A 1α,25-Dihydroxyvitamin D₃ Analog Enhances Regulatory T-Cells and Arrests Autoimmune Diabetes in NOD Mice

Silvia Gregori, Nadia Giarratana, Simona Smiroldo, Milan Uskokovic, and Luciano Adorini

Type 1 diabetes is a chronic progressive autoimmune disease characterized by mononuclear cell infiltration, dominated by interleukin-12 (IL-12)-dependent Th1 cells, of the pancreatic islets, with subsequent destruction of insulin-producing β-cells. Here, we demonstrate that treatment of adult nonobese diabetic (NOD) mice with an analog of 1α,25-dihydroxyvitamin D₃, an immunomodulatory agent preventing dendritic cell maturation, decreases lipopolysaccharide-induced IL-12 and γ-interferon production, arrests Th1 cell infiltration and progression of insulinitis, and inhibits diabetes development at nonhypercalcemic doses. Arrest of disease progression is accompanied by an enhanced frequency in the pancreatic lymph nodes of CD4⁺CD25⁺ regulatory T-cells that are able to inhibit the T-cell response to the pancreatic autoantigen insulinoma-associated protein 2 and to significantly delay disease transfer by pathogenic CD4⁺CD25⁻ cells. Thus, a short treatment of adult NOD mice with an analog of 1,25-dihydroxyvitamin D₃ inhibits IL-12 production, blocks pancreatic infiltration of Th1 cells, enhances CD4⁺CD25⁺ regulatory cells, and arrests the progression of type 1 diabetes, suggesting its possible application in the treatment of human autoimmune diabetes. Diabetes 51:1367–1374, 2002

The nonobese diabetic (NOD) mouse, which spontaneously develops type 1 diabetes with a pathogenesis similar to the human disease, represents a useful model for the study of autoimmune diabetes (1). Several effector mechanisms leading to specific β-cell destruction have been identified, including cytotoxic CD8⁺ lymphocytes and macrophages (2), both of which are regulated by interleukin-12 (IL-12)-dependent T-helper 1 (Th1) cells (3). The activation of Th1 cells specific for β-cell autoantigens could reflect defective elimination of autoreactive T-cell clones (4), inefficient mechanisms of peripheral tolerance (5), enhanced IL-12 production (6), or impaired suppressive mechanisms (7).

Studies using different autoimmune disease models have evidenced a subset of CD4⁺ T-cells, characterized by constitutive expression of CD25, with immunosuppressive activity. CD4⁺CD25⁺ T-cells fail to proliferate and to secrete cytokines in response to polyclonal or antigen-specific stimulation and inhibit the activation of responsive T-cells, probably via cell-cell contact rather than secretion of soluble factors (8,9). Transfer of CD4⁺CD25⁺ T-cells inhibits the induction of different autoimmune diseases, such as the autoimmune syndrome induced by day 3 thymectomy in genetically susceptible mice (10), inflammatory bowel disease (11), and type 1 diabetes in thymectomized rats (12) and NOD mice (7). Thus, CD4⁺CD25⁺ regulatory T-cells may control type 1 diabetes in prediabetic NOD mice.

An agent able to both inhibit IL-12 production and Th1 development and enhance CD4⁺CD25⁺ regulatory T-cells may therefore be beneficial in the treatment of type 1 diabetes. 1α,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the activated form of vitamin D₃, is a secosteroid hormone that not only plays a central role in bone and calcium metabolism, but also modulates the immune response via specific receptors expressed in antigen-presenting cells (APCs) and activated T-cells (13). 1,25(OH)₂D₃ and its analogs have been shown to inhibit autoimmune diseases in animal models, such as experimental allergic encephalomyelitis (14–16), murine lupus (17), collagen-induced arthritis (18,19), and type 1 diabetes (20). They are clinically used in the treatment of psoriasis, a Th1-mediated autoimmune disease of the skin, where they show efficacy comparable to topical steroids (21). Polymorphisms of the vitamin D receptor gene have been associated with type 1 diabetes in different populations (22,23). In addition, epidemiological studies have shown a higher incidence of the disease in northern than in southern latitudes (24), suggesting a possible involvement of a 1,25(OH)₂D₃ deficiency in the pathogenesis of type 1 diabetes. This is supported by a large population-based case-control study showing that the intake of vitamin D₃ contributes to a significantly decreased risk of type 1 diabetes development (25).

1,25(OH)₂D₃ inhibits IL-12 production (26), likely via inhibition of nuclear factor-κB (27), and IL-12 has been shown to exert an important role in the development of type 1 diabetes in the NOD mouse (3,28) and in humans (29). Thus, 1,25(OH)₂D₃ analogs could represent interest-
ing candidates to modulate APCs (30), leading to inhibition of IL-12–dependent Th1 cells and type 1 diabetes development.

Results in this paper demonstrate that a short course of treatment with the 1,25(OH)2D3 analog 1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D3 (Ro 26-2,198) inhibits IL-12 production and pancreatic infiltration of Th1 cells while increasing the frequency of CD4+CD25+ regulatory T-cells in pancreatic lymph nodes, arresting the immunological progression and preventing the clinical onset of type 1 diabetes in the NOD mouse.

RESEARCH DESIGN AND METHODS

Mice. NOD/Lt and NOD.SCID female mice were purchased from Charles River Laboratories (Calco, Italy). All mice were kept under specific pathogen-free conditions. Glucose levels in the tail venous blood were quantified using an EUROMark II glucometer (LifeScans, Issy les Moulineaux, France). A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl. Serum calcium was determined using a colorimetric assay (Sigma). Introduction of cytokines.

Cell cultures. Total spleen cells (106 cells/well) from vehicle- or Ro 26-2198–treated mice were cultured for 48 h in flat-bottom 96-well plates (Costar) with the indicated concentrations of insulinoma-associated protein 2 (IA-2). The intracellular domain (amino acids 601–970) of mouse IA-2 was expressed in Escherichia coli as a glutathione–S-transferase fusion protein and purified by affinity chromatography on glutathione-Sepharose, followed by thrombin cleavage to recover >98% pure recombinant mouse IA-2, as previously described (32). Single-cell suspensions from pancreatic lymph nodes or spleen were incubated with anti-CD4 mAbs-coated microbeads and applied onto Mini-MACS columns (Miltenyi) to obtain CD4+ cells. Splenic or pancreatic lymph node CD4+CD25+ and CD4+CD25− cells were sorted by a Multisort kit (Miltenyi). Purified CD4+ cells (6 × 106/well) were cultured for 48 h in round-bottom 96-well plates (Costar) precoated with 3 μg/ml purified anti–T-cell receptor (TCR) mAb (MB 218; American Type Culture Collection, Manassas, VA) with or without 100 units/ml IL-2 and/or 10 μg/ml anti-CD28 mAb. Cultures were performed in synthetic IL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mmol/l L-glutamine and 50 μg/ml gentamicin (Sigma). To measure cell proliferation, cultures were pulsed 8 h before harvesting with 1 μC [3H]thymidine (40 Ci/mmol; The Radiochemical Center, Amersham, U.K.). Incorporation of [3H]-thymidine was measured by liquid scintillation spectrometry.

IL-12 and IFN-γ production in vivo. Lymphokine production was induced in 8-week-old female NOD mice by intraperitoneal injection of 400 μg/mouse Salmonella abortus equi lipopolysaccharide (LPS, Sigma). Ro 26-2198 was administered twice weekly by measurement of blood glucose levels. The F values were calculated by a Mann-Whitney U test. B: Serum calcium levels (means ± SE) were measured in vehicle-treated (○) and Ro 26-2198–treated (●) NOD mice after 20 or 40 administrations. Stippled lines indicate the range of normal serum calcium levels.

FIG. 1. A: Ro 26-2198 administration to 8-week-old NOD mice inhibits type 1 diabetes development. NOD mice were treated five times per week with vehicle (○, n = 16) or with 0.03 μg/kg Ro 26-2198 per os (●, n = 12) from 8 to 16 weeks of age, or from 8 to 12 weeks of age (△, n = 10). Diabetes development was monitored twice weekly by measurement of blood glucose levels. The F values were calculated by a Mann-Whitney U test. B: Serum calcium levels (means ± SE) were measured in vehicle-treated (○) and Ro 26-2198–treated (●) NOD mice after 20 or 40 administrations. Stippled lines indicate the range of normal serum calcium levels.
RESULTS

Ro 26-2198 administration inhibits the development of insulitis and type 1 diabetes. To analyze the ability of Ro 26-2198 to inhibit type 1 diabetes development, NOD female mice were treated daily with Ro 26-2198 (0.03 µg/kg per os) or vehicle from 8 to 12 weeks of age, and glycemia levels were monitored until 38 weeks of age. The incidence of disease was significantly lower in mice treated with Ro 26-2198 compared with controls, and the longer treatment was most effective (Fig. 1). Of the vehicle-treated controls, ~90% were diabetic by 38 weeks of age, whereas Ro 26-2198 treatment from 8 to 12 weeks of age prevented diabetes in 50% of NOD mice, showing a significant disease reduction compared with controls (P = 0.006). Therefore, an effective treatment of diabetes requires administration of Ro 26-2198 at the highest nonhypercalcemic dose for at least 4 weeks.

The score and severity of insulitis was similar between 8-week-old untreated controls and Ro 26-2198–treated NOD mice at 30 weeks of age, whereas this had significantly progressed in vehicle-treated mice (Fig. 2A). These data indicate that Ro 26-2198 treatment is able to halt the

FIG. 2. Decreased insulitis in Ro 26-2198–treated NOD mice. A: Histological scoring of insulitis was performed on pancreas sections stained with hematoxylin/eosin from untreated 8-week-old mice and from NOD mice at 30 weeks of age that had been treated with Ro 26-2198 or vehicle from 8 to 16 weeks of age. Each bar represents the mean score of 40–50 islets per mouse (left panel). The results refer to the mean values ± SE of eight mice per group, except for Ro 26-2198–treated mice, where three mice were scored. The P values were calculated by Mann-Whitney U test. The severity of the infiltrate is shown in the right panel. Islets from the same mice were scored for absence of insulitis ( ), peri-insulitis (light gray), moderate insulitis with <50% infiltration of the islets (dark gray), and severe insulitis, with >50% of infiltration of the islets ( ). B: Consecutive sections of the same islets were stained with anti-CD4, anti-CD8, anti-B220, anti-CD11b (Mac-1), and anti-CD11c (N418) mAbs. In addition, islets were stained for insulin. C: Cytomfluorometric analysis of pancreas-infiltrating cells. Pancreas-infiltrating cells isolated from untreated 8-week-old NOD mice and from vehicle- and Ro 26-2198–treated NOD mice at 30 weeks of age were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD45+ cells. Bars represent the mean ± SE from three separate experiments.
recruitment of infiltrating cells into the pancreatic islets. Functional islet β-cells, demonstrated by the positive staining for insulin, were more frequent in both 8-week-old and Ro 26-2198–treated mice at 30 weeks of age, compared with vehicle-treated NOD mice at 30 weeks of age (Fig. 2B). Immunohistochemical analysis showed a similar pattern of islet infiltration in 8-week-old and Ro 26-2198–treated NOD mice at 30 weeks of age, comprising CD4⁺ and CD8⁺ T-cells, B220⁺ B cells, CD11b⁺ macrophages, and CD11c⁺ dendritic cells (DCs). Conversely, a more florid infiltrate was observed in vehicle-treated 30-week-old NOD mice (Fig. 2B). The pancreas-infiltrating cells were analyzed by flow cytometry, and no difference in the percentage of CD4⁺, CD8⁺, B220⁺, CD11b⁺, and CD11c⁺ cells was found among the three groups (Fig. 2B), indicating that Ro 26-2198 treatment blocked the progression of insulinitis without modifying the cellular composition of the infiltrate.

Ro 26-2198 administration inhibits LPS-induced IL-12 and IFN-γ production and the Th1 response to the pancreatic autoantigen IA-2. The IFN-γ production induced by LPS administration is largely IL-12 dependent, and we have previously shown that the LPS-induced serum levels of both cytokines can be inhibited by a short treatment with 1,25(OH)₂D₃ or its analogs (16). We thus tested the capacity of Ro 26-2198 to inhibit LPS-induced IL-12 and IFN-γ production in vivo. Mice were pretreated with vehicle or with 0.03 μg/kg Ro 26-2198 administered per os daily for 4 days before LPS injection. Ro 26-2198 did not induce hypercalcemia, as demonstrated by the serum calcium levels similar to controls, but inhibited significantly the LPS-induced IL-12p75 and IFN-γ production (Fig. 3A), with a potency ~100-fold higher than 1,25(OH)₂D₃ (16 and data not shown).

We have also analyzed the ability of Ro 26-2198 to inhibit the spontaneous T-cell response to the intracytoplasmic region (amino acids 601–979) of the tyrosine phosphatase–like IA-2 protein, an autoantigen associated with type 1 diabetes in both humans and the NOD mouse (32). Spleen cells from unprimed NOD mice treated with Ro 26-2198 from 8 to 12 weeks of age secreted much lower levels of IFN-γ in response to IA-2, compared with spleen cells from vehicle-treated controls (Fig. 3B). In contrast, splenic T-cells from Ro 26-2198– or vehicle-treated mice proliferated similarly to TCR ligation, indicating the lack of a generalized immunosuppressive effect.

Reduced Th1 cells in pancreatic lymph nodes and pancreas-infiltrating cells from Ro 26-2198–treated NOD mice. To identify mechanisms accounting for the reduced type 1 diabetes development in Ro 26-2198–treated NOD mice, we analyzed the cytokines produced by CD4⁺ cells of 8- and 30-week-old NOD mice treated from 8 to 16 weeks of age with Ro 26-2198 or vehicle. Splenic CD4⁺ T-cells from Ro 26-2198– or vehicle-treated NOD mice displayed a similar cytokine production pattern (data not shown). In contrast, analysis of CD4⁺ T-cells from pancreatic lymph nodes revealed an average (n = 4) threefold reduction of IL-2 and of IFN-γ–producing cells in Ro 26-2198–treated compared with either 30-week-old vehicle-treated or 8-week-old NOD mice, indicating a decrease in Th1 cells (Fig. 4). CD4⁺ pancreas-infiltrating cells showed a similar percentage of IL-2–producing cells in all three groups, but the IFN-γ–producing cells were significantly increased in vehicle-treated mice, whereas they were similarly lower in 8-week-old and Ro 26-2198–treated NOD mice. These results indicate that Ro 26-2198 can arrest the recruitment of IFN-γ–producing Th1 cells into the pancreas, and this effect is still clearly seen 14 weeks after treatment withdrawal. No increase in IL-10–producing CD4⁺ cells was observed, and a modest, though significant, increase in IL-4–producing cells (0.7 ± 0.2% in 8-week-old NOD mice and 0.9 ± 0.2% in vehicle-treated vs. 2.5 ± 0.3% in Ro 26-2198–treated NOD mice, P = 0.02 by Mann-Whitney U test, means of four experiments) was detected.

Ro 26-2198 treatment enhances the frequency of CD4⁺CD25⁺ cells. CD4⁺CD25⁺ T-cells possess regulatory activity and have been implicated in the control of autoimmune responses in several models (8,9), including type 1 diabetes in the NOD mouse (7). Analysis of pancreatic lymph node cells revealed in Ro 26-2198–treated compared with 8-week-old or vehicle-treated 20-week-old NOD mice, respectively, a significantly increased percentage of CD4⁺CD25⁺ and CD4⁺CD38⁺ cells. No difference was seen in CD152 expression between CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from either vehicle- or Ro 26-2198–treated NOD mice (data not shown). Pancreatic lymph node cells from Ro 26-2198–treated NOD mice contained a slightly lower percentage of CD45RBhigh and a significantly
higher percentage of CD45RB<sup>low</sup>/CD4<sup>+</sup> cells than 8-week-old and 20-week-old vehicle-treated NOD mice (Fig. 5A). Interestingly, double-positive CD25<sup>+</sup>/CD38<sup>+</sup>CD4<sup>+</sup> cells (17 vs. 8 or 7%, respectively) were selectively increased in the pancreatic lymph node of Ro 26-2198–treated compared with 8-week-old and 20-week-old vehicle-treated NOD mice (data not shown). These results indicate that Ro 26-2198 administration leads to a selective increase of CD4<sup>+</sup> cells with a regulatory phenotype in the pancreatic lymph node of NOD mice that persists for at least 1 month after treatment withdrawal.

**Suppressive activity of CD4<sup>+</sup>/CD25<sup>+</sup> cells.** The profound reduction of IL-2–producing cells in the pancreatic lymph nodes of Ro 26-2198–treated NOD mice was paralleled by the strongly decreased proliferative response to insolubilized anti-TCR mAb. Pancreatic lymph node CD4<sup>+</sup> cells from 20-week-old Ro 26-2198–treated NOD mice proliferated 4 times less than age-matched controls and 10 times less than cells from 8-week-old mice (Fig. 5B). The proliferation in response to TCR ligation was not restored by the addition of IL-2, alone or together with anti-CD28 mAb, indicating a profoundly anergic state of these cells. However, addition of IL-2 and/or anti-CD28 mAb restored the lower IFN-γ secretion in pancreatic lymph node cells from Ro 26-2198–treated mice. In contrast to CD4<sup>+</sup> cells from pancreatic lymph nodes, splenic CD4<sup>+</sup> T-cells isolated from Ro 26-2198–treated mice or age-matched and 8-week-old controls, stimulated with insolubilized anti-TCR mAb, showed a similar proliferative response (Fig. 3B) and IFN-γ production (data not shown). This indicates a selective effect of Ro 26-2198 treatment on IL-2 and IFN-γ secretion by pancreatic lymph node CD4<sup>+</sup> cells stimulated via TCR ligation, consistent with the results obtained by stimulation with phorbol myristic acid (PMA) and ionomycin (Fig. 3). CD4<sup>+</sup>/CD25<sup>+</sup> pancreatic lymph node cells from Ro 26-2198–or vehicle-treated NOD mice showed a similar proliferative response and IFN-γ production to TCR ligation, indicating that removal of the CD4<sup>+</sup>CD25<sup>+</sup> population was sufficient to restore T-cell responsiveness. CD4<sup>+</sup>/CD25<sup>+</sup> pancreatic lymph node cells from Ro 26-2198–treated NOD mice proliferated less to insolubilized anti-TCR mAb than age-matched vehicle-treated mice. Moreover, the proliferation of CD4<sup>+</sup>/CD25<sup>+</sup> pancreatic lymph node cells from Ro 26-2198–treated mice was not restored by the addition of IL-2 compared with vehicle treated cells, indicating that Ro 26-2198 treatment induced a long-lasting, profound anergic state selectively in CD4<sup>+</sup>/CD25<sup>+</sup> pancreatic lymph node cells. These cells could inhibit the IFN-γ production in response to IA-2 and appeared to be, in this respect, functionally similar to CD4<sup>+</sup>/CD25<sup>+</sup> cells from vehicle-treated mice (Fig. 6A). A higher frequency of CD4<sup>+</sup>/CD25<sup>+</sup> cells, as previously observed (Fig. 5A), was found in the pancreatic lymph nodes of Ro 26-2198–treated NOD mice compared with controls (Fig. 6B). The higher frequency of CD4<sup>+</sup>/CD25<sup>+</sup> cells was paralleled by a higher efficiency of total CD4<sup>+</sup> cells in delaying the capacity of CD4<sup>+</sup>/CD25<sup>+</sup> cells to transfer type 1 diabetes into NOD.SCID recipients (Fig. 6C).

**FIG. 4.** Cytokine production by pancreatic lymph node cells and pancreas-infiltrating CD4<sup>+</sup> cells. Pancreatic lymph node (A) and pancreas-infiltrating (B) CD4<sup>+</sup> cells were obtained from untreated 8- or 30-week-old NOD mice treated 5 times per week per os from 8 to 16 weeks of age with vehicle or 0.03 μg/kg Ro 26-2198. Positively selected CD4<sup>+</sup> cells (2 × 10<sup>5</sup> cells/well) were stimulated with PMA and ionomycin and analyzed by flow cytometry for IFN-γ (abscissa) and IL-2, IL-4, or IL-10 (ordinate) production. Acquisition was performed on CD4<sup>+</sup> cells. Percentage of positive cells, set according to the isotype-matched controls (not shown), are shown in the top corner of each quadrant. Dot plots are from one representative experiment out of four performed. Bar graphs represent the means ± SE of four independent experiments using pooled cells from three to four mice. The P values were calculated by Mann-Whitney U test. *P < 0.05 vs. 8-week-old NOD mice.

<table>
<thead>
<tr>
<th></th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; pancreatic lymph node cells</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt; pancreas-infiltrating cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>IL-2: 13.5</td>
<td>IL-2: 19</td>
</tr>
<tr>
<td></td>
<td>IL-4: 0.2</td>
<td>IL-4: 0.2</td>
</tr>
<tr>
<td></td>
<td>IL-10: 1.4</td>
<td>IL-10: 2.1</td>
</tr>
<tr>
<td>30 weeks</td>
<td>IL-2: 1.3</td>
<td>IL-2: 4.7</td>
</tr>
<tr>
<td></td>
<td>IL-4: 0.4</td>
<td>IL-4: 2.5</td>
</tr>
<tr>
<td></td>
<td>IL-10: 0.5</td>
<td>IL-10: 2.3</td>
</tr>
<tr>
<td>4 weeks</td>
<td>IL-2: 12.7</td>
<td>IL-2: 16</td>
</tr>
<tr>
<td></td>
<td>IL-4: 4.8</td>
<td>IL-4: 12</td>
</tr>
<tr>
<td></td>
<td>IL-10: 1.3</td>
<td>IL-10: 2.3</td>
</tr>
<tr>
<td>30 weeks</td>
<td>IL-2: 1.3</td>
<td>IL-2: 4.7</td>
</tr>
<tr>
<td></td>
<td>IL-4: 0.4</td>
<td>IL-4: 2.5</td>
</tr>
<tr>
<td></td>
<td>IL-10: 0.5</td>
<td>IL-10: 2.3</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 weeks</td>
<td>IL-2: 2.1</td>
<td>IL-2: 4.7</td>
</tr>
<tr>
<td></td>
<td>IL-4: 0.2</td>
<td>IL-4: 2.5</td>
</tr>
<tr>
<td></td>
<td>IL-10: 0.5</td>
<td>IL-10: 2.3</td>
</tr>
<tr>
<td>30 weeks</td>
<td>IL-2: 12.7</td>
<td>IL-2: 16</td>
</tr>
<tr>
<td></td>
<td>IL-4: 4.8</td>
<td>IL-4: 12</td>
</tr>
<tr>
<td></td>
<td>IL-10: 1.3</td>
<td>IL-10: 2.3</td>
</tr>
<tr>
<td>4 weeks</td>
<td>IL-2: 12.7</td>
<td>IL-2: 16</td>
</tr>
<tr>
<td></td>
<td>IL-4: 4.8</td>
<td>IL-4: 12</td>
</tr>
<tr>
<td></td>
<td>IL-10: 1.3</td>
<td>IL-10: 2.3</td>
</tr>
</tbody>
</table>

**FIG. 5.** A: IFN-γ secretion in pancreatic lymph node cells from 8-week-old controls, stimulated with phorbol myristic acid (PMA) and ionomycin, was paralleled by a higher efficiency of total CD4<sup>+</sup> cells in delaying the capacity of CD4<sup>+</sup>/CD25<sup>+</sup> cells to transfer type 1 diabetes into NOD.SCID recipients (Fig. 6C).
U Mann-Whitney SE of three separate experiments. The Pation and IFN-\(\gamma\)/H9253 CD4 delayed disease transfer by pathogenic CD4 T-cell response to the pancreatic autoantigen IA-2, and secrete IFN-\(\gamma\) as demonstrated by their impaired capacity to proliferate and secrete IFN-\(\gamma\) in response to TCR ligation, inhibited the T-cell response to the pancreatic autoantigen IA-2, and delayed disease transfer by pathogenic CD4 CD25- cells. Thus, Ro 26-2198 administration enhances the frequency of CD4 CD25- regulatory cells in pancreatic lymph nodes and arrests the progression of type 1 diabetes in NOD mice.

Two checkpoints have been defined in the pathogenesis of type 1 diabetes in the NOD mouse. The first controls the onset of insulitis, starting at \(\sim 3\) weeks of age, the second exerts its activity at \(\sim 8\)–12 weeks of age, controlling the switch to overt disease (34). The first checkpoint regulates the composition of APC populations (35) and the expres-
sion of integrins and adhesion molecules that increase the islet-homing potential of T-cells. The second checkpoint controls the transition from insulitis to diabetes via several nonexclusive mechanisms, including APCs; cytokines; Th1/Th2 balance; modulation of surface receptors such as CD152, CD25, and NK-like receptors; recruitment of pathogenic cells; and number and function of regulatory cells (34). IL-12 could play a role at both checkpoints. IL-12 administration to 2- or 8-week-old NOD mice induced premature onset of the disease mediated by pancreas-infiltrating CD4+ cells with Th1 phenotype (3,32). However, targeting IL-12 with a specific antagonist induced a deviation of pancreas-infiltrating CD4+ cells toward the Th2 phenotype and prevented type 1 diabetes when administered at 3 weeks of age, but it had little effect when administered to 8-week-old NOD mice (31). This suggests that IL-12 is critical at checkpoint 1 and dispensable at checkpoint 2. Therefore, to interfere with type 1 diabetes development in adult NOD mice, we looked for an agent able not only to inhibit IL-12, but also to exert other immunoregulatory activities.

1,25(OH)2D3 and its analogs are immunoregulatory agents able to prevent Th1-mediated autoimmune diseases (36). 1,25(OH)2D3 reduces the incidence of insulinitis (37) and prevents type 1 diabetes development (20), but only when administered to NOD mice starting from 3 weeks of age, before the onset of insulinitis. 1,25(OH)2D3 was found ineffective in preventing progression of diabetes in NOD mice when given from 8 weeks of age, when NOD mice present a well-established insulinitis (38). However, a combined treatment of 8-week-old NOD mice with the 1,25(OH)2D3 analog MC 1288 and cyclosporine A reduced the incidence of disease, although neither treatment alone was effective (39). In contrast, the 1,25(OH)2D3 analog Ro 26-2198 is able, as a monotherapy, to treat the ongoing type 1 diabetes in the adult NOD mouse, effectively blocking the disease course. This property is likely due, at least in part, to its increased metabolic stability against the inactivating C-24 and C-26 hydroxylations and the C-3 epimerization (40), resulting in a 100-fold more potent immunosuppressive activity compared with 1,25(OH)2D3.

1,25(OH)2D3, in addition to inhibiting IL-12 production, decreases antigen-induced T-cell proliferation (41) and cytokine production (42). APCs and, in particular, DCs are primary targets for the immunosuppressive activity of 1,25(OH)2D3 (30,43–46). 1,25(OH)2D3 inhibits differentiation, maturation, activation, and survival of DCs, leading to impaired activation of alloreactive CD4+ T-cells, which show downregulated CD154 and upregulated CD152 expression (30). These results may help to explain the profoundly anergic state of pancreatic lymph node CD4+ cells in Ro 26-2198–treated NOD mice. 1,25(OH)2D3 not only inhibits IL-12 but also greatly enhances IL-10 secretion by DCs (30). These two effects, coupled with the downregulation of costimulatory molecule expression by DCs (30,43–45), could account for the increase in regulatory T-cells observed in the pancreatic lymph nodes of treated mice. DCs can be not only immunogenic but also tolerogenic (47), and immature DCs have been shown to induce CD4+ cells with regulatory properties (48). Administration of 1,25(OH)2D3 induces DCs with a tolerogenic phenotype and promotes tolerance to allografts associated with an increased percentage of CD4+CD25+ regulatory cells that could adoptively transfer transplantation tolerance (49). This is consistent with the present results, suggesting that the arrest of DCs at the immature stage induced by Ro 26-2198 treatment limits T-cell costimulation, leading to enhanced frequency of CD4+CD25+ cells.

The presence of CD4+CD25+ regulatory T-cells with suppressive activity has been demonstrated in several animal models of autoimmune diseases (8,9). CD4+CD25+ regulatory T-cells appear to play an important role in controlling the progression of type 1 diabetes in NOD mice, because a low level of CD4+CD25+ T-cells correlates with exacerbation and acceleration of the disease (7). It is likely that this cell population is more relevant than Th2 cells in the control of type 1 diabetes, although both could contribute to protection from disease. 1,25(OH)2D3 can induce regulatory cells with disease-suppressive activity in the NOD mouse (20), and a disease-preventing 1,25(OH)2D3 analog could deviate pancreas-infiltrating cells to the Th2 phenotype (39). Our results show that pancreatic lymph node CD4+ cells from Ro 26-2198–treated NOD mice contain an enhanced frequency of CD25+ cells that are able to significantly delay the capacity of CD4+CD25+ pathogenic T-cells to transfer disease into NOD.SCID recipients. Conversely, Th2 cells were not observed in pancreatic lymph nodes from treated mice, although a small percentage of Th2 cells was found in the pancreas.

In conclusion, we have demonstrated that ongoing type 1 diabetes in the adult NOD mouse can be arrested by a relatively short course of treatment with a 1,25(OH)2D3 analog, which is able to induce a selective inhibition of Th1 and an increased frequency of CD4+CD25+ regulatory T-cells in pancreatic lymph nodes. These results, coupled with the recently documented presence of CD4+CD25+ regulatory T-cells in humans (50), suggest that a similar treatment may also inhibit disease progression in prediabetic or newly diagnosed type 1 diabetic patients.

ACKNOWLEDGMENTS

This work was supported in part by European Community Grant QLRT-2000-02103.

REFERENCES

self-tolerance maintained by activated T cells expressing IL-2 receptor
alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance

11. Read S, Malmstrom V, Powrie F: Cytotoxic T lymphocyte-associated
antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory
cells that control intestinal inflammation. *J Exp Med* 192:296–
311, 2000

12. Stephens LA, Mason D: CD25 is a marker for CD4+ thymocytes that
prevent autoimmune diabetes in rats, but peripheral T cells with this
function are found in both CD25+ and CD25- subpopulations. *J Immunol*
165:3105–3110, 2000

Paracrine role for calcitriol in the immune system and skin creates new
therapeutic possibilities for vitamin D analogs. *J Endocrinol* 137:1–
16, 1995

14. Lemire JM, Archer DC: 1,25-dihydroxyvitamin D3 prevents the in vivo
induction of murine experimental autoimmune encephalomyelitis. *J Clin
Invest* 87:1103–1107, 1991

blocks the progression of relapsing encephalomyelitis, a model of multiple

G, Panina-Bordignon P, Adorini L: Inhibition of Th1 development and
management of chronic-relapsing experimental allergic encephalomyelitis by
a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D3. *J Exp Med*
193:808–809, 2000

17. Lemire JM, Ince A, Takashima M: 1,25-dihydroxyvitamin D3 attenuates the
expression of experimental murine lupus of MRL/1 mice. *Autoimmunity*
12:143–148, 1992

18. Larsson P, Mattsson L, Klareskog L, Johnsson C: A vitamin D analogue (MC
1288) has immunomodulatory properties and suppresses collagen-induced
arthritis (CA) without causing hypercalcemia. *Clin Exp Immunol* 114:
277–283, 1998

the progression of arthritis in murine models of human arthritis. *J Nutr*
128:68–72, 1998

autoimmune diabetes in NOD mice by 1,25 dihydroxyvitamin D3. *Diabetes*
51:511–515, 2002

Pharm Des* 6:961–972, 2000

22. Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, Badenhoop
K: Vitamin D receptor allele combinations influence genetic susceptibility

23. Chang TJ, Lei HH, Yeh JI, Chiu KC, Lee KC, Chen MC, Tai TY, Chuang LM:
Vitamin D receptor gene polymorphisms influence susceptibility to type 1
diabetes mellitus in the Taiwanese population. *Clin Endocrinol* 52:575–
580, 2000

24. Green A, Gale EA, Patterson CC: Incidence of childhood-onset insulin-
909, 1992

25. Vitamin D supplement in early childhood and risk for type 1 (insulin-
dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group.
*Diabetologia* 42:51–54, 1999

26. Lemire JM, Archer DC, Beck L, Spiegelberg HL: Immunosuppressive
actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1

27. D’Ambrosio D, Cippitelli M, Cocciole MG, Mazzeno D, Di Lucia P, Lang R,
Sinaguglia P, Panina-Bordignon P: Inhibition of IL-12 production by 1,25-
dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in tran-

Maeda S, Kasuga M, Okumura K, Yagita H, Yokono K: Suppression and
inhibition of dendritic cell differentiation and maturation by vitamin D

Oehler L: 1,25-Dihydroxyvitamin D3 (3) that resist metabolism through C-24 oxidation and C-3 epimerization pathways (Review). *Steroids*

inhibits antigen-induced T cell activation. *J Immunol* 153:1748–1754,
1998

31. Rigby WF, Denome S, Fanger MW: Regulation of lymphokine production
and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. *Specific
inhibition at the level of messenger RNA. J Clin Invest* 79:1659–1664,
1987

P, Di Carlo V: Vitamin D3 affects differentiation, maturation, and function of
human monocyte-derived dendritic cells. *J Immunol* 164:4443–4451,
2000

2001

34. Finkelman F, Lees A, Birnbaum R, Gause W, Morris S: Dendritic cells can
present antigen in vivo in a tolerogenic or immunogenic fashion. *J Immu-

10-producing, nonproliferating CD4(+) T cells with regulatory properties

36. Griffin MD, Lutz WH, Phan VA, Bachman LA, McKean DJ, Kumfar R: Potent
inhibition of dendritic cell differentiation and maturation by vitamin D

37. Gregor S, Casorati M, Manuchastegui S, Smiroldo S, Davalli AM, Adorini L:
Regulatory T cells induced by 1,25-Dihydroxyvitamin D3 and its analogs a
vitamin D receptor-dependent pathway that promotes a persistent state of
2001