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Effects of a Vitamin D_3 Analog on Diabetes in the Bio Breeding (BB) Rat

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Abstract Non-hypercalcemic analogs of vitamin D_3 modulate the immune response through antigen-presenting cells (APCs) and activated T-cells. A large population-base case-control showed that vitamin D_3 intake significantly decreases the risk of type 1 diabetes development. The aim of this study was, therefore, to observe the in vivo effects of a vitamin D_3 analog administered to Bio Breeding (BB) rats. 1,25-Dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-219, formerly Ro 26-2198) (BioXell, Milan, Italy) was administered in vivo to BB rats from days 42 to 110 of life at 0.2 μg/Kg BW. Control animals received only vehicle (olive oil, 4.8 μl/100 g BW). The animals of these two groups were subjected to insulin treatment as they became diabetic. Insulin (Humulin, 28.6 UI/day) was administered irrespective of diabetes occurrence to another group of rats for comparison. Blood glucose, insulin levels, glycosuria, degree of islet infiltration, and the expression of some antigens were observed. Results showed that the vitamin D_3 analog reduced diabetes incidence, although limitedly, in BB rats while administration of oral insulin increased diabetes incidence. In addition, the vitamin D_3 analog did not stimulate an enhancement in the expression of CD4 and CD25 in BB rats as it does in NOD mice, which may explain the failure of this as well as other antidiabetic treatments in the BB animal model of type 1 diabetes. J. Cell. Biochem. 100: 808–814, 2007.

Key words: type 1 diabetes; vitamin D3; islets of Langerhans; insulin; cytokines

The Bio Breeding (BB) rat is a useful animal model of type 1 diabetes. In fact, these animals spontaneously develop a disease that, under several aspects, resembles that seen in humans.

Among the numerous drugs tested by researchers to prevent or suppress the disease, non-hypercalcemic vitamin D_3 analogs have been reported to be capable of modulating the immune response through specific receptors expressed on antigen-presenting cells (APCs) and activated T-cells [Bouillon et al., 1995]. In particular, the non-hypercalcemic D_3 analogs are capable of inhibiting numerous autoimmune diseases in animal models, including experimental allergic encephalomyelitis [Cantorna et al., 1996], murine lupus [Lemire et al., 1992], collagen-induced arthritis [Larsson et al., 1998], and type 1 diabetes [Mathieu et al., 1994].

In humans, vitamin receptor gene polymorphism has been associated with type 1 diabetes in different populations [Pani et al., 2000]. Moreover, a large population-base case-control has shown that the intake of vitamin D_3 significantly decreases the risk of type 1 diabetes development [Eurodiab substudy 2 study group, 1999].

Furthermore, vitamin D_3 analogs inhibit IL-12 production [Lemire et al., 1995], most probably through the inhibition of nuclear factor (NF)-kB [D'ambrosio et al., 1998]. To this regard, we have recently found a biphasic effect

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of NF-kB transcription factor in IL-1β-treated islets in vitro, which could explain the kinetics of anti-islet β-cell attack [Papaccio et al., 2005]. This is of particular significance because IL-12 seems to exert an important role in developing type 1 diabetes both in the nonobese diabetic (NOD) mouse [Fujihira et al., 2000] and in humans [Morahan et al., 2001]. Therefore, vitamin D_3 analogs are important drugs for APC modulation, capable of inhibiting differentiation, maturation, activation, and survival of dendritic cells, which are critical cells for alloreactive T-lymphocytes [Penna and Adorini, 2000; Piemonti et al., 2000].

To our knowledge, vitamin D_3 analogs have been tested in vivo in NOD mice [Casteels et al., 1998a; Smith et al., 2000], but they have not been yet administered to the BB rat model. Moreover, there is substantial disagreement with regard to the effects of the administration of oral insulin with adjuvants [Matsumoto et al., 1986; Hartmann et al., 1997; Kolb et al., 1997; Bellmann et al., 1998; Buschard et al., 2000]. Therefore, we observed the effects of a vitamin D_3 analog and insulin treatment in the BB rat model.

MATERIALS AND METHODS

Animals

BB rats (n = 60), aged 5 weeks, of both sexes (n = 30 males and 30 females), were purchased from M&B (Bomholtvej, Denmark) and maintained following the specific instructions of the supplier in a pathogen-free ambient. Animals had free access to water and food, containing a normal content of calcium. At the age of 35 days, animals were still euglycemic.

Experimental Protocol

Animals were subdivided into the following three groups. Group 1 controls: These animals were administered only the vehicle (olive oil by gavage). Group 2: vitamin D_3 analog-treated animals. These animals were administered the vitamin D_3 analog 1,25-dihydroxy-16, 23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-219, formerly Ro 26-2198) (BioXell, Milan, Italy), by gavage, three times a week at a dosage of 0.2 μg/Kg BW, dissolved in olive oil (4.8 μl/100 g BW), from day 42 up to day 110 of life. Group 3: insulin-treated animals. These animals were administered oral human insulin (Humulin R, Eli Lilly France S.A., Paris, France) at a dosage of 1 mg/kg BW corresponding to 28.6 UI, during the same period as Group 2. As animals of Groups 1 and 2 became diabetic they were subjected to insulin treatment, at doses ranging between 1 and 3 U, depending on the level of glycemia and glycosuria.

Evaluation of Insulitis and Diabetes

Diabetes was evaluated by assaying the following parameters: glycemia, determined using One-Touch profile (Lifescan Inc., Milpitas, CA) twice a week and, after the age of 90 days, daily; glycosuria, determined with Clinistix (Bayer Diagnostics, Basingstoke, UK), twice a week, and insulin levels evaluated weekly, using a R.I.A. kit (BioRad, Milan, Italy). Rats were considered hyperglycemic when their blood glucose level was higher than 12 mM/L but lower than 15 mM/L, and diabetic when their blood glucose level exceeded 15 mM/L on two successive determinations. At the end of the experiment, animals were killed by decapitation under ether anesthesia, and their pancreas removed, fixed in 4% paraformaldehyde in PBS and embedded in paraffin. Serial sections (5 μm thick on polylysine-treated slides) were used alternatively for insulitis evaluation or for immunocytochemistry. For insulitis evaluation, hematoxylin-eosin stained sections were screened blindly, by two independent researchers. Only sections containing six or more islets were selected and at least 30 islets/pancreas were observed. The level of lymphocytic infiltration in the islets was scored as follows: 0 = normal islets without infiltration; 1 = focal peri-islet or peri-ductular infiltration; 2 = peri-islet infiltration 3 = peri- and intra-islet lymphocytic infiltration invading less than 50% of the islet; 4 = intra-islet lymphocytic infiltration, invading the whole islet, associated with islet cytoarchitectural derangement; 5 = islet atrophy, due to β cell loss. The mean score for each pancreas was calculated by dividing the total score by the number of islet scored. Data, expressed in arbitrary units, are given as means ± SD.

Immunohistochemistry

Serial sections (5 μm thick) were stained by the avidin-biotin peroxidase indirect staining method. Monoclonal antibodies used in this experiment were the following: anti-CD4, anti-CD8, anti-CD25, anti-LFA-1 alpha-chain, anti-CD68 (clone PG-M1, staining macrophages in a variety of tissues and non-reacting with granulocytes or their precursor

cells), anti-lymphocyte/myeloid, anti-ICAM-1, all diluted 1:25 (goat anti-rat, Dako, Milan, Italy). The secondary antibodies were biotinylated anti-rat (Vector, Burlingame, CA). The Vectastain ABC kit, was used for stain revelation (Vector).

Determination of Calcium in the Urine and Serum

Serum calcium concentration and calciuria were determined weekly, using microcolorimetric assays (Sigma Chemicals, Co., Milan, Italy). Data, expressed in mg/ml, are given as means ± SD.

Statistical Evaluation

Data were expressed as means ± SD. Student's was used for statistical evaluations. The level of significance was set at $P < 0.05$.

RESULTS

Insulitis and Cumulative Diabetes Incidence

Control animals (Group 1), given only the olive oil vehicle, had a mean insulitis grading score of 3.9 ± 0.9 (Fig. 1). Most islets had high degree insulitis, while the remaining had focal or peri-islet infiltrations. Diabetes incidence was 65% at 80 days of life and roughly 80% at 110 days of life (Fig. 2). Insulin values were also considerably low in the majority of the animals (Fig. 3). Weights ranged between 100 and 180 g in diabetic animals but were 300–400 g in euglycemic animals.

Fig. 1. Insulitis diabetes scoring. Figure showing the mean insulitis diabetes score in control and treated groups. For insulitis evaluation hematoxylin-eosin stained serial sections (5 μm thick) were screened blindly, by two researchers. Only sections containing six or more islets were selected and at least 30 islets/pancreas were observed. The level of lymphocytic infiltration in the islets was scored as follows: 0 = normal islets without infiltration; 1 = focal peri-islet or peri-ductular infiltration; 2 = peri-islet infiltration 3 = peri- and intra-islet lymphocytic infiltration invading less than 50% of the islet; 4 = intra-islet lymphocytic infiltration, invading the whole islet, associated with islet cytoarchytectural derangement; 5 = islet atrophy, due to β cell loss. The mean score for each pancreas was calculated by dividing the total score by the numbers of islet scored. Data, expressed in arbitrary units, are given as means ± SD, $^*P < 0.01$.

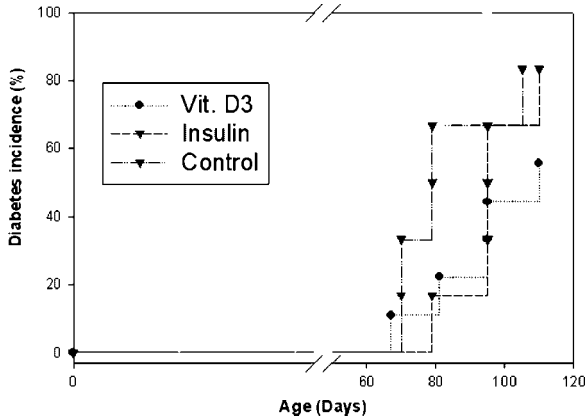

Fig. 2. Cumulated diabetes incidence. Figure showing the cumulated of IDDM, which was calculated on the basis of glycemic levels (diabetic animals presented with blood glucose levels exceeding 12 mmol/L on two consecutive determinations) in relationship with age. Values were averaged and are presented as means ± SD.

The mean insulitis score was 2.5 ± 1.1 in Group 2 (animals treated with the vitamin D_3 analog) (Fig. 1). Roughly 45% of the animals either did not have any signs of lymphocytic infiltration within their islets or presented with only a few infiltrating cells surrounding the islets, prevailingly localized around ducts or at an islet pole. The remaining animals had a diffuse or intraislet infiltration and, only in one case, islets were completely athrophized or cytoarchitecturally deranged due to β cell loss.

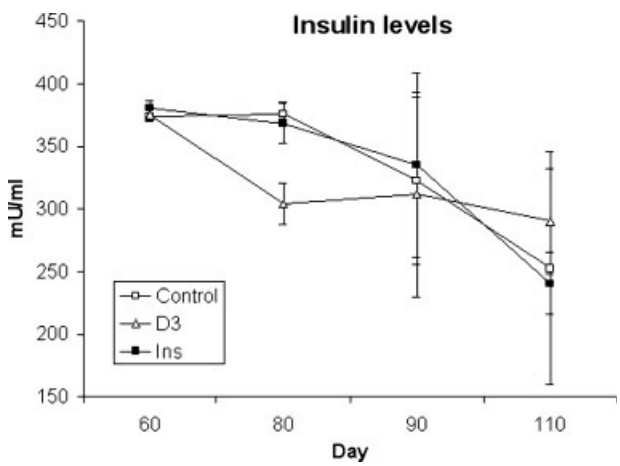

Fig. 3. Insulin levels. Figure showing insulin levels in control and treated animals. Values, expressed in mU/ml, were averaged and are presented as means ± SD, $^*P < 0.001$.

These animals were overtly diabetic: in fact, their glycemic values always exceeded 15 mM/L, already by 70–75 days of life. Insulin levels (Fig. 3) were inversely comparable to glycemic values. Animals' weights were normal in relation to their age only in 45% of cases (normoglycemic animals, whose weights ranged between 400 and 450 g) but it was significantly lower in the remaining cases (diabetic animals, whose weights ranged between 150 and 180 g). Mortality was 18% the vitamin D_3 analog exerted a slight effect on diabetes incidence with respect to controls ($P < 0.01$). In fact, already at 80 days of life, the control group had higher diabetes incidence with respect to vitamin D3 analog-treated rats. This was significantly observed at 100 and 110 days of life, when the incidence was 60%, but 80% in controls ($P < 0.0001$) (see Fig. 2).

The insulitis mean score was 4.0 ± 1.4 for Group 3 (animals treated with insulin) (Fig. 1). Also in this group, the majority of animals, showed a diffuse intra-islet infiltration and, in many cases, islets were completely athrophized or deranged. Only in a few cases intra-insulitis was present without cytoarchitectural derangement. These animals were overtly diabetic: glycemic values always exceeded 15 mM/L, by 70–75 days of life in the majority of cases and the cumulative diabetes incidence was comparable to controls (80%) at 110 days of life (Fig. 2). Insulin levels (Fig. 3) showed a progressive reduction, down to the lowest levels at the end of the experiment. These animals also had a significant decrease of their weights (ranging between 100 and 200 g) and the mortality, observed by day 90, was 30%.

Serum and Urinary Calcium Concentrations

Both serum and urinary calcium levels were normal throughout the experiment in all groups. All values were comparable during the course of the experiment (Figs. 4 and 5).

Immunohistochemistry

Results are summarized in Table I. Non-diabetic animals of the vitamin D3 analog-treated group showed immunoreactivity for CD4 (Fig. 6a) and CD25 antibodies, while diabetic animals of this group showed a high positivity for CD8 (Fig. 6b), lymphocyte/myeloid, CD68, and ICAM-1 antibodies. Islets belonging to animals treated with oral insulin showed a strong immunoreactivity for CD8, CD68, ICAM-1, (Fig. 6c) and lymphocyte/myeloid antibodies.

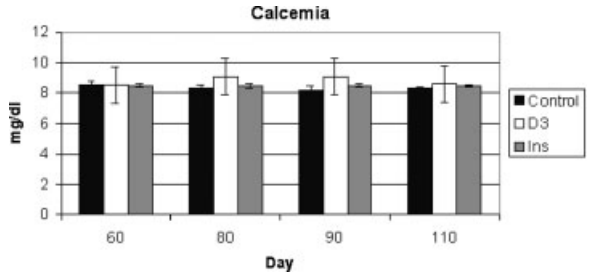

Fig. 4. Blood calcium levels. Figure showing blood calcium levels in control and treated animals. Values, expressed in mg/ml, were averaged and are presented as means ± SD.

Islets belonging to control diabetic animals were immunoreactive for CD4 and CD25 (Fig. 6d) if non-diabetic and for CD8, CD25, CD68 lymphocyte/myeloid, and ICAM-1 when diabetic.

DISCUSSION

In this study, in vivo administration of a non-hypercalcemic vitamin D_3 analog to BB rats exerts a limited preservation from diabetes occurrence with respect to controls. In fact, the mean level of islet infiltration was lower, and blood glucose and insulin levels were slightly ameliored, when compared to controls. In particular, the cumulative diabetes incidence clearly shows that the BB rats treated with the vitamin D_3 analog were diabetic in 60% of cases at 100 days of life, while control animals had a diabetes incidence of 80% at the same time. We stress that diabetes was not yet observed at the age of 100 days of life in an increased number of animals. Calcium levels in blood and urine were not increased by the drug, confirming that the analog is not hypercalcemic.

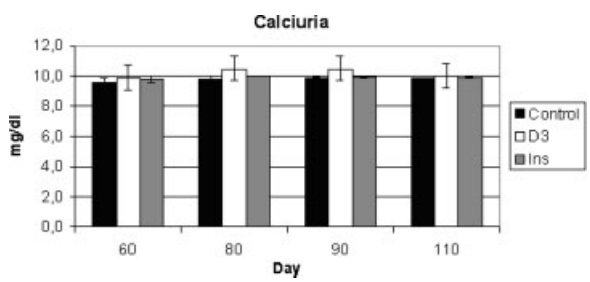

Fig. 5. Calciuria levels. Figure showing urine calcium excretion (calciuria) in control and treated animals. Values, expressed in mg/ml, were averaged and are presented as means ± SD.

TABLE I. Table Summarizing the Immunoreactivity of BB Rat Islets

GROUPS	CD4	CD8	CD25	CD68	Lymph/myeloid	ICAM-1
Group 1 non-diabetics	++	–	++	–	–	–
Group 1 diabetics	–	++	–	++	++	+
Group 2	–	++	–	++	++	++
Group 3	–	++	–	++	++	++

On the contrary, oral insulin treatment, worsened diabetes incidence when used alone. To this regard, in a multicenter double-blind trial in patients with recent onset Type 1 diabetes [Pozzilli and Gisella, 2000] oral insulin administration at the dose of 5 mg daily for 1 year starting at the time of disease onset, had no effect on residual β-cell function, as assessed by C-peptide secretion. A similar trial in the same study [Pozzilli and Gisella, 2000], performed using different doses of insulin, was carried out at the same time: it also showed no

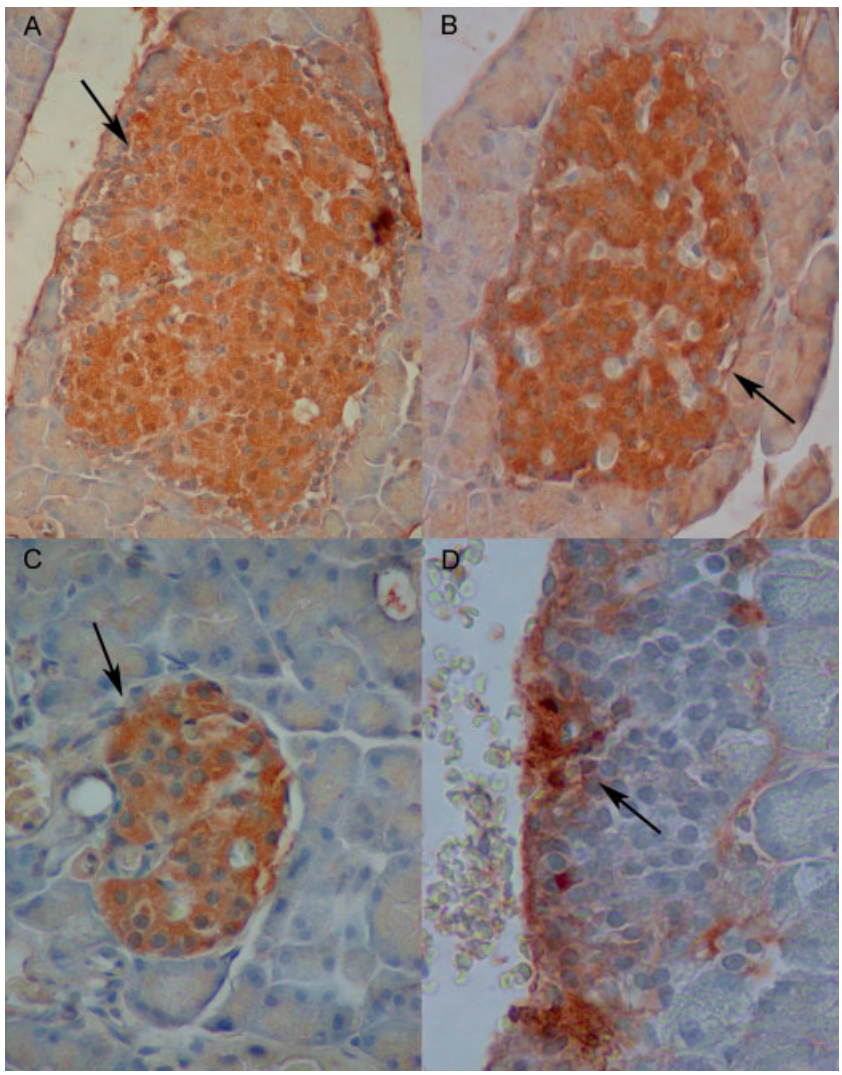

Fig. 6. Immunohistochemistry. **A**: Islet belonging to a non-diabetic animal treated with the vitamin D3 analog showing immunoreactivity for CD4 (*arrow*); (**B**) Islet belonging to a diabetic animal treated with the vitamin D3 analog showing positivity for CD8 (*arrow*); (**C**) Islet belonging to a diabetic animal treated with oral insulin showing a strong immunoreactivity for CD8 (*arrow*); (**D**) Islets belonging to a control diabetic animal showing positivity for CD25 (*arrow*).

beneficial effects on the decline of β-cell function during the first year after diagnosis. In our study, oral insulin administered at the daily dose of 28.6 UI/day increased diabetes incidence in BB rats. Our findings in this animal model and previous results in humans deeply challenge the current view that induction of tolerance can be established when the immune process is already active. Recently, it has been demonstrated that treatment of adult NOD mice with an analog of vitamin D_3 [Lemire et al., 1992], which exerts immunomodulatory activities preventing dendritic cell maturation, decreases lipopolysaccharide-induced IL-12 and γ-interferon production, arrests Th1 cell infiltration and progression of insulitis, and inhibits diabetes development at non-hypercalcemic doses [Gregori et al., 2002]. 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 brief treatment of adult NOD mice with an analog of 1,25-dihydroxyvitamin D(3) 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 [Gregori et al., 2002].

A synergy with cyclosporine has also been found and described by several authors [Mathieu et al., 1995; Casteels et al., 1998b]. Our data, in the BB rat, indicate that vitamin D_3 analogs do not exert the same activity found in the NOD mouse, but diabetes occurrence significantly enhances the expression of CD8, CD25, lymphocyte/myeloid and CD68 cytotoxic T cells, as well as LFA-1, and ICAM-1 expression. This means that in the BB rat diabetes, but not vitamin D_3 analogs, induce a selective modulation of cytotoxic T lymphocytes. Therefore, vitamin D_3 analogs do not enhance the expression of CD4 and CD25, like in NOD mice, and this may forecast treatment failure.

A short-term and early-life treatment of NOD mice with cholecalciferol or non-hypercalcaemic vitamin D_3 analogs was performed in the NOD mouse, in order to verify the possible clinical applicability of vitamin D_3 analogs in type 1 diabetic young children. Researchers [van Etten et al., 2002] found a significant protection of pancreatic β cells against autoimmune destruction in cholecalciferol-treated NOD mice, when compared with untreated controls. This short-term early-life intervention was, however, not able to protect the mice from developing diabetes for long, during their lifetime. Therefore, these authors suggested that possible solutions were longer or combined treatments with other immunomodulators that have synergistic effects with vitamin D_3 and its analogs.

Taking into consideration our data, we underline that, although the difficulties in blocking the autoimmune attack against islet β cells, which, in the BB rat, is as early as fatal, we have obtained some preservation from diabetes, even in this animal model, using this drug. Therefore, we emphasize the significance of those data for possible clinical use. In fact, the vitamin D_3 analogs should be of interest for combined therapy, mainly in young children.

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