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UNIVERSITY OF ALBERTA

AN IMPAIRMENT IN METABOLIC AVAILABILITY OF VITAMIN A IS ASSOCIATED WITH THE ONSET OF DIABETES IN BB RATS

BY

JING LU ©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN
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IN

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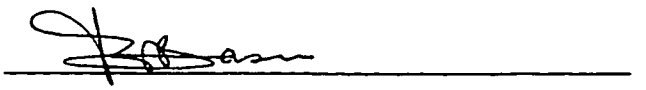
FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **AN IMPAIRMENT IN METABOLIC AVAILABILITY OF VITAMIN A IS ASSOCIATED WITH THE ONSET OF DIABETES IN BB RATS** submitted by JING LU in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in NUTRITION AND METABOLISM.



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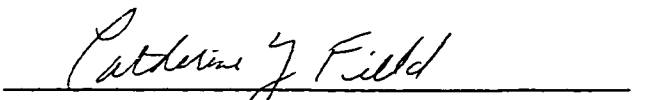

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Date January 20, 1999

ABSTRACT

The objective of this study was to investigate the metabolic availability of vitamin A (retinol) in biobreeding (BB) rats, in which diabetes mellitus develops spontaneously with many features resembling human type I diabetes. Impaired metabolic availability of vitamin A was observed in diabetic BB (BBd) rats, as evidenced by the decreased concentrations of circulatory retinol and its carrier proteins, retinol-binding protein (RBP) and transthyretin (TTR) in both the plasma and the liver, compared to those of their non-diabetic (BBn) control counterparts. A parallel decrease in the abundance of RBP mRNA in the liver of BBd rats suggests that a reduction in RBP synthesis may account for the decreased plasma retinol concentrations. Furthermore, the biochemical status of zinc, an important factor for the synthesis of RBP, was also found to be affected in BBd rats, as was indicated by a decreased circulatory level and an increased urinary concentration of this trace element. In order to determine if the biochemical evidence of vitamin A deficiency in BBd rats could be reversed, weanling diabetes-prone BB (BBdp) rats were fed a diet supplemented with vitamin A either alone or in combination with zinc; or treated subcutaneously for one week with insulin following the presence of hyperglycaemia. None of these treatments improved the reduced levels of circulatory vitamin A. It was, however, noteworthy that the hepatic abundance of RBP mRNA was significantly increased in the presence of zinc supplementation. Overall, these results suggest that an impaired metabolic availability of vitamin A, possibly caused by its decreased transport from its hepatic stores, is another metabolic derangement associated with type I diabetes. It is likely that zinc could be an important factor in preventing this metabolic abnormality.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenease
ARAT	Acyl coenzyme A: retinol acyltransferase
BB	Biobreeding
BBd	Diabetic biobreeding
BBdp	Biobreeding diabetes prone
BBM	Brush border membrane
BBn	Biobreeding non-diabetes prone
BSDREH	Bile salt-dependant retinyl ester hydrolases
BSIREH	Bile salt-independant retinyl ester hydrolases
cDNA	Complementary deoxy nucleic acid
CRABP	Cellular retinol acid binding protein
CRALBP	Cellular retinaldehyde binding protein
CRBP	Cellular retinol binding protein
CRBP(II)	Cellular retinol binding protein type II
FITC	Fluorescein isothiochanate-conjugated goat anti-mouse IgG
GAD	Glutamic acid decarboxylase
HPLC	High-performance liquid chromatography
IAA	Autoantibody to insulin
ICA	Islet cell antibody
IRBP	Inter-photoreceptor retinoid-binding protein
IU	International unit

LRAT	Lecithin: retinol acyltransferase
LU	Lytic unit
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK	Natural Killer
PE	Phycoerythrin-conjugated goat anti-mouse IgG
PEM	Protein energy malnutrition
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
RBP	Retinol binding protein
RE	Retinol equivalent
REH	Retinyl ester hydrolase
RPE	Retinal pigment epithelial cell
SAS	Statistical analysis system
SSC	Standard saline citrate
SEM	Standard error of mean
STZ	Streptozotocin
TTR	Transthyretin

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterised by hyperglycaemia resulting from defects in insulin secretion, action, or both (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1998). It is accompanied by significant nutritional alterations. Vitamin A, an essential nutrient required for normal growth, reproduction, vision and immune function, has been suggested to be of concern in uncontrolled type I diabetes.

Decreased levels of plasma vitamin A and its carriers, retinol-binding protein (RBP), accompanied by an increased urinary excretion of RBP, have been identified in human subjects with type I diabetes (Basu et al. 1989; Dubrey et al. 1997). The metabolism of Zn, an important factor for the synthesis of RBP, has also been found to be affected in the presence of diabetes as indicated by hyperzincuria (Heise et al. 1988; Cunningham et al. 1994). Studies involving Streptozotocin (STZ)-induced diabetic rats have shown that plasma levels of retinol, as well as 11-cis-retinal (an important component of rhodopsin) concentrations in the retina of the eye, are reduced in diabetes, while the hepatic storage of vitamin A is markedly elevated (Tuitoek et al. 1996a). For these rats, insulin treatment, rather than a diet supplemented with vitamin A, normalised metabolic availability of vitamin A (Tuitoek et al. 1996a & c), indicating a linkage between vitamin A status and insulin in diabetes. Indeed, vitamin A has been reported to be necessary for normal insulin secretion (Chertow et al. 1987).

Most studies linking vitamin A metabolism and type I diabetes have been carried out in STZ-induced diabetic rats. STZ, a nitrosourea derivative, is known to be toxic to the

liver and kidney (Chang 1981; Perloff et al. 1995), the two major sites involved in vitamin A metabolism. Hence, it seems possible that the impaired metabolic availability of vitamin A in these animals is a reflection of STZ-associated toxicity rather than diabetes. The present study was therefore undertaken to further investigate the metabolic availability of vitamin A in Biobreeding (BB) rats, which develop diabetes spontaneously. This genetically diabetic rat is considered a better model of human disease resulting from autoimmune attack, which is the aetiology of type I diabetes (Crisa et al. 1992). Using BB rats, the biochemical status of vitamin A, vitamin A carrier proteins (e.g., RBP and Transthyretin (TTR)) and zinc were determined at the onset of diabetes. The influence that factors such as supplementation of vitamin A and zinc as well as the administration of insulin may have on the vitamin A status of these animals was examined. In addition, the immune responses to these treatments including Natural Killer cell (NK) cytotoxic activity and mononuclear cell phenotyping in the spleen, two immune parameters relevant to the pathogenesis of diabetes in BB rats, were also studied.

1.1 VITAMIN A METABOLISM

Vitamin A refers to all compounds exhibiting the biological activity of retinol (Blomhoff et al. 1992). It occurs physiologically as the alcohol (retinol), the aldehyde (retinaldehyde), the acid (retinoic acid) and the ester (retinyl palmitate) (Figure 1-1).

Vitamin A is provided in a diet mainly as retinyl esters (e.g., retinyl palmitate), which are hydrolysed to retinol in the intestine. In the enterocytes, the free retinol is

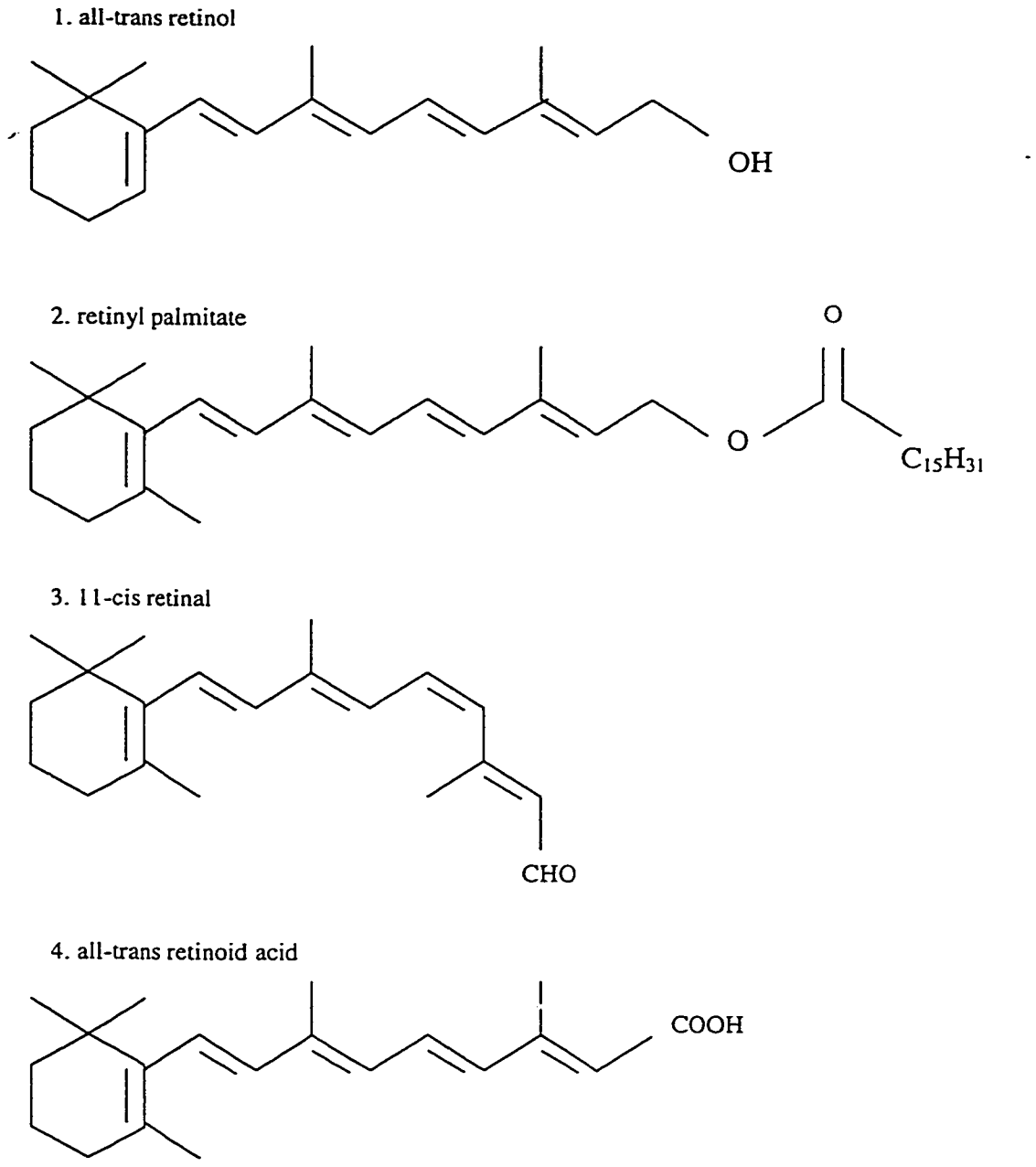


Figure 1-1. Structural formulae of some biologically active and naturally occurring forms of vitamin A

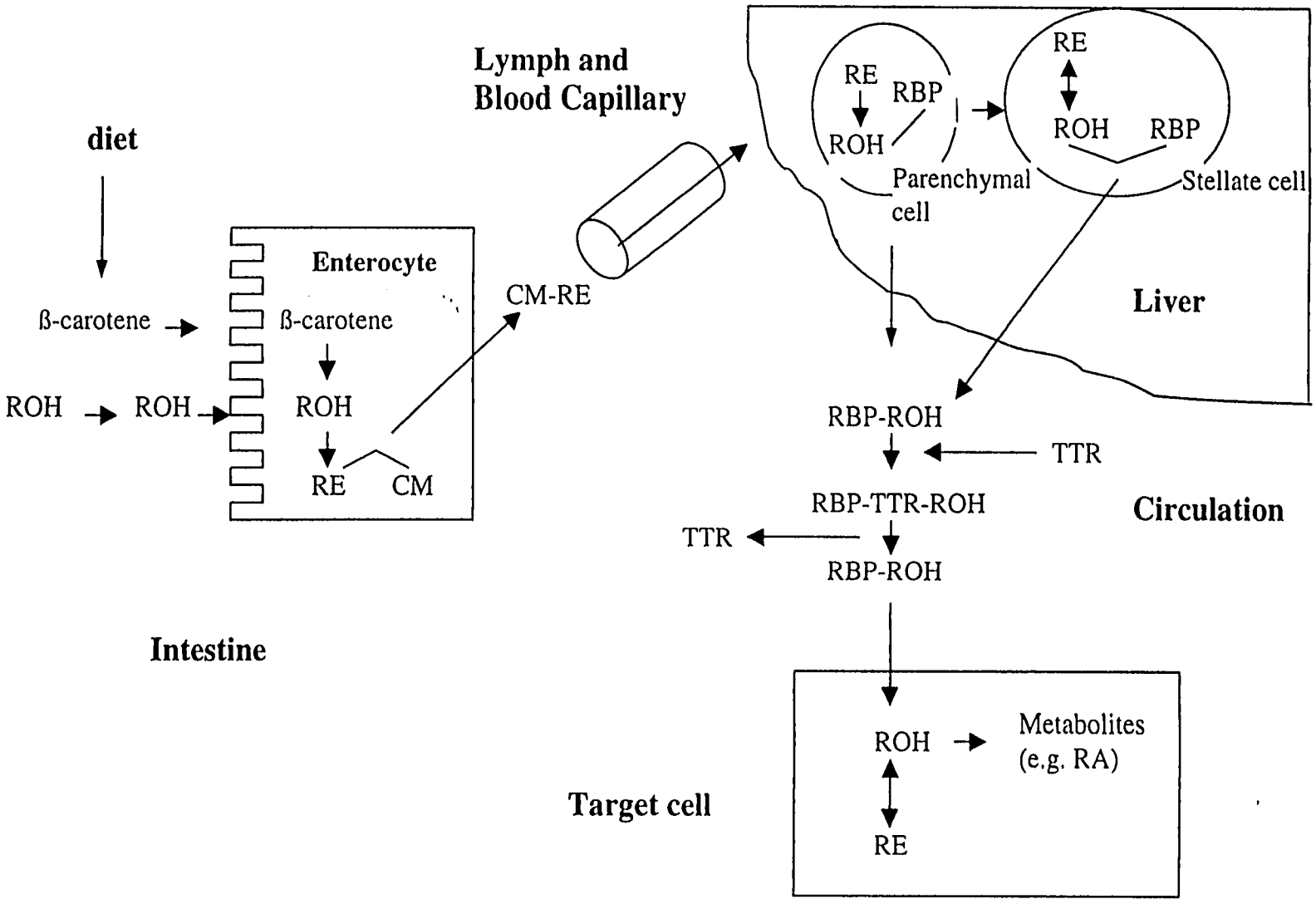
reesterified and incorporated into chylomicrons, which are subsequently transported to the liver, where it is predominantly stored in its ester form (Blaner et al. 1994). When required, retinyl esters are re-hydrolysed to retinol and subsequently carried by RBP (molecular weight 21 000 Da) into the circulation. In the blood, the retinol-RBP complex further binds to TTR (molecular weight 55 000 Da), the ratio being 1:1:1 (Soprano et al. 1994); this complex is then transported to the target tissue (Figure 1-2). Circulatory levels of retinol are homeostatically regulated over a wide range of dietary intake. The details of vitamin A metabolism are given in the following sections.

1. 1. 1 ABSORPTION

Vitamin A is provided in a diet either as provitamin A—carotenoids, or as preformed vitamin A--retinyl esters. The dietary carotenoid sources include fruits and leafy green or yellow vegetables, while retinyl ester sources include various dairy products, liver and fish oil. Approximately 80 to 90% of retinyl esters and 50% of carotenoids are absorbed by humans (Blomhoff et al. 1991; Basu 1996). Conversion of beta-carotene to vitamin A is highly regulated so that excess vitamin A is not absorbed from carotene sources.

After foods are ingested, retinyl esters and carotenoids are released from proteins by the action of pepsin in the stomach and proteolytic enzymes in the small intestine. At least three enzymes involved in the digestion of dietary retinyl esters have been identified: 1) pancreatic lipase; 2) pancreatic carboxyl ester lipase; and 3) one or more retinyl ester hydrolases associated with the brush-border membrane (Harrison et al.

Figure 1-2. Major pathways of vitamin A absorption, transport, storage and metabolism. ROH, retinol; RE, retinyl esters; RA, retinoic acid; CM, chylomicrons; RBP, retinol binding protein; TTR, transthyretin.



1993). Each of these enzymes seems to play a role through its abilities to interact with retinyl esters in different physico-chemical forms. However, the relative role of these enzymes in the digestion of the retinyl esters remains to be determined. Beta-carotene is oxidised in the cytoplasm of the intestinal cells before further reduction to retinol and esterification to retinyl esters. The conversion of beta-carotene to retinol in the enterocytes involves central oxidative cleavage of carotene to retinal by the beta-carotene-15,15'-dioxygenase and reduction of retinal to retinol by retinal reductase (Blaner et al. 1994).

In the mucosa, the resulting retinol (including that which is hydrolysed from retinyl ester and reduced from beta-carotene) is re-esterified and further incorporated into chylomicrons together with triacyglycerols. Two enzymes, acyl coenzyme A: retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT), have been recognised to be involved in the esterification of retinol in enterocytes. It has been suggested that LRAT requires the binding of cellular retinol binding protein type II (CRBP II) to retinol for the esterification reaction, whereas ARAT esterifies free retinol (MacDonald et al. 1988). Therefore, it has been suggested that esterification via LRAT occurs during the absorption of a normal load of retinol, while ARAT seems to be involved in esterification when a large amount of retinol is ingested and CRBP II becomes saturated.

1. 1. 2 TRANSPORT AND STORAGE

After esterification, retinyl esters are packaged with lipid and other fat-soluble vitamins into chylomicrons, which are transported via the thoracic duct lymph into the general circulation and to the liver for metabolism and storage.

In circulation, chylomicrons undergo lipolysis, catalysed by lipoprotein lipase, resulting in chylomicron remnants (Blaner et al. 1994). The liver takes up most of chylomicron retinyl esters, although many extrahepatic tissues, such as adipose tissue, kidney, testes, lungs and bone marrow, also can take up and store vitamin A. It is estimated that more than 90% of the body's vitamin A is stored in the liver (Basu 1996).

In the liver, two different cell types are involved in vitamin A storage and metabolism: the parenchymal cell (the predominant cell type of the liver) and the stellate cells (fat-storing cells). Parenchymal cells, which contain 90% of all protein present in the liver, are directly involved in the uptake of chylomicron remnants and in the synthesis and secretion of RBP (Blaner et al. 1994). The much smaller and less abundant stellate cells, which account for approximately 6 to 8% of all cells, are the major storage cells for retinyl esters.

The uptake of the chylomicron remnants by the liver requires the participation of a cell surface receptor that recognises the apolipoprotein (apoB and apoE) of the chylomicron remnants (Mahley et al. 1991). Rapid hydrolysis then takes place, catalysed by retinyl ester hydrolases (Harrison et al. 1989). It has been suggested that

bile-salt-independent retinyl ester hydrolases (BSIREH) might well play a role in the hydrolysis of retinyl esters from the chylomicron remnants, in view of its subcellular location in the plasma membrane of the liver (Harrison et al. 1989; Gad et al. 1991). Chylomicron retinyl esters must first be hydrolysed to retinol prior to being transferred to stellate cells. The resulting retinol binds to RBP, which is then either secreted into the circulation or transferred to the stellate cells for storage. It has been reported that the relative proportion of newly absorbed vitamin A being either secreted into the circulation for delivery to the tissues or transferred to the stellate cells for storage depends on the vitamin A nutritional status of the animals (Olson et al. 1987). That RBP is responsible for the intracellular transfer and release of retinol from the cells is supported by the observations that: 1) antibodies to RBP inhibit the transfer of retinol from parenchymal to stellate cells; and 2) RBP-retinol complex can be taken up by purified stellate cells (Blaner et al. 1994).

It has been shown that about 90% of the hepatic vitamin A is present in the stellate cells mainly as retinyl esters (Trøen et al. 1994). Two enzymes similar to those found in the enterocytes, ARAT and LRAT, have been described in the esterification of retinol in the stellate cells. The relative role of ARAT and LRAT in the esterification of retinol is thought to be regulated by vitamin A nutritional status (Randolph et al. 1991). The LRAT reaction appears to be the predominant activity when retinol and fatty acyl CoA are present at normal physiological concentrations, whereas ARAT may well play a larger role if the hepatic concentrations of retinol or fatty acyl CoA are markedly increased.

When required, retinyl esters stored in the liver are first hydrolysed and released as the alcohol form, retinol. This process is important since it regulates the release of retinol from its liver storage. Unlike the hydrolysis of retinyl esters in chylomicron remnants, the hydrolysis of retinyl esters in stellate cells takes place with the bile-salt-dependent retinyl ester hydrolases (BSDREH), in view of their highly specific activity in hepatic stellate cells (Blaner et al. 1994). Almost no activity of BSDREH is observed in the absence of bile salts. It has been reported that protein malnutrition could significantly reduce the activity of BSDREH in rat liver (Tsin et al. 1986). Rats fed a diet containing 3% casein as the only source of protein for 8 weeks had markedly reduced activity of BSDREH accompanied by a parallel reduction in plasma retinol.

Following hydrolysis, the free retinol is subsequently conjugated with RBP and secreted as RBP-retinol complex into the circulation. RBP is a polypeptide chain with a molecular weight of 21,000 Da and is mainly synthesized in the hepatic parenchymal cells (Goodman 1984). In serum, RBP-retinol complex combines with another protein, transthyretin (TTR), in a 1:1 molar ratio. The TTR is a tetrameric protein with a molecular weight of 54,900 Da. The formation of a complex between retinol and RBP may solubilize the vitamin A in serum and protects the retinol against oxidative damage. Further binding of RBP-retinol complex to TTR serves to stabilise the association of retinol with RBP and also prevents glomerular filtration and renal catabolism of RBP (Goodman, 1984). The half–life of RBP once it forms a complex with TTR, is approximately 12 hours, whereas the half-life of free RBP is only 4 hours (Vahlquist et al. 1973). It is estimated that approximately 95% of plasma retinol are

present as TTR-RBP-retinol complex, 4.4% as RBP-retinol complex and only 0.1% as unbound retinol (Blomhoff et al. 1991).

Circulatory levels of retinol remain constant despite normal fluctuation in daily intake. In situations of inadequate intake of vitamin A, its hepatic stores are drawn upon in order to maintain relative constant circulatory levels, until the stores are nearly exhausted. In the case of prolonged excessive intake of vitamin A, the hepatic storage capacity exceeds its limits, retinyl ester but not retinol is secreted into the circulation in association with lipoprotein (Smith et al. 1976). Retinyl esters transported by lipoprotein are available to the cell membranes, which may lead to toxicity and damage of these membranes (Goodman et al. 1984).

1. 1. 3 CELLULAR UPTAKE

Target cells without a concomitant uptake of either RBP or TTR, take up circulatory retinol. However, the underlying mechanism by which target cells take up retinol from plasma still remains to be elucidated. According to the hypothesis elucidated by Goodman (1984), cellular uptake of retinol is mediated by a specific cell membrane receptor that recognises the RBP but not retinol. Using cultured human retinal pigment epithelial cells (RPE), a study demonstrated that the specific binding of [^{125}I] RBP occurred at the apical surface of these cells and [^{3}H] retinol was taken up specifically by RPE cells from [^{3}H] retinol-RBP (Pfeffer et al. 1986). It has also been suggested that retinol is delivered to the membrane receptor by RBP rather than RBP-TTR. TTR has been shown to inhibit the binding of the retinol-RBP complex and reduce the transfer of retinol to cells (Sivaprasadarao et al. 1988a and b). To date, retinol binding

protein receptors have been identified in RPE, hepatic parenchymal and stellate cells, testis, brain barriers, placental brush border membranes and keratinocytes.

Once retinol enters a target cell, it is bound by the intracellular retinol-binding protein (CRBP) (15,000 Da), which does not cross-react immunologically with RBP (Olson 1991). To date, many other intracellular vitamin A binding proteins, including intracellular retinol-binding protein II (CRBPII), intracellular retinol acid-binding protein (CRABP), intracellular retinaldehyde-binding protein (CRALBP), and inter-photoreceptor retinoid-binding protein (IRBP), have been identified in various tissues. Our understanding of these intracellular vitamin A binding proteins is far from clear. However, it is believed that they facilitate the transfer of specific forms of vitamin A to the nucleus of the cells, where they can participate in the regulation of gene expression for controlling cell differentiation and growth (Chytil et al. 1987).

Recently two types of nuclear receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs), have been identified by gene cloning (Wolf 1993). Both of them exist as at least three different subtypes, designated alpha, beta, gamma, and bind retinoic acid. They become transcriptionally active upon ligand binding and transactivate their target genes by binding to hormone response elements.

1. 1. 4 BIO-TRANSFORMATION AND EXCRETION

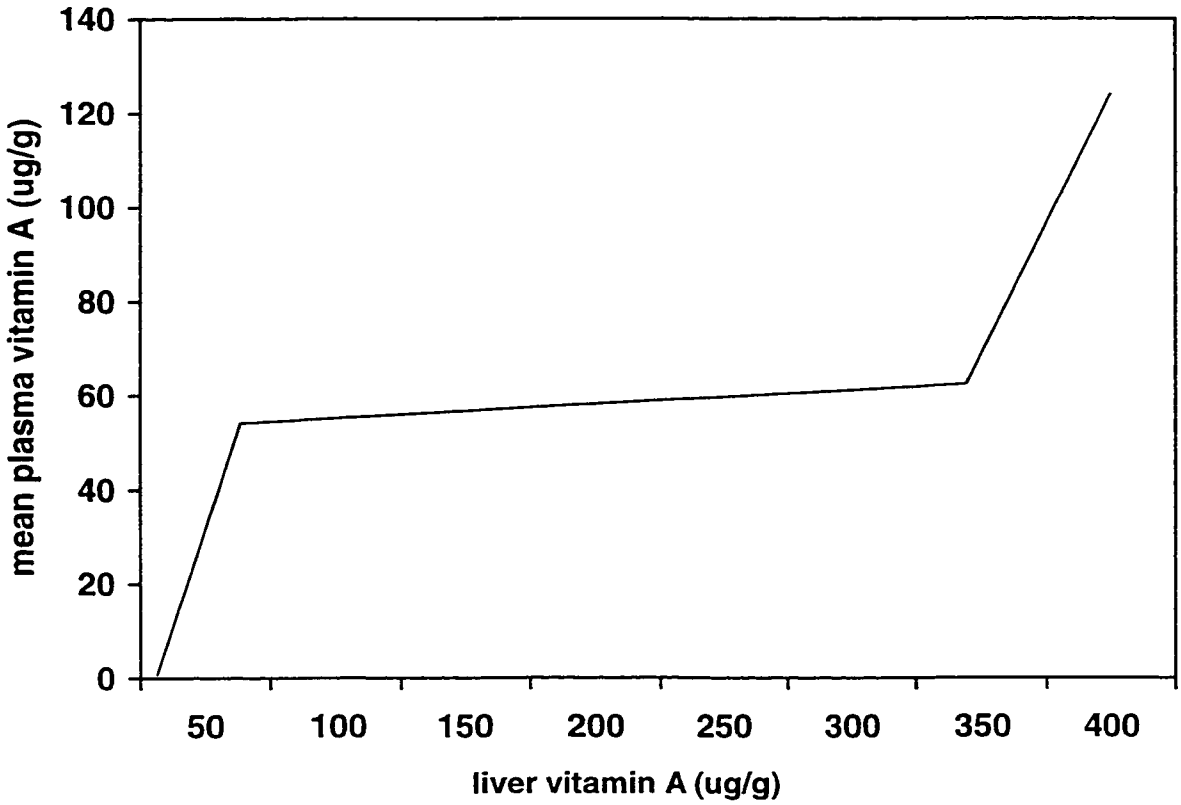
The biologically active form of vitamin A in mammalian cells is all-*trans* retinol. It could be further oxidized to retinal by nonspecific retinol dehydrogenases which are present in many tissues including the eye, liver and intestine (Ganguly 1989). This

reaction in the eye provides retinal for rhodopsin regeneration, and in the liver, retinal could, through a further irreversible step, produce retinoic acid by aldehyde dehydrogenase or xanthine oxidase. Most oxidized products are either excreted in the urine or conjugated with glucuronic acid in the liver. The newly formed glucuronides are excreted in the bile and may be partially reabsorbed in the intestinal tract while the rest is excreted in the faeces. It is estimated that 10 to 20% of the dietary vitamin A is not absorbed. Of the remaining amount, 20% appears in the faeces through the bile, 17% is secreted in the urine, 3% is secreted as CO_2 and 40 to 50% is stored in the liver (Olson 1994).

1. 2 VITAMIN A HOMEOSTASIS AND REGULATION

It has been demonstrated that the plasma retinol level is maintained within a normal range of concentrations as long as there is some minimal level of vitamin A in the liver. This may suggest that there are mechanisms that regulate plasma retinol input and output. Only in the conditions of severe hypo- and hypervitaminosis A, do plasma levels of vitamin A correlate with its liver storage (Olson 1984). When the liver's reserve is depleted (20 μg/g liver), plasma retinol levels fall rapidly. When the storage of hepatic vitamin A exceeds the limit of 300 μg/g liver, the homeostatic control over plasma vitamin A concentration is lost. A hypothetical relationship between plasma vitamin A level and hepatic concentrations of vitamin A is shown in Figure 1-3.

Figure 1-3. Hypothetical relationship between mean plasma vitamin A levels and liver vitamin A concentrations. Source: Olson (1987)



A number of endogenous and exogenous factors have been identified that could alter the homeostasis of vitamin A. These include vitamin A, protein, zinc and fat nutriture, kidney and liver dysfunction as well as stress and hormone status.

1. 2. 1 DIETARY FACTORS

1. 2. 1. 1 Vitamin A

Plasma vitamin A levels remain constant over a wide range of dietary intake, except in a state of severe deficiency or toxicity. This presupposes that the hepatic function is intact and allows synthesis of the RBP.

Secretion of RBP from the liver is strictly regulated by the availability of its ligand, retinol. In vitamin A deficiency, the secretion of RBP from the hepatocyte is specifically inhibited, leading to decreased plasma RBP levels and accumulation of RBP in the liver (Muto et al. 1972). Repletion of dietary vitamin A rapidly increases the RBP secretion into the plasma. When protein inhibitor is given to vitamin A deficient rats, repletion of vitamin A increases the plasma RBP (Smith et al. 1973), suggesting that vitamin A deficiency is associated with the hepatic secretion but not the synthesis of RBP.

The sole indicator of excessive vitamin A supplies is an increase of retinyl esters in the plasma. Administration of large doses of vitamin A results in an increased serum concentration of retinyl esters whereas retinol levels remains unchanged (Smith et al. 1976). Under normal conditions, the retinyl esters content is approximately 5 to 8%

that of plasma retinol, while a massive overdose gives rise to more than twice as much retinyl esters as retinol (Ellis et al. 1986). Retinyl esters are thought to be transported by lipoproteins that are more readily taken up by cell membranes. Thus the increased levels of retinyl esters in the plasma may lead to the damage of cell membranes and clinical signs of vitamin A toxicity.

1. 2. 1. 2 Zinc

Cumulative evidence has demonstrated the importance of zinc in the metabolism of vitamin A, especially its role in the mobilisation of vitamin A from the liver to the plasma and synthesis of hepatic vitamin A carrier proteins. Zinc deficiency in both animals and humans has been found to be associated with low plasma retinol levels; zinc supplementation restores the level of plasma retinol to normal (Smith 1980). In addition, the plasma RBP is depressed in zinc-deficient rats (Riordan 1972). The exact mechanism by which zinc influences vitamin A homeostasis is not entirely clear at present.

Zinc is essential for at least 40 to 50 enzyme systems in the body, some of which are directly or indirectly involved in vitamin A metabolism. Significantly reduced activity of alcohol dehydrogenase (ADH) is observed to be associated with zinc deficiency (Boron et al. 1988). ADH, whose highest concentrations are found in the liver, retina, kidneys and gastic mucosa (Mezey et al. 1971), is the enzyme involved in the conversion of retinol to retinaldehyde. Moreover, the eye and testis, which have specific requirements for the more-reduced forms of vitamin A for their normal functions, appear to be more sensitive than the liver to zinc deficiency.

A link between vitamin A and zinc has been further supported by the fact that both RBP and TTR, two important carrier proteins in vitamin A metabolism, have been found to be decreased in the plasma and the liver during zinc deficiency (Smith et al. 1974; Smith et al. 1976 & 1980; Bate et al. 1981). This could be due to either a decrease in the rate of synthesis, an increase in the rate of degradation, or both. A recent study investigating the liver mRNA content of RBP and TTR in zinc-deficient, pair-fed, and *ad libitum* fed rat, suggests that zinc can regulate the synthesis of RBP and TTR in rat liver through changes in the relative abundance of mRNA (Kimball et al 1995).

1. 2. 1. 3 Other Nutrients

Protein and lipid also could affect vitamin A homeostasis. Dietary protein in the maintenance of vitamin A homeostasis is essential. Patients with protein-energy malnutrition (PEM) are thus associated with a decrease in plasma RBP, TTR and retinol concentrations (Goodman 1984). This may be due to a decreased synthesis of RBP and TTR in the liver. Treatment of PEM with protein, but without dietary vitamin A supplementations, increases the concentration of RBP, TTR and retinol in plasma. These findings suggest that depressed plasma retinol concentrations in PEM patients is largely a reflection of a functional impairment in the hepatic release of vitamin A. In addition, both RBP and TTR are rapid turn-over proteins (half-life of 5 to7 hr and 12 to 16 hr for RBP, and 10 to 11 hr and 3 to 4 days for TTR, in rats and humans, respectively). Thus they are both sensitive markers of PEM status (Polberger et al. 1990; Wade et al. 1988).

Vitamin A is a fat-soluble vitamin. Before its absorption to the mucosal phase of the small intestine, dietary vitamin A requires solubilization into a micellar solution which is formed with bile salt. Therefore, when a diet is low in fat or an obstruction of the bile duct occurs, the absorption of vitamin A is impaired. It has been demonstrated that when diets contain fat at levels below 5g/day, the absorption of vitamin A is markedly reduced (Olson 1987).

1. 2. 2 DISEASES

Various diseases have been reported to be associated with altered plasma vitamin A, RBP and TTR levels. These include acute and chronic diseases of the liver and kidneys, measles, diabetes and gastrointestinal disorders (Smith and Goodman 1971; Sommer et al. 1986; Basu et al. 1989). Among them, liver and kidney diseases have been extensively studied.

1. 2. 2. 1 Liver Disease

In the face of large fluctuations in dietary vitamin A intake, controlled secretion of retinol by the liver is a key regulatory element for maintaining plasma retinol concentrations.

During the progression of hepatic fibrosis, loss of vitamin A has been shown to be one of the major changes in stellate cells, which contain 80 to 90% of the total hepatic vitamin A (Yamane et al. 1993). An alteration of hepatic REH, ARAT and CRBP,

which are thought to participate in intracellular transport and esterification of retinol may be responsible for this impaired vitamin A metabolism (Kent et al. 1976).

RBP bound retinol is the only form of retinol to be secreted by the liver under physiological conditions. Retinol secretion by the liver is largely regulated by the process that controls the rates of hepatic RBP synthesis and/or its secretion. Altered vitamin A metabolism has also been observed in alcohol abuse patients (Adachi et al. 1991). The possible reason for this altered vitamin A metabolism might be the fact that alcohol abuse causes damage of the parenchymal cells, the major site for RBP synthesis.

1. 2. 2. 2 Kidney Disease

The kidneys play a major role in the catabolism of RBP; thus the alteration of this clearance mechanism could significantly account for an abnormality of retinol and RBP in the circulation. Either increased or decreased plasma concentrations of retinol and RBP have been reported in the presence of renal disorders (Basu 1996).

Patients with defects in glomerular filtration display an elevated circulatory level of RBP, whereas those with impaired tubular function are associated with an increased urinary loss of RBP (Soprano et al. 1994). In addition, some patients with type I diabetes have been reported to be accompanied by alterations in both circulatory and urine RBP levels, which might be partly due to a combination of tubular and glomerular malfunction (Holm et al. 1988; Soprano et al. 1994).

1. 2. 3 STRESS AND HORMONES

Acute and chronic stress, originating from physical, psychological or pathological conditions, alters the vitamin A status both in human and experimental animals. Fever, surgical operations, burn injury and prolonged immobilisation have been linked with decreased serum levels of retinol and RBP (Arroyave et al. 1961; Cynober et al. 1985; Takase et al. 1992). Stress, induced by a prolonged immobilisation causes an accumulation of vitamin A hepatic storage and a decrease in plasma levels of vitamin A and RBP in rats (Takase et al. 1992). Impaired mobilisation of vitamin A from the liver may be the key event in the changes in vitamin A status in these animals.

It is thought that these changes may be related to the secretion of stress-related hormones. Administration of glucocorticoid enhances the release of vitamin A from hepatic stores with an elevation of plasma retinol (McGullvray 1961). Adrencorticoids stimulate RBP-retinol complex secretion from the liver *in vivo* and *in vitro* by increasing RBP synthesis (Georgieff et al. 1991).

1. 3 DIABETES MELLITUS AND VITAMIN A METABOLISM

Diabetes mellitus is characterised by chronic hyperglycaemia resulting from defects in insulin secretion, action, or both (The Expert Committee on the Diagnosis and Classification of diabetes mellitus 1998). Insulin, secreted by the pancreatic beta cell, is an anabolic hormone and required for the utilisation of glucose by insulin-dependent

tissues such as skeletal muscle and adipose tissue. Thus, diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels. Due to the increased risk of complications in diabetes, including retinopathy, nephropathy and neuropathy, this disease represents a severe burden to patients and society.

Diabetes is classified into two major categories: type I diabetes and type II diabetes. Type I diabetes is an autoimmune disease resulting from a progressive destruction of insulin-producing pancreatic beta-cells, while type II diabetes is a complex, multifactorial disease resulting from decreased insulin secretion and/or insulin resistance. Epidemiological studies suggest a world-wide, steady rise in the incidence of diabetes. Using World Health Organisation criteria, the prevalence of all forms of diabetes in the U. S. has increased from 11.4% in 1976-1980 to 14.3% in 1988-1994 (Clark 1998). It is hypothesised that this increased prevalence is due to lifestyle changes.

Diabetes mellitus is a chronic disorder accompanied by a vast array of metabolic and nutritional alterations. Metabolism of many nutrients is homeostatically regulated and subject to factors such as hormones and other physiological influences (Cousin 1985). Thus, dietary issues are integral to diabetes care and management. However, due to the gaps in our knowledge of both the nutritional status of diabetic patients and the optimal dietary guidelines, a host of questions still remain unanswered.

Several recent studies have suggested that uncontrolled chronic hyperglycaemia can cause a significant change in vitamin A status. The prevalence of hyperzincuria and

hypercarotenemia in the presence of diabetes strongly supports this hypothesis (Mosenthal et al. 1944; Heise et al. 1988; Cunningham et al. 1994). Impaired conversion of carotenes to vitamin A, along with the perturbed metabolism of zinc, an important factor for the synthesis of vitamin A carrier proteins, may indeed affect the metabolic availability of vitamin A in diabetes. In addition, increased urinary excretion of RBP has been reported in both type I and type II diabetes as an early sign of nephropathy (Holm et al. 1993; Dubrey et al. 1997). *In vivo* studies using isolated islets and in *vivo* studies with depletion and repletion of vitamin A in rats have shown that vitamin A is required for insulin secretion (Cherton et al. 1987).

Both vitamin A deficiency and diabetic retinopathy have the same ultimate consequence--loss of vision. Thus, it is conceivable that diabetic retinopathy could be further aggravated if diabetes is associated with vitamin A deficiency. Nearly all patients with type I diabetes and more than 60% of patients with type II diabetes have some degree of retinopathy after 20 years of the disease. It is estimated that diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years (American Diabetes Association 1998). Thus, total understanding of vitamin A metabolism in diabetes will be of significance in therapy and nutritional care as well as in the prevention and management of diabetes.

1. 3. 1 TYPE I DIABETES AND VITAMIN A METABOLISM

Type I diabetes is characterised by a low level or absence of circulating endogenous insulin, resulting from the destruction of insulin-producing pancreatic beta-cell (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1998).

Genetic factors, environmental factors (virus, infections, diet, toxins), and autoimmunity have been suggested to be possible causes of beta-cell destruction. Markers of the immune destruction of the beta-cell include islet cell autoantibodies (ICAs), autoantibodies to insulin (IAAs), autoantibodies to glutamic acid decarboxylase (GAD), and autoantibodies to the tyrosine phosphatases IA-2 and IA-2 ß. 85 to 90% of type I diabetes patients have one or more of these autoantibodies present when fasting hyperglycaemia is initially detected. However, precise mechanisms for the initiation and progression of beta-cell destruction are poorly understood.

1. 3. 1. 1 Animal Models of Type I diabetes

Good animal models exist for studying type I diabetes and have contributed much to our understanding of the pathogenesis and treatment of diabetes. These can be categorised as animals with spontaneously developing diabetes (e.g. BB rats and NOD mice) and as animals with chemically induced diabetes (e.g. STZ-induced diabetic rats).

1. 3. 1. 1. 1 Chemically induced diabetic animal model

STZ is the most common diabetogenic chemical used to induce permanent diabetes in animals. It is a nitrosourea derivative, isolated from the mould *Streptomyces griseus*. STZ induces hyperglycaemia through its effect on pancreatic beta cells, apparently by damaging the beta cell membrane and inducing DNA strand breaks (Bone et al. 1997).

Type I diabetes induced by a single intravenous or intraperitoneal injection of STZ is most widely used in experimental animals.

Hepatotoxic and nephrotoxic effects of STZ have also been reported (Perloff et al. 1995). Effects on both bile flow, biliary excretion and hepatic biotransformation at 1 to 2 weeks after STZ administration which were not completely reversible by insulin treatment, suggested that the damage may be owing to toxicity of the diabetogen rather than diabetes itself (Carnovale et al. 1991 & 1992). Thus, interpretation of studies in STZ-induced diabetic animal models must consider the toxicity of the STZ.

1. 3. 1. 1. 2 Spontaneous diabetic animal model

Given our current understanding of the pathogenesis of type I diabetes, animal models with spontaneous diabetes (e. g., BB rat and NOD mouse), are considered better and more physiologically accurate models of the human disease. This is because the beta-cell destruction resulting from an autoimmune attack, rather than with chemically-induced disease (Yoon et al. 1995; Crisa et al. 1992).

The spontaneously diabetic BB rat syndrome was recognised in 1974 at the Bio Breeding Laboratories, Canada. The affected animals were eventually inbred to create what is now the diabetes prone (BBdp) BB rat line. Early in the program of inbreeding, those animals that failed to develop diabetes were selected to start a control line of nondiabetic BB rats, now designated as the nondiabetes-prone (BBn) BB rat line. As in human type I diabetes, disease in a BBdp rat occurs spontaneously during adolescence followed by selective destruction of beta-cells. In BBdp rats of

both sexes, diabetes typically shows an abrupt onset with glycosuria, hyperglycaemia, ketoacidosis and hypoinsulinemia. Death occurs if insulin therapy is not administered.

The development of diabetes in BBdp rats is associated with insulitis, an inflammatory lymphocytic infiltration in the islets of Langerhans, which is similar to that observed in human type I diabetes (Crisa et al. 1992). T cells, B cells, macrophages, dendritic cells and NK cells have been found in human and BB rat islets at the time of diabetes onset. Most evidence supports a model which T cells are playing a central role in the beta-cell destruction by nonspecific effector mechanisms, which may include cytokine release from T-cells, macrophages, or NK cells, or direct killing by macrophages or NK cells.

Both genetic susceptibility and environmental factors contribute to the expression of autoimmune diabetes in BB rats. Disease susceptibility appears to be associated with specific alleles of the major histocompatibility complex (MHC), as well as other genes (Yoon et al. 1995). Not all BBdp rats develop diabetes, suggesting that environmental factors interact with genetic susceptibility in the BB rats. Environmental factors that have been studied in the BB rat include diet and viral infection (Crisa et al. 1992). The major characteristics of type I diabetes in human and BB rats are shown in Table 1-1.

The immune abnormalities in the BB rat distinguish it from human type I diabetes, in which abnormalities are more subtle. This may make it easier to dissect immunopathogenetic mechanisms in the BB rat, but also suggests caution in the transfer to humans of therapeutic interventions successful in the rat.

Table 1-1. Characteristics of type I diabetes syndromes in human and BB rats (Bone et al. 1996).

Feature	Human	BB rat
Aetiology		
Genetic predisposition	Yes	Yes
MHC association	Yes	Yes
Environmental factors	Yes	Yes
Immune phenomena		
Insulitis	Yes	Yes
ICA/CSA	Yes/Yes	No/Yes
GAD antibodies	Yes	Yes
Lymphopenia	No	Yes*
T-lymphocyte dysfunction	No	Yes*
Other autoimmune disease (thyroiditis, etc.)	Yes	Yes
Clinical manifestations		
Insulin-dependent, ketosis prone	Yes	Yes
Long prediabetic period	Yes	Yes
Obesity	No	No
Sex difference	No	No

*Not in all BB rat colonies.

1. 3. 1. 2 CURRENT KNOWLEDGE OF VITAMIN A METABOLISM IN TYPE I DIABETES

Impaired metabolic availability of vitamin A has been identified in human subjects with type I diabetes, as evidenced by decreased levels of plasma vitamin A and RBP, accompanied by an increased urinary excretion of the RBP (Basu et al. 1989; Dubrey et al. 1997).

Investigations involving STZ-induced diabetic rats have shown that circulatory levels of retinol as well as 11-cis-retinal (an important component of rhodopsin) concentrations in the retina of the eye are reduced when diabetes is present, while the hepatic storage of vitamin A is markedly elevated (Tuitoek et al. 1996a). The intestinal absorption of vitamin A in these animals has been shown to be unaffected (Tuitoek et al 1994), and the intake of food and hence vitamin A is increased in STZ-induced diabetic rats. Despite these characteristics, significant reductions in the concentrations of both RBP and TTR in the kidney and plasma have been observed in these animals (Tuitoek et al. 1996b). The absolute amount of RBP in the liver is decreased markedly in diabetic rats. These results suggest that depressed circulatory vitamin A levels in STZ-induced diabetic rats may reflect an impaired mobilisation of vitamin A from liver storage, due to inadequate availability of vitamin A carrier proteins. Insulin treatment rather than dietary vitamin A supplementation was found to normalise the metabolic availability of vitamin A in these rats (Tuitoek et al. 1996c), indicating a linkage between vitamin A status and insulin in diabetes. Indeed, vitamin A has been reported to be required for normal insulin secretion (Chertow et al. 1987).

Insulin release from islets of vitamin A-deficient rat in vitro has been shown to be markedly impaired in response to glucose. In vivo study has also demonstrated that vitamin A-deficient rats are associated with a decreased acute insulin release and that this defect is reversed by retinyl palmitate repletion. Thus, it appears that altered vitamin A metabolism in type I diabetes is primarily due to an insulin deficiency.

The liver contributes amino acids for gluconeogenesis and synthesis of most of the plasma proteins, including RBP and TTR. Secretary proteins are primarily synthesized by ribosomes in the rough endoplasmic reticulum, which appears as orderly rows of membrane-bound polyribosome structures (Jefferson 1980). This ordered structure, however, is severely disrupted in STZ-induced diabetic rats (Reaven et al. 1973). In addition, plasma RBP and TTR are known to be sensitive to changes in protein nutritional status (Wade et al. 1988; Polberger et al. 1990). Patients with type I diabetes are in a catabolic state in the absence of insulin replacement. It is therefore possible that a disruption of this structure in liver cells of diabetic rats might be an indication of an impairment in the synthesis and secretion of proteins. This hypothesis is further supported by the fact that diabetes results in a marked reduction in the levels of albumin, a specific indicator of protein status, and total hepatic protein in the perfused liver of a diabetic rat (Peavy et al. 1978). These alterations are restored to normal levels by insulin. At a molecular level, alteration in hepatic albumin protein levels also corresponds with changes in albumin mRNA levels.

1. 3. 2 TYPE II DIABETES AND VITAMIN A METABOLISM

Unlike type I diabetes, type II diabetes is a consequence of reduced insulin sensitivity or insulin resistance, resulting in high or normal blood insulin levels (Olefesky et al. 1995). No evidence of vitamin A deficiency has been found in type II diabetes patients (Havivi et al. 1990; Basualdo et al.1997). Using a select group of First Nations peoples, a study demonstrated that metabolic availability of vitamin A was normal in type II diabetes subjects (Basualdo et al.1997). The underlying mechanisms for the differences in vitamin A status between type I and type II diabetes can not be explained at the present time. However, it has been suggested that this difference might be associated with the insulin availability involved in the two diseases. Individuals with type II diabetes may actually have hyperinsulinemia, a consequence of reduced insulin sensitivity or insulin resistance.

1. 4 CONCLUSIONS AND OBJECTIVES OF THE PRESENT STUDY

Diabetes is associated with a vast array of metabolic and nutritional alterations, and dietary issues are integral to overall diabetes care and management. Several recent studies have clearly pointed out that vitamin A in type I diabetes is of concern. Studies involving STZ-induced diabetic rats have demonstrated that type I diabetes in these animals is associated with depressed vitamin A status due to an impaired availability of its carrier proteins (Tuitoek 1996 a & b). These diabetes-associated alterations in vitamin A metabolism are reversed by insulin treatment and not dietary vitamin A

supplementation, suggesting that the observed changes are primarily due to an insulin deficiency (Tuitoek et al. 1996c). This has been further substantiated by the fact that patients with insulin-resistant type II diabetes, having sufficient insulin, display normal metabolic availability of vitamin A (Basualdo et al. 1997). Most of the experimental studies linking vitamin A metabolism and type I diabetes, however, have been carried out in STZ-induced diabetic rats. Since STZ is toxic to the kidney and the liver, the effects on vitamin A metabolism in these animals may be partially a reflection of toxicity but not solely diabetes.

In order to fully understand the vitamin A metabolism in type I diabetes, the present study was undertaken to investigate the metabolic availability of vitamin A in BB rats, which develop diabetes spontaneously and are known to be suitable animal models for human type I diabetes (Crisa et al. 1992).

Using BB rats, this study was carried out to test the following objectives:

1. To determine that vitamin A metabolism is affected in the presence of diabetes;

2. To ascertain the responses of vitamin A metabolism to dietary vitamin A and zinc supplementations as well as to insulin administration;

3. To ascertain immune function and phenotypic changes in response to vitamin A and zinc supplementations, with particular reference to NK cell cytotoxic activity and mononuclear cell phenotyping in the spleen.

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2. VITAMIN A (RETINOL) METABOLISM IS ALTERED IN BB RATS WITH THE ONSET OF DIABETES

2.1 INTRODUCTION

Impaired metabolic availability of vitamin A has been reported in patients with type I diabetes, as evidenced by decreased levels of plasma vitamin A and RBP, accompanied by an increased urinary excretion of RBP (Basu et al. 1989; Dubrey et al. 1997). The metabolism of zinc, an important factor for the synthesis of vitamin A carrier proteins, has also been shown to be disturbed in the presence of diabetes, as indicated by hyperzincuria (Heise et al. 1988; Cunningham et al. 1994). These findings provide important new information with respect to the interrelationship between type I diabetes and vitamin A deficiency.

Recent studies involving STZ-induced diabetic rats demonstrated that circulatory levels of retinol as well as 11-cis-retinal (an important component of rhodopsin) concentrations in the retina of the eye were reduced, while the hepatic storage of vitamin A was markedly elevated (Tuitoek et al. 1996a). These results may imply an impairment in mobilization of vitamin A from the liver to the circulation in the presence of type I diabetes. In addition, this diabetes-associated changes of vitamin A metabolism was reversed by insulin treatment but not by dietary vitamin A supplementation (Tuitoek et al. 1996a & c), indicating a linkage between vitamin A status and insulin deficiency. Indeed, vitamin A has been reported to be required for normal insulin secretion (Chertow et al. 1987). However, STZ is known to be toxic to

the liver and kidney (Perloff et al. 1995), the two major sites involved in vitamin A metabolism. The possibility that vitamin A metabolic derangement is a reflection of STZ toxicity rather than of diabetes, cannot be excluded.

The objective of the present study is to verify whether or not this impaired metabolic availability of vitamin A exists in type I diabetes using BB rats. These animals are known to produce the highest incidence of diabetes by feeding NIH-07 diet, consisting of unrefined and natural ingredients (Scott 1994). After feeding this diet, we examined vitamin A status, including its carrier proteins, at the onset of diabetes. The biochemical status of zinc, which is important in the maintenance of vitamin A homeostasis, was also determined.

2. 2 MATERIALS AND METHODS

Animals and Diets

BBdp and BBn rat dams originally from Health Canada (Animal Resources Division, Health Protection Branch, Ottawa, ON, Canada) were obtained from Department of Agricultural, Food and Nutritional Science of the University of Alberta breeding colony. Approximately 50 to 80% of the BBdp rat lines develop Type I diabetes within 60 to 120 days after birth, while BBn rat lines do not develop diabetes spontaneously and are similar to normal rats. Animals were housed in temperature and humidity-controlled room with a 12-hour light/dark cycle. Principles of laboratory animal care

were followed. All studies were reviewed and approved by the University of Alberta Animal Welfare Committee.

Weanling (21 d) litter- and gender- matched BBdp and BBn rats (n=12/group) were given free access to NIH-07 diet (Table 2-1) and water up to 120 days of age. After diabetes was diagnosed, the diabetic rats and their paired BBn rats were killed within 24 hours. Food intake and body weight 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 by Glucometer II (Ames Miles, Toronto, ON, Canada). Diabetes was diagnosed on the basis of glycosuria >2+ and hyperglycaemia (blood glucose >200 mg/dL). Once these animals manifested hyperglycemia, they were described as BBd rats. Animals were killed using carbon dioxide after overnight fasting. Blood was collected in heparinized tubes. In order to avoid light-induced oxidation of vitamin A, separated plasma was protected from light and stored at –20°C. Livers were removed, immersed in liquid nitrogen and stored at –72°C for later analysis.

Determination of Vitamin A

Plasma (Nierenberg et al. 1985) and liver (Forlik et al. 1984) vitamin A were assayed by high-performance liquid chromatography (HPLC), performed on a Supercosil LC-18 (15.0 cm X 4.6 mm) reverse phase column (Supelco, Mississauga, ON, Canada) with 3 μm packing.

Table 2-1. 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 (Zeigler brothers Inc. Gardmers. PA) for lot number used].

† Pre-mixes content of diet (/ 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.

For extraction of retinol from plasma, duplicate 200 μL samples were mixed with 50 μL of acetonitrile containing 200 ng of retinyl acetate as an internal standard. After vortexing, 250 μL of butanol:ethyl acetate (1:1) was added and vortexed for another 60 seconds. Finally 150 μL of an aqueous solution of dipotassium monohydrogen phosphate (1.2 g/mL) was added; the solution was vortexed for 30 seconds and centrifuged at 9000 rpm for 1 minute. The organic layer was collected. Standards for retinol were exposed to the same extraction procedure as samples and prepared daily.

Liver total vitamin A was extracted by a saponification method. Liver homogenate was digested with an equal volume of 5% potassium hydroxide in methanol (w/v) at 50°C for 1 hour and extracted with n-hexane. After centrifugation, the upper layer of hexane extract was removed and evaporated to dryness under nitrogen. Residues were dissolved into 200 μL acetonitrile and then injected into the HPLC. A set of standards with retinyl acetate were carried out identically and used for calibration. Free retinol in the liver was extracted without saponification.

Radioimmunoassay

Plasma and liver RBP and TTR were assayed by radioimmunoasssay (Tuitoek et al. 1996b). Liver samples were homogenized in 19 volumes of 250 mM-sucrose with a Polytron homogenizer (Brikmann, Wesbury, N.Y., USA) at speed 5 for 15 seconds and then diluted with an equal volume of an aqueous Triton X-100 solution (20 g/L) to release the proteins trapped in subcellular organelles. The plasma samples and Triton X-100-treated liver homogenates were diluted properly with assay buffer (50 mM-Tris-HCl containing 10g bovine serum albumin/L, PH 8.6).

Purified rat RBP and TTR was iodinated with ^{125}I using a carrier-free $Na^{125}I$ and separated by gel filtration. The protein was then eluted with 0.07 M barbital buffer. After addition of labeled proteins, assay buffer, anti-rat antiserum for the protein, all samples were incubated in the dark at 4 °C for 3 days. After being mixed with goat anti-rabbit gamma globulin antiserum for RBP and polyethylene glycol for TTR, all samples were incubated at 4 °C to precipitate the antibody-bound ^{125}I-protein and collected by centrifugation at 7,000 rpm for 15 min at 4 °C. The precipitates were washed with barbital albumin buffer, and the wash supernatants were combined. The precipitates representing antibody-bound RBP-^{125}I or TTR-^{125}I, and the wash-supernatants representing free protein, were assayed for ^{125}I separately in a Packard 500C AutoGamma Counter (Packard Instrument Co., Meriden, CT, USA). After calculation of the bound/free ratios of RBP-^{125}I or TTR-^{125}I, the amount of the proteins was determined from the standard curve.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from the liver samples using Trizol™ (Gibco BRL, Burlington, ON, Canada) according to the method provided with the reagent. Briefly, approximately 200 mg of liver was mixed with 2 ml of Trizol™ reagent and homogenized for 30 seconds. To each sample, 0.4 ml chloroform were added and incubated at room temperature for 5 minute. After centrifugation at 12,000 x g for 15 minutes at 4°C, the aqueous upper phase containing RNA was transferred. one ml of isopropanol was added to precipitate the RNA overnight at -20 °C. RNA was then isolated by centrifugation at 12,000 x g for 10 minutes at 4 °C followed by 2 washes with 75% ethanol. Finally,

isolated RNA was dissolved in RNase-free water by gentle vortexing. RNA quantification and quality determination were carried out by ultraviolet spectrophotometry at 260 and 280 nm.

Northern blot analysis was performed by denaturing 15 μg total RNA, which was dissolved in 10 μl gel loading buffer [50% deionized formamide (v/v), 2 mol/L formaldehyde, 1.3% glycerol (v/v), 0.02 mol/L morpholinopropanesulphonic acid, 5 mmol/L sodium acetate, 1 mmol/L EDTA, 0.1% bromophenol blue (w/v)], followed by electrophoresis in 1% (w/v) agarose gel (Reimer et al 1997). RNA was then transferred to a MSI Nitropure nitrocellulose membrane (MSI Laboratories, Westboro, Mass., USA.) and baked at 80 °C for 2 hours. All membranes were prehybridized at 65°C for 2 hours in prehybridization buffer [6 x SSPE (0.18 mol/L NaCl, 0.01 mol/L sodium phosphate, 1 mmol/L EDTA, PH 7.4), 0.1% SDS (standard saline citrate) (w/v), 5x Denhardt's solution (0.5 g Ficoll 400, 0.5 g polyvinylprodrolidone), 0.5 g bovine serum albumin]. After prehybridization, membranes were hybridized in the same condition as the prehybridization for 16 hours with the addition of labeled cDNA probe for rat RBP (kindly provided by Dr. Dianne R. Soprano, Temple University, Philadelphia, Pennsylvania), which was labeled using Random Primers DNA Labeling System (Life Technologies, Burlington, ON, Canada) with [^{32}P] dATP (3000 Ci/mmol, Amersham Canada, Oakville, ON, Canada). Following hybridization, membranes were washed 3 times for 20 minutes each at room temperature with 0.1 x SDS and 2 x SSPE followed by a 20 minutes wash at 65 °C with 0.1 x SSC and 0.1 x SDS and then exposed at -70°C with intensifying screen to KODAK XAR5 film (Eastman Kodak, Rochester, N.Y., USA.). The appropriate exposures of radioautograms were quantified using laser

densitometry [Model GS-670 Imaging Densitometer, BioRad Laboratories (Canada), Mississuaga, ON, Canada]. The 28S and 18S ribosomal RNA bands were used to check the integrity of RNA and to compensate for any loading discrepancies.

Determination of Zinc

Zinc levels in the plasma (Butrimovitz, 1977), liver (Luterotti 1992) and urine (Kiilerich et al., 1980) were determined using flame atomic absorption spectrophotometry (Perkin Elmer 4000) with a zinc standard (Fisher Scientific Ltd., Edmonton, AB, Canada). All glassware was rinsed with a 20% nitric acid solution and deionized water to avoid any contamination.

Plasma zinc was diluted 1/5 with deionized water. Working zinc standard for plasma was prepared in 5% glycerol to approximate the viscosity characteristics and aspiration rates of the diluted plasma sample.

Urine was acidified to pH 0-1 with the addition of 10% HCl. All urine samples and standard were then diluted with deionized water.

Weighed liver samples were cut into small pieces using stainless-steel surgical scissors and mixed. The pooled material was then randomly divided into equal weighed portions. A portion of pooled liver sample was homogenized with five-fold (by mass) of deionized water in a Polytron homogenizer for 1 minute. The aqueous homogenate was then digested in HCl and shaken for 30 min, followed by centrifugation at 12,000 g. An additional dilution of deionized water was carried out to give a final dilution of 20-folds. Standards were prepared by diluting the stock solution with deionized water.

Another portion was used to determine the dry mass by drying the sample in preweighed porcelain crucible for 23 hours at 110 °C, cooling in a dessicator and weighing.

Determination of Plasma Glucose, Insulin and Glucagon

Plasma glucose was measured by the Glucose-UV detection method of Abbott Laboratories (Lot #. 37243 HW) and performed on Molecular Devices Corporation's Microwell Plate Reader. Insulin was analyzed by a solid phase two-site enzyme immunoassay kit for rat insulin (Mercodia AB, Sweden, Lot #.1732). Plasma glucagon was assayed by the competitive enzyme immunoassay on Zeus Scientific Inc.'s Microwell Plate Reader using the Peninsula Lab procedure (Lot #. 980443). All assay runs were calibrated. Duplicate sets of calibrators, positioned at random along with the test samples, were used for the calibration curve.

Determination of Plasma Total Cholesterol and Triglyceride

The plasma samples were analyzed for total cholesterol and triglyceride using enzymatic kits obtained from Sigma Biochemicals (Catalog # 7921 and 336, respectively).

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analyses were performed by SAS computer program (Version 6.12, SAS Institute, Cary, NC). The level of significance was set at $p<0.05$. Paired student-t test was used to determine the differences between

age-paired BBd and BBn rats. The Pearson's correlation coefficient was used to quantify the strength of the association between plasma concentrations of vitamin A and its carrier proteins.

2. 3 RESULTS

No significant differences in the mean body weight gain (239 ± 32.5 vs 230 ± 40.1 g) and food intake (17.6 ± 0.8 vs 16.9 ± 1.1 g/day) between BBd and BBn rats were observed. All BBd rats exhibited the characteristic signs of diabetes, including elevated blood and urinary glucose, water intake and volume of daily urinary excretion. Besides hyperglycaemia, BBd rats were also characterized by hyperglucagonemia and hyperlipidemia (Figure 2-1). Glucagon, triglyceride and total cholesterol in plasma were increased by 57.5 %, 161% and 52.5% in BBd rats, respectively. However, the plasma insulin levels remained unaffected in BBd rats. The onset age of diabetes and its incidence rate among BBdp rats were 89 ± 4.4 days and 75%, respectively.

The BBd rats had decreased concentrations of plasma retinol in parallel with its carrier proteins, RBP and TTR, which were reduced in both the plasma and the liver compared to those of BBn rats (Table 2-2). A significant decrease in the abundance of the hepatic RBP mRNA was also observed in the liver of BBd rats (Figure 2-2). The hepatic concentrations of retinol in its free state decreased while the total vitamin A levels were similar to the control. According to a regression analysis, the plasma concentrations of RBP but not TTR were significantly correlated with plasma levels of vitamin A in both diabetic and control animals (Table 2-3).

At the onset of diabetes, the BBd rats exhibited biochemical evidence of zinc deficiency, as indicated by a decreased circulatory level accompanied by an increased urinary excretion of this trace element (Figure 2-3). Liver zinc concentrations, however, were not affected in the presence of hyperglycemia.

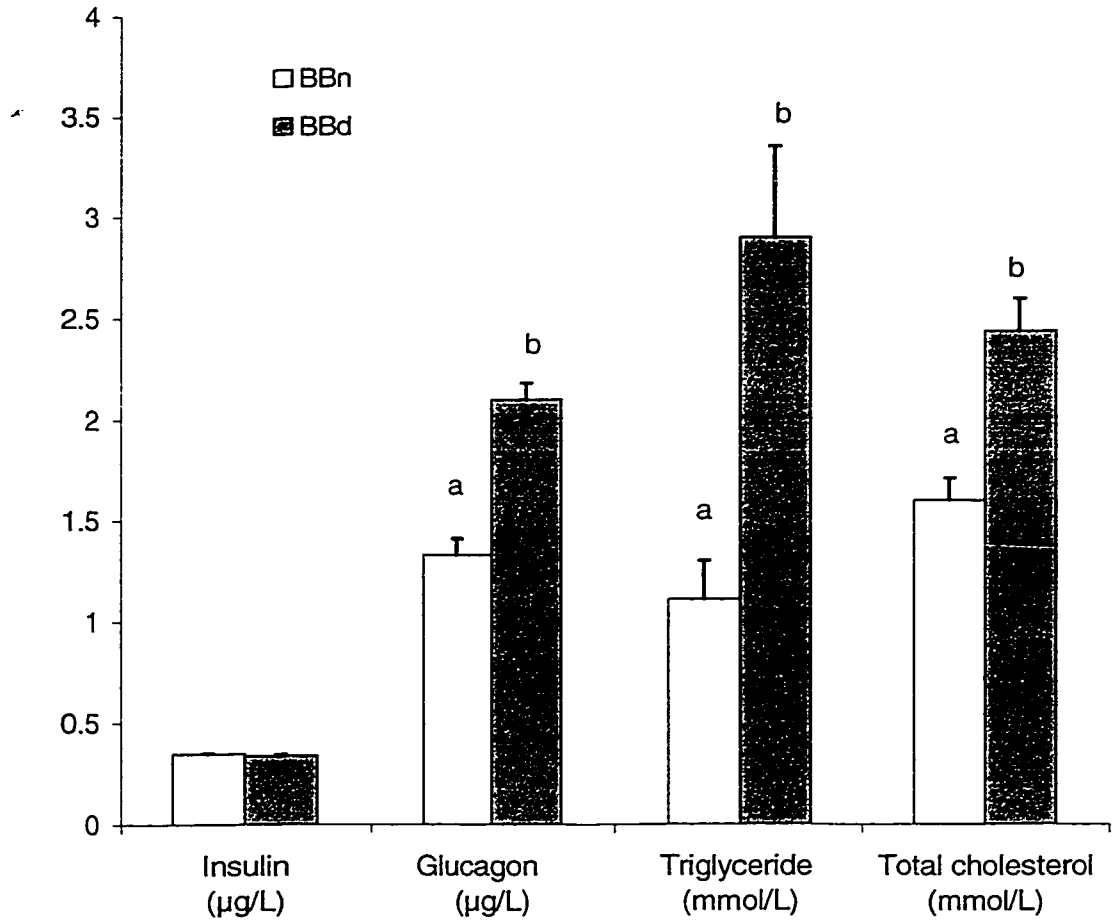

Figure 2-1. General plasma characteristics of age-paired BBn and BBd rats. Results are expressed as mean + SEM (n=6). Within each parameter, bars with different letters are significantly different at $p<0.05$ as analyzed by paired student-t test.

Table 2-2. Vitamin A status of age-paired BBn and BBd rats

	BBn	BBd	P-value
Plasma vitamin A (μmol/L)	1.10 ± 0.17	0.70 ± 0.16	0.01
Free retinol (μmol/g liver)	0.1605 ± 0.037	0.056 ± 0.014	0.01
Hepatic vitamin A (μmol/g liver) *	1.23 ± 0.17	1.08 ± 0.18	NS †
Plasma RBP (μmol/L)	4.26 ± 0.91	2.72 ± 0.43	0.04
Plasma TTR (μmol/L)	8.08 ± 1.29	6.03 ± 1.03	0.04
Liver RBP (nmol/g liver)	2.18 ± 0.22	1.29 ± 0.18	0.009
Liver TTR (nmol/g liver)	1.58 ± 0.32	0.81 ± 0.07	0.03

Results are expressed as mean ± SEM (n=6). Paired student-t test was used to determine differences between group means.

* Hepatic vitamin A includes free retinol + retinyl esters.

† NS = P>0.05.

Table 2-3. Correlation between the concentrations of vitamin A and its carrier proteins in plasma of BBn and BBd rats

	Pearson's correlation coefficient (r)	P-value
BBn (n=8)		
Plasma Vitamin A with RBP	0.79	0.02
Plasma Vitamin A with TTR	0.42	0.30
BBd (n=8)		
Plasma Vitamin A with RBP	0.88	0.004
Plasma Vitamin A with TTR	0.55	0.16

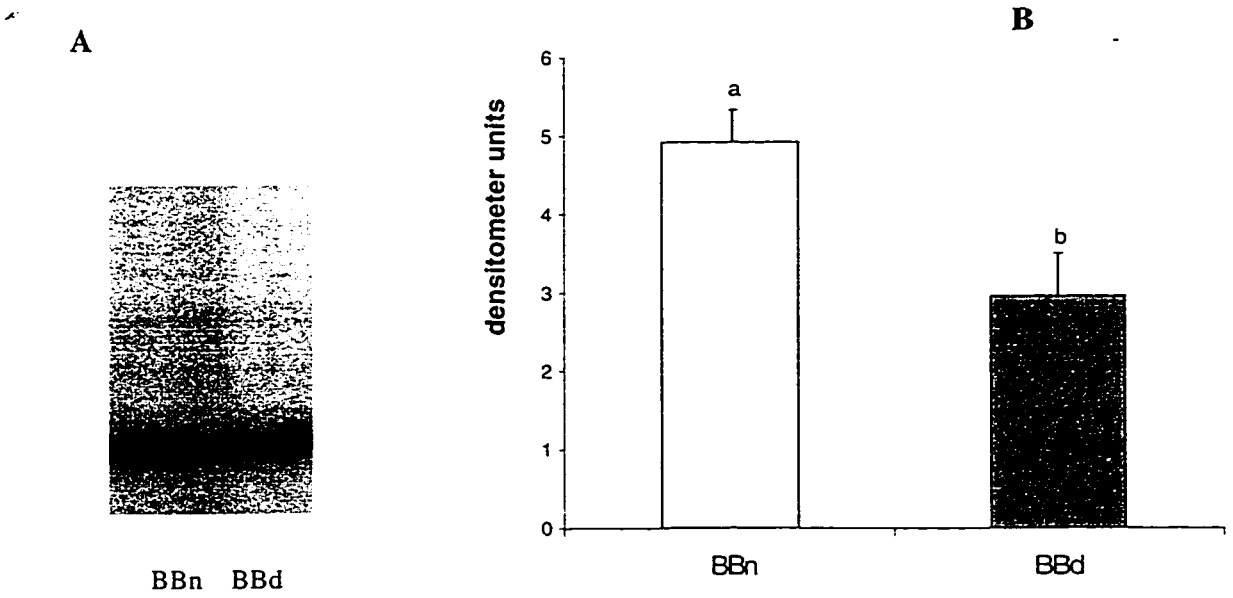

Figure 2-2 (A, B) Quantification of hepatic RBP mRNA of age-paired BBn and BBd rats by northern blotting

A. Northern blot analysis of RBP mRNA isolated from the livers of BBn and BBd rats.

B. Densitometric determination of changes of hepatic RBP mRNA. Bars are means + SEM (n=6). [a, b] Significantly different at $p<0.05$ as analyzed by paired student-t test.

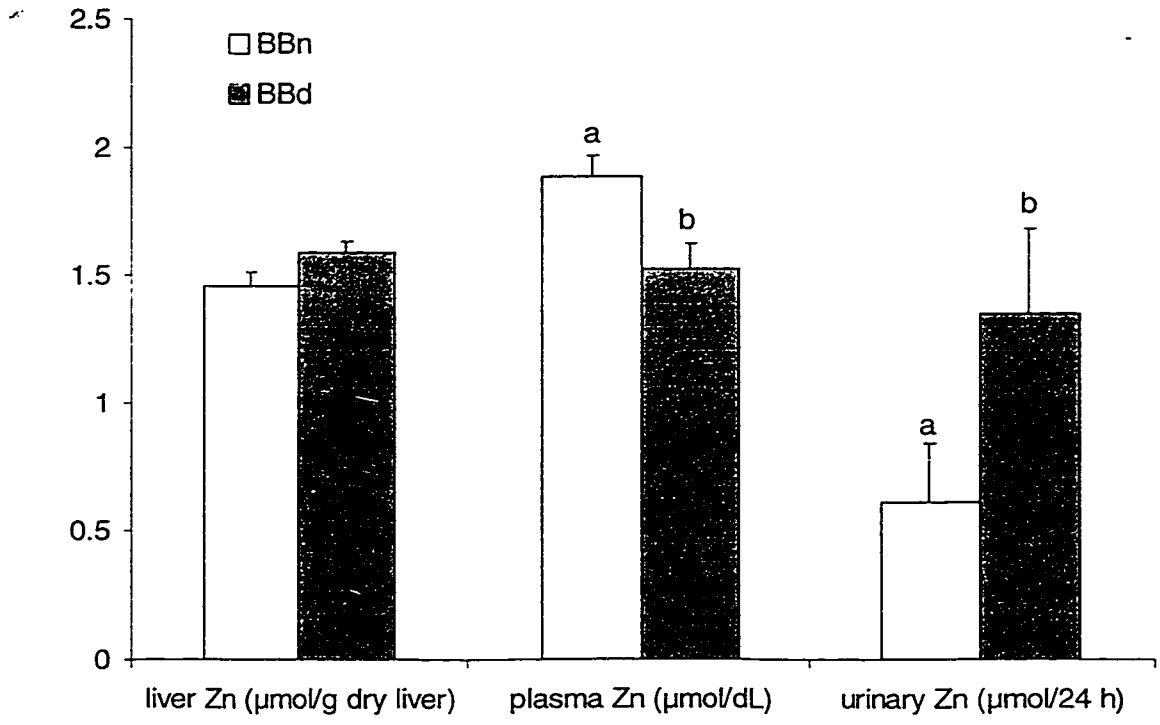

Figure 2-3. Tissue zinc content of age-paired BBn and BBd rats. Bars are means + SEM (n=6). Within each parameter, bars with different letters are significantly different at $p<0.005$ as analyzed by paired student-t test.

2. 4 DISCUSSION

These results clearly demonstrate that the presence of the hyperglycaemic state affects vitamin A metabolism in BB rats as indicated by decreased levels of plasma retinol and its carrier proteins in the plasma and the liver. These results agree with the previous studies involving type I diabetes patients (Basu et al. 1989) and STZ-induced diabetic rats (Tuitoek et al. 1996a & b), in which the plasma levels of retinol, RBP and TTR were all markedly reduced.

Onset of diabetes in BB rats is known to be associated with hyperglycaemia, hypoinsulinemia and hyperglucagonemia (Crisa et al. 1992). In the present study, the BB rats with the onset of diabetes also exhibited hyperglycemia and hyperglucagonemia. However, no significant difference in plasma insulin was found between BBd and their paired BBn rats. One possible explanation for this unanticipated finding is that diabetic rats in our study were at an early stage of diabetes and were killed within 24 hours after hyperglycemia was detected. According to a time-course study (Tannenbaum et al, 1981), plasma insulin levels in BB rats appear to decline gradually to an unmeasurable value following 4 to 8 days after the onset of diabetes. In our study, the time-lapse following the onset of diabetes may not be long enough to have an appreciable effect on insulin levels.

At the onset of diabetes, the BB rats had significantly reduced levels of plasma vitamin A, while its hepatic concentrations remained unaffected compared with those of their paired BBn rats. Both BBd and BBn rats had a similar daily food intake; previous

studies have shown that using STZ-induced diabetic rats the intestinal absorption of retinol is unaffected in the presence of diabetes (Tuitoek et al. 1994). Hence, the depressed plasma levels of vitamin A and its control levels in the liver is unlike due to differences in the intake of vitamin A or its bioavailability in diabetic rats. Vitamin A is mainly stored in the liver; its circulatory levels account for less than 1% of the total body pool of vitamin A. Thus, it is possible that a reduction of plasma vitamin A levels in BBd rats may not be enough to affect the total hepatic storage pool of vitamin A.

While the total vitamin A concentrations in the liver remained unaffected, the hepatic levels of retinol in its free state were markedly reduced in BBd rats. These results are different from the study involving STZ-induced diabetic rats, in which an elevated level of free retinol in the liver was observed (Tuitoek et al. 1996a). In the STZ studies, these animals ate 50% more food compared with their non-diabetic control counterparts. The raised free retinol levels in the STZ-induced diabetic rats may be a reflection of an increased food intake and hence the higher vitamin A intake. This was not the case, however, with BBd rats that had food intake levels similar to the control rats. The transformation of retinyl esters to free retinol in the liver is regulated by the hepatic retinyl ester hydrolases, including bile salt-dependent (BSDREH) and bile salt-independent retinyl ester hydrolases (BSIREH) (Blaner et al. 1994). BSIREH is thought to be involved in the initial hydrolysis of dietary retinyl esters delivered to the liver from the gut. BSDREH, due to its high specific activity in vitamin A storage site (e.g., hepatic stellate cells), is believed to be more essential for the later retinyl esters hydrolysis and retinol release in the liver (Harrison, 1993). Biliary structure and hepatic function are affected in diabetes (Watkins et al. 1995). In addition, it has been reported

that BBd rats have alterations in bile flow, biliary secretion of bile acid and cholesterol metabolism (Gonzalez et al. 1992). It is thus possible that diabetes-induced modification of bile salt metabolism may account for the impaired vitamin A hydrolysis activity in BBd rats.

It has been suggested that homeostasis of plasma retinol is essentially regulated through synthesis and secretion of RBP (Basu 1996). Thus, as expected, a significant correlation between plasma retinol and RBP in both BBd and BBn rats was observed. BBd rats had a marked reduction in plasma and hepatic RBP concentrations. A parallel decrease in its hepatic mRNA abundance suggests that a reduction in RBP synthesis may account for the decreased retinol concentrations in plasma. The underlying mechanism for this decreased hepatic RBP synthesis at the onset of diabetes in BB rats, is not clear. According to an early study, a zinc deficiency diet resulted in significantly reduced plasma vitamin A, RBP and hepatic RBP concentrations, suggesting a linkage between zinc status and hepatic RBP synthesis (Brown et al. 1976). The role of zinc in regulating gene expression has been well documented (Clegg et al. 1989). Zinc is required for nucleic acid metabolism and stabilization. Both DNA polymerase and RNA polymerase are zinc metalloenzymes. Reduced activities of these two enzymes accompanied by a zinc deficiency have been reported (Wu et al. 1983). Type I diabetes is characterized by hyperzincuria which has been also demonstrated in our study. BBd rats were thus associated with markedly hyperzincuria and a reduction in plasma zinc levels. It is therefore reasonable to suggest that the reduced circulatory and hepatic levels of RBP in BBd rats may be a metabolic consequence of zinc deficiency.

Increased urinary excretion of RBP has been reported in type I diabetes patients (Dubrey et al. 1997). RBP is a low-molecular-weight protein which could be freely filtered through the glomerulus and reabsorbed by the proximal tubule. An increased urinary RBP excretion in diabetes has been suggested to indicate an early sign of diabetic nephropathy. Therefore, this loss of RBP may also contribute to the depressed circulatory RBP levels in BB rats.

In summary, the present study revealed that metabolic availability of vitamin A is impaired in BB rats with the onset of diabetes. The mechanism for this abnormality is not yet fully understood. Possible factors may include alteration in hepatic retinyl ester hydrolase activity, synthesis and renal clearance of retinol carrier proteins and perturbed zinc metabolism.

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3. EFFECTS OF DIETARY VITAMIN A, ZINC SUPPLEMENTATIONS AND INSULIN ADMINISTRATION ON THE METABOLIC AVAILABILITY OF VITAMIN A IN BB RATS

3. 1 INTRODUCTION

Type I diabetes has been reported to be associated with biochemical evidence of vitamin A deficiency, as evidenced by decreased levels of plasma vitamin A and RBP, accompanied by an increased urinary excretion of RBP (Basu et al. 1989; Dubrey et al. 1997). The metabolism of Zn, an important factor for the synthesis of vitamin A carrier proteins, has also been found to be disturbed in the presence of diabetes, as indicated by hyperzincuria (Heise et al. 1988; Cunningham et al. 1994). Using STZ-induced diabetic rats, it has been demonstrated that circulatory levels of retinol are reduced in diabetes, while its hepatic storage is markedly elevated (Tuitoek et al. 1996a). Insulin administration to these rats normalized the metabolic availability of vitamin A, suggesting that the metabolic derangement of vitamin A in STZ-induced diabetic rats was primarily due to the lack of insulin (Tuitoek et al. 1996c). However, the nephrotoxic and hepatoxic effects of STZ in these studies cannot be totally excluded. In addition, STZ-induced diabetic rats, although hypoinsulinemic, are not dependent on exogenous insulin for survival and hence their conditions does not entirely resemble human type I diabetes. Thus the altered vitamin A metabolism observed in STZ-induced diabetic rats needs to be re-examined using a better animal model for human type I diabetes.

The BB rat is a useful model for type I diabetes. As in humans, diabetes syndrome in BB rats is spontaneous and results in the autoimmune destruction of the insulin-producing betâ cells of the pancreas (Crisa et al. 1992). Using these rats, vitamin A metabolism has been shown to be affected as reported in STZ-induced diabetic rats, as indicated by decreased plasma retinol and its carrier proteins in the plasma as well as in the liver in BB rats at the onset of diabetes (chapter 2).

In order to determine if the biochemical evidence of vitamin A deficiency in diabetic BB rats could be normalized, the present study was undertaken to examine the responses of BB rats to dietary vitamin A and zinc supplementations. This study was further extended to examine the effect of insulin administration on the metabolic availability of vitamin A in diabetic BB rats. The immune responses, with particular reference to NK cell cytotoxic activity and mononuclear cell phenotyping in the spleen, to these treatments were also determined. The purpose of the latter study was to examine the modifying effects of vitamin A and zinc supplementations on the pathogenesis of diabetes.

3. 2 MATERIALS AND METHODS

Animals and Diets

BBdp and BBn 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

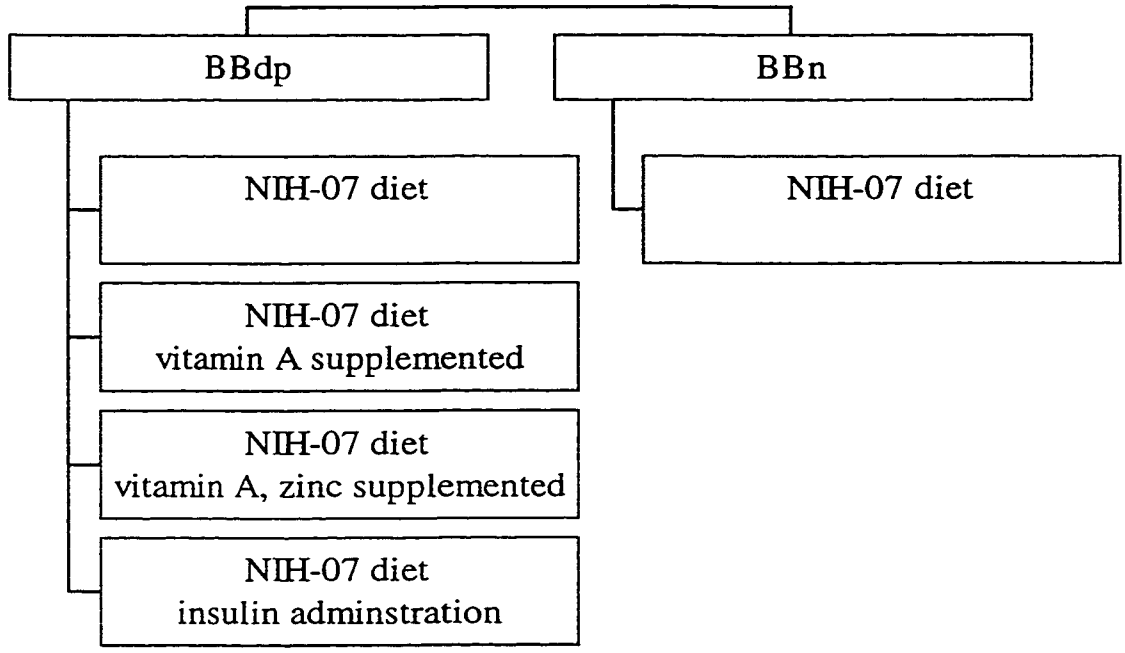
colony. Approximately 50 to 80% of the BBdp rat lines develop Type I diabetes within 60 to 120 days after birth, while BBn rat lines do not develop diabetes spontaneously and are similar to normal rats. All studies were reviewed and approved by the University of Alberta Animal Welfare Committee. Animals were housed in temperature and humidity-controlled room with a 12-hour light/dark cycle.

Group 1, including BBn rats receiving NIH-07 diet (Table 2-1, chapter 2), was used as controls; groups 2 to 5 were all BBdp rats. Weanling (21 d) BBdp pups from each litter were evenly distributed to 4 treatment groups of 12 rats each (Figure 3-1). Group 2 received NIH-07 diet only. Groups 3 and 4 received basal diet supplemented with retinyl palmitate (60.5 IU/g diet) either alone or in combination with zinc (180 ug/g diet), respectively. Group 5 received basal diet with a daily single dose of insulin injected subcutaneously (2-4 unit/day) at noon for one week to maintain plasma glucose at 300-400 mg/dL after the onset of diabetes, and were killed in the morning following one week treatment. All animals in other groups were killed within 24 hours after the diagnosis of diabetes. By 120 days of age, BBdp rats that had not developed diabetes were also killed.

Food intake and body weight were monitored throughout the studies. BBdp rats older than 50 days of age were tested for glycosuria by Testape (Eli Lilly, Indianapolis, IN, U.S.A.), 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 hyperglycaemia (blood glucose >200 mg/dL); these rats were described as BBd rats. Animals were killed using carbon dioxide after overnight fasting. Blood was collected in heparinized tubes. In order 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. Livers were removed, immersed in liquid nitrogen and stored at –72°C for later analysis.

Figure 3-1. Experimental design



Determination of Vitamin A

Plasma (Nierenberg et al. 1985) and liver (Forlik et al. 1984) vitamin A were assayed by HPLC as described in chapter 2.

RNA Isolation and Northern Blot Analysis

Total RNA isolation and Northern blot analysis (Reimer et al. 1997) were performed from the liver samples as described in chapter 2.

Determination of Zinc

Zinc levels in the plasma, liver and urine were determined using flame atomic absorption spectrophotometry (Perkin Elmer 4000) with a zinc standard (Fisher Scientific Ltd., Edmonton, AB, Canada) as described in chapter 2 (Butrimovitz 1977; Luterotti 1992; Kiilerich 1980). All glassware was rinsed with a 20% nitric acid solution and deionized water to avoid any contamination.

Preparation of splenocytes

Splenocytes were prepared by pressing slices through a nylon mesh in cold Krebs-Ringer HEPES Buffer (KRH) (pH 7.4) supplemented with 0.5% (w/v) bovine serum albumin (fraction V; Gibco, Burlington, ON, Canada). After centrifugation at 4°C, red blood cells were lysed with a solution containing 155 mM NH_4CL, 0.1mM disodium EDTA and 10 mM $KHCO_3$, followed by three washes with KRH buffer. Isolated cells were suspended in cell-culture medium RPMI 1640 supplemented with 4% (v/v) heat-inactivated fetal

calf serum (ICN, Montreal, Canada), penicillin ($1x10^5$ U/L), streptomycin (100 mg/L), amphotericin B (25 mg/L), glutamine (4 mmol/L), 2-mercaptoethanol (2.5 μmol/L), and HEPES (25 mmol/L). Cell viability was assessed by Trypan Blue exclusion.

Mononuclear cell phenotyping

Mononuclear cell subsets from the spleen were characterized by an immunofluorescence assay (Field 1995), 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 recognises a surface glycoprotein found on rat T-helper cells (CD4); and OX8, which recognises T cytotoxic/suppressor lymphocytes (CD8) and NK cells. For identifying B lymphocytes, T lymphocytes, macrophages and NK cells, one-color staining method was used. Aliquots of 4 x 10^5 splenocytes were incubated for 30 minutes 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 for another 30 minutes in 50 μL of a 1:300 dilution of fluorescein isothiochanate-conjugated goat anti-mouse IgG (FITC) (Organon-Teknika, Scarborough, Canada). T lymphocyte subset was identified by two-color staining method. OX19 and W3/25, recognises rat T-helper cells (CD4), OX19 and OX8 recognises T cytotoxic/suppressor lymphocytes (CD8), and OX8 and W3/25 recognises $CD4^+CD8^+$ T

cells. Aliquots of 4 x 10^5 splenocytes were incubated with first monoclonal antibodies (OX19, OX19 and OX8 respectively). After FITC binding and washing, second phenotypic antibodies (W3/25, OX8, W3/25 respectively) were added, incubated and washed as before. Finally, 10 μL of a 1:25 dilution of phycoerythrin-conjugated goat-anti-mouse IgG(PE) (Cedarlane Laboratorlies) was added to each well and incubated for 30 minutes at 4°C.

After the last incubation with antibodies, cells were washed three times with assay buffer and fixed in PBS containing 1% (w/v) 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 FITC or PE only.

NK cell cytotoxic activity

NK cell cytotoxic activity was measured using a 4-hour ^{51}Cr release assay (Field 1995) in isolated splenocytes 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 cell ratios, which were between 2:1 and 100:1. After 4 hours of 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 cytotoxic activity was calculated as % Specific Lysis= 100 x

[(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. 1 LU is the number of NK cells (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). The level of significance was set at $p<0.05$. Data were analyzed by two-way ANOVA, which 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 cytotoxic activity was analyzed by a one-way split-plot procedure. Fisher's exact test was used to analyze the cumulative incidence rate of diabetes.

3. 3 RESULTS

All BBd rats exhibited the characteristic signs of diabetes, including elevated blood and urinary glucose, water intake and the volume of daily urinary excretion. The onset age of diabetes in different treatment groups is shown in Table 3-1; it remained unaffected by dietary supplementations with vitamin A alone or in combination with zinc. The incidence rate of diabetes in these animals was 54%, 50% and 67%, respectively.

However, the differences among them were not statistically significant. No significant differences in the mean body weight gain and food intake were observed among the groups (Table 3-2).

In order to identify whether vitamin A deficiency in BBd rats is due to genetic or disease factors, vitamin A concentrations in the plasma and the liver were examined in BBdp rats that remained diabetes-free by 120 days of age and were fed NIH-07 diet. No significant differences were observed in BBdp rats compared to those of controls (Figure 3-2).

Following vitamin A supplementation up to 3 months, the hepatic concentrations of vitamin A were highly elevated in the face of its depressed circulatory status, compared with those of rats fed a basal diet (Figure 3-3, Figure 3-4). Neither zinc supplementation nor insulin treatment altered this trend. It was noteworthy that although vitamin A plus zinc supplemental intake had no modifying effect on the plasma vitamin A, this dietary regime sufficed to increase the abundance of RBP mRNA in the liver of the BBd rats. On the other hand, insulin treatment failed to change the lower abundance of RBP mRNA in the liver (Figure 3-5).

Neither vitamin A alone nor its combination with zinc supplementation improved the diabetes-associated depressed circulatory zinc concentrations of BBd rats (Figure 3-6). Urinary and hepatic concentrations of zinc, however, were markedly increased in the presence of its supplemental intake. Nevertheless, the differences between intakes with and without zinc supplement were not statistically significant. When BBd rats were treated with insulin subcutaneously for one week after the onset of diabetes, only hyperzincuria was decreased; depressed plasma zinc was not reversed.

Neither diet nor insulin treatment significantly affected splenic cell subset distribution in BBd rats (Table 3-3). Calculated as % specific lysis, the NK cell cytotoxic activity was significantly greater for splenocytes in all BBd rats than in BBn rats (Figure3-7). It was higher in the presence of vitamin A supplementation alone or in combination with zinc, compared with that of BBd rats on the basal diet; however, the differences among them were not statistically significant. The degree of NK cell cytotoxic activity was markedly decreased in response to insulin treatment, but still higher than that of BBn rats. The lytic unit which expressed on a per-cell basis, was not affected by either diet or insulin administration and was lower for all BBd rats than for BBn rats (Figure 3-8).

Table 3-1. Rate and onset age of diabetes in BBdp rats receiving NIH-07 diet, supplemented with vitamin A alone, or in combination with zinc.

	Number	Incidence rate (%)*	Age of onset of diabetes (d)†
NIH-07	12	50%	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 analysed by Fisher's exact test.

† Results are expressed as means ± SEM

Table 3-2. Effect of vitamin A supplementation alone or in combination with zinc, and insulin treatment on animal characteristics *

	BBn NIH-07 only	BBd insulin treated	BBd NIH-07 only	BBd NIH-07+vitamin A	BBd NIH-07+vitamin A+Zn
Food intake (g/day)	18.4 ± 0.8	20.0 ± 0.8	19.8 ± 1.4	18.9 ± 1.3	18.7 ± 1.0
Body weight gain (g)	265.2 ± 25.8	269.8 ± 22.0	265.5 ± 24.2	240.8 ± 27.5	237.4 ± 28.0

*Results are expressed as means ± SEM of at least five animals. No significant differences were found.

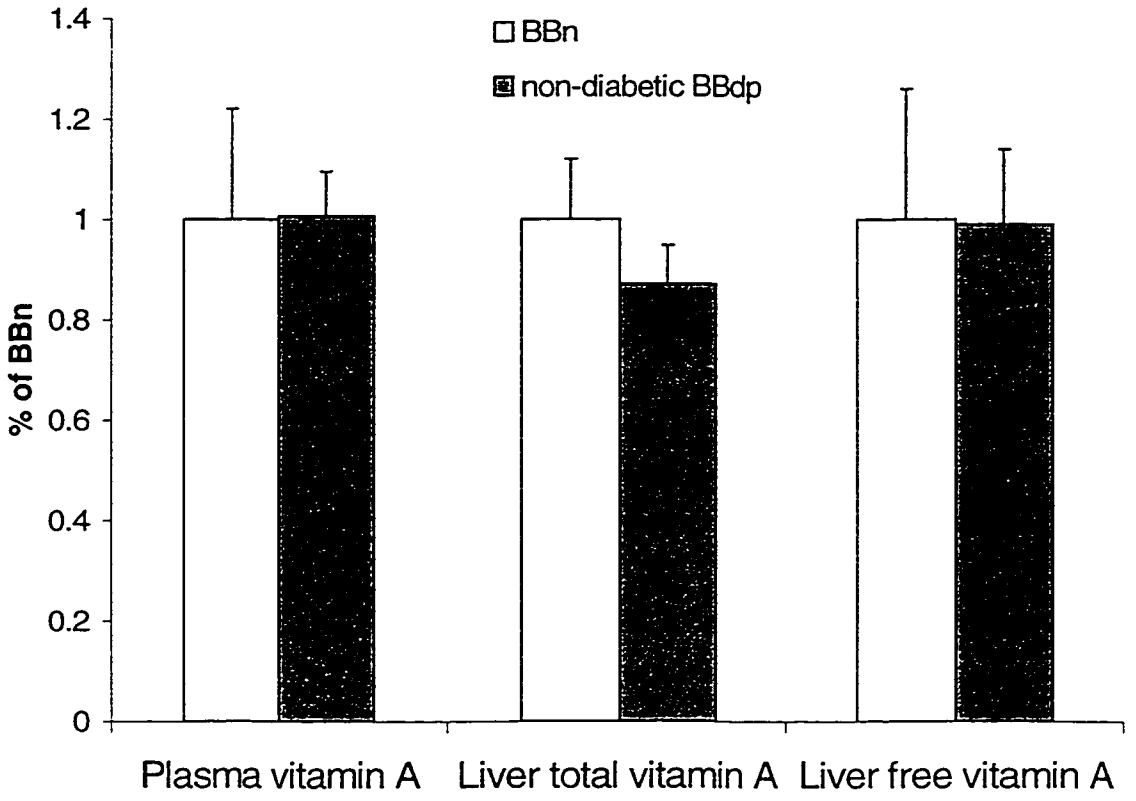


Figure 3-2. Plasma and liver vitamin A levels in non-diabetic BBdp to BBn rats at 120 days of age. Results are expressed as means + SEM of at least five animals. No significant differences were found for each comparison.

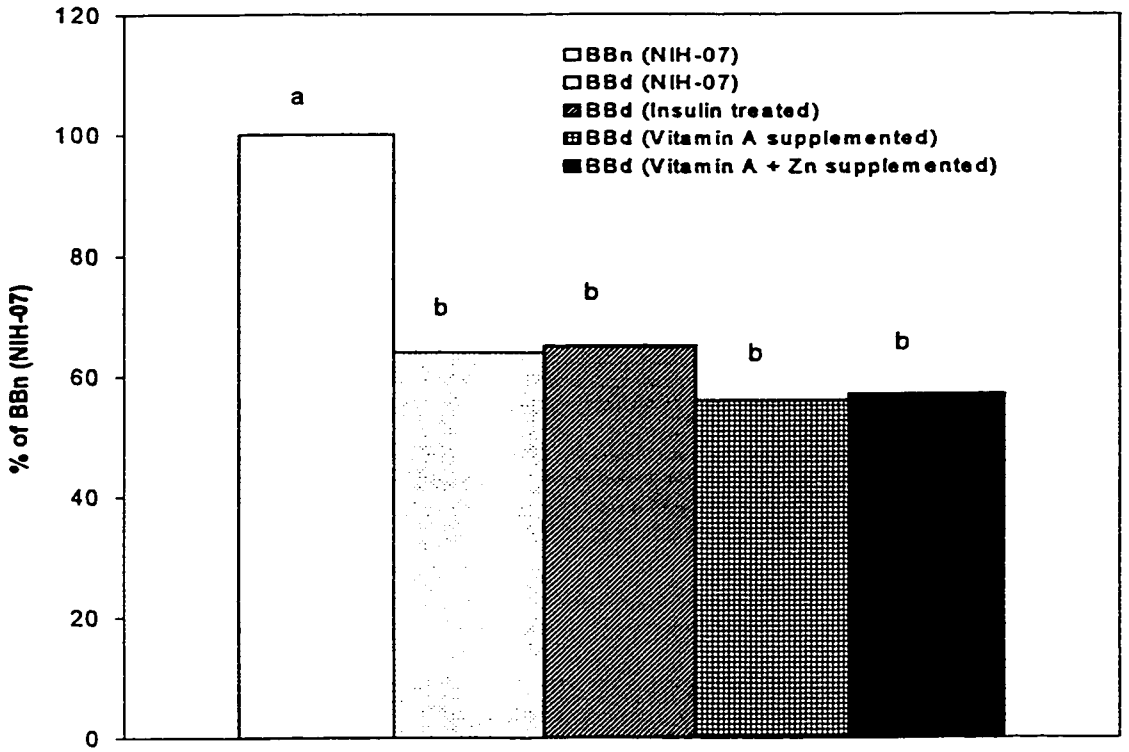


Figure 3-3. Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on the plasma vitamin A levels of BBd rats. Results are expressed as means of at least five animals. Letters not shared show significance ($p<0.05$).

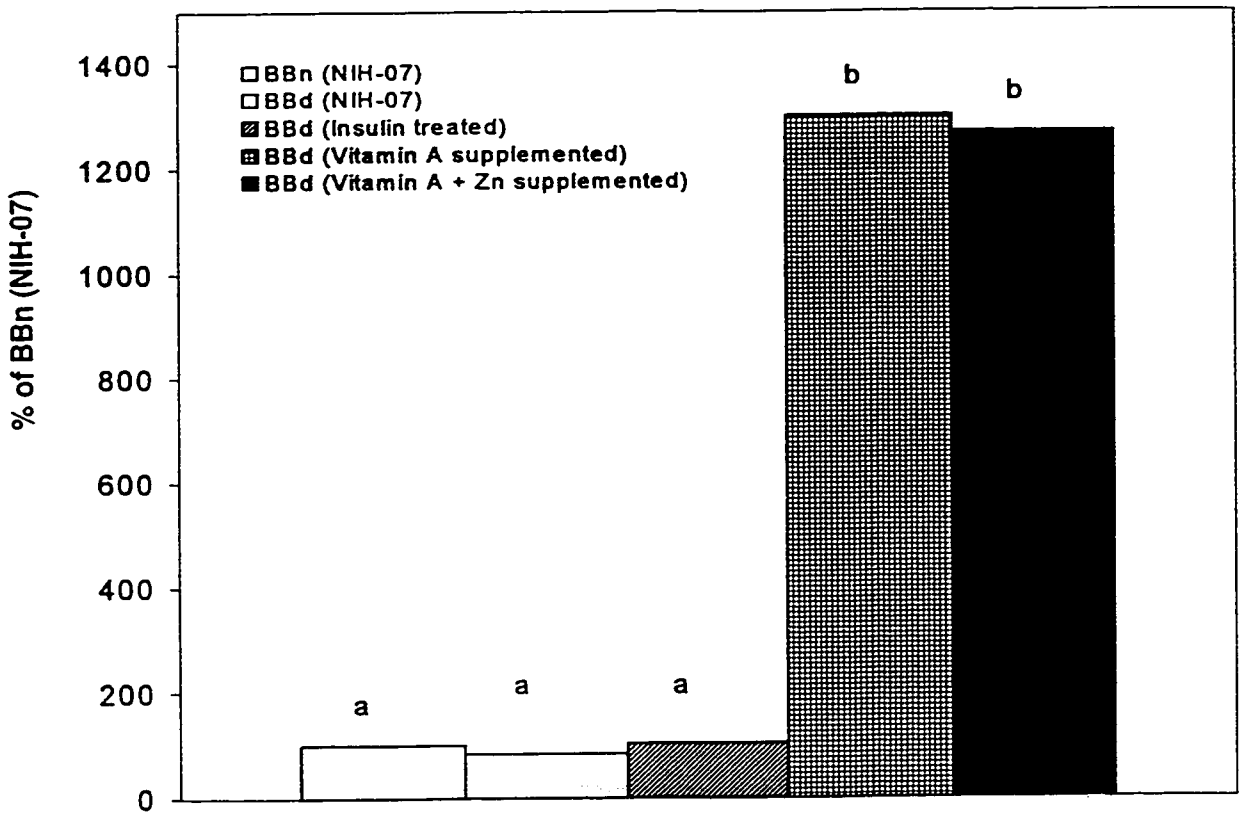


Figure 3-4. Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on the liver total vitamin A levels of BBd rats. Results are expressed as means of at least five animals. Letters not shared show significance ($p<0.05$).

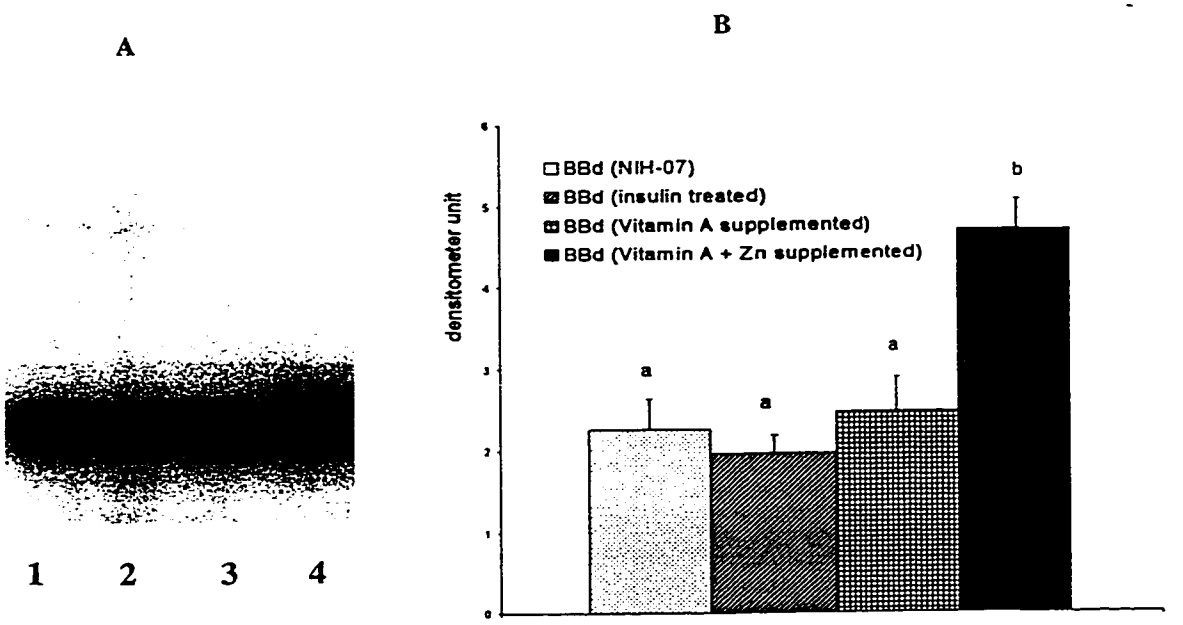


Figure 3-5 (A, B) Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on the abundance of liver RBP mRNA content of BBd rats.

A. Northern blot analysis of RBP mRNA isolated from the livers of BBd rats. Lane 1 control; Lane 2 insulin treated; Lane 3 vitamin A supplemented; Lane 4 vitamin A + Zn supplemented.

B. Densitometric determination of changes of hepatic RBP mRNA of BBd rats. Bars are means + SEM at least five animals. Bars with different letters are significantly different at $p<0.05$.

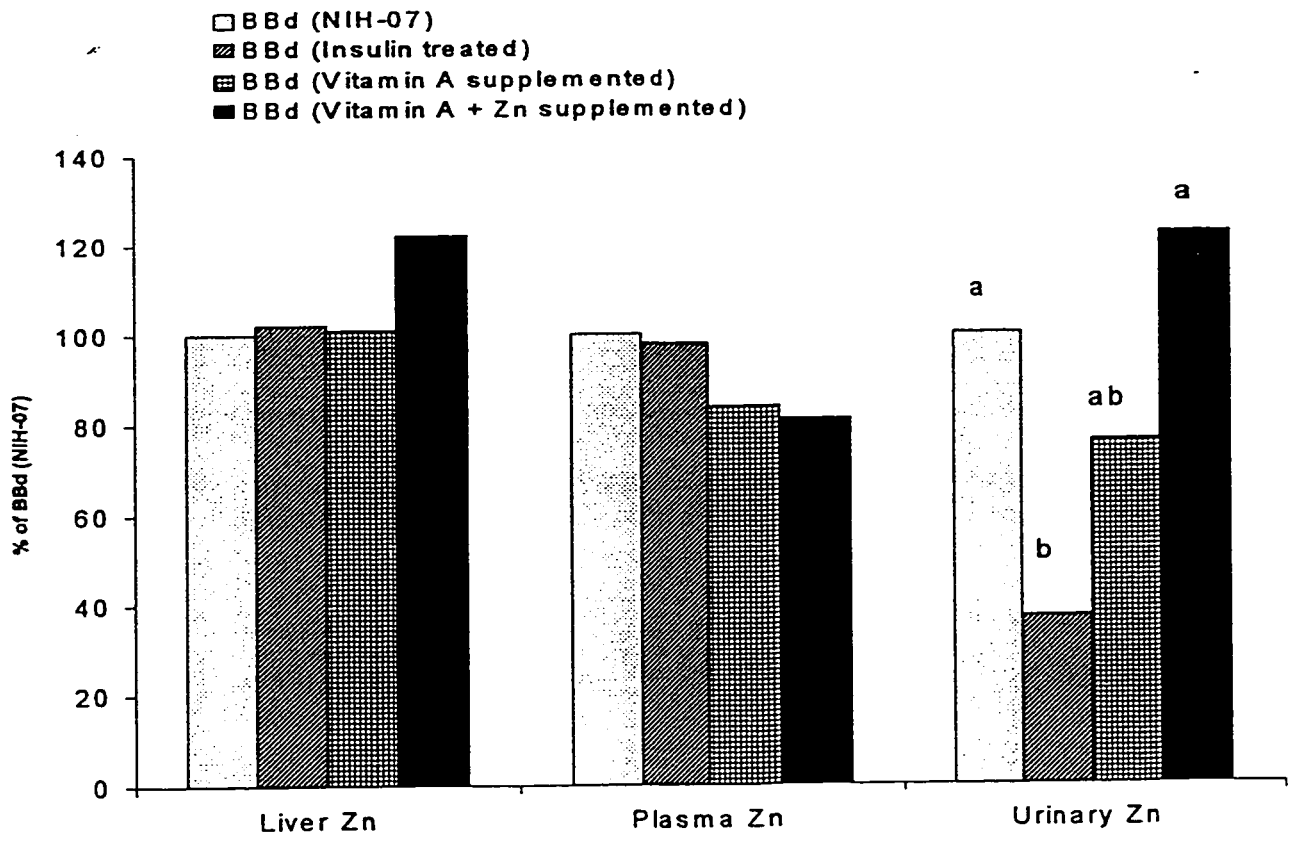


Figure 3-6. Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on tissue zinc levels of BBd rats. Results are expressed as means of at least five animals. Within each parameter, letters not shared show significance ($p<0.05$).

Table 3-3. . Effect of vitamin A supplementation alone or in combination with zinc, and insulin treatment on monoclonal antibody identified phenotypes in the rat spleen

	BBn NIH-07 only	BBd insulin treated	BBd NIH-07 only	BBd NIH-07+vitamin A	BBd NIH-07+vitamin A+Zn
B cells ($OX12^+$)	25.8 ± 2.3^a	53.5 ± 2.4^b	60.4 ± 3.0^b	58.2 ± 2.6^b	58.6 ± 2.0^b
T cells ($OX19^+$)	55.2 ± 1.7^a	5.7 ± 1.1^b	4.8 ± 0.8^b	5.2 ± 0.9^b	5.5 ± 0.6^b
$CD4^+$ ($OX19^+$ $W3/25^+$)	33.1 ± 1.1^a	3.9 ± 0.9^b	2.4 ± 0.5^b	3.0 ± 0.8^b	2.4 ± 0.4^b
$CD8^+$ ($OX19^+$ $OX8^+$)	15.0 ± 1.5^a	1.4 ± 0.6^b	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	1.1 ± 0.3^b	0.7 ± 0.1^b	0.9 ± 0.2^b	1.0 ± 0.1^b
NK cells ($3.2.3^+$)	3.5 ± 0.4	3.9 ± 1.4	3.7 ± 0.4	4.2 ± 0.8	4.1 ± 0.6
Macrophages ($OX42^+$)	11.0 ± 0.9^a	16.6 ± 2.4^{ab}	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 ($p<0.05$).

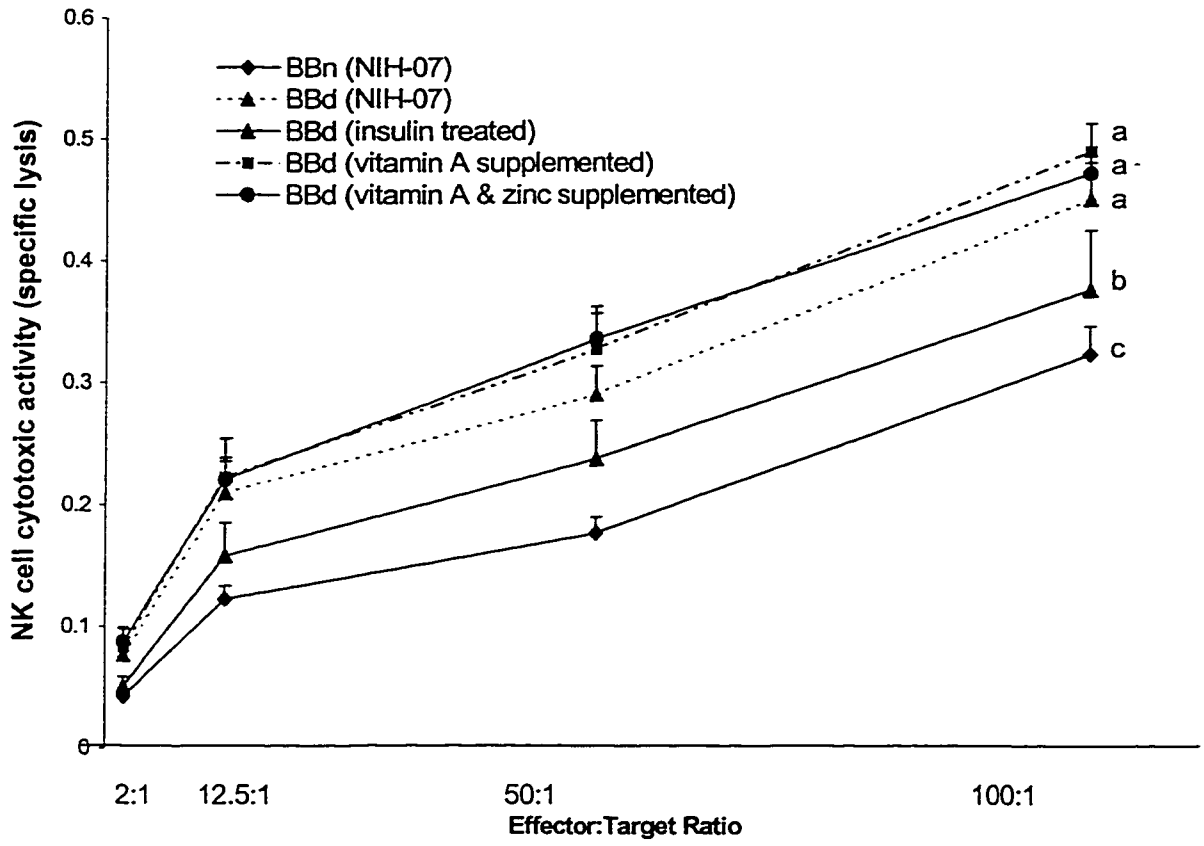


Figure 3-7 Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on NK cell cytotoxic activity in the rat spleen, which is expressed as % Specific lysis=100 x [(experimental release of ^{51}Cr - spontaneous release)/(maximum release - spontaneous release)].

Values are mean + SEM of at least five animals. Lines that do not share a common letter are significantly different at $p<0.05$.

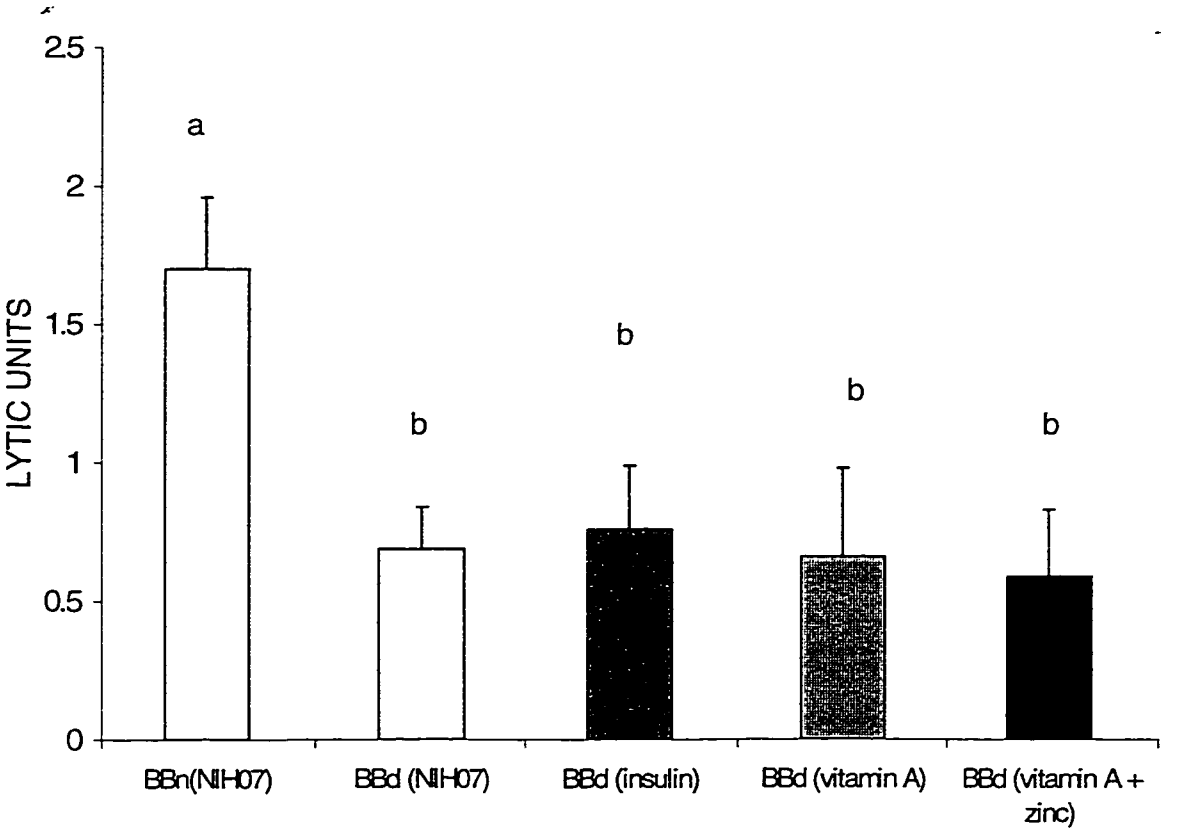


Figure 3-8 Effect of insulin treatment, vitamin A supplementation and vitamin A plus zinc supplementation on NK cell cytotoxic activity in the rat spleen, which is expressed as Lytic Units (LU) on a per-cell basis. 1 LU is the number of NK cells (10^{-3}) required to cause 20% lysis of target cells.

Values are mean + SEM of at least five animals. Bars that do not share a common letter are significantly different at $p<0.05$.

3. 4 DISCUSSION

The results of the present study show that BB rats with the onset of diabetes are associated with an impaired metabolic availability of vitamin A. Vitamin A concentrations in the plasma and the liver in BBdp rats without diabetes are similar to those of control rats, suggesting that vitamin A deficiency in BBd rats is primarily due to the presence of hyperglycaemia. Neither vitamin A supplementation alone nor its combination with zinc reversed vitamin A deficiency in BBd rats. These results agree with earlier studies involving STZ-induced diabetic rats (Tuitoek et al 1996a & c).

Despite the markedly elevated total hepatic storage of vitamin A in BBd rats following vitamin A supplementation, plasma levels of retinol remained lower. Vitamin A is predominantly stored in the liver. The total hepatic storage of vitamin A is proportional to its intake (Hick et al. 1984). The increased liver concentration of vitamin A may thus be explained in part by the increased vitamin A intake. This is consistent with the previous (chapter 2), which demonstrated that food intake and total hepatic storage of vitamin A were not affected in BB rats with the onset of diabetes.

The transportation of free retinol from the liver to target cells is accomplished by RBP (21,000 Da) which circulates in a 1:1 ratio with TTR (55,000 Da) (Blaner et al. 1994). A significant reduction of RBP and TTR concentrations was observed in the plasma and liver of BBd rats (chapter 2). A parallel decrease in RBP mRNA abundance in the liver (chapter 2), suggests that decreased hepatic concentrations of RBP are at least partly, due to its insufficient synthesis. Zinc has been identified as an important factor for the

synthesis of RBP and its metabolism is affected in diabetes (Smith 1982; Cunningham et al. 1994). The role of zinc in regulating gene expression has been well established (Clegg et al. 1989). Zinc is required for nucleic acid metabolism and stabilization. Both DNA polymerase and RNA polymerase are zinc metalloenzymes (Wu et al. 1983). In the present study, the BBd rats were associated with hyperzincuria in parallel with a reduction in plasma zinc levels. These results suggest that the reduced circulatory and hepatic levels of RBP in BBd rats are probably a metabolic consequence of a zinc deficiency. The evidence that reduced RBP mRNA was improved following zinc supplementation supports this hypothesis.

Despite increased RBP mRNA abundance in BBd rats in the presence of zinc supplementation, the circulatory vitamin A levels remained unaffected compared with those of BBd rats on a basal diet or a vitamin A supplemented diet. Two factors may contribute to this finding. First, RBP secretion is strictly regulated by the availability of its ligand, vitamin A, in the liver (Soprano et al. 1994). Lower hepatic levels of free retinol, a consequence of reduced activity of retinyl ester hydrolases, have been demonstrated in BBd rats (chapter 2). It is thus possible that the reduced circulatory levels of vitamin A in BBd rats were unaffected if the availability of vitamin A in the liver did not change. Second, BBd rats have been reported to be associated with an altered bile salt metabolism (Gonzalez et al. 1992). It has been demonstrated that abnormal bile salt metabolism could cause an altered hepatic subcellular distribution of RBP mRNA and subsequently altered protein levels (Imamine et al. 1996).

Altered vitamin A metabolism has been suggested to be primarily due to an insulin deficiency, as evidenced by the normalization of vitamin A concentrations in the plasma and the liver in STZ-induced diabetic rats after 4 weeks of insulin administration through implantation (Tuitoek et al. 1996c). However, our study failed to change this diabetes-associated vitamin A abnormality. This inconsistency may be explained by the duration and the mode of insulin treatment as well as the status of hyperglycaemic control. Unlike the STZ-induced diabetes, the development of hyperglycaemia in BB rats was sudden and severe. An adjustment of the insulin dose for a few days is needed to determine the optimum dosage for maintenance of these animals. In addition, hyperglycaemia in BBd rats will lead to death, while STZ-induced diabetic rats will survive if not treated with exogenous insulin. Therefore, one week of insulin injections to BBd rats may not be a sufficient time to have any reversing effect on the depressed plasma vitamin A status. This is in agreement with a study that showed a single dose of insulin injection to STZ-induced diabetic rats for 3 weeks only restored the plasma retinol to normal levels without any effect on the elevated hepatic vitamin A levels (Leichter et al. 1991). Moreover, insulin-treated BBd rats in our study maintained plasma glucose at 300-400 mg/dL, whereas the STZ-induced diabetic rats with insulin implants had normal plasma glucose at 115-180 mg/dL (Tuitoek et al. 1996c). Thus, further studies are needed to examine the metabolic availability of vitamin A in BBd rats with a longer period of insulin treatment, perhaps delivering of insulin through an implanted copula.

Type I diabetes is an autoimmune disease resulting in a progressive destruction of the beta cell of the islets of Langerhans (Yoon et al. 1995). Both genetic susceptibility and environmental factors appear to contribute to the expression of the disease. Dietary

factors have been identified as the major determinants of diabetes development in BB rats (Virtanen et al. 1994). Vitamin A plays an important role in the regulation of immune function. Vitamin A deficiency in experimental animals has been reported to be associated with broad alterations in the immune system, including changes in organ morphology, cell numbers, and the response to specific pathogens and antigens, as well as nonspecific protection through phagocytic and cytotoxic mechanisms (Ross 1992). In a recent study (Driscoll et al., 1996), vitamin A deficiency in BB rats has been shown to be associated with a marked reduction in both insulitis and the development of clinical diabetes. However, in the present study, vitamin A supplementation failed to show any statistically significant effect on the incidence of diabetes in BBdp rats. Neither NK cytotoxic activity nor phenotypes in the rat spleen were changed in response to this dietary supplementation, compared with that of BBdp rats on a basal diet. The possible explanation for the contradiction of disease incidence in two studies is that the BB rats in Driscoll's study were at a state of severe vitamin A deficiency. These animals were born from pregnant rats on a diet deficient in vitamin A from midgestation, and continued on the same diet at weanling. Thus, it is possible that vitamin A deficiency in these animals resulted in a severe immunosuppressive condition, which can delay or prevent diabetes in BBdp rats.

Although not significantly different, it is noteworthy that the incidence of diabetes of BBdp rats on a vitamin A plus zinc supplemented diet shows a trend towards a higher diabetes incidence compared with that of BBdp rat on a basal or vitamin A supplemented diet. Zinc is a crucial nutritional component required for normal development and maintenance of immune function (Rundles 1996). Zinc supplementation has been shown

to induce regrowth of the thymus and to increase the production of thymulin, a thymic hormone that promotes the development and differentiation of T-lymphocytes (Dardenne et al.1993). T cells have been suggested to play a central role in the pathogenesis of diabetes in the BB rat. Thus, it is possible that a higher incidence of diabetes may occur when vitamin A and zinc are supplemented together. However, the biological function of zinc supplementation in these animals is difficult to interpret since type I diabetes results in an increased risk of developing zinc deficiency; in addition, the diabetic state might be involved in the modulation of the immune system. Further study is needed to address the effect of dietary zinc supplementation to BB rats on the T-lymphocytes differentiation and activation, and subsequently on the development of diabetes.

In summary, impaired metabolic availability of vitamin A in BBd rats is primarily due to the presence of hyperglycaemia. Neither vitamin A supplementation alone or in combination with zinc, nor a short period of insulin treatment improved the decreased levels of circulatory vitamin A in these rats. It is important to note that increased RBP mRNA abundance was observed in BBd rats in the presence of zinc supplementation, and that circulatory vitamin A levels remained unaffected. Thus, future studies are needed to address the effect of type I diabetes on the hepatic metabolism of vitamin A and RBP, including retinyl ester hydrolase activity, cellular synthesis and regulation of hepatic RBP.

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4. GENERAL DISCUSSION AND CONCLUSIONS

Uncontrolled diabetes mellitus is associated with a host of alterations in micronutrient status. One of these alterations involves an impairment in metabolic availability of vitamin A in type I diabetes. The present study was the first to investigate the modifying effect of type I diabetes on vitamin A metabolism in BB rats, which develop type I diabetes spontaneously.

At the onset of diabetes, BBd rats had decreased levels of plasma vitamin A as well as its carrier proteins, RBP and TTR, in both the plasma and the liver (chapter 2). These results closely resembled the observations reported in STZ-induced diabetic rats (Tuitoek et al. 1996a & b). While biochemical evidence of vitamin A deficiency exists in BBd rats, the vitamin A status including the concentrations of vitamin A in the plasma and total vitamin A and free retinol in the liver, remained normal in those of BBdp rats without overt diabetes by 120 days of age (chapter 3). Hence, it is unlikely that vitamin A deficiency in BBd rats was due to a genetic makeup.

The level of vitamin A in the plasma is homeostatically regulated by the release from its hepatic storage over a wide range of its intake. However, this presupposes that the absorption is intact. It is unlikely that vitamin A absorption is affected in the presence of type I diabetes. An *in vitro* study, which demonstrated that uptake of [^{3}H] retinol by the jejunum and ileum was unaffected in STZ-induced diabetic rats, suggests that impaired vitamin A metabolism in type I diabetes is not caused by its absorption (Tuitoek et al. 1994). The current study, in which no difference in food consumption and hepatic total

vitamin A storage between BBd rats and their control counterparts was observed (chapter 2 & 3), further supports this hypothesis.

Vitamin A is mainly stored in its ester form in the liver. When required, retinyl esters are hydrolyzed and released as retinol, the alcohol form. The mobilization of free retinol from the liver is accomplished by RBP that circulates in a 1:1 ratio with TTR. Decreased concentrations of hepatic free retinol, RBP and TTR in BBd rats (chapter 2) suggest that the secretion of vitamin A from the liver is impaired. One possible explanation for the lower levels of hepatic free retinol in these rats is that a reduced activity of retinyl ester hydrolase, due to an altered bile salt metabolism, may exist. Diabetes has been reported to be associated with alterations in biliary structure and hepatic function (Watkins et al. 1995). An abnormality in bile salt metabolism was found in BBd rats as indicated by alterations in bile flow, biliary secretion of bile acid, cholesterol, phospholipid, sodium, potassium, chloride and bicarbonate (Gonzalez et al. 1992). The transformation of retinyl esters to free retinol requires BSDREH, a bile salt-dependent enzyme (Blaner et al. 1994). Almost no activity was observed in the absence of bile salts or in the presence of several detergents (Harrison 1993). It is thus, possible that these modifications of bile salt metabolism may account for an impaired retinyl ester hydrolase activity and subsequently reduced free retinol in the liver of BBd rats.

A parallel decrease in hepatic abundance of RBP mRNA in BBd rats (chapter 2) suggests that the reduced secretion of RBP is due to a low synthesis rate. Both RBP and TTR have short half-lives, the synthesis rate in the liver is known to affect markedly their plasma levels (Soprano et al. 1994). Hence, in order to obtain information regarding the abnormal

metabolism of RBP, we next measured the status of zinc, an important factor for the synthesis of RBP as well as the mobilization of vitamin A from the liver (Brown et al. 1976). Hyperzincuria, accompanied by a reduction in plasma zinc levels were found to be present in BBd rats (chapter 2). Thus the reduced hepatic abundance of RBP mRNA in BBd rats may be a metabolic consequence of zinc deficiency. The evidence that reduced RBP mRNA was improved following zinc supplementation strongly supports this hypothesis (chapter 3). However, in the present study, despite improved RBP mRNA abundance in response to a zinc supplemented diet, circulatory vitamin A levels remains unaffected compared with those of BBd rats on a basal diet. The underlying mechanisms are not known. Future studies may be needed to address the effect of zinc supplementation on the RBP translation and the vitamin A hydrolase activity, two important factors in the regulation of vitamin A secretion from the liver.

Altered vitamin A metabolism in type I diabetes has been suggested primarily due to an insulin deficiency, as evidenced by the normalization of vitamin A concentrations in the plasma and the liver in STZ-induced diabetic rats after 4 weeks of insulin administration through implantation (Tuitoek et al. 1996c). This agrees with the fact that vitamin A status is not affected in type II diabetes (Basualdo et al. 1997). Type II diabetes, a consequence of insulin resistance or reduced insulin sensitivity, is associated with either high or normal blood insulin levels. However, our study failed to change this diabetes-associated vitamin A abnormality (chapter 3). This inconsistency may be explained by the duration and mode of insulin treatment, as well as by the status of hyperglycaemic control. Unlike STZ-induced diabetes, the development of hyperglycaemia in BB rats was

sudden and severe. An adjustment of the insulin dose for a few days is needed to determine the optimum dosage for maintenance of these animals. Therefore, one week of insulin injections to BBd rats may not be sufficient time to have any reversing effect on the depressed plasma vitamin A status. In addition, insulin-treated BBd rats in our study maintained plasma glucose at 300-400 mg/dL, whereas STZ-induced diabetic rats with insulin implants had the normal plasma glucose (115-180 mg/dL) (Tuitoek et al. 1996c). Insulin implant has been suggested to be a better way for the long time management of diabetes in BB rats.

Type I diabetes is an autoimmune disease resulting in a progressive destruction of the beta cell of the islets of Langerhans (Yoon et al. 1995). Both genetic susceptibility and environmental factors appear to contribute to the expression of the disease. Dietary factors have been identified as a major determinant of diabetes development in BB rats (Virtanen et al. 1994; Crisa et al. 1992). Both vitamin A and zinc play an important role in the regulation of immune function (Ross 1992; Rundles 1996). Hence, the incidence of diabetes and NK cell cytotoxic activity as well as mononuclear cell phenotyping in the spleen, the two immunological parameters are known to contribute to the pathogenesis of diabetes in BB rats, were also examined in the present study (chapter 3). vitamin A deficient BBdp rats have been reported to be associated with a marked reduction in the development of clinical diabetes (Driscoll et al. 1996). In the present study, however, the incidence of diabetes remained statistically unaffected in BBdp rats supplemented with vitamin A. Neither NK cytotoxic activity nor mononuclear cell phenotyping in the spleen was changed in response to this dietary supplementation. The possible explanation for

this contradiction is that BBdp rats in Driscoll's study were at a state of severe vitamin A deficiency, which might cause an immunosuppressive effect and hence delay or prevent diabetes in BBdp rats. Although not significantly different, the incidence of diabetes in BBdp rats eating a vitamin A plus zinc supplemented diet showed a higher trend compared with that of the BBdp rat on a basal or vitamin A only supplemented diet. Zinc has been shown to induce re-growth of the thymus, and to increase the production of thymulin, a thymic hormone that promotes the development and differentiation of T-lymphocytes (Dardenne et al.1993), which play a central role in the pathogenesis of diabetes in the BB rat. Thus, it is possible that a higher incidence of diabetes may occur when vitamin A and zinc are supplemented together. Further study is needed to address the effect of dietary zinc supplementation to BB rats on the T-lymphocytes differentiation and activation, and subsequently on the development of diabetes.

In summary, results of the present study suggest that metabolic availability of vitamin A is impaired in BBd rats, as shown by the decreased concentrations of circulatory retinol and its carrier proteins, RBP and TTR. No significant difference in food intake and subsequently hepatic total vitamin A storage between BBd and BBn rats suggests that vitamin A absorption remained unaffected in the presence of diabetes. However, the secretion of vitamin A from the liver seems dramatically altered due to: 1) a decreased transformation from retinyl esters to free retinol in the liver; 2) lower concentrations of hepatic RBP and TTR; 3) lower abundance of hepatic RBP mRNA. The possible explanation for this impaired vitamin A secretion from the liver is that BBd rats may be associated with an insufficient RBP synthesis as well as a reduced hepatic retinyl ester

hydrolase activity. Furthermore, decreased circulatory levels and the increased urinary excretion of zinc, an important factor for the synthesis of vitamin A carrier proteins, suggest that altered vitamin A metabolism in BBd rats, at least partly, is due to a zinc deficiency. The fact that hepatic abundance of RBP mRNA was increased in the presence of zinc supplementation strongly supports this hypothesis. It is unlikely that vitamin A deficiency in BBd rats is due to a genetic makeup since vitamin A concentrations in the plasma and the liver of diabetes-free BBdp rats were similar to those of BBn rats. Neither vitamin A supplementation alone nor its combination with zinc normalized the biochemical evidence of vitamin A deficiency in BBd rats.

It was noteworthy that circulatory vitamin A levels in BBd rats remained lower despite an increase in RBP mRNA abundance in the presence of zinc supplementation. A short period of insulin treatment also failed to improve this diabetes-associated vitamin A abnormality. The underlying mechanisms remained unclear. Thus, further studies in BB rats are needed: 1) to elucidate RBP metabolism in the liver, including the subcellular distributions of its mRNA and subsequently translation; 2) to examine the hepatic retinyl ester hydrolase activity; 3) to determine the effect of longer term insulin administration on the vitamin A homeostasis.

At present, the underlying mechanism of impaired vitamin A metabolism in uncontrolled type I diabetes is still not fully understood. Although a recent study has suggested a need for vitamin A supplementation in type I diabetes patients with marginal serum retinol levels in view of its possible preventive effect on certain long-term diabetes complications (Granado et al. 1998), there is little evidence to confirm that such therapy

has any benefit (American Diabetes Association 1995). In STZ-induced diabetic rats, 12-fold increase in vitamin A intake did not show any effect on the degree of hyperglycemia and glycosuria (Seifer et al 1981). Considering the impaired hepatic secretion of vitamin A existing in type I diabetes and hence the potential toxicity, supplementation of vitamin A is not recommended.

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