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Novel insights in the immune function of the vitamin D system: Synergism with interferon-beta

Evelyne van Etten [a], Conny Gysemans [a], Dumitru D. Branisteanu [b], Annemieke Verstuyf [a], Roger Bouillon [a], Lut Overbergh [a], Chantal Mathieu [a,*]

[a] *Laboratory for Experimental Medicine and Endocrinology (LEGENDO), Katholieke Universiteit Leuven, Herestraat 49, O&N1-bus 902, B-3000 Leuven, Belgium*
[b] *Clinic of Endocrinology, University of Medicine and Pharmacy "Gr. T. Popa", Str. Vasile Conta 18, 6600 Iasi, Romania*

Abstract

The $1,25(OH)_2D_3$ analog, TX527 (19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$), has an interesting dissociation profile between its potent immunomodulatory and its calcemic effects in vivo. The strong immunomodulatory potency of TX527 is reflected by its ability to attenuate experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis (MS). At present most MS patients are being treated with systemic IFN-β administration. The aim of this study was to investigate whether combining IFN-β with TX527 could empower its EAE-protective effects. We evaluated also combinations with the standard immunosuppressant cyclosporin A (CsA). EAE was induced in SJL mice by PLP immunization, treatment was started 3 days before disease induction. The TX527 + IFN-β combination resulted in significant disease protection which was superior to the effect of both treatment separately. No disease amelioration, even aggravation, was obtained with the IFN-β + CsA combination. By adding TX527 to the IFN-β + CsA combination near complete protection from EAE was achieved (100% protection from paralysis, mean maximal score of 1.8 ± 1.5, both $p < 0.05$ versus controls and all individual treatments). From these data we conclude that adding TX527 to an IFN-β and/or CsA treatment results in clear additional immunomodulatory effects in EAE prevention and is therefore a potentially interesting candidate to be considered in clinical intervention trials in MS.

Keywords: $1,25(OH)_2D_3$; IFN-β; Experimental autoimmune encephalomyelitis; Synergism; Multiple sclerosis

1. Introduction

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has besides its effects on calcium and bone homeostasis, long recognized immunomodulatory effects [1,2]. $1,25(OH)_2D_3$ exerts its immunomodulation both on the antigen-presenting cell as well as on the T cell level thereby influencing all aspects of an immune reaction in a coherent way. A major drawback of using $1,25(OH)_2D_3$ in clinical immune therapy are its adverse side effects on calcium and bone. Structural analogs of $1,25(OH)_2D_3$, such as TX527 (19-nor-14,20-bisepi-23-yne-$1,25(OH)_2D_3$), have been developed showing reduced calcemic activity in association with enhanced in vitro and in vivo immunomodulatory capacity [3]. One of the in vivo models frequently used for evaluating the immunomodulatory effects of $1,25(OH)_2D_3$ analogs is experimental autoimmune encephalomyelitis (EAE), a murine model of human multiple sclerosis (MS). In the treatment of human MS, IFN-β is, because of its potent immunomodulatory properties, for most patients the treatment of choice nowadays [4]. The immunomodulatory properties of IFN-β are mainly focused on counteracting the effects of IFN-γ [5,6]. MHC class II antigen expression of antigen-presenting cells is decreased [7] and proliferation of T lymphocytes is inhibited [8] by IFN-β. By reducing the secretion of Th1 cytokines, IFN-γ and TNF-α [9], and by enhancing the production of the Th2 cytokine IL-10 [10], IFN-β induces a shift from Th1 towards Th2

Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; CsA, cyclosporin A

* Corresponding author. Tel.: +32 16 346023; fax: +32 16 345934.
E-mail address: chantal.mathieu@med.kuleuven.be (C. Mathieu).

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mediated immune responses. Besides that, treatment with IFN-β causes a down-regulation of adhesion molecules like very late antigen 4 and matrix degrading enzymes such as matrix metalloproteinase 9 [11,12], both molecules involved in the trans-endothelial migration of leukocytes, an inherent step of inflammation.

Consequently, when clinical trials using $1,25(OH)_2D_3$ analogs would be considered in human MS, combination treatments with IFN-β would be the most obvious choice. Combining IFN-β with the $1,25(OH)_2D_3$ analog TX527 has already been shown to prevent the recurrence of autoimmune diabetes after syngeneic islet transplantation in spontaneously diabetic NOD mice [13]. An important issue that needs to be known before performing combinations of TX527 with IFN-β therapy in human MS, is whether they lead to advantageous and certainly not to antagonistic immune effects in this disease model. Therefore, the aim of this study was to investigate the cooperative immunomodulatory effects between the TX527 and IFN-β in the murine EAE model. To correlate our results with known immune cooperativity, we also combined TX527 with the well-established immunosuppressant cyclosporine A (CsA), a combination previously shown to be effective in EAE [14]. Finally, we also evaluated the EAE-protective effects of the triple combination of TX527, IFN-β and CsA.

2. Materials and methods

2.1. EAE model

Inbred female SJL mice (Harlan CPB, Zeist, The Netherlands) of 8–10 weeks of age were used to induce EAE as described previously [14]. Briefly, on day 0, mice were immunized with an encephalitogenic peptide of murine proteolipid protein (100 μg/mouse, $PLP_{139–151}$, Peptides international, Louisville, KY). On the same day and on day +2, Pertusigen (200 ng/mouse, Sigma, St. Louis, MO) was administered intravenously. Data were cumulated from a total of 76 mice randomized in eight treatment groups: control (vehicle peanut oil) treatment (CTR, $n = 17$), TX527 (first synthesized by P. Declercq and M. Vandewalle, Laboratory of Organic Chemistry, University of Ghent, Belgium and further provided by J.C. Pascal and N. Adje, Théramex, Monaco) treatment at 7.5 μg/kg (TX, $n = 6$), mouse recombinant IFN-β (kind gift of E. Croze, Berlex Biosource, Richmond, CA) treatment at 500 IU (IFN, $n = 18$), CsA (Sandoz Pharma, Basel, Switzerland) treatment at 4 mg/kg (CsA, $n = 12$), and the combinations TX527 + IFN-β (TX + IFN, $n = 5$), TX527 + CsA (TX + CsA, $n = 6$), IFN-β + CsA (IFN + CsA, $n = 6$) and TX527 + IFN-β + CsA (TX + IFN + CsA, $n = 6$). All compounds were injected intraperitoneally (i.p.), daily from day −3 onwards. All experiments were ended on day +20 at which time serum and a femur from each individual mouse was collected and stored at −20 °C for further analysis.

2.2. Endpoints

Mice were evaluated for their disease evolution, their change in body weight and their calcium and bone parameters, all as described previously [15]. Briefly, from day +9 onwards mice were assessed daily for signs of paralysis by two independent observers. A clinical score on a scale from 0 (no signs of paralysis) to 5 (complete paralysis or dead after signs of paralysis) was used to quantify the severity of disease. End point evaluation included mean severity of disease over time, paralysis-free survival over time (score <4 throughout the experiment), mean day of disease onset (first day of score >0) and mean maximal disease score (maximal score in the course of the experiment). For each individual mouse, the difference in body weight between day +9 and day −3 was evaluated. Serum calcium content was measured using a photocolorimetric method (Sigma). Serum osteocalcin levels were evaluated using an in house radioimmunoassay technique [16]. Femur dry weight and ash weight were measured after 16 h on 100 °C and on 800 °C, respectively. Femur calcium content was determined on HCl-dissolved bone ash, using the same method as for the serum.

2.3. Statistical analysis

ANOVA was used for statistical comparison of the results. When ANOVA was significant, Fischer's LSD multiple-comparison test was applied. Significance was defined at the 0.05 level. Results were expressed as mean ± S.D.

3. Results

3.1. Disease evolution

All but one of the control mice developed EAE with a mean time of disease onset on day 14 and a mean maximal disease score of 3.8 (Table 1). Mice treated with CsA showed

Table 1
The effects of the different treatments on EAE development

Groups[a] name (n)	PFS (%)	Start[b] (day)	Max[c] (score)
CTR (17)	24	14 ± 2	3.8 ± 0.7
TX (6)	50	15 ± 1	3.5 ± 0.5
IFN (18)	17	13 ± 2	4.4 ± 0.9
CsA (12)	17	13 ± 3	4.3 ± 1.2
TX + IFN (5)	80[*,$]	16 ± 1[$]	3.2 ± 0.4[$]
TX + CsA (6)	67[§]	16 ± 4[§]	2.2 ± 2.0[*,#,§]
IFN + CsA (6)	0	12 ± 1[*]	4.8 ± 0.4[*]
TX + IFN + CsA (6)	100[*,#,$,§]	17 ± 2[*,#,$,§]	1.8 ± 1.5[*,#,$,§]

[a] The drug codes are as follows: CTR, control treatment; TX, TX527 7.5 μg/kg day; IFN, IFN-β 500 IU/day; CsA, cyclosporin A 4 mg/kg day; with n the number of mice in each treatment group.
[b] Start, mean day of disease onset.
[c] Max, mean maximal disease score.
[*] $p < 0.05$ vs. control treatment.
[#] $p < 0.05$ vs. treatment with TX527 alone.
[$] $p < 0.05$ vs. treatment with IFN-β alone.
[§] $p < 0.05$ vs. treatment with CsA alone.

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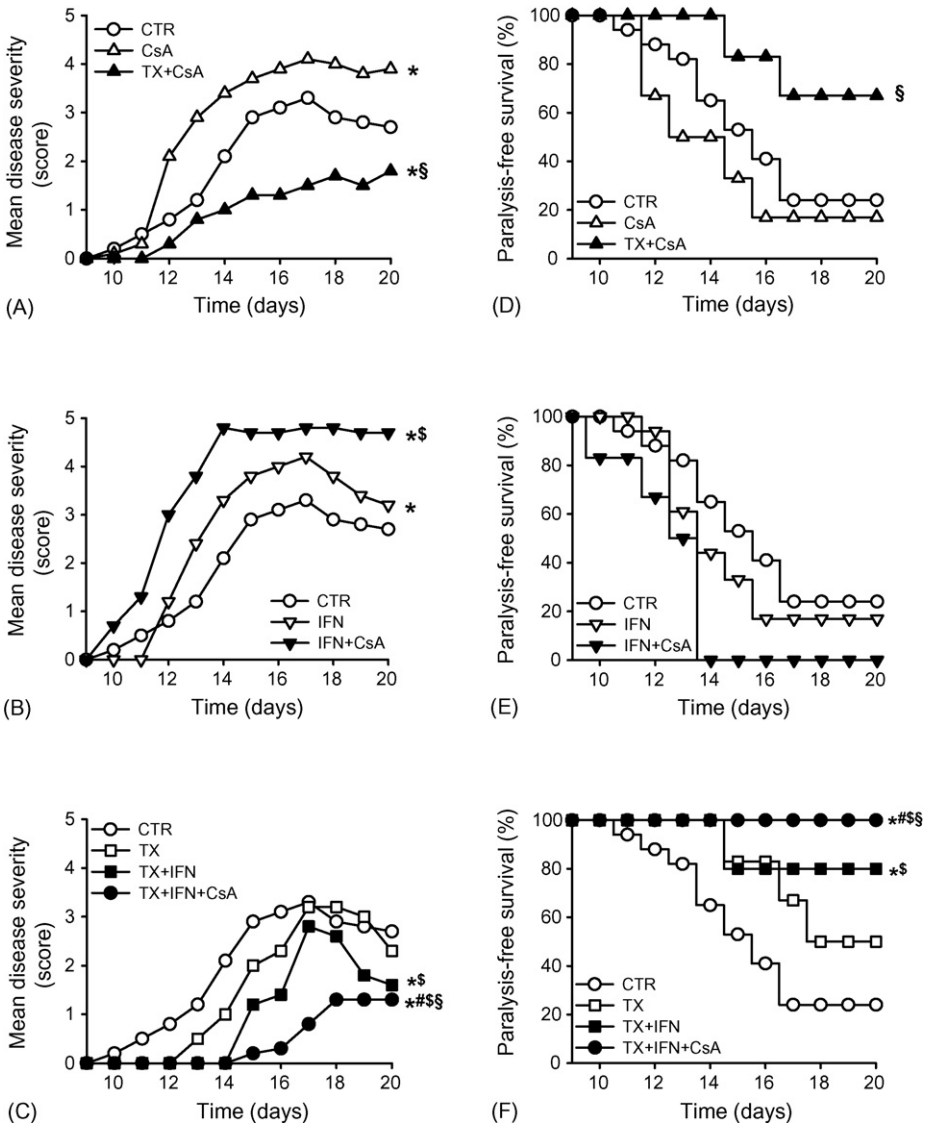

Fig. 1. Prevention of EAE by different treatments, expressed as the evolution in time of mean disease severity (A–C) and of paralysis-free survival (D–F). (◯) Control group (CTR); (□) TX527-treated group (TX); (△) CsA-treated group (CsA); (▽) IFN-β-treated group (IFN); (■) mice treated with a combination of TX527 and IFN-β (TX + IFN); (▲) mice treated with a combination of TX527 and CsA (TX + CsA); (▼) mice treated with a combination of IFN-β and CsA (IFN + CsA); (●) mice treated with a combination of TX527 and IFN-β and CsA (TX + IFN + CsA). $^{*}p < 0.05$ vs. control treatment; $^{\#}p < 0.05$ vs. treatment with TX527 alone; $^{\$}p < 0.05$ vs. treatment with IFN-β alone; $^{\S}p < 0.05$ vs. treatment with CsA alone.

a comparable disease evolution than the control group with a slightly lower paralysis-free survival (Fig. 1D, NS) and earlier disease onset (Table 1, NS). The mean disease severity of CsA-treated mice was significantly higher compared to controls (Fig. 1A). This disease pattern could also be observed for mice treated with IFN-β only (Table 1 and Fig. 1B and E). These results indicate that both treatments, 4 mg/kg day CsA and 500 IU/day IFN-β, were set at a subtherapeutical level. Treatment with 7.5 μg/kg day TX527 resulted in a clear, although not significant, disease protection with an increased paralysis-free survival (Fig. 1F), a delayed disease onset (Table 1) and a decreased mean disease severity (Fig. 1C). Although suboptimal, this dose of TX527 still conferred protection from EAE.

Combining TX527 with IFN-β (TX + IFN) provided substantial protective effects with especially an increased paralysis-free survival (Fig. 1F) and a delayed disease onset (Table 1). Also disease severity was decreased as expressed by the mean maximal disease score (Table 1) and the mean disease evolution in time (Fig. 1C). Mice treated with a combination of TX527 and CsA (TX + CsA) experienced protective effects of the same order of magnitude (Table 1 and Fig. 1A and D). The combination of IFN-β and CsA did not result in any disease protection. On the contrary, a trend towards a more aggressive disease could be noted with an earlier onset (Table 1) and a higher severity (mean maximal disease severity (Table 1) and evolution in time of mean disease severity (Fig. 1B)). Finally, all mice treated with

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the triple combination TX527 + IFN-β + CsA were protected from paralysis (Fig. 1F). Disease severity (Fig. 1C) was significantly decreased as compared to control mice and also in comparison with each treatment separately (TX, IFN and CsA). Mean time of disease onset was delayed to day 17 and mean maximal disease score dropped to 1.8 (both Table 1).

3.2. Side effects on calcium and bone

Treatment of mice with TX527 resulted in significant hypercalcemia (Fig. 2B). CsA and IFN-β, when used alone or in combination with each other, had no effect on serum calcium levels. Also, no additional effects on calcium levels could be observed when the latter immunosuppressants were combined with TX527. These effects on serum metabolism are reflected on body weight evolution before disease onset (Fig. 2A). Control mice showed a slight gain in body weight on day 9 compared to the beginning of the experiment as would be expected from normal, healthy mice. A gain in body weight was also seen for mice treated with the subtherapeutic doses of CsA and IFN-β. Treatment with TX527 resulted in a major loss of body weight as could be expected based on previous dose finding experiments [14]. All mice treated with combinations including TX527 showed similar loss of body weight, indicating again that no additional effects on toxicity resulted from adding IFN-β and/or CsA to the TX527 treatment. For none of the treatments, a significant change in serum osteocalcin level was observed (data not shown), indicating no increase in bone turnover. Additionally, none of the treatments resulted in loss of mass or calcium content of bone (data not shown).

4. Discussion

IFN-β has been shown to be efficacious in the treatment of patients with relapsing-remitting MS [17–19] and to inhibit the progression of EAE in mice [20]. IFN-β has multiple immunomodulatory activities including inhibition of lymphocyte activation, down-regulation of MHC II surface expression on antigen-presenting cells such as dendritic cells and decrease of leukocyte transmigration through the blood–brain barrier. The imbalance between Th1 and Th2 cytokines in MS is also corrected by IFN-β [21]. These properties have made IFN-β the present treatment of choice for most MS patients.

We and others have demonstrated that $1,25(OH)_2D_3$ and its analogs have protective effects in the animal model of MS, EAE [22–26,14,15] and in some studies indirect evidence suggests that $1,25(OH)_2D_3$ could be protective in MS [27–29]. The geographic distribution of MS strongly indicates a role of UV light in the protection from MS. The link between UV light-exposure and protection from MS could be the production of the immunomodulatory $1,25(OH)_2D_3$, knowing that UV light is necessary to produce previtamin D_3 from 7-dehydrocholestoral in the epidermis [30]. Therefore, clinical trials using analogs of $1,25(OH)_2D_3$ in MS can be taken under serious consideration and combinations with IFN-β are the obvious treatment of choice. However, in a first step it has to be proven that, upon combination, analogs of $1,25(OH)_2D_3$ does not counteract the immunomodulatory effects of IFN-β.

In the present study, treatment with IFN-β alone did not result in amelioration of disease symptoms. This is in line with an earlier study were IFN-β had also no effect on EAE in SJL mice [31]. Combinations of TX527 with IFN-β resulted in substantial synergistic immunomodulatory effects in EAE. The amelioration of EAE symptoms could be observed as an increase in paralysis-free survival, a delay of disease onset and a decrease of disease severity. This combined treatment was as potent as the previously tested TX527 + CsA combination. TX527 being, like its parent molecule $1,25(OH)_2D_3$, a strong inhibitor of antigen presentation and CsA being a specific suppressor of TCR-mediated T cell activation, their combined use targets both APCs as well as T lymphocytes explaining the strong protective effects in EAE. On the other hand, IFN-β exert similar immunomodulatory effects as TX527, and

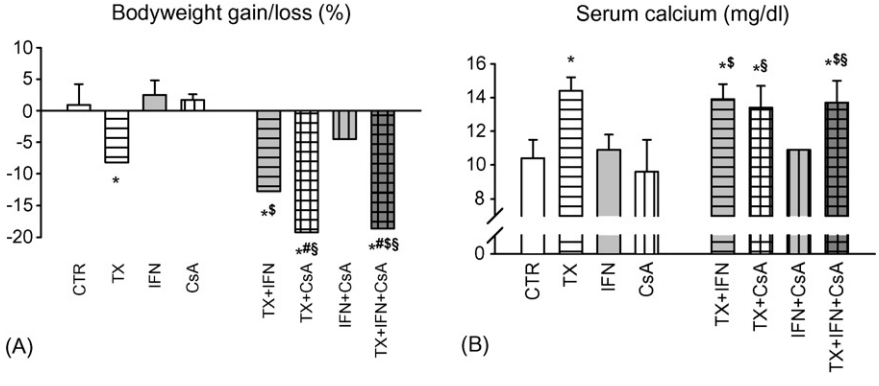

Fig. 2. The effects of the different treatments on body weight variation (A) and serum calcium (B). Values for control mice (CTR) and mice treated with TX527 (TX), IFN-β (IFN), CsA, TX + IFN, TX + CsA, IFN + CsA and TX + IFN + CsA are expressed as mean ± S.D. $^{*}p < 0.05$ vs. control treatment; $^{\#}p < 0.05$ vs. treatment with TX527 alone; $^{\$}p < 0.05$ vs. treatment with IFN-β alone; $^{\S}p < 0.05$ vs. treatment with CsA alone.

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combining them still results in ameliorated EAE protection.

In contrast with the previous protective combinations (TX + IFN and TX + CsA), the combination of IFN-β with CsA significantly aggravated disease symptoms compared to controls. All animals developed severe signs of paralysis and mean disease onset was accelerated. These results show that, although both TX527 and IFN-β exert similar immunomodulatory effects (mainly inhibition of antigen presentation and induction of a Th2 directed immune shift), adding TX527 to a treatment regimen of CsA results in a substantial additional protection from the autoimmune disease, whereas IFN-β does not.

In several studies, CsA has proven its efficacy in EAE [32,24,33]. However, in clinical trials using CsA for the treatment of relapsing-remitting and chronic progressive MS, the adverse effects of CsA (especially nephrotoxicity) were serious enough to outweigh its modest beneficial effects [34,35]. The reasons for this discrepancy in CsA protection between the animal model and the human disease are not known. One reason could be that human MS is mostly treated in a later stage of the disease, when symptoms become visible, long after the autoimmune attack is initiated. CsA, being a immunomodulator acting on very early IL-2-dependent processes of T cell activation [36], loses most of its efficacy at this stage of the autoimmune attack. In the animal models, as in this study, treatment is started before or at the time of disease induction, when the immunomodulatory effects of CsA can be fully explored.

Finally, the use of the triple combination TX527 + IFN-β + CsA indicates that there is indeed a potential role for CsA in the treatment of EAE. Mice treated with this triple combination were all protected from paralysis and the mean disease onset was delayed to day 17. Besides some overlapping effects (especially for TX527 and IFN-β), each of the drugs have additional inhibitory effects on different parts of the immune system. Therefore, a broader range of the autoimmune attack can be blocked, resulting in a better protection from EAE than observed with any of the double combinations used.

From these results we have concluded that the $1,25(OH)_2D_3$ analog TX527 and IFN-β can be combined successfully in EAE without inhibiting each others immunomodulatory effects. Therefore, a clinical trial in multiple sclerosis using combinations of TX527 and IFN-β can be taken under serious consideration.

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