Diets Rich in Polyphenols and Vitamin A Inhibit the Development of Type I Autoimmune Diabetes in Nonobese Diabetic Mice

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Abstract
Type 1 juvenile diabetes mellitus is characterized by the infiltration of activated T lymphocytes and monocytes into the islets of Langerhans of the pancreas, resulting in inflammation and progressive destruction of the insulin-producing β cells. We hypothesized that feeding nonobese diabetic (NOD) mice diets rich in polyphenols or vitamin A, both known modulators of immune function, would decrease the autoimmune inflammatory process associated with type I diabetes. NOD mice were fed a control diet (C) and diets containing either 1% freeze-dried grape powder (GP) or 250 IU vitamin A/g (VA) or 25% grape powder; NOD, nonobese diabetic; ORAC, oxygen radical absorbance capacity; T1d and T2d, T lymphocyte helper 1 and 2; trollex, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; VA, vitamin A.

Introduction
Type 1 juvenile diabetes mellitus (also known as insulin-dependent diabetes mellitus [IDDM]) is a T-cell-mediated, inflammatory autoimmune disease that currently affects ~0.5% of the population in developed countries (1). Onset of type 1 diabetes usually occurs in children and young adults and is characterized by the infiltration of activated T lymphocytes into the islets of Langerhans of the pancreas (insulitis), resulting in the inflammation and progressive destruction of the insulin-producing β cells. The etiology of this disease remains unknown with both genetic and environmental factors playing a role in triggering the inflammatory response. Destruction of the β cells leads to severe depletion of insulin, resulting in hyperglycemia, increased breakdown in fat, fatty acid oxidation, and excessive production of ketones. Without exogenous insulin, patients with this disease succumb to severe metabolic disturbances resulting in coma and death.

Several factors have been proposed to be involved in the inflammatory response leading to β cell destruction, including cytolytic lymphocytes and proinflammatory cytokines, as well as the local generation of chemokines and cellular adhesion molecules (2–6). Animal models for IDDM, such as the nonobese diabetic (NOD) mouse, have revealed that CD4+ T lymphocyte helper 1 (Th1) and CD8+ lymphocytes from diabetic mice promoted type 1 diabetes when adoptively transferred to nondiabetic recipients (7,8). Several proinflammatory cytokines have been shown to be involved in the pathogenesis of IDDM, including IFN-γ, TNF-α, and IL-1 (4). Production of these proinflammatory cytokines in turn induces the expression of the chemokines and adhesion molecules that may further promote the recruitment of cytolytic lymphocytes and macrophages into the islets (5,6).

Grapes contain numerous polyphenols that have antiinflammatory and antioxidant activity, including the stilbene, resveratrol, and the flavanoids quercetin, catechin, and anthocyanins. Resveratrol, quercetin, and (+)-catechin can inhibit the activation of the transcription factor nuclear factor (NF)-κB, an important mediator of inflammatory responses, as well as several enzymes involved in signal transduction pathways (9–11). Resveratrol, quercetin, and anthocyanins inhibit the enzyme cyclooxygenase-2, which plays a key role in inflammatory processes and is induced by mitogens, cytokines, and bacterial LPS (9,12,13). Due to their antioxidant activities, quercetin and catechins can protect β cells from oxidative stress and damage in vitro and in rodent models of chemically induced diabetes (14,15).
Vitamin A modulates the adaptive and innate immune systems by a variety of methods, including modulation of Th1/Th2 lymphocyte helper 2 (Th2) development and production of cytokines by inflammatory cells such as macrophages (16,17). For example, high-level dietary vitamin A can diminish development of Th1 and promote development of Th2 responses (18). Such immune modulation by vitamin A could decrease the severity of autoimmune diseases. It has been demonstrated that the vitamin A metabolite retinoic acid decreases the severity of disease in experimental autoimmune encephalomyelitis, apparently by diminishing production of proinflammatory cytokines by microglia at the site of inflammation (19), as well as by promoting Th2 development (20). Similar mechanisms may be induced by high dietary levels of vitamin A that could decrease the development of diabetes in NOD mice.

An increase of polyphenols and/or vitamin A levels in the diet may have profound effects on suppressing inflammatory immune cells and reducing the oxidative damage in the islets that contributes to loss of β cells. In this study, 2 dietary interventions that act by different mechanisms were used to alter immune responsiveness. High levels of dietary vitamin A were fed to NOD mice to determine whether vitamin A would switch Th1 cells to a Th2 phenotype and, therefore, would diminish the cytotoxic T cell destruction of the islet β cells. We also used a diet enriched with freeze-dried grape powder to determine whether a diet rich in polyphenols would reduce the inflammatory processes responsible for β cell loss in the NOD mice.

Materials and Methods

Mice and Diets. Forty-five female NOD/Lt mice between the ages of 3 and 5 wk were purchased from the Jackson Laboratory. Three modified diets were prepared by Harlan Teklad and γ irradiated to sterilize. The standard diet was a commercial NIH-31 fixed formula diet (Harlan-Teklad Diet 7013) containing ~18% crude protein, 6% crude fat, and 5% crude fiber. This diet was chosen because it is used by the Jackson Laboratory for maintenance of the NOD/Lt mouse colony where up to 80–90% incidence of diabetes in female mice is routinely observed. Control (C) diet was the standard diet supplemented with 0.45% fructose and 0.45% glucose to control for sugars found in the grape powder. Vitamin A (VA) diet was standard diet supplemented to contain a total of 262 μmol vitamin A/kg food (as retinyl acetate) and 0.45% fructose and 0.45% glucose were also added to the VA diet so that control diet could be used for the study. The grape powder (GP) diet was standard diet supplemented with 1% (wt:wt) freeze-dried grape powder obtained from the California Table Grape Commission. The total vitamin A concentrations were 26 μmol and 32 μmol retinol/kg diet (as retinyl acetate) for the C and GP diets, respectively. The powder was prepared from a mixture of seeded and seedless fresh red, green, and blue-black California grape cultivars, which were frozen, ground, freeze-dried, and reground using good manufacturing practices. The nutritional composition of the grape powder (Table 1) was determined by the California Table Grape Commission as described (21–23). Mice were randomly divided into the C, VA, and GP groups (15 mice per group) and maintained on the modified diets for the duration of the experimental period. Mice were housed under pathogen-free conditions in a temperature controlled environment with 12 h light/dark and were allowed to consume food and water ad libitum. All experimental procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee.

Diabetes assessment and tissue preparation. Blood was collected from the tail artery once per week for monitoring hyperglycemia using an Accucheck glucose meter (Roche Diagnostics). Mice were considered diabetic when the blood glucose level was equal to or >13.9 mmol/L and they were killed by carbon dioxide asphyxiation. All remaining mice that did not become diabetic were killed when they reached ~28 wk of age.

### TABLE 1 Nutritional composition of the grape powder

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Concentration (g/kg powder)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>35</td>
</tr>
<tr>
<td>β Carotene</td>
<td>0.0018</td>
</tr>
<tr>
<td>Vitamin A&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.0009</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0.03</td>
</tr>
<tr>
<td>Thiamin</td>
<td>0.0015</td>
</tr>
<tr>
<td>Folate</td>
<td>0.26</td>
</tr>
<tr>
<td>Total phenols</td>
<td>0.2</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.90</td>
</tr>
<tr>
<td>Anthocyanins&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.70</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.028</td>
</tr>
<tr>
<td>Myricetin</td>
<td>0.006</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>0.0023</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>0.00091</td>
</tr>
</tbody>
</table>

<sup>1</sup> 3.14 μmol; 1 μg retinol = 0.003491 μmol.

<sup>2</sup> Malvidin monoglucoside.

The pancreatic tissue, spleen, liver, and blood were removed under aseptic conditions. For each mouse, the pancreas was fixed in 4% paraformaldehyde in PBS (Sigma-Aldrich) and stored at 4°C. Splenocytes were placed in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma), 60 mg/L penicillin, 100 mg/L streptomycin, 1 mmol/L sodium pyruvate, and 2 mmol/L l-glutamine (Invitrogen). The spleen cells were isolated by perfusing the tissue with medium and subsequent shredding. The livers were removed and stored at ~80°C for analysis of vitamin A content. The remaining blood was collected and the serum was stored at ~20°C.

Histological examination. For each mouse, the fixed pancreatic tissue was immersed in sequential solutions of 10, 20, and 30% sucrose in PBS over 3 d before freezing in Tissue-Tek OCT embedding medium (Electron Microscopy Sciences). Serial sections were prepared at a thickness of 6 μm using a Leica 3050M cryostat and stained with hematoxylin (Sigma) and eosin Y (Fisher Scientific) to visualize the insulitis present in the islets. Insulitis was scored for each islet as follows: 0, no insulitis; 1, peri-insulitis; 2, <25% insulitis; 3, 25–<50% insulitis; 4, 50–~75% insulitis; and 5, >75% insulitis. For each mouse, the overall insulitis score was calculated by dividing the total score by the number of islets evaluated (24).

Vitamin A content of liver. The livers from 4–5 mice per dietary group were analyzed for vitamin A levels, as described (25). 6-Tocopherol was added as an internal control. The hexane was evaporated in a rotary evaporator and the residue was dissolved in equal parts of acetonitrile and isopropanol. Samples were analyzed by HPLC (Model 2695, Waters Associates) using a 150-mm × 4.6-mm column (Adsorbosphere H5 S5a) with a mobile phase of acetonitrile and water. Retinol was detected by UV absorbance at 325 nm and 6-tocopherol at 298 nm (Model 2487, Waters).

Oxygen radical absorbance capacity assay of serum. The oxygen radical absorbance capacity (ORAC) assay was used to determine the antioxidant capacity of the serum for mice from each dietary group. Serum ORAC values were measured, as described 26. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was used for the standard curve. The loss of fluorescein signal (excitation wavelength = 490 nm, emission wavelength = 515 nm) was monitored for 2 h at 37°C on a SpectraMAX GeminiXS spectrofluorometer using SoftMax PRO v4.0 software (Molecular Devices). Serum trolox equivalents were calculated by comparing the area under fluorescence intensity vs. time curves of trolox standards to serum samples.

Cytokine production by activated splenocytes. Splenocytes were washed and erythrocytes were removed by hypotonic lysis as described (17). Cells were diluted to a concentration of 1 × 10<sup>6</sup>/L in Russ-10...
medium containing 10% fetal bovine serum and plated in a 48-well plate at 1.0 mL/well. The cells were treated with either anti-CD3 plus anti-CD28 antibodies (BD Pharmingen) at a final concentration of 1 mg/L each or with LPS (Sigma) at a final concentration of 10 μg/L and incubated at 37°C in 5% carbon dioxide. After 1 and 3 d, the cells were centrifuged at 200 × g; 10 min at 4°C and supernatants were stored frozen at −20°C for later analysis. Cytokine concentrations in the supernatants were measured in duplicate using the Bio-Rad Multiplex bead array instrument and Luminex cytokine kits from Upstate Biotechnology according to the manufacturer’s protocol. For splenocytes stimulated with anti-CD3/anti-CD28, the supernatants were analyzed for the Th1 and Th2 cytokines, TNF-α, IFN-γ, IL-2, IL-4, and IL-10. For splenocytes stimulated with LPS, the supernatants were analyzed for the monocyte/macrophage cytokines, TNF-α, IL-1β, and IL-6.

Statistics. Statistical analyses were performed with Sigma Stat (version 3.10, Systat Software) and Graphpad software (Graphpad Software). Kaplan-Meier survival curves were used to determine differences in survival (incidence in diabetes). The Mann-Whitney U-test was used to evaluate differences in islet scores among the groups (27). We compared liver vitamin A and serum ORAC scores by 1-way ANOVA using the Holm-Sidak method to adjust for multiple comparisons. Two-way ANOVA was used to determine whether diet or diabetes status affected cytokine production on each day of culture (d 1 and d 3). IL-10 data on d 1 and IL-4 data on d 3 did not have equal variance (vs. C) were made using the Holm-Sidak method when the diet effect was significant. The VA and GP groups were not compared with one another in the post hoc analysis. Values in the text are means ± SEM. Differences were considered as significant at P < 0.05.

Results

Incidence of diabetes and severity of insulitis. Survival of VA and GP mice was >50% greater than that of C mice (P < 0.05) (Fig. 1). Individual islets from diabetic C mice and nondiabetic VA or GP mice (7 mice per group) were given a score for the severity of insulitis (Fig. 2; Supplemental Table 1). The severity of the insulitis was less in nondiabetic mice fed either the GP (2.89 ± 0.24) or the VA (2.74 ± 0.07) diets compared with the diabetic C mice (3.34 ± 0.13) (P < 0.05). The severity of insulitis in diabetic GP, VA, or C mice did not differ.

Liver vitamin A and serum antioxidant capacity. The liver vitamin A concentration was greater (P < 0.001) in the VA mice (5.86 ± 0.68 μmol retinol/g liver) than in the C (2.66 ± 0.08) or GP (3.02 ± 0.29) mice, which did not differ from one another.

Antioxidant capacity was greater in the sera from GP mice (9.62 ± 0.58 mmol trolox equivalents; n = 13) compared with the C mice (7.42 ± 0.26, n = 10, P < 0.05). Serum antioxidant capacity did not differ between the C and VA mice.

Production of cytokines by activated splenocytes. No interactions between diet and diabetes status were observed in splenocytes stimulated with anti-CD3 and anti-CD28, but the concentrations of TNF-α, IFN-γ, and IL-2 were significantly higher in the diabetic mice compared with nondiabetic mice for all 3 groups on d 1 and d 3. IL-10 and IL-4 concentrations were higher in diabetic mice compared with nondiabetic mice for each group on d 1 (data not shown). The only diet effect was a 15% higher IFN-γ concentration in the GP mice compared with the C mice on d 1 (1.32 ± 0.04 vs. 1.15 ± 0.04 μg/L; P = 0.006).

IL-1β concentrations were undetectable in many cultures (data not shown). IL-6 and TNF-α production were higher in diabetic than nondiabetic C mice after stimulation with LPS (Fig. 3; P < 0.05). In addition, on both days, IL-6 concentrations were higher in nondiabetic mice from both the VA and GP groups compared with the C mice, but this difference was not observed in diabetic mice. TNF-α concentrations were reduced in the VA mice on both d 1 and 3 compared with C mice (P < 0.05). TNF-α production was also reduced in the diabetic GP mice on d 1 compared with diabetic C mice (P < 0.05).

Discussion

In this study, we utilized dietary approaches to alter the immune response in the NOD mice to inhibit the progression of type 1 diabetes. We found that dietary interventions with grape powder and high levels of vitamin A inhibited the development of type 1 diabetes in these mice. Diabetes-free survival of the mice over a 6-mo period increased >50% in the groups of mice fed GP or VA compared with the C group. The extent of insulitis correlated with the incidence of diabetes in these groups. The histological analyses of the islets showed that both of these dietary interventions reduced or delayed the infiltration of immune cells into the islets and, thus, resulted in prolongation of life. The presence of insulitis in the islets of mice receiving GP or VA showed that these 2 dietary interventions did not completely inhibit the

FIGURE 1 VA and GP diets increased survival (decreased the incidence of diabetes) of NOD mice compared with the C diet, n = 13/group, P < 0.05.

FIGURE 2 Representative sections showing the different stages of insulitis used to score each NOD mouse for comparison of the severity of insulitis among the dietary groups. Magnification: (A,E), 400×; (B,C), 600×; (D,F), 200×.
significant differences, and cell mass. Diet with high levels of vitamin A did not decrease inflammatory cytokines contribute to the progressive destruction of the beta cells, recruitment of macrophages, and production of inflammatory cytokines. Increases in the Th2 cytokines IL-4 and IL-10 were not observed. We did observe a decrease in TNF-\(\alpha\) production in the monocyte/macrophage population of splenocytes from both the GP and VA groups of mice compared with C. The decrease in TNF-\(\alpha\) production by macrophages could contribute to a decrease in the expression of the chemokines IP-10, RANTES, and MCP-1B, as well as intracellular adhesion molecule-1, which promote the recruitment of immune cells into the islets (5,6). Previous work using this high-level vitamin A diet showed decreased antigen-specific IFN-\(\gamma\) production (18) and such decreases may well have been seen in this study if the appropriate \(\beta\)-cell-specific antigen response could have been examined.

IL-6 is a pluripotent cytokine, primarily produced by the monocyte/macrophage lineage and regulates immune response, hematopoiesis, the acute phase response, and inflammation (29). It is unclear why IL-6 production was elevated in LPS-stimulated splenocytes from nondiabetic mice receiving the VA and GP diets. However, IL-6 has been shown to delay the onset of diabetes and prolong survival of NOD mice overexpressing IL-6 in the \(\beta\) cells (30). Both in vitro analysis and an islet transplantation model showed that IL-6 could protect pancreatic \(\beta\) cells from cell death (31). IL-6 appears to be produced at the greatest level during the recruitment phase of inflammatory cells into the islets (32). Therefore, IL-6 may have been induced as a protective cytokine in the mice receiving the VA and GP diets or production was due to the general delay in the onset of insulitis and diabetes in these groups.

Oxidant molecules have been implicated in damaging the islet \(\beta\) cells and appear to play a role in promoting the pathogenesis of type I diabetes. IFN-\(\gamma\), TNF-\(\alpha\), and IL-1 have been proposed to increase the presence of inducible nitric oxide synthetase in the islets, resulting in increased production of nitric oxide from macrophages (2,33). Nitric oxide, other reactive nitrogen species such as peroxynitrite, as well as reactive oxygen species were found to promote \(\beta\) cell destruction (34–37). Furthermore, increased expression of antioxidant enzymes (catalase, manganese superoxide dismutase, glutathione peroxidase, and thioredoxin) was found to protect \(\beta\) cells from destruction by cytokines in vitro and diabetogenic T cells in vivo (38–40). Although TNF-\(\alpha\) was the only cytokine reduced in the splenocytes from mice fed the GP diet, these mice also displayed an elevation in serum antioxidant capacity. Elevation in antioxidant activity in these mice after prolonged feeding of grape powder may be part of the mechanism of inhibition of diabetes in this group of mice. Quercetin and catechins from grapes and other foods have been shown to protect \(\beta\) cells from oxidative stress (14,15). Local accumulation of polyphenolic antioxidants in the highly vascularized islet could protect the \(\beta\) cells from oxidative damage induced by proinflammatory cytokines from activated T cells and macrophages. It is interesting that quercetin and anthocyanins have also been shown to enhance insulin secretion and sensitivity both in vitro and in diabetic rodents (41,42). Our hypothesis was that the polyphenols in the grapes would inhibit diabetes. Further experiments are warranted to determine whether these components of grapes are responsible for reducing the insulitis and increasing survival of the NOD mice.

The inhibition of inflammation, increasing insulin secretion and/or sensitivity, and preservation of \(\beta\) cell mass are critical components in the prevention and treatment of type I diabetes. Intervention strategies for treatment of type I diabetes have included immunotherapies with monoclonal antibodies, immunosuppression with glucocorticoids, calcineurin inhibitors, and azathioprine, and immunomodulatory therapies, such as cytokine administration, plasmapheresis, and adoptive cellular gene pathogenesis of type I diabetes in these mice. However, the increased survival of the mice in the GP and VA groups strongly suggests that dietary interventions can play an important role in the regulation and progression of this disease.

Although no other studies have, to our knowledge, examined the effect of high-level vitamin A on development of type I diabetes in an animal model, I previous study did examine the effect of vitamin A deficiency on development of diabetes in BioBreeding/Worcester rats (28) and found that deficiency decreased the occurrence of diabetes relative to a control diet similar to the one used here. The deficient group in that study had a high mortality rate resulting from the deficiency itself before diabetes developed, but a retinoic acid-treated control group that was also considered vitamin A-deficient also had a reduced incidence of diabetes. Because vitamin A deficiency can directly impair cytotoxic T cell responses (16), it is plausible that both vitamin A deficiency and excess can affect the development of diabetes via different effects on the \(\beta\)-cell-specific immune response.

Type I diabetes is an autoimmune disease mediated by CD4+ and CD8+ T lymphocytes that are specifically reactive with antigens on the \(\beta\) cells in the pancreas. Infiltration of T lymphocytes, recruitment of macrophages, and production of inflammatory cytokines contribute to the progressive destruction of the \(\beta\) cell mass. Diet with high levels of vitamin A did not decrease the production of the Th1 proinflammatory cytokines TNF-\(\alpha\) and IFN-\(\gamma\) in the splenocyte population. Vitamin A was used in these experiments due to its ability to induce a switch from Th1 to Th2 lymphocytes. Increases in the Th2 cytokines IL-4 and IL-10 were not observed. We did observe a decrease in TNF-\(\alpha\)
therapy (43). However, none of these therapies has produced long-term remissions in patients with newly diagnosed disease and a number of therapies have several side effects. Dietary intervention with foods or food constituents may prove to be beneficial in the prevention and/or management of type 1 diabetes. Increasing polyphenol or vitamin A levels in the diet may have profound effects on suppressing inflammatory immune cells and reducing the oxidative damage in the islets that contributes to loss of β cells. Furthermore, dietary interventions such as those in this study may be useful for treatment of other autoimmune and inflammatory diseases.

**Literature Cited**


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