Journal of Neuroscience Research 71:575–582 (2003)

# Vitamin D3 Inhibits Proinflammatory Cytokines and Nitric Oxide Production by the EOC13 Microglial Cell Line

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In recent years, a neuroimmunomodulatory role for 1,25-dihydroxyvitamine $D_3$ [1,25$(OH)_2D_3$] has emerged. Microglial cells present a potential target for the effects of this hormone in the brain. This study focuses on the effect of 1,25$(OH)_2D_3$ on the expression and production of inflammatory cytokines and nitric oxide (NO) by the EOC13 microglial cell line. The presence of the vitamin D3 receptor in microglia was demonstrated by RT-PCR. 1,25$(OH)_2D_3$ inhibited the production of tumor necrosis factor-α, interleukin-6, and NO by stimulated microglia in a concentration-related fashion. The production of transforming growth factor-β1 (TGF-β1), an anti-inflammatory cytokine, was not modified in the presence of 1,25$(OH)_2D_3$, indicating that the effects of 1,25$(OH)_2D_3$ may not involve TGF-β1 regulation. These results show that 1,25$(OH)_2D_3$ has direct anti-inflammatory properties on microglia. It further supports the hypothesis that 1,25$(OH)_2D_3$ could be involved in the maintenance of the brain homeostasis and may have a therapeutic potential in inflammatory pathologies of the central nervous system. 

**Key word:** vitamin D3; VDR; microglia; TNF; IL-6; NO; TGFβ

In addition to its classical role in calcium homeostasis, the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3 [1,25$(OH)_2D_3$], exerts a large spectrum of tissue-specific biological activities. It is now well established that 1,25$(OH)_2D_3$ displays immunomodulatory properties in vitro, as well as in vivo, via its specific receptor, the vitamin $D_3$ receptor (VDR), expressed in antigen-presenting cells (APC) and activated lymphocytes (Lemire, 2000). In particular, 1,25$(OH)_2D_3$ inhibits Th1 functions and suppresses the production of interleukin-12 (IL-12) by myelomonocytic cells (Lemire et al., 1995; D'Ambrosio et al., 1998). 1,25$(OH)_2D_3$ also strongly inhibits a full differentiation of monocytes to dendritic cells and their terminal maturation into a potent APC (Penna and Adorini, 2000; Piemonti et al., 2000). Whereas 1,25$(OH)_2D_3$ enhances phacocytosis and antigen capture (Xu et al., 1993), it inhibits accessory cell function of monocytes and dendritic cells by down-regulating the costimulatory B7-2 and CD40 molecules (Clavreul et al., 1998; Penna and Adorini, 2000; Piemonti et al., 2000) and by suppressing cytokine production (Muller et al., 1992; Cohen et al., 2001).

There is also accumulating evidence supporting an involvement of vitamin $D_3$ in brain function (Veenstra et al., 1998; Garcion et al., 2002). Indeed, VDR is expressed in neurons (Prufer et al., 1999), in astrocytes (Neveu et al., 1994b), and in oligodendrocytes (Baas et al., 2000). The hormone may also exert neuroprotective effects, by inducing an increase in the expression of neurotrophic factors and of γ-glutamyl transpeptidase by macroglial cells (Neveu et al., 1994a,b; Baas et al., 2000; Garcion et al., 2002) or via the modulation of neuronal $Ca^{2+}$ homeostasis (Brewer et al., 2001). It also presents immunosuppressive properties in experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis: 1,25$(OH)_2D_3$ inhibited EAE disease induction (Cantorna et al., 1996), and 1,25$(OH)_2D_3$ curative treatment results in a significant improvement of the clinical signs, in particular through its effects on the inflammatory infiltrates

Contract grant sponsor: "la Ligue Nationale Contre le Cancer, comité départemental du Maine et Loire."

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Received 1 July 2002; Revised 10 September 2002; Accepted 11 September 2002

(Nataf et al., 1996; Nashold et al., 2000). In addition, inducible nitric oxide synthase (iNOS) expression is inhibited by $1,25(OH)_2D_3$ in the EAE model (Garcion et al., 1997) and in a model of brain inflammation (Garcion et al., 1998). Interestingly, $1,25(OH)_2D_3$ can be synthesized from its precursor by activated microglia (Neveu et al., 1994c) and, thus, be available in situ.

In addition to the cells from the immune system, the possible targets of $1,25(OH)_2D_3$ immunomodulatory effects in the CNS are astrocytes and microglia. Microglial cells, the resident macrophages of the brain derived from myelomonocytic precursors, are considered as the main mediators of immune responses in the CNS. Microglia are present in the CNS in a quiescent state and can become rapidly activated in response to various injuries (Streit et al., 1999). The most conspicuous functional changes include the acquisition of immunoregulatory functions such as antigen-presenting activity to T lymphocytes as well as phagocytic and cytotoxic potentials (Streit et al., 1999; Aloisi et al., 2000). At the site of inflammation, activated microglia can express de novo or enhance the levels of major histocompatibility (MHC) molecule and of B7 costimulatory molecules (Menendez Iglesias et al., 1997). They can produce soluble factors involved in the host defense against CNS infection, including tumor necrosis factor (TNF), IL-1, IL-6, and NO (Chao et al., 1992a,b). However, an overproduction of these factors may be harmful and thus play an important role in the pathogenesis of several immune-mediated CNS diseases, including multiple sclerosis, AIDS infection, and some neurodegenerative diseases (Gonzalez-Scarano and Baltuch, 1999). For these reasons, a better understanding of the mechanisms regulating cytokine secretion by microglial cells is of particular interest.

The aim of the present study was thus to determine the potential regulatory role of $1,25(OH)_2D_3$ in the release of inflammatory mediators (TNF, IL-6, and NO) by microglial cells. We also investigated whether $1,25(OH)_2D_3$ might exert its effect on microglia either directly via the VDR or indirectly through the modulation of a well-known immunosuppressor, transforming growth factor-β1 (TGF-β1). For this purpose, we used a well-characterized mouse microglial cell line derived from brain microglial precursors of normal young mice using a nonviral immortalization procedure (Walker et al., 1995; Askew et al., 1996; Askew and Walker, 1996).

## MATERIALS AND METHODS

### Cytokines and Reagents

$1,25(OH)_2D_3$ was provided by Leo Pharmaceuticals Products (Ballerup, Denmark). Recombinant TNFα and interferon-γ (IFNγ) were obtained from R&D Systems (Abingdon, Oxon, United Kingdom) and recombinant IL-6 from Endogen (Cliniscience, Paris, France). Lipopolysaccharide (LPS), concanavalin A, 3-(4,(–)dimethylthiazol-2-yl)2,5diphenyltetrazolium bromide (MTT), actinomycin D, and crystal violet were purchased from Sigma (St. Quentin, Fallavier, France).

### Microglial Cell Lines

The EOC microglial cell lines were kindly provided by Dr. W.S. Walker (St. Jude Children's Research Hospital, Memphis, TN) and were derived from brain microglial precursors of C3H/HeJ (H-2k) normal young mice using a nonviral immortalization procedure (Walker et al., 1995). These cells were cultured in polymethylpentene dishes (Nalgen, Rochester, NY) and maintained in complete Dulbecco's modified Eagle's medium (2 mM glutamine, 10% heat-inactivated fetal calf serum) supplemented with 20% of a bone marrow stromal cell line (LADMAC) conditioned medium containing colony stimulating factor-1 (CSF-1; Walker et al., 1995).

### Cytokine Bioassays

Microglial cells were seeded in 24-well plates at a density of $5 \times 10^4$ cells/well. Cells were primed or not for 24 hr with 50 U/ml IFNγ and then stimulated with 5 μg/ml LPS for 4, 6, 8, 12, 24, or 48 hr. For $1,25(OH)_2D_3$ studies, cells were primed for 24 hr with IFNγ, in the presence or absence of $10^{-9}$–$10^{-7}$ M $1,25(OH)_2D_3$ during the last 12 hr. Cells were then stimulated with 5 μg/ml LPS for 8 or 48 hr. The supernatants were then harvested, centrifugated, and stored at −80°C until use.

For the cytokine bioassays, serial dilutions of samples were distributed in 96-well plates. TNF was then measured by a cytotoxicity assay using the L929 mouse fibrosarcoma cell line ($5 \times 10^4$ cells/well) as previously described (Lefebvre d'Hellencourt et al., 1996).

IL-6 productions were determined by MTT cell proliferation assay using the B9 cell line ($5 \times 10^3$ cells/well) as previously described (van der Schoot et al., 1989). Cytokine levels were calculated using standard curves obtained with recombinant cytokines. The detection limit of the bioassay was 5 pg/ml for TNF and 10 pg/ml for IL-6. $1,25(OH)_2D_3$ did not affect the cytotoxic activity of recombinant TNF (1–500 pg/ml) in this assay or the proliferation of B9 cells as previously described (Muller et al., 1992).

### Nitrite Production

Microglial cells were seeded in 24-well plates at a density of $5 \times 10^4$ cells/well. LPS, IFNγ, and TNF were added at 5 μg/ml, 50 U/ml, and 1,000 U/ml, respectively. Cells were stimulated at the same time with LPS or LPS and IFNγ or TNFα and IFNγ for 48 hr. For $1,25(OH)_2D_3$ studies, cells were stimulated at the same time with LPS and IFNγ or TNFα and IFNγ in the presence or absence of $10^{-9}$–$10^{-7}$ M $1,25(OH)_2D_3$.

NO is rapidly oxidized to nitrite in culture medium, and determination of nitrite concentrations is used as a measure of NO production. Briefly, equal volumes (150 μl) of cell supernatants (without phenol red) and Greiss reagent (1% sulfanilamide, 0.1% naphthylethyline diamine dihydrochloride, 2.5% $H_3PO_4$) were added to individual wells of a 96-well plate. After a 10-min incubation at room temperature, the absorbance was read at 570 nm. Nitrite concentrations were calculated from a sodium nitrite standard curve (0.5 and 60 μM).

### TGF-β1 ELISA

Microglial cells were stimulated as described above under Cytokine Bioassays. The assay was performed as previously

described by Danielpour et al. (1989) using an affinity-purified mouse anti-TGF-β1 capture antibody (Ab) at 100 ng/well (Danielpour et al., 1989). The detection Ab was an affinity-purified turkey anti-TGF-β1 (100 μl/well of a 1/200 dilution). The reaction was developed with a phosphatase-linked anti-turkey IgG (50 ng/well), followed by addition of the phosphatase substrate solution (Kirkegaard and Perry, Gaithersburg, MD). The reaction was allowed to proceed for 2 hr at room temperature, and then the plate was read in an ELISA reader at 410 nm with a reference wavelength at 450 nm. Recombinant (r)TGF-β1 (0–200 pM) was used as a standard curve. The capture and detection Abs as well as the rTGF-β1 were kindly provided by Dr K. Flanders (NCI, NIH, Bethesda, MD).

### Northern Blot Analysis

Total RNA was extracted from each cell culture by the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Forty micrograms of glioxal-treated RNA were separated on a 1.4% agarose gel, transferred to a Hybond N membrane, and serially hybridized with α-$^{32}$P-dCTP-labelled probes for TNFα, IL-6, iNOS, and TGF-β1 according to standard protocol (Sambrook et al., 1989). Standardization of RNA loading was controlled by hybridization of the blots with the housekeeping gene HS 26 (Vincent et al., 1993). Band intensities were quantified using a phosphoimager (Molecular Dynamics, Bondoufle, France) and analyzed by densitometric tracing (Image Quant).

### RT-PCR

One microgram of total RNA was reverse transcribed by incubating at 37°C for 10 min and 42°C for 90 min in 20 μl of a reaction mixture containing 0.1 μM oligo-$(dT)_{12-18}$, 1× PCR buffer, 1 mM of each dNTP, 2.5 mM $MgCl_2$, 0.01 M dithiothreitol (DTT), and 200 U Moloney's murine leukemia virus reverse transcriptase (Life Technologies, Bethesda, MD).

For PCR, the murine VDR primers were sense 5′-CCGCATCACCAAGGAGAACC-3′ and antisense 5′-TAGCTCCCTGTACTTACGTC-3′ as previously described (Liu et al., 1996); the primer set for the mouse housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was sense 5′-ACCACAGTCCATGCCA-3′ and antisense 5′-TCCACCACCCTGTTGC-3′ (Clontech, Palo Alto, CA). Amplification of 100 ng cDNA was performed in a total volume of 50 μl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM or 0.5 μM of each VDR or G3PDH primer, respectively, and 1 U of Goldstar Taq DNA polymerase (Eurogentech, Seraing, Belgium). The reaction mixture was subjected to 40 amplification cycles on a PCR express thermocycler (Hybaid) consisting of 95°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec. The final extension lasted for 7 min at 72°C. The L929 mouse fibrosarcoma cell line was used as a positive control for VDR in the RT-PCR experiment. RNA from the human monocytic cell line THP-1 was used as a negative control. PCR products were then visualized under ultraviolet (UV) light after 2% agarose gel electrophoresis containing ethidium bromide.

### Statistical Analysis

Data are expressed as mean ± SEM. Statistical significance was assessed by an ANOVA, followed by Tukey-Kramer post hoc analysis using the Jmp software (SAS Institute Inc., Cary, NC). A value of $P < 0.05$ was considered statistically significant.

## RESULTS

### Inhibition of TNF and IL-6 Microglial Production by $1,25(OH)_2D_3$

To characterize better the release parameters of proinflammatory cytokines by the EOC13 cell line, bioassays for TNF and IL-6 were performed. After LPS stimulation, substantial amounts of TNF (Fig. 1A) and IL-6 (Fig. 1B) were observed, whereas these cytokines were undetectable in the supernatant of unstimulated microglial cells. As previously described (Chao et al., 1992a), we observed that stimulation of microglial cells with IFNγ did not induce the production of TNF, but, when the cells were preincubated with IFNγ and then stimulated with LPS, a marked enhancement of TNF and IL-6 release was recorded. Among the different periods of treatment (data not shown), a maximal effect was obtained with a 24 hr preincubation period with IFNγ before LPS stimulation (Fig. 1A,B). TNF secretion after IFNγ plus LPS stimulation peaked at 8–12 hr and decreased thereafter, whereas IL-6 release was gradual and reached its maximal level at 48 hr.

On the basis of these results, the effect of $1,25(OH)_2D_3$ on TNF and IL-6 production was examined after priming with IFNγ (24 hr), followed by stimulation with LPS for 8 hr (TNF) and 48 hr (IL-6). As shown in Figure 1C,D, $1,25(OH)_2D_3$ inhibited the release of TNF and IL-6 by stimulated microglia in a dose-dependent manner. Maximal inhibition of the release of TNF (80%) and IL-6 (50%) by microglial cells was achieved in the presence of $10^{-7}$ M $1,25(OH)_2D_3$.

### $1,25(OH)_2D_3$ Inhibits NO Production by Microglia

Several studies have previously shown that stimulation by LPS induces moderate levels of NO expression in macrophages and microglia. However, if these stimulated cells are also exposed to IFNγ, which by itself only induces low levels of NO, there is a synergistic effect of the two signals that leads to a greater secretion than that observed with either molecule alone (Lorsbach et al., 1993). In a similar pattern, a substantial amount of NO (evidenced by nitrite measurements) was detected in the EOC13 microglial cell line upon LPS treatment. This production was greatly increased by the combination of LPS with IFNγ and to a lesser extent by TNF with IFNγ (Fig. 2A).

To test the ability of $1,25(OH)_2D_3$ to interfere with NO secretion, an incubation period of 48 hr in the presence of a combination of LPS with IFNγ or of TNF with IFNγ was chosen. As shown in Figure 2B, nitrite production by microglia stimulated by these two combinations of cytokines was inhibited by $1,25(OH)_2D_3$ in a dose-dependent manner. At a concentration of $10^{-7}$ M, the

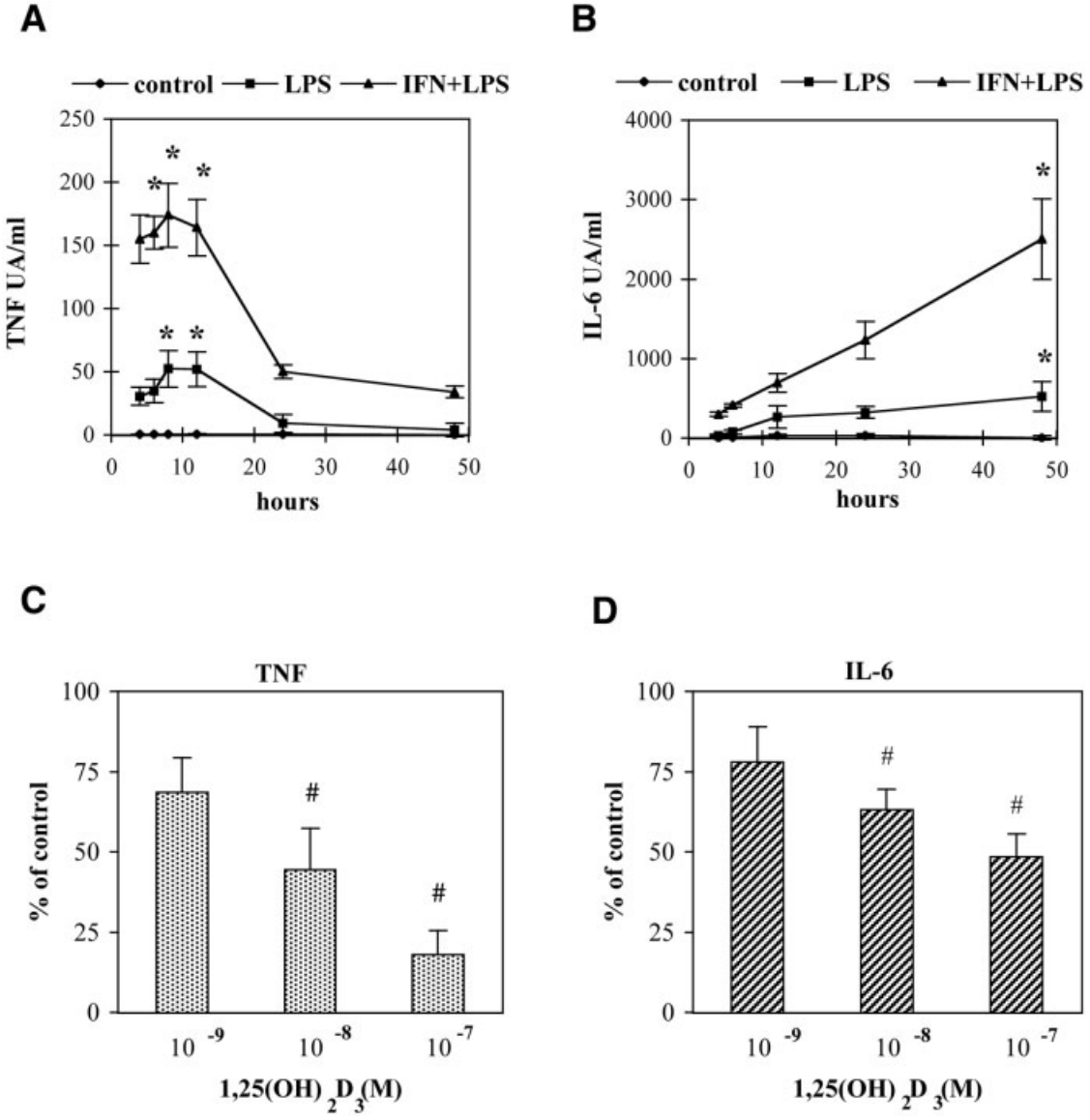


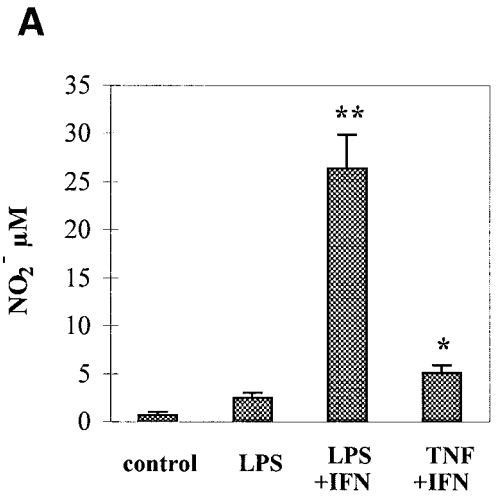

Fig. 1. Kinetics of TNF (**A**) and IL-6 (**B**) release by microglia. Cells were left unstimulated, exposed to LPS, or primed with IFNγ for 24 hr and then exposed to LPS for 4–48 hr. Effect of $1,25(OH)_2D_3$ on TNF (**C**) and IL-6 (**D**) production by microglial cells primed with IFNγ for 24 hr and stimulated with LPS for 8 hr (for TNF) or 48 hr (for IL6). Results are expressed as the percentage of change compared with the stimulated control without $1,25(OH)_2D_3$ (100%). Bars denote mean ± SEM (N = 4). *Significantly different from unstimulated microglia at $P < 0.05$. #Significantly different from the stimulated control at $P < 0.05$.

hormone induced from 30% to 50% inhibition of NO release, compared with the untreated control, depending on the stimulation conditions.

## Expression of VDR mRNA in Microglial Cells

Given that $1,25(OH)_2D_3$ elicits its genomic effects via its intracellular receptor, VDR mRNA expression was investigated in microglial cells. The use of the RT-PCR technique clearly demonstrated the presence of VDR mRNA in the EOC13 cell line treated or not with $1,25(OH)_2D_3$ (Fig. 3).

The ligand-receptor complexes may interact with appropriate consensus DNA sequences (vitamin D-responsive element; VDRE) located in the regulatory region of target genes and may exert transcriptional effects (Ross et al., 1992). Northern blot analyses were performed to determine whether the observed $1,25(OH)_2D_3$ inhibition of IL-6, TNF, and NO production occurred at the transcriptional level. Figure 4A shows the expression of TNFα and IL-6 mRNA in the EOC13 cell line stimulated with LPS after priming with IFNγ and of iNOS mRNA after LPS plus IFNγ stimulation. However, there is no



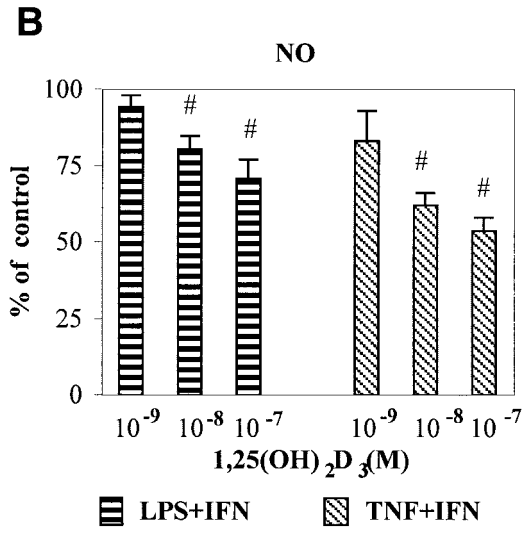




Fig. 2. **A:** NO production by microglia either unstimulated or incubated at the same time with LPS, LPS + IFNγ, or TNF + IFNγ for 48 hr. **B:** Effect of $1,25(OH)_2D_3$ on NO production by microglial cells stimulated with LPS + IFNγ or TNF + IFNγ for 48 hr. Results are expressed as the percentage of change compared with the stimulated control without $1,25(OH)_2D_3$ (100%). Bars denote mean ± SEM (N = 4). Significantly different from unstimulated microglia at $^{\star\star}P < 0.01$ or $^{\star}P < 0.05$. $^{\#}$Significantly different from the corresponding stimulated control at $P < 0.05$.

significant modification of mRNA expression in the presence of $1,25(OH)_2D_3$, even with the highest concentration ($10^{-7}$ M) used.

### Expression and Production of TGF-β 1

Previous reports indicate that $1,25(OH)_2D_3$ stimulates the production of TGF-β1 (Koli and Keski-Oja, 1993, 1995; Weinreich et al., 1999), which is known to be an inhibitor of the production of proinflammatory cytokines by microglia (Suzumura et al., 1993; Chao et al., 1995). We therefore investigated whether $1,25(OH)_2D_3$ could increase the production of TGF-β1 by stimulated microglia. We observed, at 4 and 8 hr after $1,25(OH)_2D_3$ ($10^{-7}$ M) treatment of stimulated microglia, a slight increase of TGF-β1 production, which was not statistically significant (Fig. 5). Furthermore, Northern blot analysis showed that the level of TGF-β1 transcript was not modified in the presence of $1,25(OH)_2D_3$ (Fig. 4B).

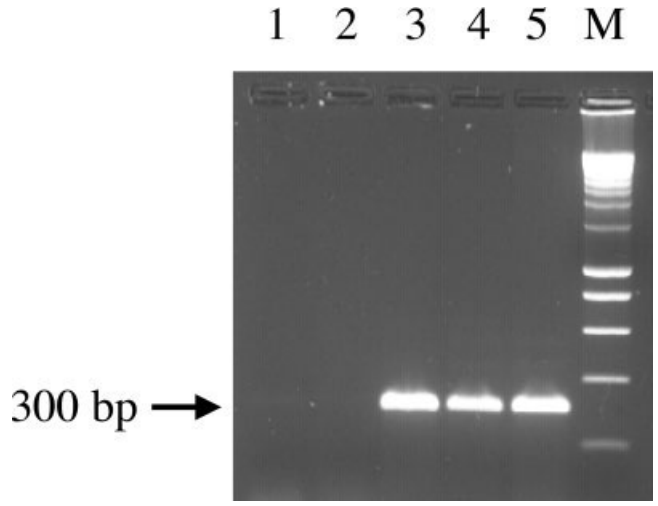



Fig. 3. Ethidium bromide-stained gel electrophoresis of RT-PCR products of VDR. **Lane 1:** Control (no RNA). **Lane 2:** THP-1 cells (negative control). **Lane 3:** L929 cells (positive control). **Lane 4:** EOC13 cells. **Lane 5:** EOC13 cells incubated with $1,25(OH)_2D_3$. M, molecular weight markers.

## DISCUSSION

In this study, we demonstrate that the active form of vitamin $D_3$ down-regulates the production of some proinflammatory molecules by stimulated microglia. This is the first evidence of a direct effect of $1,25(OH)_2D_3$ on microglial cells.

Activated microglia are present in some neurological diseases, such as human immunodeficiency virus dementia, Alzheimer's disease, and multiple sclerosis (for review see Munoz-Fernandez and Fresno, 1998; Streit, 2000). They represent an important source of proinflammatory factors (TNF, IL-6) and of iNOS, which are overexpressed during these pathologies. Efforts have concentrated on finding drugs capable of inhibiting microglial activation and the production of these harmful factors. Indeed, even though NO production is necessary under physiological conditions, an overproduction of this molecule has detrimental effects in the CNS. We show that $1,25(OH)_2D_3$ significantly inhibits TNF, IL-6, and NO production by microglia in a dose-dependent manner. Furthermore, we found that the mRNA synthesis of these cytokines was not affected by the presence of $1,25(OH)_2D_3$. Thus, it is likely that the $1,25(OH)_2D_3$-induced inhibitory effect on TNF, IL-6, and NO produc-

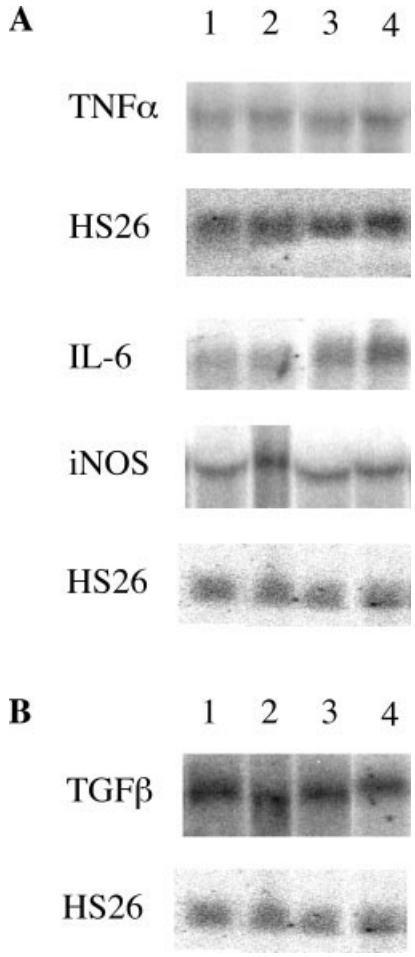



Fig. 4. Northern blot analysis of TNFα, IL-6, iNOS (**A**) and TGF-β1 (**B**) mRNA. Microglial cells were stimulated by LPS and IFNγ as described in Materials and Methods and incubated for 4 hr (TNFα) or for 24 hr (IL-6, iNOS, and TGF-β1). **Lane 1:** No $1,25(OH)_2D_3$. **Lane 2:** $1,25(OH)_2D_3$, $10^{-9}$ M. **Lane 3:** $1,25(OH)_2D_3$, $10^{-8}$ M. **Lane 4:** $1,25(OH)_2D_3$, $10^{-7}$ M. HS26 was used as a housekeeping gene.

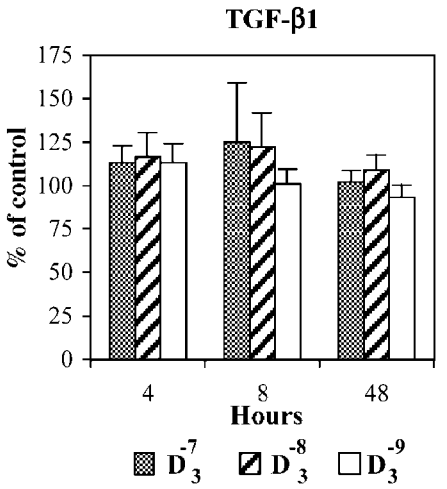



Fig. 5. Effect of $1,25(OH)_2D_3$ on TGF-β1 production by microglial cells preincubated for 24 hr with IFNγ and stimulated with LPS. Supernatants were harvested after the indicated period following LPS stimulation. Results are expressed as means ± SEM of the percentage of change compared with the stimulated control without $1,25(OH)_2D_3$ (100%) and correspond to four separate experiments.

tion by the EOC13 cell line is mediated by a posttranscriptional mechanism. In agreement with our results, it has been proposed that $1,25(OH)_2D_3$ inhibits the secretion of TNF and IL-6, by a posttranscriptional mechanism, in an in vitro model using human monocytes (Muller et al., 1992), which are closely related to microglial cells (Streit, 2001). These immunoregulatory effects on microglia suggest a potential therapeutic use of $1,25(OH)_2D_3$ (or of less hypercalcemiant analogues) in immune-mediated CNS pathologies. Moreover, $1,25(OH)_2D_3$ has also been described to down-regulate surface molecules expressed by activated microglia. Indeed, expression of MHC class II and accessory molecules B7 and CD4, involved in the antigen presentation by microglia or by monocytes, is inhibited in the presence of $1,25(OH)_2D_3$ (Nataf et al., 1996; Clavreul et al., 1998).

$1,25(OH)_2D_3$ exerts its effect mainly by binding to the VDR, a specific intracellular receptor belonging to the steroid-thyroid receptor superfamily. VDR can act as ligand-inducible transcription factor and thus directly modulate the transcription of genes having a functional binding site for the VDR in their regulatory region (Carlberg and Polly, 1998).

We have demonstrated here, in line with the reported presence of VDR in monocytes, the expression of VDR transcripts in microglia. The EOC13 cells constitutively express the VDR, and this expression is not limited to this particular cell line; we also detected the VDR mRNA in two other murine microglial cell lines, EOC20 and EOC9, and in the human microglial cell line CHME (data not shown), which was established by Janabi et al. (1995). The presence of VDR mRNA in microglial cells supports the hypothesis that $1,25(OH)_2D_3$ could act directly via the VDR on these cells. However, we cannot exclude an independent or additional indirect effect of $1,25\ D_3$, insofar as it has been reported that this hormone induces TGF-β1 expression in keratinocytes (Koli and Keski-Oja, 1993), breast carcinoma cells (Koli and Keski-Oja, 1995), and mouse renal proximal tubular cells (Weinreich et al., 1999). Indeed, TGF-β1 is a known immunosuppressor that inhibits the production of inflammatory cytokines by stimulated microglia (Suzumura et al., 1993; Chao et al., 1995). In our study, no modifications of TGF-β1 levels were observed in the presence of $1,25(OH)_2D_3$, suggesting that the inhibitory effect of $1,25(OH)_2D_3$ on microglia is not mediated by an increase in TGF-β1 production.

In vivo, $1,25(OH)_2D_3$ has been shown to have a protective effect in EAE. A curative treatment for EAE rats with $1,25(OH)_2D_3$ induces inhibitory effects on cellular and molecular events inside the CNS, which are correlated

with the improvement in clinical signs (Nataf et al., 1996; Garcion et al., 1997). In the EAE paradigm, the participation of TGF-β1 in the beneficial effects exerted by $1,25(OH)_2D_3$ is not clear. Depending on the species and on the type of treatment used, these beneficial effects may either be mediated by a direct effect of $1,25(OH)_2D_3$ on the expression of TGF-β1 and IL-4 (Cantorna et al., 1998) or be independent of the modulation of TGF-β1 expression by the hormone (Garcion, personal communication). The results of the present study argue for a direct anti-inflammatory effect of $1,25(OH)_2D_3$ on microglia.

Interestingly, recent data demonstrating the brain localization of the two enzymes, vitamin $D_3$ 25-hydroxylase and 25-hydroxyvitamin $D_3$-1α-hydroxylase, necessary for the bioactivation of vitamin $D_3$ to $1,25(OH)_2D_3$ suggest that this hormone may be locally produced in the CNS (Hosseinpour and Wikvall, 2000; Zehnder et al., 2001). Moreover, in vitro, activated microglia may be committed to produce $1,25(OH)_2D_3$ from its precursor (Neveu et al., 1994c). Thus, in addition to a potential therapeutic use of this vitamin, it is also possible that the endogenous production of $1,25(OH)_2D_3$ may contribute to maintain the homeostasis in the brain.

## ACKNOWLEDGMENTS

We are very grateful to Dr. W.S. Walker for the kind gift of EOC13 and LADMAC cell lines, to Dr. Fontana for TGF-β1 cDNA, to Dr. K. Flanders for TGF-β1 antibodies, and to Dr. L. Binderup (Leo Pharmaceuticals) for the $1,25(OH)_2D_3$. We also thank Drs. F. Darcy and G. Jean Harry for critical reading of the manuscript.

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