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Treatment of Experimental Autoimmune Prostatitis in Nonobese Diabetic Mice by the Vitamin D Receptor Agonist Elocalcitol

Giuseppe Penna,* Susana Amuchastegui,* Chiara Cossetti,* Francesca Aquilano,* Roberto Mariani,* Francesca Sanvito,† Claudio Doglioni,† and Luciano Adorini2*

On the basis of the marked inhibitory activity of the vitamin D receptor agonist Elocalcitol on basal and growth factor-induced proliferation of human prostate cells and on its potent anti-inflammatory properties, we have tested its capacity to treat experimental autoimmune prostatitis (EAP) induced by injection of prostate homogenate-CFA in nonobese diabetic (NOD) mice. Administration of Elocalcitol, at normocalcemic doses, for 2 wk in already established EAP significantly inhibits the intraprostatic cell infiltrate, leading to a profound reduction in the number of CD4+ and CD8+ T cells, B cells, macrophages, dendritic cells, and I-Aβ7-positive cells. Immunohistological analysis demonstrates reduced cell proliferation and increased apoptosis of resident and infiltrating cells. Significantly decreased production of the proinflammatory cytokines IFN-γ and IL-17 is observed in prostate-draining lymph node T cells from Elocalcitol-treated NOD mice stimulated by TCR ligation. In addition, Elocalcitol treatment reduces IFN-γ production by prostate-infiltrating CD4+ T cells and draining lymph node T cells specific for an immunodominant peptide naturally processed from prostate steroid-binding protein, a prostate-specific autoantigen. Finally, CD4+ splenic T cells from Elocalcitol-treated NOD mice show decreased ability, upon adoptive transfer into NOD.SCID recipients, to induce autoimmune prostatitis, paralleled by a reduced capacity to produce IFN-γ in response to prostate steroid-binding protein. The results indicate that Elocalcitol is able to interfere with key pathogenic events in already established EAP in the NOD mouse. These data show a novel indication for vitamin D receptor agonists and indicate that treatment with Elocalcitol may inhibit the intraprostatic inflammatory response in chronic prostatitis/chronic pelvic pain syndrome patients. The Journal of Immunology, 2006, 177: 8504–8511.

The activated form of vitamin D, 1,25-dihydroxyvitamin D3, is a secosteroid hormone that has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types and pronounced immunoregulatory and anti-inflammatory properties (1–3). The biological effects of this hormone are mediated by the vitamin D receptor (VDR)3, a member of the superfamily of nuclear hormone receptors (4), functioning as an agonist-activated transcription factor that binds to specific DNA sequence elements in vitamin D-responsive genes and ultimately influences the rate of RNA polymerase II-mediated gene transcription (5).

VDR agonists can act directly on T cells, but dendritic cells (DCs) appear to be their primary targets (6). The capacity of VDR agonists to modulate DC and T cell functions is mediated by VDR expression in both cell types and by the presence of common targets in their signal transduction pathways, such as NF-κB, which is down-regulated by VDR agonists in APCs and in T cells (3). The near abrogation of IL-12 production and the strongly enhanced production of IL-10 highlight the important functional effects of VDR agonists on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties (7).

These intriguing actions of VDR agonists have been demonstrated in several experimental models of autoimmune diseases (8). Distinct regulatory mechanisms may predominate in different autoimmune disease models, although a common pattern, characterized by inhibition of Th1 cell development, has been frequently observed (8). VDR agonists can prevent systemic lupus erythematosus in MRL/lpr/lpr mice (9, 10), experimental allergic encephalomyelitis (11–13), collagen-induced arthritis (14, 15), Lyme arthritis (15), inflammatory bowel disease (16), and autoimmune diabetes in nonobese diabetic (NOD) mice (17, 18). VDR agonists are able not only to prevent but also to treat ongoing autoimmune diseases, as demonstrated by their ability to inhibit type 1 diabetes development in adult NOD mice (18) and the recurrence of autoimmune disease after islet transplantation in the NOD mouse (19), or to ameliorate significantly the chronic-relapsing experimental allergic encephalomyelitis induced in Biozzi mice by spinal cord homograft (13).

In the present study, we have examined the capacity of Elocalcitol (BXL-628), a VDR agonist tested preclinically (20) and clinically (21) for the treatment of benign prostatic hyperplasia (BPH), to treat autoimmune prostatitis in the NOD mouse, a strain genetically prone to develop different organ-specific autoimmune diseases, including type 1 diabetes, thyroiditis, sialilitis, oophoritis, adenitis, and orchitis (22). The male NOD mouse is susceptible to induction of experimental autoimmune prostatitis (EAP) by injection of mouse prostate homograft in complete Freund’s adjuvant. This protocol induces EAP in 100% of male NOD mice, characterized by a florid leukocyte infiltrate into the prostate (23). Subsequent work has shown that EAP can also be induced in NOD mice by injection of prostatic steroid-binding protein (PSBP), also known as prostatein (24), a major autoantigen in rodent EAP (25, 26). The male NOD mouse

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3 Abbreviations used in this paper: VDR, vitamin D receptor; DC, dendritic cell; EAP, experimental autoimmune prostatitis; NOD, nonobese diabetic; PSBP, prostate steroid-binding protein; BPH, benign prostatic hyperplasia; CP/CPPS, chronic prostatitis/chronic pelvic pain syndrome; iNOS, inducible NO synthase.
responds to PSBP by developing specific cellular and humoral autoimmune responses associated with inflammatory infiltrates in the prostate (24). In addition, using NOD mice lacking expression of β2-microglobulin or MHC class II β-chain, an essential role for CD4+ T cells has been shown in the development of EAP in this model (24).

We show herein that Elocalcitol treats established autoimmune prostatitis in the NOD mouse. These data extend the potential application of VDR agonists to a novel indication that represents an important unmet medical need. In addition, they support the autoimmune pathogenesis of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS), and indicate that treatment with the nonhypecalcemic VDR agonist Elocalcitol may prove clinically beneficial in this condition.

Materials and Methods

Animals

Male CD rats were purchased from Charles River Breeding Laboratories (Calco, Italy). Male NOD mice were purchased from The Jackson Laboratory. Male NOD.SCID and CD1 mice from The Jackson Laboratory were isolator reared at Charles River Breeding Laboratories. All animals were kept under specific pathogen-free conditions. All animal studies have been approved by the Institutional Review Board.

Treatments

The VDR agonist 1α,25-dihydroxy-16,23E-diene-26,27-bishomono-20-epi-cholecalciferol (Elocalcitol, formerly BXL-628) was provided by Dr. Milan Uskokovic (BioXell Inc., Nutley, NJ). Elocalcitol was dissolved in ethanol, diluted in vehicle (miglyol 812), and administered orally by gavage daily (5 days/week) in a volume of 100 μl of vehicle, typically at a dose of 100 μg/kg/day from day 14 to day 30 after immunization, for a total of 13 administrations, or as indicated. Vehicle alone (miglyol 812) was used as control. Dexamethasone (Sigma-Aldrich) was administered orally at 0.25 mg/kg/day from day 14 to day 30 after immunization.

Cell cultures

Single-cell suspensions from periaortic lymph nodes were cultured for 48 h in round-bottom 96-well plates (Costar) precoated with the indicated concentrations of purified anti-TCR mAb (ATCC HB 218; American Type Culture Collection; ATCC). To detect Ag-specific responses, popliteal lymph node cells or spleen cells from vehicle or Elocalcitol-treated mice were cultured for the indicated time in flat-bottom 96-well plates (Costar) with the indicated concentrations of PSBP or PSBP21-40 peptides. Cultures of lymph node cells were performed in synthetic HL-1 medium (Ventrex Laboratories), supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma-Aldrich), whereas spleen cells and prostate-infiltrating CD4+ cells were cultured in round-bottom 96-well plates in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Serono), 50 μM 2-ME, 2 mM L-glutamine, and 50 μg/ml gentamicin (Sigma-Aldrich). To purify prostate-infiltrating CD4+ cells, pooled prostate tissues (five per group) were digested in 3 ml of HBSS containing 3 mg/ml collagenase IV (Sigma-Aldrich), by shaking (140 rpm) at 37°C for 30 min. Cell suspensions were collected after diluting the enzyme with ice-cold HBSS containing 5% FCS and removing aggregates by filtration with Cell Strainer (BD Biosciences). Single-cell suspensions were incubated with anti-CD4 mAb-coated MicroBeads (Miltenyi Biotec) and applied onto MiniMACS separation columns (Miltenyi Biotec). APCs were obtained by Thy 1.2+ cell depletion of NOD splenocytes, using Thy 1.2-coated MicroBeads (Miltenyi Biotec). Prostate-infiltrating CD4+ cells were cultured with Thy 1.2-depleted splenocytes in the presence of 1 μM PSBP21-40 and IL-2 (100 U/well).

IFN-γ was quantified by two-site ELISA using paired mAbs from BD Pharmaning as previously described (27). IL-17 was quantified using an Optielix kit (BD Pharmaning). Detection limits were 15 pg/ml for both cytokines.

Inducible NO synthase expression and NO production

For the detection of inducible NO synthase (iNOS) protein, resident peritoneal CD11b+ macrophages were permeabilized with PBS, 5% FCS, 0.5% saponin, and 0.1% sodium azide (PBS-FCS-saponin) for 10 min and then stained with FITC-conjugated anti-iNOS mAb (Transduction Laboratories; BD Pharmaning) or with an isotype control. The cells were washed, and the cell surface was stained with PE-conjugated anti-CD11b mAb (BD Pharmaning). Analysis was performed on CD11b+ gated cells using a LSR flow cytometer (BD Biosciences) equipped with CellQuest software. For the detection of NO, resident peritoneal cell culture supernatants in macrophages by adherence, were cultured for 24 h in flat-bottom 96-well plates in complete RPMI medium. For quantification of NO, 100 μl of tetrated culture supernatants were incubated with 100 μl of Griess reagent, and optical density was read at 550 nm.

Quantification of prostate infiltrates

Prostates were snap-frozen in Tissue Tek (Miles Laboratories), and 50 μm cryostat sections/mouse, covering the entire prostate, were scored blindly by a pathologist, after staining with H&E, for the presence of intraprostatic lymphomononuclear cell infiltrates organized in nodules. Single infiltrating cells were disregarded. Data are reported as number of infiltrates/cross-section.

Immunohistochemistry

Prostates were snap-frozen in Tissue Tek and stored at −80°C. Frozen sections (5 μm thick) were air-dried and then fixed in acetone for 10 min. Endogenous peroxidase activity was blocked with 2% hydrogen peroxide and 0.1% sodium azide in cold Tris-buffered saline. Endogenous biotin was blocked by incubation with an avidin solution mixed in 1% BSA in PBS for 15 min and followed by a biotin solution mixed in 1% BSA in PBS for 15 min (Vector Laboratories). Sections were stained with biotinylated mAbs against CD4, CD8, CD11b, and I-Ag7 (all purchased from BD Pharmaning), or CD11c (N418; ATCC), followed by streptavidin-peroxidase conjugate. To detect proliferating cells, sections were stained with polyclonal rabbit anti-mouse Ki67 Ab (Abcam), followed by blocking with normal goat serum (Santa Cruz Biotechnology) and staining with biotinylated goat anti-rabbit IgG (Santa Cruz). 3-Amino-9-ethylcarbazole (DAKO) was used as chromogen, and hematoxylin was used as a counterstain. For the TUNEL assay, 10-μm-thick sections were stained with a TACS.XL DAB in situ apoptosis detection kit (Trevigen), according to the manufacturer's instructions.

Cell transfer

Spleen cells from 24-wk-old NOD mice, treated from 6 to 14 wk of age with vehicle or Elocalcitol (100 μg/kg orally, daily for 5 days/week), were incubated with anti-CD4 mAb and negatively selected with a single-cell isolation kit (Miltenyi Biotec). Purified CD4+ T cells were adoptively transferred (4 × 106/reipient) by i.v. injection into male NOD.SCID mice. NOD.SCID recipients were analyzed 45 days after cell transfer. Glucose
levels in the tail venous blood were quantified using a EUROFlash glucometer (Lifescan). A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl.

Statistical analysis

Differences between groups were evaluated using the two-tailed Mann-Whitney U test or an unpaired two-tailed Student t test, as appropriate. Differences were considered to be statistically significant at \( p < 0.05 \).

Results

**Elocalcitol treats established EAP**

NOD mice develop EAP after administration of mouse prostate homogenate emulsified in CFA (23). EAP induction was monitored by histological analysis of lymphomononuclear cell infiltrates into the prostate, scored blindly by a pathologist. Prostate-infiltrating lymphomononuclear cells, organized in distinct nodules, are already observed 2 wk after immunization, and their number increases significantly \(( p = 0.01)\) 4 wk after priming (Fig. 1A). Elocalcitol was administered orally 5 days/wk at 100 \( \mu g/kg \) from day 14 to day 30 postimmunization with mouse prostate homogenate in CFA. At the end of treatment, NOD mice were sacrificed, and prostate sections, stained with H&E, encompassing the entire prostate were scored for the number of infiltrating nodules/cross section. Elocalcitol treatment was able to reduce by 67\% \(( p = 0.0002)\) the number of intraprostatic infiltrates compared with vehicle-treated mice (Fig. 1B, left). For comparative purposes, an optimal dose of dexamethasone (0.25 mg/kg; Ref. 28) was administered daily (5 days/wk) from day 14 to day 30 postimmunization. Administration of dexamethasone also reduced the number of intraprostatic infiltrates compared with vehicle-treated mice (Fig. 1B, left), but only by 33\%, without reaching statistical significance \(( p = 0.7)\). All mice were normocalcemic at the end of treatment, with serum calcium levels below 10.7 mg/dl (Fig. 1B, right).

Histological analysis of prostate sections stained with H&E (Fig. 1C) shows, in mice treated with vehicle only, dense inflammatory aggregates with a nodular appearance, mainly composed of lymphoid cells infiltrating the prostatic stroma, surrounding and disrupting glands (Fig. 1C1). In mice treated with dexamethasone, sparse nodular lymphoid infiltrates are present in the prostatic stroma (Fig. 1C2); whereas in mice treated with Elocalcitol, rare, single lymphoid cells are scattered in the prostatic stroma, and the overall architecture of the gland is well preserved (Fig. 1C3). In mice with established EAP treated with vehicle, dense leukocyte infiltrates are observed also in the ganglia and in the nerve bundles of the prostate peripheral nervous system (Fig. 1C4). Rows of lymphoid cells infiltrate and dissociate nerve fibers in the prostate of mice treated with dexamethasone (Fig. 1C5), whereas the ganglia and the nerve fibers are mostly free of infiltrating cells in mice treated with Elocalcitol (Fig. 1C6). This finding may suggest an effect of Elocalcitol on the pain component of autoimmune prostatitis.

A dose-response analysis of Elocalcitol treatment (Fig. 1D) shows that \(~60\%) reduction in the number of intraprostatic

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**FIGURE 1.** Elocalcitol treats established EAP. A, Kinetics of EAP development in the NOD mouse. Male NOD mice were immunized with 300 \( \mu g/mouse \) prostate homogenate in CFA and sacrificed 14 \(( n = 6), 21 \(( n = 4), \) and 28 \(( n = 29)\) days after immunization. Mean numbers \( \pm SE\) of intraprostatic infiltrates/cross-section are reported. \( * p = 0.011 \) by Mann-Whitney U test. B, NOD mice were immunized with 300 \( \mu g/mouse \) prostate homogenate in CFA and treated orally, daily (5 days/wk) from day 14 to day 30 postimmunization with vehicle (miglyol 812, \( n = 25), \) dexamethasone (Dex, 0.25 mg/kg, \( n = 5), \) or Elocalcitol (100 \( \mu g/kg, n = 26)\). Mean numbers \( \pm SE\) of intraprostatic infiltrates/cross-section (left) and serum calcium levels (right) are reported. The line in the right panel indicates the higher normal limit of serum calcium levels (10.7 mg/dl), \( ** p = 0.0002 \) by Mann-Whitney U test. C, H&E staining of representative prostate sections from mice treated as in B show several lymphoid aggregates in EAP treated with vehicle (C1), sparse nodular infiltrates in dexamethasone-treated (C2), and only scattered stromal lymphoid cells following Elocalcitol treatment (C3). Sections in the lower row highlight the more intense lymphomononuclear infiltrate in nerve structures in vehicle (C4) compared with dexamethasone (C5) and Elocalcitol (C6)-treated EAP. The arrow in C4 indicates a ganglion cell. Magnifications: C1–C3, \( \times 200; \) C4–C6, \( \times 400. \) D, Titration of Elocalcitol activity. NOD mice were immunized with 300 \( \mu g/mouse \) prostate homogenate in CFA and treated orally, daily (5 days/wk) from day 14 to day 30 postimmunization with vehicle (miglyol 812, \( n = 27) \) or Elocalcitol at 3 \(( n = 4), 10 \(( n = 10), 30 \(( n = 14), \) or 100 \(( n = 27)\) \( \mu g/kg. \) Percentages of intraprostatic infiltrates compared with vehicle-treated mice (○) and serum calcium levels (red) are shown. The red line indicates the higher normal limit of serum calcium levels (10.7 mg/dl).
infiltrates, compared with vehicle-treated mice, is already induced by 3 μg of the VDR agonist per kg, a dose 30-fold lower than the maximum tolerated dose of this compound in terms of hypercalcemia, indicating its wide therapeutic window in the treatment of EAP.

**Inhibition of intraprostastic lymphomononuclear cell infiltration by Elocalcitol treatment**

Immunohistochemical analysis shows florid infiltrates in prostate sections from NOD mice injected with prostate homogenate in CFA and treated with vehicle compared with mice injected with CFA alone (Fig. 2). Prostate-infiltrating cells include CD4+ and CD8+ T cells, B220+ B cells, CD11c+ DCs, and CD11b+ macrophages. Conversely, fewer and smaller infiltrates were observed in Elocalcitol-treated mice, in line with the results shown in Fig. 1, with a considerable reduction of all cell types analyzed. Administration of dexamethasone reduced cell infiltration to a lower extent, compared with Elocalcitol, with higher numbers of CD4+ and CD8+ T cells, B220+ B cells, CD11c+ DCs, and CD11b+ cells present in the infiltrates, but an overall similar composition of the different cell types (Fig. 2). The inflammatory and prostatic epithelial component express high and homogeneous levels of I-Aδ+ MHC class II molecules in vehicle-treated mice, in contrast with I-Aδ+ expression limited to scattered inflammatory cells in Elocalcitol-treated mice with established EAP (Fig. 2), suggesting a reduced potential to activate CD4+ T cells. Again, dexamethasone appears to be inferior to Elocalcitol in the capacity to inhibit I-Aδ+ expression.

Staining for the proliferation marker Ki67 showed reduced proliferation of prostate-infiltrating cells in Elocalcitol-treated compared with vehicle-treated NOD mice immunized with prostate homogenate in CFA, indicating Elocalcitol-induced inhibition of infiltrating cell proliferation (Fig. 3). Conversely, apoptotic cells, detected by TUNEL assay, were more frequent in Elocalcitol-treated compared with vehicle-treated mice (Fig. 3). Increased apoptosis was apparent not only in prostate-infiltrating cells but also in resident epithelial and stromal prostate cells, consistent with the enhanced apoptosis induced in prostate cells by Elocalcitol treatment in intact and in castrated, testosterone-repleted rats (20).

**Elocalcitol treatment inhibits inflammatory markers and polyclonal T cell responses**

Next, functional analysis of inflammatory markers and T cell responses was conducted. Therapeutic administration of Elocalcitol in NOD mice with established EAP leads to decreased iNOS expression (Fig. 4A) and NO production (Fig. 4B), important mediators of acute and chronic inflammation, by resident peritonal macrophages. Elocalcitol treatment also leads to decreased IFN-γ production by anti-TCR-stimulated lymph node cells (Fig. 4C), indicating inhibition of Th1-type responses in prostate-draining periaortic lymph nodes. Interestingly, in vivo treatment with Elocalcitol inhibits also ex vivo production of IL-17 (Fig. 4D), a proinflammatory cytokine produced by pathogenic T cells in autoimmune diseases (29), by anti-TCR-stimulated cells from popliteal lymph nodes.

**Elocalcitol treatment inhibits CD4+ T cell responses to an immunodominant PSBP peptide**

PSBP is a well-defined prostate autoantigen in the NOD mouse (24). Using 20-mers overlapping by 10 amino acid residues covering the entire PSBP sequence, we have recently identified PSBP21–40 as an immunodominant, naturally processed epitope presented by I-Aδ+ to NOD CD4+ T cells, able to induce autoimmune prostatitis in NOD mice (G. Penna et al., manuscript in preparation). To determine the capacity of Elocalcitol to inhibit T cell

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**FIGURE 2.** Inhibition of intraprostastic lymphomononuclear cell infiltration by Elocalcitol treatment. Immunohistochemical staining for the indicated markers in cryostatic sections of prostates from NOD mice immunized with CFA alone or with 300 μg/mouse prostate homogenate in CFA and treated orally, daily (5 days/wk) from day 14 to day 30 postimmunization with vehicle, dexamethasone (Dex; 0.25 mg/kg) or Elocalcitol (100 μg/kg). The mixed nature of lymphomononuclear infiltrate in EAP is highlighted by the high number of T cells (CD4, CD8), B cells (B220), and dendritic and mononuclear cells (CD11c, CD11b) in vehicle-treated mice. Reduced numbers of lymphomononuclear cells are evident after Elocalcitol treatment, in line with the results shown in Fig. 1, with a considerable reduction of all cell types analyzed. Administration of dexamethasone reduced cell infiltration to a lower extent, compared with Elocalcitol, with higher numbers of CD4+ and CD8+ T cells, B220+ B cells, CD11c+ DCs, and CD11b+ cells present in the infiltrates, but an overall similar composition of the different cell types. Original magnification, ×200.

**FIGURE 3.** Inhibition of cell proliferation and enhanced apoptosis in intraprostastic lymphomononuclear cell infiltrates by Elocalcitol treatment. Immunohistochemical staining for the indicated markers in cryostatic sections of prostates from NOD mice immunized with 300 μg/mouse prostate homogenate in CFA, and treated orally, daily (5 days/wk) from day 14 to day 30 postimmunization with vehicle or Elocalcitol (100 μg/kg). A high percentage of inflammatory intraprostastic cells are Ki67 positive, and a few epithelial and inflammatory cells are marked in the TUNEL assay in vehicle-treated mice. Conversely, fewer Ki67-positive inflammatory cells and a higher percentage of TUNEL-positive epithelial (blue arrow), stromal (red arrow), and inflammatory (green arrow) cells are present in the prostatic gland of Elocalcitol-treated mice. Original magnification, ×400.
FIGURE 4. Eocalcitol treatment inhibits inflammatory markers and polyclonal T cell responses. NOD mice were immunized with 300 μg/mouse prostate homogenate in CFA and treated orally, daily (5 days/wk) from day 14 to 30 postimmunization with vehicle or Eocalcitol (100 μg/kg). A, Intracytoplasmic NO synthesis, shown as ∆ geometric mean fluorescence intensity (MFI), in resident peritoneal CD11b+ macrophages from vehicle (n = 11) or Eocalcitol-treated (n = 10) mice. B, NO production in 24-h culture supernatant by resident peritoneal cells (3 x 10^6 cells/well) enriched in macrophages by adherence from vehicle (n = 7) or Eocalcitol-treated (n = 9) mice. C, Periaortic lymph node cells (2 x 10^6/well) were cultured in 96-well plates coated with the indicated amount of anti-TCR mAb. After 72 h, IFN-γ was determined in culture supernatants by two-site ELISA. Data are means ± SE from three mice per group. D, Periopiteal lymph node cells (2 x 10^5/well) were cultured in 96-well plates coated with 5 μg/ml anti-TCR mAb. After 72 h, IFN-γ and IL-17 were determined in culture supernatants by two-site ELISA. Data are means ± SE from three mice per group. *p < 0.05 and **p < 0.005 by two-tailed Student’s t test.

responses to prostate autoantigens, NOD mice have been immunized with PSBP_21–40 peptide and treated with vehicle or Eocalcitol for 9 days. Results in Fig. 5A demonstrate that lymph node cells from NOD mice immunized with PSBP_21–40 and treated with vehicle respond vigorously to the peptide, as determined by IFN-γ production, whereas the response is significantly reduced in lymph node cells from Eocalcitol-treated NOD mice. A significant inhibition induced by Eocalcitol treatment is also observed following restimulation of lymph node cells from PSBP_21–40-primed mice with purified native PSBP, indicating its capacity to inhibit T cell responses to naturally processed autoantigen epitopes (Fig. 5B). To assess the capacity of Eocalcitol to inhibit CD4+ T cell responses in the target organ, pooled prostate-infiltrating CD4+ T cells from PSBP_21–40-primed NOD mice treated for 4 wk with vehicle or with Eocalcitol were cultured with splenic Thy 1.2-negative APCs from vehicle-treated NOD mice in the presence of 1 μM PSBP_21–40 peptide. Results in Fig. 5C show a marked inhibition of IFN-γ production in response to PSBP by prostate-infiltrating CD4+ T cells from Eocalcitol-treated compared with vehicle-treated NOD mice, demonstrating the capacity of this VDR agonist to inhibit autoantigen-specific CD4+ T cell responses in prostate-infiltrating T cells.

FIGURE 5. Eocalcitol treatment inhibits CD4+ T cell responses to an immunodominant PSBP peptide. A and B, NOD mice were immunized with 2.5 nmol/mouse PSBP_21–40 peptide in CFA into the hind footpads and treated orally, daily (5 days/wk) until day 10 postimmunization with vehicle (○, □) or Eocalcitol (100 μg/kg, ●, ■). Eleven days after immunization, popliteal lymph node cells (6 x 10^5/well) were cultured with the indicated concentrations of PSBP_21–40 peptide (A), or PSBP (B). After 72 h, IFN-γ was determined in culture supernatants by two-site ELISA. Data are means ± SE from three mice per group. C, NOD mice (five mice per group) were immunized with 5 nmol/mouse PSBP_21–40 peptide in CFA into the hind footpads and at the tail base and treated orally, daily (5 days/wk) from day 14 to day 30 postimmunization with vehicle or Eocalcitol (100 μg/kg). Thirty days after immunization, pooled prostate-infiltrating CD4+ T cells (2 x 10^5/well) were cultured with 2 x 10^5 Thy 1.2-depleted splenocytes from vehicle-treated NOD mice in the presence of 1 μM PSBP_21–40 peptide. After 96 h, IFN-γ was determined in culture supernatants by two-site ELISA.

Impaired capacity of CD4+ T cells from Eocalcitol-treated NOD mice to transfer autoimmune prostatitis and type 1 diabetes into NOD.Scid recipients

Aging NOD mice spontaneously develop the intraprostatic lymphomononuclear cell infiltrate characteristic of autoimmune prostatitis that becomes well established by 20 wk of age (G. Penna et al., manuscript in preparation). To determine whether the disease could be transferred by lymphoid cells and if Eocalcitol treatment could affect the spontaneous development of autoimmune prostatitis, NOD mice were treated with vehicle alone or containing Eocalcitol from 6 to 14 wk of age and then rested until 24 wk of age. At this time point, after 10 wk of Eocalcitol withdrawal, CD4+ T cells were adoptively transferred into NOD.Scid recipients, which were analyzed 45 days later for intraprostatic lymphomononuclear cell infiltration (Fig. 6A). To assess whether the transfer was able to induce autoimmune prostatitis, NOD mice (five mice per group) were immunized with PSBP (Fig. 6B), a prostate autoantigen able to induce a T cell response in spleen cell cultures from naive NOD mice, indicating lack of tolerance to this self Ag (G. Penna et al., manuscript in preparation). As a control, in the same recipient mice, glycerin was measured to assess the development of type 1 diabetes. The majority (9 of 13, 71%) of NOD.Scid recipients transferred with splenocytes from vehicle-treated NOD mice developed type 1 diabetes, whereas this was observed only in 1 of 11 (9%, p = 0.005) NOD.Scid recipients receiving spleen cells from Eocalcitol-treated NOD mice (Fig. 6C). The incomplete induction of type 1 diabetes NOD.Scid recipients transferred with CD4+ T cells from vehicle-treated NOD mice reflects the lower ability of T cells from male, compared with female, NOD mice to transfer type 1 diabetes, as well as the transfer
of CD4+ T cells only (30). Thus, Elocalcitol treatment impairs the capacity of NOD spleen cells to transfer both autoimmune prostatitis, associated with a reduced capacity of transferred spleen cells to respond to the prostate autoantigen PSBP, and type 1 diabetes.

Discussion

Results in this study show that the VDR agonist Elocalcitol is able, at normocalcemic doses, to treat established EAP induced in the NOD mouse by injection of prostate homogenate in CFA. The data show a marked decrease of intraprostatic inflammatory cell infiltration, characterized by reduced proliferation and enhanced activation-induced cell death. The reduction in inflammatory infiltrates is associated with inhibition of inflammatory markers and polyclonal T cell activation. Inhibition of CD4+ T cell responses, in the target organ and in draining lymph nodes, to an immunodominant peptide of PSBP and to the native PSBP protein, a well-defined autoantigen in EAP (25, 26), are also induced by Elocalcitol treatment. In addition, CD4+ T cells from Elocalcitol-treated NOD mice show an impaired capacity to transfer autoimmune prostatitis into NOD.SCID recipients, associated with a reduced response of transferred spleen cells to PSBP.

CP/CPPS (National Institutes of Health category III) is a highly prevalent syndrome of suspected autoimmune origin. This syndrome affects 5–14% of the general population (31) and represents a major unmet medical problem (32). The pathogenesis of CP/CPPS is still poorly understood, but neurological, immunological, and endocrine dysfunctions have been proposed to be involved in disease development (33). Recently, evidence indicating an autoimmune component in the pathogenesis of CP/CPPS has begun to emerge. Autoreactive CD4+ and CD8+ T cells specific for prostate Ags exist in normal individuals (34). Moreover, PBMC and CD4+ T cells from CP/CPPS patients proliferate in response to seminal plasma (35, 36) and to specific prostate Ags (37, 38), indicating expression of the autoreactive T cell repertoire in disease pathogenesis. High titer IgG autoantibodies to prostate-associated proteins are found in patients with CP/CPPS (39), further indicating a T cell-dependent autoimmune process. In addition, patients with a clinical diagnosis of CP/CPPS show higher levels, compared with controls, of proinflammatory cytokines, like IL-1β, IL-6, and TNF-α, in seminal plasma (40–43), and we have recently documented significantly increased levels of the chemokines CCL3, CCL4, CCL17, CCL22, and CXCL8 (44).

Further support for the autoimmune nature of CP/CPPS is provided by EAP models in rodents (45–47). They have been extensively characterized, demonstrating that immunization of rats or mice with prostate gland extracts can induce T cell and Ab responses to prostate Ags, associated with histological evidence of prostate inflammation. EAP, typically quite severe, can be also induced in the NOD mouse, a strain genetically prone to developing different autoimmune diseases, by injection of mouse prostate homogenate (23) or PSBP (24). In NOD EAP, T cells specific for prostate Ag are IFN-γ-producing Th1 cells, which have an essential role in disease induction (24). Therefore, EAP in the NOD mouse can be considered a Th1-dependent autoimmune disease, similar to other well-defined organ-specific autoimmune pathologies in this mouse strain, such as type 1 diabetes or autoimmune thyroiditis.

The vitamin D endocrine system is involved in a variety of biological processes able to modulate immune responses, and the tolerogenic properties of VDR agonists (6, 48) render this class of compounds particularly suitable for the treatment of autoimmune diseases (8). Treatment of EAP by a VDR agonist represents a novel application for these hormone-like agents. The VDR is not only expressed by cells from classic target tissues as bone, bowel, and kidney but also in several others, including those derived from the urogenital sinus, such as prostate (49) and bladder (50). Interestingly, epithelial prostate cells express 1α-hydroxylase, which is required for 1,25-dihydroxyvitamin D3 synthesis (51). The extra-renal synthesis of 1,25-dihydroxyvitamin D3 in the prostate could have a local growth-regulating physiological role, as suggested by the marked decrease of 1α-hydroxylase activity in prostate cancer cell lines (52), and possibly also an anti-inflammatory one. Although the prostate has been long recognized as a target organ of VDR agonists (49), its capacity to respond to these agents has thus far been probed only for the treatment of prostate cancer (53) and BPH (20, 21).

Our present results show that treatment with normocalcemic doses of the VDR agonist Elocalcitol for 2 wk in already established EAP is able to inhibit significantly the intraprostatic cell infiltrate, leading to a profound reduction in the number of infiltrating leukocytes, whereas dexamethasone administration is much less effective. Interestingly, in NOD mice with established EAP, numerous leukocyte infiltrates are observed adjacent to the ganglia of the prostate peripheral nervous system, whereas ganglia are mostly free of infiltrating cells in mice treated with Elocalcitol, suggesting a possible effect on the pain component of autoimmune prostatitis (54). Indications supporting this possibility could be obtained by functional analysis, for example by evaluating nerve-induced and frequency-dependent contractile responses in isolated prostates. EAP-induced changes of the Rho kinase pathway might also be involved in altered regulation of emission in NOD mice, and this...
pathway could be inhibited by Elocalcitol treatment, as recently shown in the bladder of spontaneously hypertensive rats (55).

Therapeutic administration of Elocalcitol in NOD mice with established EAP decreases IFN-γ production by anti-TCR-stimulated lymph node cells, indicating inhibition of Th1 cell responses in prostate-draining periaortic lymph nodes. Treatment with Elocalcitol inhibits also ex vivo production of IL-17, a proinflammatory cytokine recently shown to be produced by pathogenic T cells in autoimmune diseases (29). Inhibition of IL-17 by Elocalcitol treatment may be relevant to the therapy of CP/CPPS, because this cytokine has been found to be elevated in situ in prostate specimens from patients affected by BPH (56), a prostate condition characterized by an inflammatory component (57). In addition, a significantly decreased expression of iNOS, a key enzyme required for the synthesis of the inflammatory agent NO, and a markedly decreased production of NO itself, is observed in peritoneal macrophages from Elocalcitol-treated NOD mice with established EAP. A number of studies have associated excessive NO production with acute and chronic inflammation in model systems, and have also demonstrated that administration of NO synthase inhibitors can induce beneficial anti-inflammatory effects (58). The capacity of Elocalcitol to inhibit iNOS expression and NO production may thus represent an additional mechanism explaining its anti-inflammatory properties.

Elocalcitol treatment inhibits CD4+ T cell responses in prostate-infiltrating cells and in prostate-draining lymph node cells; to PSBP, a well-defined Ag in NOD EAP (24); and to a PSBP epitope immunodominant in the NOD mouse, defined by peptide PSBP21–40. Thus, treatment with Elocalcitol can inhibit CD4+ T cell responses characterized by IFN-γ secretion to inciting autoantigens in EAP, in agreement with the known capacity of VDR agonists to selectively inhibit Th1 cell responses to disease-inducing epitopes in experimental allergic encephalomyelitis (13). A reduced response to PSBP is also observed in NOD.SCID recipients transferred with CD4+ T cells from Elocalcitol-treated NOD mice, and is associated with their reduced capacity to transfer autoimmune prostateitis. In the same NOD.SCID recipients, a reduced development of type 1 diabetes is also observed, consistent with the ability of VDR agonists to inhibit pathogenic mechanisms in several Th1 cell-dependent autoimmune diseases (8), and in particular in type 1 diabetes (17, 18, 59).

A potentially very important activity of VDR agonists is their capacity to induce in vitro and in vivo tolerogenic DCs able to enhance CD4+CD25+ suppressor T cell responses, which, in turn, inhibit Th1 cell responses (18, 60). These mechanisms of action could explain some of the immunoregulatory properties of VDR agonists in the treatment of EAP, considering that day 3 thymectomy, leading to depletion of the CD4+CD25+ regulatory T cell pool (61), can induce autoimmune prostateitis (62). Indeed, prostate-specific CD4+ suppressor T cells appear to be activated extrathythymically and to maintain peripheral tolerance to prostate autoantigens (63, 64). Studies addressing the capacity of Elocalcitol to induce CD4+CD25+ regulatory T cells in EAP are currently in progress.

The optimal medical treatment of CP/CPPS is not known (65), and effective therapies for CP/CPPS patients are warranted. Our results, showing that Elocalcitol interferes with key pathogenic events in already established autoimmune prostateitis, reducing by ~70% intraprostatic lumphomononuclear cell infiltration, indicate that treatment with this compound may inhibit prostatic inflammation in CP/CPPS patients. VDR agonists have widespread clinical applications, notably in the treatment of osteoporosis, secondary hyperparathyroidism, and psoriasis (66), but hypercalcemia is a dose-limiting effect that prevents sustained systemic administration. However, hypocalcemic, tissue-selective VDR agonists, with a wider therapeutic index compared with the natural hormone 1,25-dihydroxyvitamin D3, have been identified (67), and Elocalcitol has been shown to induce tissue and cell type-selective VDR-mediated activation, likely via differential recruitment of coactivators and corepressors by the VDR-Elocalcitol complex in different cell types (68). Indeed, as demonstrated here, Elocalcitol can inhibit EAP at doses considerably lower than those required to induce hypercalcemia, confirming the significant therapeutic index already shown by this VDR agonist in animal models of osteoepenia (69). Elocalcitol has been selected for a phase II clinical study in patients affected by BPH, based on its capacity to inhibit prostate cell proliferation in vitro and in vivo (70). Interestingly, also in BPH models Elocalcitol treatment has demonstrated the capacity to decrease prostate cell proliferation, assessed by reduced expression of the proliferation marker Ki67, and to enhance apoptosis, shown by increased staining in the in situ end-labeling assay (20). BPH patients treated with 150 μg/day BXL-628 for 90 days experienced a placebo-like incidence of adverse events and did not show any pathological alteration in calcium and phosphate metabolism (21), confirming the excellent safety profile of this VDR agonist. Based on the preclinical evidence presented here, a randomized, double-blind, placebo-controlled, parallel group study to determine the effects of Elocalcitol in CP/CPPS patients is currently ongoing.

Disclosures
F. Sanvito and C. Doglioni are employees of the Scientific Institute San Raffaele, and L. Adorini and G. Penna have equity interests in Bio Xell S.p.A.

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