factors, e.g., IL-1, (20) and more inhibiting factors, e.g., prostaglandin E2 (23). All these data thus indicate that the negative effect of calcitriol on T lymphocyte activation can at least partly be mediated via a suppression of the monocytic accessory function.

Some authors attempted to purify T lymphocytes and their subsets in order to study direct effects of calcitriol on these cells (20–23). In their functional studies, Lemire *et al.* stated that only the CD4 lymphocytes were inhibited by calcitriol, both in their proliferative response to PHA and in their helper function for antibody production (21). Rigby *et al.*, on the contrary, concluded that calcitriol impaired the activation of both CD4 and CD8 subsets, since the hormone inhibited the progression in the cell cycle, the expression of TfR, and the incorporation of [^{3}H]thymidine in both subsets (22). Although both authors used several procedures to purify T lymphocytes and their subsets, the resulting cell population proliferated very well after the addition of PHA alone, indicating that accessory cells were still present in the culture. In the one experiment where T subsets were unresponsive to PHA alone, MO were added back to induce proliferation (22). Consequently, from all these experiments no firm conclusions can be drawn about the primary cellular target for calcitriol. Moreover, results concerning subset sensitivity to calcitriol differed (21, 22). Rigby explained this discrepancy by invoking kinetic considerations and differences in MO contamination (22). Recently, Ravid *et al.* stated that purified T lymphocytes, cul-

TABLE 2

Influence of Calcitriol on PHA-Induced Proliferation of T Lymphocyte Subsets[a]

Culture addition[b]		[^{3}H]Thymidine incorporation (cpm)[c]		
Accessory signal	Calcitriol (10^{-8} M)	CD_3(+) cells	CD_4(+) cells	CD_8(+) cells
—	–	251	173	156
5% MO	–	123,414	150,460	27,872
	+	21,876	28,508	2,400
10% MO sup	–	8,706	2,576	142
	+	384	471	98
IL-2 10 U/ml	–	27,777	8,350	8,811
	+	17,552	4,720	11,300

[a] T lymphocyte subsets were negatively selected by an immunomagnetic separation.
[b] Culture conditions were as in the experiments of Figs. 3 and 4.
[c] Lymphocytes were harvested on Day 5. Results represent the means of five microcultures from one donor.

tured with PHA and IL-2, were still moderately sensitive to the anti-proliferative action of calcitriol (23). The authors claimed a high degree of T lymphocyte purity, but they showed no data on the proliferative response of their T lymphocytes to PHA alone.

In order to study direct effects of calcitriol on T lymphocytes, we eliminated all accessory cells by a mixture of mAb, reactive with a large variety of non-T membrane markers and lytic complement. The resulting negatively selected population was highly enriched in CD3(+) lymphocytes and was also free of CD14(+) and HLA-DR(+) cells. Moreover, these T lymphocytes showed essentially no proliferative response to PHA alone, but in the presence of MO they proliferated again. Kinetic experiments on this mixed T–MO population demonstrated that the anti-proliferative effect of calcitriol only started at Day 3 and progressively increased until Day 5.

Monocytes can be replaced by their soluble factors (a crude MO sup or a combination of purified monokines) in order to provide a cell-free accessory signal (13, 24–26). The inhibitory action of calcitriol in this system started earlier (Day 2) and was relatively stronger than when MO (cells) were present. The fact that inhibition of T lymphocyte growth occurred in the absence of MO (cells) proves the direct sensitivity of T lymphocytes to calcitriol. The progressive increase in the strength of the inhibition from Day 2 to Day 5 probably reflects the known progressive rise in T lymphocyte calcitriol-receptor concentration (17). The anti-proliferative effect of calcitriol was equally pronounced when MO sup was replaced by a combination of pure IL-1 and IL-6. Moreover, the inhibition by calcitriol could not be neutralized by increasing the dose of the stimuli (MO sup and/or PHA). Dose–response curves for calcitriol indicated that the minimal effective concentration of the hormone in this system was around 10^{-10} M. Since we always used complete serum (containing the vitamin D binding protein) as a medium supplement, we can deduce from our previous data (11) that a 100-fold lower concentration (10^{-12} M) of the free hormone is sufficient to reduce MO sup-induced T lymphocyte proliferation. This concentration is in the physiological range (36, 37). We thus conclude that calcitriol renders pure T lymphocytes hyporesponsive to monocyte-derived activation factors.

In PBMC cultures, it was repeatedly shown that IL-2 production is strongly inhibited by calcitriol (3–5, 7), but that the expression of the IL-2 receptor is not altered (6, 17). Moreover, the addition of IL-2 to PHA-stimulated PBMC treated with calcitriol did not restore the full proliferative response (2, 6, 9), suggesting the possibility of a postreceptor action of calcitriol on T lymphocytes. Since PHA is known to induce IL-2 responsiveness in pure T lymphocytes (24, 26, 30), we added IL-2 as a growth factor to induce T lymphocyte proliferation. Calcitriol had a moderate anti-proliferative effect when IL-2 was used at a suboptimal concentration (2 U/ml) but this inhibition was significantly less pronounced than in paired MO sup-treated T lymphocyte cultures. Since [^{3}H]thymidine incorporation induced by PHA and 2 U/ml IL-2 was in the same cpm range as that induced by PHA and 5% MO sup, the differential sensitivity to calcitriol could not be ascribed to a difference in the level of stimulation in both systems. Moreover, the inhibition by calcitriol on IL-2-driven proliferation could on average be eliminated by using an optimal concentration of the growth factor (10 U/ml) while the calcitriol effect on MO sup-induced proliferations is independent of the MO sup concentration.

Two recent reports confirm that IL-2-driven growth of purified T lymphocytes is not very sensitive to calcitriol (20, 23). From all these data we thus conclude that postreceptor phenomena in IL-2-dependent T lymphocyte proliferation are not an important target for calcitriol action.

An alternative system to overcome the requirement for monocytes (or monocytic factors) in the stimulation of pure T lymphocytes is the use of the mAb 9.3 (anti-CD28). This pathway of T lymphocyte activation is known to involve IL-2 production (27, 28), IL-2 receptor expression (27, 28), and IL-2-dependent T lymphocyte proliferation (38). T lymphocyte growth induced by PHA and mAb 9.3 was essentially insensitive to calcitriol's suppressive effect, in contrast to the situation when monocytes (or their factors) provide the accessory signal. The differential effect of calcitriol on MO-dependent vs anti-CD28-dependent T lymphocyte activation resembles the effects of cyclosporin and of prostaglandin E_2. These agents were reported to inhibit IL-2 production and T lymphocyte proliferation when MO provided the second signal (39–42) and to have essentially no influence when anti-CD28 was the comitogen (43, 44). It was also shown by Manolagas that IL-2 production and T lymphocyte proliferation were resistant to the inhibitory action of calcitriol when the phorbol ester PMA was used as the accessory signal instead of MO (20). So, clearly the sensitivity of T lymphocytes for the suppressive action of calcitriol (and other immunomodulators) is dependent on the nature of the activation signal.

The appearance of TfR is a sensitive marker for T lymphocyte activation (45). TfR expression was shown to be an IL-2-dependent phenomenon (46). We confirm that PHA alone is unable to induce TfR in pure T lymphocytes, but we obtained high levels of TfR after stimulation with a combination of PHA and MO sup or PHA and IL-1 + IL-6. Calcitriol is able to suppress the TfR expression in activated PBMC and in partly purified T lymphocytes (6, 22). Using a pure T lymphocyte population stimulated with PHA and monocytic factors for 5 days, we analyzed the influence of calcitriol on TfR expression in T lymphocyte *subsets* by means of two-color immunofluorescence: TfR was dramatically suppressed in both the CD8(+) and CD8(−) subsets. However, we cannot claim a *direct* effect on both T lymphocyte subsets separately in this system. A diminished expression of TfR on CD8 lymphocytes could, for instance, be entirely secondary to calcitriol–CD4 interactions, resulting in a decreased CD4 cell-derived activation signal for CD8 lymphocytes.

In order to study the direct sensitivity of both subsets to calcitriol, we separated them using an immunomagnetic method. When costimulated with PHA and MO or IL-2 both CD4 and CD8 populations showed a good proliferative response. In contrast, MO sup could provide an accessory growth signal only to CD4 lymphocytes, but not to CD8 lymphocytes. Since calcitriol was able to inhibit proliferation of CD4 lymphocytes when stimulated with cell-free MO sup, this subset is a direct target for the hormone. The inhibitory influence of calcitriol on CD8 lymphocytes, on the other hand, could only be demonstrated in the presence of monocytes. As a consequence, no conclusion about the cellular target for calcitriol (CD8 vs MO) can be drawn from this system.

In summary, pure T lymphocytes and their CD4 subset are direct targets for the inhibitory action of calcitriol on TfR expression and proliferation after stimulation with PHA and cell-free monocytic factors. PHA-stimulated T lymphocytes, however, are relatively resistant to calcitriol inhibition when other accessory or growth signals, like mAb 9.3 and IL-2, are used.

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