1,25 Dihydroxyvitamin D₃ Exerts Regional Effects in the Central Nervous System during Experimental Allergic Encephalomyelitis

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Abstract. 1,25-dihydroxyvitamin D₃ (1,25-D₃) is already known to prevent clinical signs of experimental allergic encephalomyelitis when animals are treated during the immunization phase. In the present work we have evaluated the ability of 1,25-D₃ to inhibit chronic relapsing experimental allergic encephalomyelitis (EAE) of the Lewis rat, when administered after the beginning of clinical signs. We observed a significant clinical improvement in 1,25-D₃-treated rats. This effect was accompanied by a profound inhibition of CD4 antigen expression by central nervous system (CNS) infiltrating monocytes/macrophages and parenchymal microglia. In addition, immunohistochemical analysis performed at the time of the second attack evidenced a region-specific distribution of inflammatory cells. In the same way, some aspects of the effects exerted by 1,25-D₃ appeared to vary depending on the region considered, namely spinal cord, brainstem, cerebellum, midbrain or anterior brain. Thus, in 1,25-D₃-treated rats, we observed an almost complete inhibition of CD4 antigen expression in the granule cell layer and the adjacent white matter of the cerebellum as well as a marked decrease in the number of OX-42-positive cells (macrophages and activated microglia) in anterior brain sections. We conclude that 1,25-D₃ can exert immunomodulatory effects inside the CNS during an ongoing immune process and may thus represent a promising therapy for multiple sclerosis.

Key Words: Calcitriol; CD4 antigen; Encephalomyelitis; Lymphocyte; Microglia; Multiple sclerosis; Vitamin D.

INTRODUCTION

Vitamin D₃ is a seco-steroid generated in the skin by the cleavage of 7-dehydrocholesterol, or obtained from the diet. Vitamin D₃ gives two main metabolites consisting of 25-hydroxyvitamin D₃ (25-OH D₃), the inactive hormonal precursor, and 1,25-dihydroxyvitamin D₃ (1,25-D₃), the biologically active hormone. 1,25-D₃ binds to a specific intracellular receptor, Vitamin D Receptor (VDR), which belongs to the steroid/thyroid receptor superfamily and acts as a ligand-dependent transcription factor (1). Initially considered the main hormone involved in calcium metabolism and bone mineralization, 1,25-D₃ exerts a large spectrum of tissue-specific biological activities. Among these, the immunomodulatory properties of 1,25-D₃, and particularly its inhibitory effects on Interleukin-2 (IL-2) and Interferon gamma (IFN-γ) secretion by T lymphocytes (2) have been clearly established. Furthermore, 1,25-D₃ prevents immunoglobulin secretion by B lymphocytes (3) and in a more general way affects the proliferation of both B and T lymphocytes (4, 5). The effects exerted by 1,25-D₃ on monocytes/macrophages are less clear and conflicting results have been reported regarding, for instance, Interleukin-1 (IL-1) production (2, 6). However, 1,25-D₃ exerts an immunosuppressive effect in vivo, and prevents in the mouse the appearance of autoimmune diseases, such as lupus or type I diabetes (7, 8). Likewise, experimental allergic encephalomyelitis (EAE) in SJL mice is inhibited by the administration of 1,25-D₃ at the time of immunization (9). This immunosuppressive effect is not an indirect consequence of the hypercalcemic activity of the hormone, but is likely to rely on VDR-mediated mechanisms (10).

Recently, several studies have demonstrated that the central nervous system (CNS) constitutes a target tissue of 1,25-D₃. Autoradiographic and immunohistochemical studies have identified 1,25-D₃ binding sites or VDR-like immunoreactivity in different structures of the rodent CNS such as spinal cord, hippocampus and cerebellum (11, 12). Vitamin D receptor mRNA was found in the human hippocampal formation (13) and 1,25-D₃ as well as 25-D₃ are present in human cerebrospinal fluid (14). Furthermore, in vitro studies have demonstrated that 1,25-D₃ is a potent inducer of VDR gene expression and of NGF synthesis in primary cultures of rat brain astrocytes (15). The involvement of 1,25-D₃ in some CNS functions is also supported by the fact that microglial cells (16), like macrophages (17), can convert 25-D₃ into 1,25-D₃, upon IFN-γ or LPS stimulation. From these results, it was hypothesized that during an inflammatory process, microglia-derived 1,25-D₃ could participate in microendocrine regulation (16).

This possibility has been investigated in the present work, using a model of chronic relapsing EAE of the Lewis rat. This cell-mediated autoimmune disease is induced by the immunization of rats with total guinea pig spinal cord tissue (GPSC) in the presence of enriched complete Freund’s adjuvant (CFA) (18, 19). The clinical course is characterized by the appearance of at least two predictable paralytic attacks which peak respectively 12

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days and 23 days after immunization (18, 19). As in the mouse model, most of the infiltrating cells comprising CD4-positive T cells and monocytes/macrophages invade the spinal cord at the first attack, whereas the lesions extend to rostral locations, particularly the cerebellum, during the second attack (19–21). The mechanism by which this time- and region-dependent localization of inflammatory cells occurs is not understood. However, it has been proposed that tissue factors controlling immune response could in part explain this phenomenon (21).

Here we report on the effects of 1,25-D₃ administered after the onset of EAE clinical signs. Immunohistochemical analysis performed on day 23 post-immunization, at the time of the second attack, showed a region-specific distribution of inflammatory cells, which was modified following 1,25-D₃ treatment. Thus, the CD4 antigen expression was markedly inhibited in CNS infiltrating monocytes/macrophages and parenchymal microglia, especially in the cerebellum. In addition, 1,25-D₃ induced a clinical improvement which reached significance on the first attack.

**MATERIALS AND METHODS**

**Animals**

Lewis female rats (8 to 9 weeks old) were obtained from Charles River France (Cléon) and bred in our animal facilities.

**EAE Induction**

Chronic Relapsing EAE was induced in rats as previously described (19). GPSC was obtained from female Dunkin-Hartley guinea pigs. One gram (g) GPSC was homogenized with 1 ml saline. The homogenate was then emulsified with 2 ml of Difco's complete Freund's adjuvant (CFA) supplemented with 40 mg of Mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI). Rats were injected intradermally under ether anesthesia into each footpad with 0.1 ml of this emulsion. The intradermal mode was preferred to subcutaneous injection in the tail or flanks since a previous work has shown that, in this model, lymphoid populations derived from popliteal lymph nodes play a key role in the recovery periods (22).

**Treatment Protocol**

A total of 41 age-matched Lewis female rats were used for this study. The six experimental groups comprised (a) control rats which were not immunized for EAE (control rats, n = 3); (b) control rats not immunized, treated with 1,25-D₃ (control 1,25-D₃-treated group, n = 4); (c) rats immunized for EAE (EAE control group, n = 15); (d) rats immunized for EAE and treated with vehicle alone (EAE vehicle-treated group, n = 7); (e) rats immunized for EAE and treated with 1,25-D₃ (EAE 1,25-D₃-treated group, n = 7); (f) rats immunized for EAE and treated with 25-D₃ (EAE 25-D₃-treated group, n = 5). 1,25-D₃ was a kind gift of L. Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). 1,25-D₃ and 25-D₃ were dissolved in propylene glycol and disodium phosphate and administered by intraperitoneal route (i.p.). Since 25-D₃ is the inactive hormonal precursor of 1,25-D₃, a different protocol using higher doses was applied to EAE 25-D₃-treated rats. It consisted of daily doses of 6 µg/kg 25-D₃ administered intraperitoneally. Regarding clinical outcome, this group was compared to pooled EAE control rats and EAE vehicle-treated rats. For EAE 1,25-D₃-treated rats and control 1,25-D₃-treated rats, a two-step protocol was applied. This consisted of two injections of high doses (5 µg/kg) on days 11 and 13 during the first attack, followed by three injections of lower doses (1 µg/kg) during the second attack, on days 19, 21 and 23. The treatment was interrupted from day 14 to day 18, a period which includes the remission phase. In parallel, EAE vehicle-treated rats were injected intraperitoneally with propylene glycol and disodium phosphate without 1,25-D₃.

This therapeutic protocol allowed us to study the effects of 1,25-D₃, after the neuroimmunological interactions had begun. Furthermore, it resembles therapeutic protocols currently used for the treatment of multiple sclerosis (MS) acute relapses by anti-inflammatory hormones structurally related to 1,25-D₃, namely corticosteroids (23, 24). Here, a short course of high doses of 1,25-D₃ was also applied at a time where the clinical signs were obvious.

**Clinical Assessment**

Rats were weighed and examined daily for grading of clinical signs as follows: 1 = loss of tail tonicity; 2 = weakness of one or both hind legs or mild ataxia; 3 = severe ataxia or paralysis; 4 = severe hind leg paralysis accompanied by urinary incontinence. In the control group and the group treated with the vehicle alone, clinical symptoms appeared on days 10 to 11 and all animals developed a severe paralysis of grade 4 on day 12. They completely recovered on days 17 to 18 and most animals (78%) experienced a second paralytic attack of grade 2 to 3 which peaked on day 23.

**Immunohistochemical Procedure**

Animals were killed by CO₂ inhalation on days 12, 18, or 23 for the EAE control group, or on day 23 for EAE 1,25-D₃-treated group and EAE vehicle-treated group. Control 1,25-D₃-treated rats were also killed on the last day of the therapeutic protocol corresponding to the third injection of 1 µg/kg 1,25-D₃.

Brain, brainstem, cerebellum and lumbosacral spinal cord were surgically removed, snap-frozen in liquid nitrogen-chilled isopentane and stored at −80°C until immunostaining was performed. Ten to 14 µm transverse sections of anterior brain (1.2 mm anterior from the bregma), midbrain (3.8 mm posterior from the bregma), brainstem, cerebellum, and lower thoracic or upper lumbar spinal cord were cut with a cryostat. The sections were fixed in cold absolute ethanol for 10 minutes, then washed in phosphate-buffered saline (PBS) and incubated in a solution of 10% normal goat serum in PBS. The sections were incubated overnight at 4°C with the primary antibodies directed against specific cell markers (lymphocytes and macrophages/microglia) and MHC class II antigens (Table 1). After rinsing in PBS, sections were incubated for 40 minutes with a rat-absorbed biotinylated horse anti-mouse antibody, then washed again, exposed to an avidin-biotinylated-peroxidase complex for 60 minutes at room temperature (Vectastain ABC kit, Vector, Burlingame, CA) and reacted with 0.04% diaminobenzidine in

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TABLE 1
Antibodies Used for Immunohistochemistry

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Molecule identified</th>
<th>Antibody dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC-OX42</td>
<td>Complement receptor type 3; present on monocytes/macrophages and microglia</td>
<td>1/100</td>
<td>Serotec (Kidlington, UK)</td>
</tr>
<tr>
<td>ED1</td>
<td>Cytoplasmic antigen present on monocytes/macrophages and reactive microglia</td>
<td>1/100</td>
<td>Serotec (Kidlington, UK)</td>
</tr>
<tr>
<td>OX6</td>
<td>Monomorphic determinant on rat class II MHC molecules</td>
<td>1/100</td>
<td>Cedarlane (Ontario, Canada)</td>
</tr>
<tr>
<td>MRC-OX33</td>
<td>Leucocyte common antigen; present on B cells</td>
<td>1/300</td>
<td>Serotec (Kidlington, UK)</td>
</tr>
<tr>
<td>OX19</td>
<td>CD5: present on T cells</td>
<td>1/100</td>
<td>Serotec (Kidlington, UK)</td>
</tr>
<tr>
<td>W3/25</td>
<td>CD4: present on helper T cells, some macrophages and on activated microglia</td>
<td>1/200</td>
<td>Serotec (Kidlington, UK)</td>
</tr>
<tr>
<td>OX8</td>
<td>CD8: present on cytotoxic/suppressor T cells and on some NK cells</td>
<td>1/500</td>
<td>Cedarlane (Ontario, Canada)</td>
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PBS with 0.01% H2O2 for 6 to 10 minutes. In all cases, sections without primary antibodies were used as controls.

RESULTS

Controls

Age-matched control rats were first studied. In all sections (anterior brain, midbrain, brainstem, cerebellum and lower thoracic or upper lumbar spinal cord), immunostaining was performed with OX42, W3/25, ED1, OX6, OX33, OX19, and OX8 antibodies (Table 1). The OX42 mAb stained dendritic resting microglia (Fig. 1A) and occasional round cells in meninges and ventricles. W3/25, OX6 and OX8 gave no staining for CNS parenchymal cells. No cells were stained with ED1.

Control 1,25-D3-treated rats were killed on the last day of the therapeutic protocol as described in the Materials and Methods section. Immunohistochemical analysis was performed on CNS sections with OX42, W3/25, and ED1. The staining pattern with the different antibodies was similar to that of the control group.

EAE Control Group Rats

For each CNS region investigated, immunostaining with OX42, ED1, OX19, and OX33 antibodies was carried out on tissue sections obtained from 11 of 15 EAE control group rats randomly chosen and killed on day 12 (n = 4), 18 (n = 4) (Table 2), and 23 (n = 3) (Table 3).

On day 12, the OX42 mAb gave a widespread and intense staining located on both round cells and cells of dendritic morphology corresponding to invading monocytes/macrophages and activated microglia, respectively. These cells were essentially localized in the spinal cord (Fig. 1B) and were less abundant in brainstem and cerebellum sections (Fig. 1C and Table 2). OX42-positive cells observed in the cerebellum were predominantly localized inside the granule cell layer and the adjacent white matter (Fig. 1C). Several foci of stained cells were observed in anterior brain and midbrain sections. ED1 staining was less intense but displayed a similar pattern of distribution. Numerous T-lymphocytes labeled with OX19 mAb were present in the white matter of the spinal cord while OX33-positive cells, corresponding to B lymphocytes, were sparse. OX33-positive cells were absent in the other areas studied.

On day 18, the number of OX19-positive cells decreased in the spinal cord compared to day 12 (Fig. 1G, H and Table 2) while it increased in the cerebellum (Fig. 1E, F and Table 2). Cell foci immunostained with OX19, OX42, and ED1 were observed in the cerebellum, but very few OX33-positive cells were detected. Surprisingly, as observed on day 12, in all rats studied (n = 4) these foci were almost exclusively localized in the granule cell layer and the adjacent white matter (Fig. 1D). In the same manner, in anterior brain sections, most OX42-positive cells were localized in the striatum. Some OX42- and ED1-positive cells were observed in the corpus callosum in anterior brain sections as well as in midbrain sections.
Fig. 1. EAE control rats were killed on day 12, at the peak of clinical signs during the first attack (B, C, E, G), or on day 18, during the remission phase (D, F, H). The microphotographs show a widespread OX42 immunoreactivity in the spinal cord on day 12 (B) while sections obtained from control rats are poorly immunostained (A). ×20. On day 18, the number of OX42-positive cells increases in the cerebellum (D) compared to day 12 (C). ×50. These OX42-positive cells are mostly localized inside the granule cell layer and adjacent white matter (C, D). In the cerebellum, the number of OX19-positive cells increases on day 18 (F) compared to day 12 (E). ×50. In contrast, OX19 immunoreactivity in the spinal cord decreases on day 18 (H) compared to day 12 (G). ×100.
TABLE 2
Immunohistochemical Analysis of EAE Control Rats

<table>
<thead>
<tr>
<th>Marker–time</th>
<th>Spinal cord</th>
<th>Cerebellum</th>
<th>Brainstem</th>
<th>Midbrain</th>
<th>Anterior brain</th>
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<tr>
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<td>+/–</td>
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<tr>
<td>ED1–D18</td>
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<tr>
<td>OX19–D12</td>
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<td>+/-</td>
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<td>+</td>
<td>0</td>
<td>0</td>
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</table>

Animals were killed on day 12 (n = 4) during the first attack or on day 18 (n = 4) during the clinical remission.

On day 23, the number of OX19-positive cells increased in the spinal cord compared to day 18, while the number of OX33-positive cells remained constant (Table 3). Concerning the cerebellum, most of the infiltrating cells were still localized in the granule cell layer and the adjacent white matter (Fig. 3A), although inflammatory cells also invaded the molecular layer. In the anterior brain, as observed on day 18, most of OX42-positive cells were essentially located in the striatum (Fig. 2A) and only a few cells were stained with OX19 mAb. No OX33-positive cells were detected in anterior brain and midbrain sections.

EAE Vehicle-Treated Rats

Three rats randomly chosen in the EAE vehicle-treated group (n = 7) were killed on day 23, at the time of the second attack. Results obtained with OX42, OX33, OX19, and ED1 mAb were similar to the immunostaining pattern described in EAE control group rats on day 23.

In addition, an analysis of OX8, W3/25, and OX6 immunostainings was performed. Two morphologically distinct cell types were immunostained with the OX8 mAb. The predominant cell type consisted of small round cells probably corresponding to CD8-positive lymphocytes. Only a few large round CD8-positive cells considered as natural killer cells were observed. Although detected in all the areas of the CNS, CD8-positive cells were predominantly found in the brainstem and the cerebellum and especially around the fourth ventricle. Staining with W3/25 and OX6 antibodies gave a pattern of distribution similar to OX42 mAb, indicating that the great majority of microglial cells were CD4-positive and expressed MHC class II molecules. This observation was confirmed by immunolabeling with OX42 and W3/25 antibodies on adjacent sections (Fig. 3A, B).

TABLE 3
Immunohistochemical Analysis of EAE Vehicle-Treated Rats and EAE 1,25-D3-Treated Rats

<table>
<thead>
<tr>
<th>Marker</th>
<th>Treatment</th>
<th>Spinal cord</th>
<th>Cerebellum</th>
<th>Brainstem</th>
<th>Midbrain</th>
<th>Anterior brain</th>
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<tr>
<td>OX42</td>
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Three animals of each group were killed on day 23, during the second attack.

Fig. 2. EAE vehicle-treated rats and EAE 1,25-D$_3$-treated rats were killed on day 23 at the peak of clinical signs, during the second attack. OX42 immunostaining in anterior brain sections is less intense in 1,25-D$_3$-treated rats (B) compared to vehicle-treated rats (A). ×50. Widespread OX42 immunoreactivity is observed in the spinal cord of both EAE vehicle-treated rats (C) and EAE 1,25-D$_3$-treated rats (D). ×50. OX6-positive cells are less numerous in midbrain sections obtained from EAE 1,25-D$_3$-treated rats (F) than from EAE vehicle-treated rats (E). ×50.
Fig. 3. EAE vehicle-treated rats and EAE 1,25-D$_3$-treated rats were killed on day 23, at the peak of clinical signs, during the second attack. Adjacent sections of the cerebellum obtained from EAE vehicle-treated rats were stained with OX42 (A) or W3/25 (B) antibodies. The majority of OX42-positive cells (A) are CD4-positive cells (B) of both round and dendritic morphology. ×150. Adjacent sections of the cerebellum (white matter adjacent to the granule cell layer) obtained from EAE 1,25-D$_3$-treated rats were immunostained with OX42 (C) or W3/25 (D) antibodies. ×150. While OX42 immunoreactivity is intense, no CD4-
Fig. 4. Rats immunized for EAE were treated either with 1,25-D$_3$ ( ■ ) (n=7) or vehicle alone (○) (n=15) (*: EAE control rats and EAE vehicle-treated rats are pooled). Rats were monitored daily and a mean clinical score was assigned for each group. 1,25-D$_3$ was administered on days 11, 13 (5 μg/kg) and 19, 21 and 23 (1 μg/kg) (arrowheads). EAE 1,25-D$_3$-treated rats present a reduced clinical score during the first and the second attack.

W3/25 labeling also showed a striking decrease in the number of CD4-positive cells in all regions studied. This effect was especially marked in the brainstem and the cerebellum, particularly in the granule cell layer and the adjacent white matter (Fig. 3D). The staining of adjacent sections with W3/25 and OX42 showed that the expression of the CD4 antigen was decreased in cells displaying a round morphology (macrophages, amoeboid microglia or monocytes) or a dendritic morphology corresponding to activated microglial cells (Fig. 3C–F). Similarly, the use of OX19 and W3/25 antibodies on adjacent sections revealed a complete inhibition of W3/25 labeling in some OX19-positive cell foci, indicating that some T lymphocytes could be concerned by this phenomenon (data not shown). However, we cannot exclude the possibility that these cells were in fact CD4-negative, CD8-positive T lymphocytes.

**Effects of 1,25-D$_3$ and 25-D$_3$ on EAE Clinical Course**

1,25-D$_3$, 25-D$_3$ or vehicle alone were administered from day 11, one day before clinical signs became maximal during the first attack (Fig. 4). Since the clinical outcome was similar in EAE control rats and EAE vehicle-treated rats, these two experimental groups were pooled for statistical analysis of clinical data. EAE control rats (n = 15), EAE vehicle-treated rats (n = 7), and EAE 25-D$_3$-treated rats (n = 5) all experienced a severe hind leg paralysis accompanied by urinary incontinence (grade 4 on clinical scale) on day 12. Among EAE 1,25-D$_3$-treated rats (n = 7), only 57% gained to grade 4 on day 12 compared to 100% for EAE 25-D$_3$-treated rats and pooled EAE control and EAE vehicle-treated rats (p<0.05) (Table 4). In addition, although not statistically significant, a reduced incidence in the occurrence of a second paralytic attack (57% instead of 78%) was observed in EAE 1,25-D$_3$-treated rats as well as a reduced mean severity score on day 23 (1.6 instead of 2.5). Considering these parameters, 25-D$_3$ treatment had no effects on the clinical course of EAE (Table 4). Otherwise, as previously described (19), the rats immunized for EAE that were not treated or treated with either vehicle alone or 25-D$_3$, all experienced a weight loss ranging from 15 to 20% of their body weight on day 12 and from 5 to 10% body weight on day 23. The weight curve was the same for EAE 1,25-D$_3$-treated rats except during the second attack when the weight loss reached 10 to 15% on day 23. However, the differences did not reach significance when compared to EAE control, EAE vehicle-treated or EAE 25-D$_3$-treated rats (data not shown). Similarly, all the control 1,25-D$_3$-treated rats displayed a weight loss from 5 to 10% of their body weight, probably related to the dehydration induced by the hypercalcemic effects of 1,25-D$_3$ (1).

**DISCUSSION**

1,25-D$_3$ is already known to prevent EAE induction in the mouse model if animals are treated before and during

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*EAE vehicle-treated group and EAE control group are pooled.
**p < 0.05.
the time course of immunization (9). This action emphasizes the immunosuppressive effect of the hormone which was detected in other animal models of autoimmune disease. In the present work, we have investigated the effect of 1,25-D$_3$ administered after the immunization phase, when clinical signs become obvious. By monitoring the importance and nature of cells infiltrating the CNS, we show unambiguously that 1,25-D$_3$ can exert immunomodulatory effects inside the CNS even during an ongoing immune disorder. Thus, 1,25-D$_3$, administered after the beginning of clinical signs, induces a profound downregulation of the CD4 antigen expression by infiltrating cells. This process takes place not only in invading monocytes/macrophages but also in parenchymal activated microglia. A downregulation of the CD4 antigen expression by 1,25-D$_3$ has been already described in vitro for the human promyelocytic cell line HL60 (25) or for human monocytes (26) but, to our knowledge, the present finding is the first demonstration that 1,25-D$_3$ exerts in vivo a similar effect in rat microglial cells. Since it has been proposed that the CD4 antigen expression is subject to a specific regulation inside the CNS (27), our results suggest that 1,25-D$_3$ could be involved in this phenomenon. In addition, 1,25-D$_3$ downregulates, although to a lesser extent, the expression of MHC class II molecules. This effect is reminiscent of that observed in the case of human monocytes and thyroid follicular cells (26, 28). During interactions between antigen-presenting cells (APC) and lymphocytes, the CD4 antigen acts as an accessory molecule (29, 30) which strengthens adhesion to MHC class II proteins, the molecular support of antigen presentation (31–33). Therefore, a decreased expression of the CD4 antigen and of MHC class II molecules should limit the ability of monocytes, macrophages, and especially microglial cells to function as APC. Moreover, it has been demonstrated that CD4-blocking strategies using monoclonal antibodies or synthetic analogues could inhibit EAE even if administered after the onset of clinical signs (34, 35). Thus, it is tempting to speculate that the clinical improvement observed in 1,25-D$_3$-treated rats is related to the downregulation of CD4 antigen expression inside the CNS.

The mechanism of CD4 downregulation induced by 1,25-D$_3$ in HL60 cells and monocytes has been ascribed to a direct effect of the hormone on the differentiation state or the phenotype of these cells (25, 26). Similarly, the decreased ability of monocytes for antigen presentation in the presence of 1,25-D$_3$ appeared independent of soluble factors such as IL-1, Interleukin-6 (IL-6), Prostaglandins (PGE2), or IFN$_\gamma$ (26). Therefore, our results raise the possibility that during an inflammatory process, 1,25-D$_3$ exerts direct effects on microglial cells. This assumption implies that like other tissue macrophages (36), activated microglial cells express VDR, since they respond to 1,25-D$_3$. Moreover, since microglial cells can also convert 25-D$_3$ to 1,25-D$_3$ upon IFN$_\gamma$ or LPS stimulation (16), it appears that activated microglia could play a prominent role in a microendocrine regulation of 1,25-D$_3$ inside the CNS. The fact that administration of the hormonal precursor 25-D$_3$ did not modify the clinical course of EAE is not in contradiction with this assumption. 25-D$_3$ has a higher affinity than 1,25-D$_3$ for plasma vitamin D-binding protein and this might limit its availability in the CNS. Furthermore, local 1,25-D$_3$ synthesis by activated infiltrating macrophages or microglia is likely to induce vitamin D$_3$-24 hydroxylase, an enzyme which inactivates 1,25-D$_3$ and whose gene can be expressed by astrocytes (37). Therefore, intracerebral production of 1,25-D$_3$ might not be sufficient to prevent a generalized autoimmune attack such as that induced in EAE.

Another body of information collected in this study concerns the chronic localization of inflammatory cells during EAE. In agreement with previous studies (18, 20, 21), we observed a caudo-rostral evolution of the lesion topography during EAE. The analysis of each immunological marker investigated (OX42, OX19, OX33, ED1), revealed a region- and time-specific evolution. Furthermore, we found that activated microglia, B-lymphocytes or T-lymphocytes are not uniformly distributed inside each area. It seems unlikely that a systemic control of the immune response, induced for instance by suppressor cells or the idiotypic antibody network, can exert such regional effects. As previously suggested (21), we propose that tissue factors provided by CNS cells confer to each region its own immunological status. Several lines of evidence indicate that neurons (38, 39) as well as astrocytes and microglial cells interact during a CNS immunological disorder. These cells can form regional networks which, by producing or responding to a variety of cytokines or neurotrophic factors, are able to shape the immune response (40). In addition, microglial cells and astrocytes express receptors to numerous neuromediators. These could modulate in a region-specific manner the ability of these cells to produce or respond to proinflammatory cytokines (41, 42). Regional differences in microglial cells as well as astrocytes have been already reported. Brain macrophages localized in the vicinity of blood vessels (perivascular cells) or in circumventricular regions have been proposed to present specific immune properties (43, 44). These observations, taken together with our results, are of particular interest in the case of MS since the distribution of lesions is also region-specific in this pathology. Thus, demyelination is frequently observed in periventricular areas, optic nerves, and the spinal cord of MS patients (45). Likewise, the primary chronic progressive form of MS appears to evolve as a pure, slowly progressing spinal cord disease.
REGIONAL EFFECTS OF VITAMIN D3 DURING EAE

Some aspects of the effects induced by 1,25-D3 on the characteristics of the immune reaction are also region-specific. This is the case of OX42-positive cells, which notably decreased in number in the anterior brain, while the number remained unchanged in the spinal cord and cerebellum. Likewise, the downregulation of CD4 antigen expression, although observed in all regions, was more pronounced in the cerebellum and the brainstem.

Such regional differences could result from the functional heterogeneity of the neurons. Autoradiographic studies have identified subpopulations of neurons bearing a high density of 1,25-D3 binding sites; for instance, in the forebrain or brainstem (10). VDR-like immunoreactivity was observed by immunohistochemical methods in the granular layer of the cerebellum (11). Therefore some regional effects elicited by 1,25-D3 could result from neuron-mediated mechanisms. Neuronomodulators themselves might transduce selective responses, since agonists of the dopamine D1 subtype can switch on a VDR-mediated transcription in some cells (46). Finally, regional differences might exist, in the level of VDR expression and/or 1,25-D3 availability. In this respect, it has been proposed that in the rat CNS, glucocorticoids (GCC) could mediate regional effects depending on the distribution and binding characteristics of the adenocorticoid receptors (47). The fact that, during an autoimmune disorder, CNS regions such as spinal cord and cerebellum differ in their immune status and response to 1,25-D3 could be of significance in the case of MS. Attempts to use an endocrine therapy involving nuclear hormone receptor ligands should be adequately addressed. 1,25-D3 or less hypercalcemic analogues might have a therapeutic potential in the cerebellar forms of MS, such as that initially described in Charcot’s triad (48).

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