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The immune responses to diabetes in BB rats supplemented with vitamin A

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A substantial amount of evidence suggests that in type I diabetes, vitamin A and zinc status could be of concern because of their impaired metabolic availability. Because both vitamin A and zinc play important roles in the regulation of immune function, the present study was undertaken to examine the immune responses to vitamin A and zinc supplements in diabetic-prone Bio-Breed rats (BBdp), and if the supplements increase the incidence of diabetes. Weanling BBdp rats were fed a NIH-07 diet supplemented with vitamin A either alone or in combination with zinc up to 120 days of age. A greater percentage of rats developing diabetes was found in rats that had supplements of vitamin A and zinc (67%) than those on the basal diet (55%) or with vitamin A supplementation alone (50%). The B cells and macrophages were all markedly increased, whereas CD_4^+ and CD_8^+ T cells were decreased at the onset of diabetes. However, this immune status was not changed by vitamin A and zinc supplements. The plasma vitamin A levels were significantly decreased in the presence of diabetes and the vitamin A status did not improve when the rats were given vitamin A and zinc supplements. The Natural Killer cell cytotoxicity on a per-cell basis was significantly decreased in the presence of diabetes, irrespective of supplements with vitamin A and zinc. Overall, results indicated that vitamin A and immune status are both affected by type I diabetes; these effects, however, are not responsive to supplemental intakes of vitamin A either alone or in combination with zinc. (J. Nutr. Biochem. 11:515–520, 2000)

Keywords: BB rats; type I diabetes; vitamin A; zinc; immune response

Introduction

Vitamin A (retinol) is an essential nutrient required for normal growth, reproduction, vision, and immune function.[1] The role of vitamin A in maintaining immune function has been broadly studied within the last two decades. Both clinical and experimental studies have shown that vitamin A deficiency is associated with decreased resistance to infection, leading to the breakdown of epithelial barriers or changes in immune system defenses that normally counteract environmental pathogens.[2,3] On the other hand, vitamin A administration markedly stimulates the cellular immune responses and nonspecific immunity including cytokine production, lymphocytes transformation, and phagocytosis in normal animals.[4] The mechanism of retinol in altering immune function has not been established. It has been suggested that the immune-stimulating effect of vitamin A is mediated through its metabolites (e.g., 14-hydroxy-retro-retinol), which may play a role in lymphocytes proliferation, signaling, and activation. Retinoic acid is another metabolite that has been demonstrated to induce the T-cell apoptosis by binding to both retinoic acid receptor and retinoid X receptor, which become transcriptionally active upon ligand binding and transactivate their target genes.[5]

An impairment of vitamin A metabolism has been identified in type I diabetes patients and streptozotocin (STZ)-induced diabetic rats.[6,7] Depressed circulatory levels in association with elevated hepatic concentrations of vitamin A in STZ-induced diabetic rats were returned to normal only by insulin treatment, but not dietary vitamin A supplementation alone.[8,9] The metabolic availability of vitamin A has also been found to be affected in Bio-Breed (BB) rats,[10] which like humans, spontaneously develop autoimmune diabetes.[11] On the other hand, vitamin A deficiency has been shown to markedly reduce the incidence of diabetes

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Table 1 Composition of NIH-07 diet*

Ingredient	g/kg diet
Dried skim milk	50
Fish meal	100
Soybean meal	120
Alfalfa meal	40
Corn gluten meal	30
Ground corn	245
Ground wheat	230
Wheat middling	100
Brewers dried yeast	20
Dry molasses	15
Soybean oil	25
Sodium chloride	5
Calcium phosphate	12.5
Ground limestone	5
Pre-mixes†	2.5

*NIH-07 diet contains (g/kg diet): protein 215; carbohydrate 514; fat 52; fiber 32; water 125 (provided by supplier for lot number used).
†Pre-mixes content of diet (per kg diet): vitamin A 6,050 IU; vitamin D_3 5,060 IU; vitamin K 3.1 mg; tocopheryl acetate 22 IU; choline 0.6 g; folic acid 4 mg; niacin 33 mg; d-pantothenic acid 20 mg; riboflavin 3.7 mg; thiamin 11 mg; vitamin B_{12} 4 μg; pyridoxine 1.9 mg; biotin 0.15 mg; cobalt 44 mg; copper 4.4 mg; iron 132 mg; manganese 6 mg; zinc 18 mg; iodine 1.5 mg.
Vitamin A supplement, 60.5 IU/g diet. Zinc supplement, 180 mg/kg diet.

and insulitis in BB rats.[12] Thus, the role of vitamin A in autoimmune-mediated diabetes needs to be elucidated.

Using BB rats, the present study was designed to explore the effect of vitamin A on the incidence of diabetes, the vitamin A status, and the immune indices including the Natural Killer (NK)-cell cytotoxicity and mononuclear cells phenotypes. Zinc is an important factor for the maintenance of vitamin A homeostasis;[13] like vitamin A, this trace element is thought to promote immune function.[14] The present study was therefore extended to investigate the synergistic effects of vitamin A and zinc in terms of their action in promoting immune functions and the occurrence of diabetes.

Methods and materials

Animals and diets

Diabetes-prone BB (BBdp) and non-diabetes-prone (BBn) BB rat dams originally from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, Ontario, Canada), were obtained from the Department of Agricultural, Food, and Nutritional Science of the University of Alberta breeding colony. Approximately 50–80% of the BBdp rat lines develop type I diabetes within 60–120 days after birth, whereas BBn rat lines do not develop diabetes spontaneously. Animals were housed in a temperature and humidity-controlled room with a 12-hr light/dark cycle.

BBn rats receiving the NIH-07 diet (Zeigler Brothers Inc., Gardmers, PA USA) (*Table 1*) were used as controls (Group 1). Weanling (21-day-old) BBdp rats from each litter were randomly distributed to three treatment groups of 12 rats each. Group 2 received the NIH-07 diet only. Groups 3 and 4 received the NIH-07 diet supplemented with retinyl palmitate (60.5 IU/g diet) either alone or in combination with zinc (180 μg/g diet), respectively. All animals were killed within 24 hr after the onset of diabetes.

Food intake and body weights were monitored throughout the study. BBdp rats older than 50 days of age were tested for glycosuria by Testape (Eli Lilly, Indianapolis, IN USA) three times a week. Glucose levels were determined in blood samples taken from the tail vein (Glucometer II, Ames Miles, Toronto, Canada). Diabetes was diagnosed on the basis of glycosuria $> 2+$ and subsequently, hyperglycemia (blood glucose > 11 mmol/L). When these animals manifested hyperglycemia, they were described as BBd rats. Animals were killed using carbon dioxide after an overnight fast, and blood was collected in heparinized tubes. To avoid light-induced oxidation of vitamin A, separated plasma was protected from light and stored at −20°C. Spleens were removed and cells were isolated under sterile conditions as previously described.[15] Livers were removed and immersed in liquid nitrogen and stored at −72°C for later analysis.

Vitamin A determination

Plasma[16] and liver[17] vitamin A were assayed by high performance liquid chromatography (HPLC) as described by Tuitoek et al.[9] Chromatography was performed on a LC-18 (15.0 cm × 4.6 mm, Supelco, Mississauga, ON, Canada) reverse phase column with 3 μm packing, with a mobile phase consisting of methanol/water (95:5, v/v). Detection was carried out by UV absorption at 325 nm. Quantification was obtained by the injection of known amounts of retinol and calculations with the use of Shimadzu Class-VP Chromatography Data System (Schimadzu, Columbia, MD USA). The standard added to plasma, taken through the extraction procedure, and injected into the HPLC resulted in the recovery of 82–88%.

Preparation of splenocytes

Splenocytes were prepared by pressing slices through a nylon mesh in cold Kreb-Ringe Hepes Buffer (pH 7.4) supplemented with 0.5% (w/v) bovine serum albumin (Fraction V; Gibco, Burlington, ON, Canada), as previously described.[15] Cell viability was assessed by Trypan Blue exclusion.

Mononuclear cell phenotyping

Mononuclear cell subsets from spleen were characterized by an immunofluorescence assay[15] using supernatants from hybridomas-secreting mouse monoclonal antibodies specific for the different rat mononuclear cell subsets. The following monoclonal antibodies were used: OX12, which recognizes a determinant on the rat kappa chain of immunoglobulins on B lymphocytes; OX42, which reacts with a receptor found on most monocytes, granulocytes, and macrophages; 3.2.3, which reacts with rodent NK cells; OX19, which recognizes a glycoprotein on the surface of thymocytes and T-lymphocytes (CD5); W3/25, which recognizes a surface glycoprotein found on rat T-helper cells (CD4); and OX8, which recognizes T cytotoxic/suppressor lymphocytes (CD8) and NK cells.

For identifying B lymphocytes, T lymphocytes, macrophages, and NK cells, one-color staining method was used. Briefly, aliquot of cells was incubated for 30 min at 4°C with OX12, OX19, OX42, and 3.2.3, respectively, and then washed three times in 200 μL of PBS containing fetal calf serum (40 g/L) and further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FIT-C) for another 30 min. Two-color staining method was used to identify T lymphocytes subset. OX19 and W3/25 recognize rat T-helper cells (CD4), OX19 and OX8 recognize T cytotoxic/suppressor lymphocytes (CD8), and OX8 and W3/25 recognize $CD4^+CD8^+$ T cells. Aliquot of cells was incubated with first monoclonal antibodies (OX19, OX19, and OX8). After FIT-C binding and washing, second phenotypic antibodies (W3/25, OX8,

and W3/25) were added, incubated, and washed as before. Finally, 10 μL of a 1:25 dilution of phycoerythrin-conjugated goat-anti-mouse IgG (PE) was added to each well and incubated for 30 min at 4°C.

After the last incubation with antibody in both one-color and two-color staining, cells were washed three times with assay buffer and fixed in PBS containing 1% paraformaldehyde and analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA, USA) according to relative fluorescence intensity. Resulting percentages were corrected for background fluorescence as determined by incubating cells with FIT-C or PE only.

NK-cell cytotoxicity

NK-cell cytotoxicity was measured using 4-hr ^{51}Cr release assay as previously described[15] in splenocytes isolated from BBd and BBn rats. Briefly, the NK-sensitive YAC-1 cells were incubated with (^{51}Cr) sodium chromate and seeded into 96-well V-bottom microtiter plates. Triplicate splenocytes were then added to establish different effector-to-target ratios, which were between 2:1 to 100:1. After 4-hr incubation at 37°C and brief centrifugation, 75 μL of the supernatant was counted in a Gamma counter (Beckman gamma 8000, Beckman Instruments, Mississauga, ON, Canada) to determine the extent of target cell lysis. Spontaneous release was determined by incubation of target cells in the absence of effector cells. Maximum release was determined for target cells incubated with detergent. The NK-cell cytotoxic activity was calculated as percent specific lysis = 100 × ([experimental release of ^{51}Cr − spontaneous release]/[maximum release − spontaneous release]). Results were also expressed as lytic units (LU) on a per cell basis using the number of NK cells present as determined by immunofluorescence assay using monoclonal antibody 3.2.3. One LU is the number of effector cells ($\times 10^{-3}$) required to cause 20% lysis of target cells.

Statistical analysis

Statistical analyses were performed by SAS computer program (Version 6.12, SAS Institute, Cary, NC USA). The level of significance was set at $P < 0.05$. Data were analyzed by two-way ANOVA that included the effect of gender. If no effect of gender was found, groups classified according to different treatments were compared using one-way ANOVA. Multiple comparisons were carried out using Student-Newman-Keuls test. The NK-cell cytotoxicity was analyzed by a one-way split-plot procedure. Fisher's exact test was used to analyze the cumulative incidence rate of diabetes.

Results

All BBd rats exhibited the characteristic signs of diabetes, including elevated blood, urinary glucose, water intake, and volume of daily urinary excretion. No significant differences in mean body weight gain and food intake were observed among the groups. The onset age of diabetes was similar in BBdp rats receiving basal diet and in rats fed the diet supplemented with vitamin A either alone or in combination with zinc (*Table 2*). The incidence rate of diabetes in these animals was 55%, 50% and 67%, respectively; however, the differences among them were not statistically significant.

All BBd groups had decreased plasma levels of vitamin A compared to those of BBn rats (*Figure 1*). There were no differences in plasma vitamin A levels between BBd groups, irrespective of the treatment. However, supplementation with vitamin A alone or in combination with zinc resulted in increased hepatic concentrations of vitamin A (*Figure 2*).

Dietary treatment did not significantly affect spleen cell subset distribution in BBd rats (*Table 3*). Calculated as percent specific lysis, the NK-cell cytotoxicity for splenocytes was significantly greater in all BBd rats than in BBn rats (*Figure 3*). Lytic unit, which expressed on a per cell

Table 2 Rate and onset age of diabetes in BBdp rats receiving NIH-07 diet supplemented with vitamin A either alone or in combination with zinc treatment

Group	Number	Incidence rate (%)*	Age of onset of diabetes (days)†
NIH-07	12	55%	96.2 ± 3.6
NIH-07 + vitamin A	12	50%	97.8 ± 5.3
NIH-07 + vitamin A + zinc	12	67%	94.1 ± 1.4

*Cumulative diabetes incidence by 120 days of age. Significant difference was analyzed by Fisher's exact test.
†Results are expressed as means ± SEM.

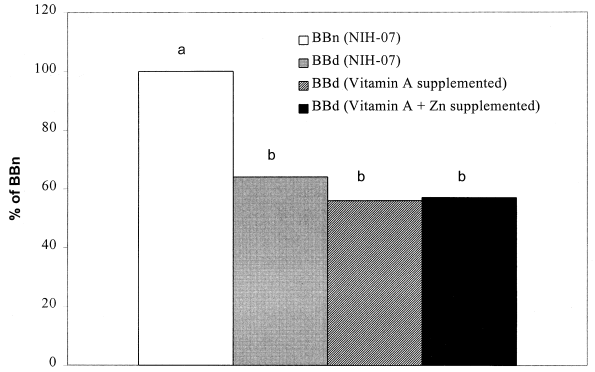

Figure 1 Effect of vitamin A supplementation and vitamin A plus zinc supplementation on the plasma vitamin A levels of BBd rats. Results are expressed as means of at least five animals. Letters not shared show significance ($P < 0.05$).

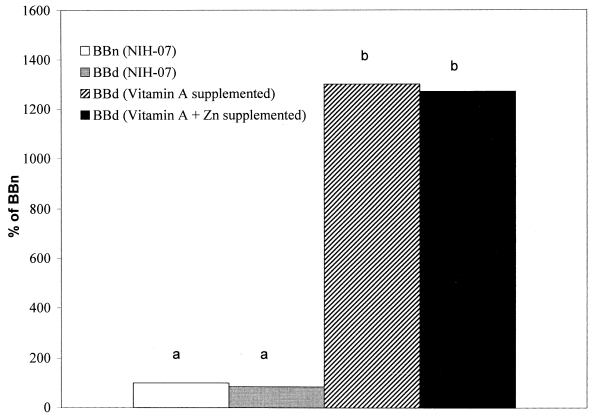

Figure 2 Effect of vitamin A supplementation and vitamin A plus zinc supplementation on the liver total vitamin A levels of BBd rats. Results are expressed as means of at least five animals. Letters not shared show significance ($P < 0.05$).

Table 3 Effect of supplementation of vitamin A either alone or in combination with zinc on monoclonal antibody-identified phenotypes in rat spleen

	BBn	BBd		
	NIH-07 only	NIH-07 only	NIH-07 + vitamin A	NIH-07 + vitamin A + Zn
B cells ($OX12^+$)	25.8 ± 2.3^a	60.4 ± 3.0^b	58.2 ± 2.6^b	58.6 ± 2.0^b
T cells ($OX19^+$)	55.2 ± 1.7^a	4.8 ± 0.8^b	5.2 ± 0.9^b	5.5 ± 0.6^b
$CD4^+$ ($OX19^+$ $W3/25^+$)	33.1 ± 1.1^a	2.4 ± 0.5^b	3.0 ± 0.8^b	2.4 ± 0.4^b
$CD8^+$ ($OX19^+$ $OX8^+$)	15.0 ± 1.5^a	0.6 ± 0.1^b	0.9 ± 0.2^b	1.0 ± 0.1^b
$CD4^+CD8^+$ ($W3/25^+$ $OX8^+$)	4.0 ± 1.2^a	0.7 ± 0.1^b	0.9 ± 0.2^b	1.0 ± 0.1^b
NK cells ($3.2.3^+$)	3.5 ± 0.4	3.7 ± 0.4	4.2 ± 0.8	4.1 ± 0.6
Macrophages ($OX42^+$)	11.0 ± 0.9^a	18.3 ± 4.8^b	19.0 ± 3.7^b	20.0 ± 3.3^b

Values are means ± SEM of at least four animals. Within each parameter, letters not shared show significance ($P < 0.05$).
BBn–non-diabetes-prone Bio-Breed rats. BBd–diabetic Bio-Breed rats. NK–Natural killer.

basis, was not affected by diet and was lower for all BBd rats compared to BBn rats (*Figure 4*).

Discussion

Type I diabetes is an autoimmune disease resulting in a progressive destruction of the beta cell of the islets of Langerhans.[18] According to clinical and animal studies, both genetic susceptibility and environmental factors appear to contribute to the clinical expression of type I diabetes. Recently, studies using animal models for type I diabetes such as BB rats have provided an opportunity for enhancing our understanding of pathogenic mechanisms for the disease with particular reference to environmental factors.[19] Dietary factors have been shown to be major determinants of diabetes development in BB rats.[20] It has been shown that replacing cereal-based rodent chow with a number of defined semi-purified, purified, and elemental diets decreases the incidence of diabetes in these animals.[21] Dietary protein source seems to be the most important factor, and intact protein appears to be required for full expression of type I diabetes in these rats. However, the effect of micronutrients, which are essential for normal immune function on the expression of the diabetic syndrome in BB rats has not been well studied. In addition, most of the protein sources tested to date were impure and contained various levels of micronutrients.[22] Thus, the impact of micronutrients on the pathogenesis of diabetes can not be neglected. A recent study reported that vitamin A-deficient BB rats were associated with a marked reduction in both insulitis and the development of clinical diabetes, linking the micronutrient and type I diabetes.[12] Vitamin A plays an important role in the regulation of immune function. However, the immune suppression due to vitamin A deficiency, or acting as an immune stimulator via vitamin A supplementation, are two different approaches. Therefore, the impact of vitamin A on the stimulation of immune response and, consequently, the development of type I diabetes in BB rats was explored in this study.

Our study confirmed previous observations in STZ-induced diabetic rats[8,9] and type I diabetic patients[6] that the presence of a hyperglycemic state affects vitamin A metabolism, as indicated by decreased levels of plasma vitamin A

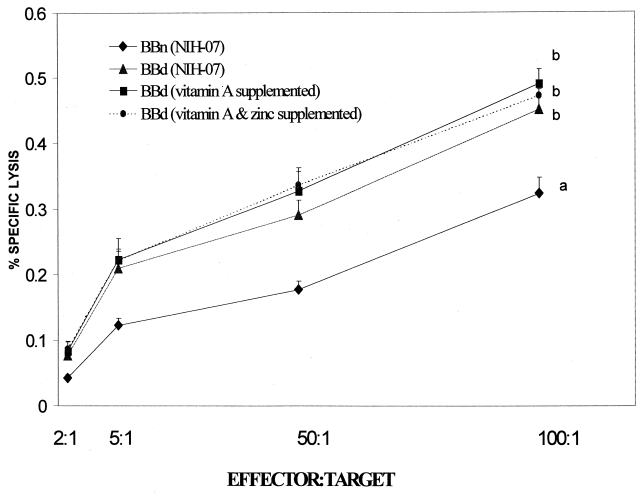

Figure 3 Effect of vitamin A supplementation alone or in combination with zinc on NK-cell cytotoxic activity in the rat spleen, which is expressed as percent specific lysis = 100 × ([experimental release of ^{51}Cr − spontaneous release]/[maximum release − spontaneous release]). Values are mean ± SEM of at least five animals. Lines that do not share a common letter are significantly different at $P < 0.05$.

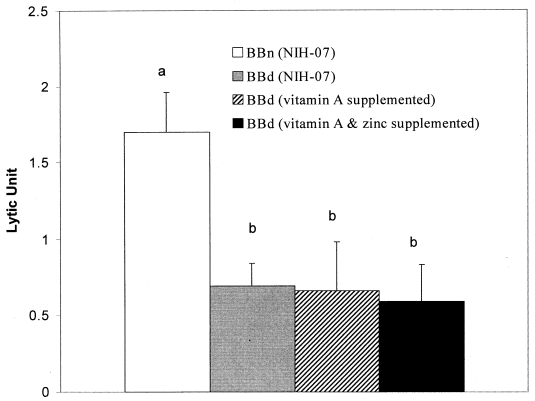

Figure 4 Effect of vitamin A supplementation and vitamin A plus zinc supplementation on NK-cell cytotoxic activity in the rat spleen, which is expressed as lytic units (LU) on a per-cell basis. One LU is the number of NK cells (10^{-3}) required to cause 20% lysis of target cells. Values are mean ± SEM of at least five animals. Bars that do not share a common letter are significantly different at $P < 0.05$.

at the clinical onset of diabetes in BB rats. Neither vitamin A supplementation alone nor its combination with zinc reversed this reduced level of circulatory vitamin A in BBd rats. The control levels of total hepatic storage of vitamin A in BBd rats without dietary vitamin A supplementation suggest that depressed plasma levels of vitamin A in BBd rats are unlikely due to lack in intestinal absorption. This is in agreement with a previous study involving STZ-induced diabetic rats that found intestinal absorption of vitamin A is unaffected in the presence of diabetes.[23] Vitamin A supplement-associated increase in its hepatic storage without changing the decreased plasma vitamin A was suggested to be caused by its impaired metabolic availability.

Vitamin A deficiency affects the nonspecific arm of the immune system, possibly by altering the functional capacity of cells to produce lymphokines needed for the generation of an appropriate cytolytic response.[4] Vitamin A supplementation to BB rats in our study tended to increase the number of NK cells without changing the cytotoxicity per NK cell. Vitamin A has been shown to be essential for maintaining basal NK-cell number and activity, but not for NK-cell activation.[24] In BB rats, beta cell is destroyed through an inflammatory process, in which NK cells are a major cell population present in the inflamed islet during the prediabetes period.[25] In addition, BBdp rats have a relative increase in the number and activity of NK cells in blood and spleen.[26] However, a study using 3.2.3, a monoclonal antibody that selectively depletes NK cells, found that incidence of diabetes in 3.2.3-treated and control BB rats was similar.[27] This finding may implicate that NK cells may be involved in the pathogenesis of diabetes, but are not necessary for autoimmune islet destruction.

In our BBdp rats, the incidence of diabetes tended to be higher in the presence of vitamin A and zinc supplementation than in those without any supplementation or with vitamin A supplementation alone. This was not associated with any changes in NK-cell cytotoxicity, nor in phenotypes in spleen. Zinc is a crucial nutritional component required for normal development and maintenance of the immune functions.[14] Zinc supplementation has been shown to induce growth of the thymus as well as to increase the production of thymulin, a thymic hormone promoting development and differentiation of T-lymphocytes.[28] Currently, most evidence supports T cells as playing a central role in the pathogenesis of diabetes in the BB rat. This may explain the higher incidence of diabetes in BB rats when supplemented with vitamin A plus zinc. However, the biological function of zinc supplementation to these animals is difficult to interpret because type I diabetes is at a risk of developing zinc deficiency and that the diabetic state might be involved in the modulation of immune system. Further study may be needed to address the effect of dietary zinc supplementation to BB rats on T-lymphocytes differentiation and activation.

In summary, the expression of autoimmune diabetes in BB rats is associated with decreased circulatory levels of vitamin A, abnormal NK-cell cytotoxicity, and splenocyte phenotypes. Neither vitamin A supplementation alone nor its combination with zinc changed this depressed circulatory vitamin A. The reason for this diabetes-associated vitamin A abnormality remains unknown. It is noteworthy that BBdp rats on the zinc-supplemented diet showed a trend toward a higher diabetes incidence compared with that of BBdp rats on a diet without zinc supplementation. More studies are needed to examine the effects of vitamin A and zinc on T-lymphocytes differentiation and activation and, consequently, the disease development.

Acknowledgments

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