



## INFANT FORMULA INGESTION IS ASSOCIATED WITH THE DEVELOPMENT OF DIABETES IN THE BB/WOR RAT

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### Summary

The association between early exposure to cow's milk products in infancy and risk for insulin dependent diabetes mellitus (IDDM) is controversial. We examined whether the ingestion of cow's milk-based infant formula altered the expression of the diabetic syndrome in the BB/Wor rat, an animal model of IDDM. Pregnant BB/Wor dams were obtained from the NIH contract colony at the University of Massachusetts and housed under semi-barrier conditions. Rat pups were intubated with 1 to 2 ml of commercially available cow's milk-based infant formula (Enfamil® or Nutramigen®) or sham intubated (controls) daily from day 12 to day 25 of life. Pups were weaned at day 25 and monitored for glucosuria daily through 120 days of life. All rats including dams consumed a milk-free rat chow and acidified water ad libitum throughout the study. The mean age of disease onset was 4 to 10 days earlier in Nutramigen®-fed and Enfamil®-fed rats relative to controls ( $84 \pm 3$ ,  $78 \pm 2$  and  $88 \pm 4$  days, respectively); the mean age of disease onset was significantly different between controls and Enfamil®-fed animals ( $p < 0.05$ ). At 120 days, 60% (12/20) of control rats developed diabetes versus 100% of animals fed either type of infant formula prior to weaning (15/15:Enfamil®-fed; 19/19:Nutramigen®-fed) ( $p < 0.05$ ). These data indicate that direct, early ingestion of cow's milk-based formula was related to the expression of diabetes in the BB/Wor rat.

*Key Words:* infant formula, diabetes, BB/Wor rat

Early exposure to cow's milk-based formulas in human infancy has been associated with the later development of IDDM in a number of retrospective investigations (1-7). Together these reports suggest that, as infants, IDDM patients were breast-fed for a shorter duration and exposed to cow's milk products at a younger age than control subjects. Other reports, however, do not support these associations (8-10). Similarly, prospective dietary investigations utilizing animal models for human IDDM yield conflicting results (11-14), and any association between early ingestion of cow's milk products and IDDM remains controversial (15-17).

Infant formulas derived from cow's milk contain various bovine proteins which are the proposed dietary triggers for IDDM, including bovine serum albumin,  $\beta$ -lactoglobulin and  $\beta$ -casein (4-6,18,19). Early dietary exposure to these proteins may allow for intestinal passage of intact

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peptides across the immature mucosa (20). Investigators have postulated that these peptides share antigenic determinants with host antigens expressed on pancreatic  $\beta$  cells (18,21) and that antibodies induced against dietary antigens may cross-react with self antigens and destroy  $\beta$  cells leading to IDDM. Recently, Vaarala et al. (22) have postulated that bovine insulin in cow's milk based formulas may induce insulin-binding antibodies in children. A cross-reaction between these antibodies and human insulin would result in the breakdown of immune tolerance to pancreatic  $\beta$  cells.

As a further consideration, genetic factors related to the HLA class II gene region are implicated in the risk for IDDM (23,24). Recent reports suggest that a strong humoral response to cow's milk proteins is limited to specific genotypes of the HLA-DQ/DR loci (19,25). Thus, if molecular mimicry to a dietary antigen in cow's milk is operating in the development of IDDM, genotype, as well as the timing of dietary exposure to cow's milk protein, are critical factors, i.e., early exposure to cow's milk proteins may be linked to the development of IDDM only in genetically susceptible individuals.

These factors, genetic predisposition and precise timing of the introduction of cow's milk products, are difficult variables to assess retrospectively in human studies. Furthermore, the cost and commitment necessary to conduct a long-term prospective trial would be high, and the ethics of feeding an infant with genetic risk for IDDM cow's milk-based infant formula must be considered. The diabetes-prone BB/Wor rat displays clinical and pathological features closely resembling human IDDM. As in humans, diabetes develops during adolescence, between 60 and 100 days of age, and clinical onset is abrupt and characterized by weight loss, hyperglycemia, glycosuria and ketonuria (26). The mean incidence of diabetes in BB/Wor rats by 120 days of age is 86%. Genes associated with the class II region of the major histocompatibility complex are related to diabetes susceptibility in these animals, and this gene loci, RT1.B/D, is homologous to the human HLA-DQ/DR (27). The impact of cow's milk protein ingestion on the incidence of diabetes in the BB rat is unresolved (11,13), and the discrepancies in the literature may be related to study design and the timing of the introduction of cow's milk products to neonatal rats.

In the rat, gut closure (i.e., the inhibition of transcellular transport of intact proteins across the intestine) occurs by 22 days of age (28). Hence, to address the hypothesis that early ingestion of cow's milk-based infant formula is related to risk for diabetes in the rat model, formula would need to be administered to neonatal rats prior to day 20 of life. We examined whether the ingestion of commercially available cow's milk-based infant formulas from day 12 to day 25 of life altered the expression of diabetes in the BB/Wor rat.

### **Methods**

Pregnant, diabetes-prone BB/Wor dams were obtained from the National Institutes of Health contract colony at the University of Massachusetts (University of Massachusetts Medical Center, Worcester, MA) and housed under semi-barrier conditions. Dams consumed a milk-free non-purified diet (#7012, Teklad LM-485 Mouse/Rat Sterilizable diet, Harlan Teklad, Madison, WI) and acidified water ad libitum during gestation and throughout lactation. Pups were breast-fed through the 25th day of life. From days 12 to 25, pups were intubated with 1 to 2 ml of either the cow's milk-based formula Enfamil® (Mead Johnson, Evansville, IN) (n=15) or the hypoallergenic, protein hydrolysate formula, Nutramigen® (containing casein peptides of a molecular weight less than 1200, Mead Johnson) (n=19). Based on our past experience feeding rat pups, this 1 to 2 ml aliquot of formula represented between 10 to 25% of total daily intake depending on the size of the rat pup. Infant formulas were obtained from commercial outlets and prepared as directed with acidified water. Control pups (n=20) were sham intubated during the same period of life. Using power analysis, a sample size of 17 was appropriate for detecting a statistically significant effect

with 6% error. All pups were separated from the dams at day 25 and had ad libitum access to the milk-free non-purified diet and acidified water. The study was approved by the Institutional Animal Care and Use Committee at Arizona State University.

Offspring were weighed and monitored for glucosuria daily for 120 days. Diabetes diagnosis was based on consistent weight loss and glucosuria (++) for three consecutive days, 'TesTape', Eli Lilly & Co, Indianapolis, IN) at which time the animal was killed under CO<sub>2</sub> anesthesia and blood collected by heart puncture. Diabetes was confirmed by blood glucose analysis; non-fasting blood glucose greater than 8.9 mmol/L is indicative of diabetes (29). Blood glucose was analyzed by the glucose oxidase method (#510-A, Sigma Chem. Co., St. Louis, MO).

Data are reported as the mean  $\pm$  SEM. Oneway analysis of variance was used to examine differences between group means, and the  $\chi^2$  analysis was used to identify significant differences in the incidence of disease among groups. All analyses were performed using the Statistical Package for Social Sciences (SPSS/PC+; SPSS, Inc, Chicago, IL). The 0.05 level was used as the criterion for statistical significance.

TABLE 1

Infant formula ingestion, diabetes incidence, and diabetes indices (mean $\pm$ SEM) at disease diagnosis in BB/Wor rats

Diet Treatment	Diabetes incidence	Age at diagnosis (d)	Weight loss at diagnosis (g)	Blood glucose at diagnosis mmol/L
Control	12/20 (60%) <sup>a</sup>	88 $\pm$ 4 <sup>a</sup>	14.3 $\pm$ 1.5	22.8 $\pm$ 1.3
Enfamil®	15/15 (100%) <sup>b</sup>	78 $\pm$ 2 <sup>b</sup>	12.7 $\pm$ 2.8	21.9 $\pm$ 1.2
Nutramigen®	19/19 (100%) <sup>b</sup>	84 $\pm$ 3 <sup>ab</sup>	16.1 $\pm$ 2.5	21.9 $\pm$ 1.1

Different superscripts in same column denote statistical differences ( $p < 0.05$ )

### Results

Growth over the experimental period was not impacted by dietary treatment. Diabetes was confirmed in all animals diagnosed with diabetes by blood glucose analysis. The mean age at disease diagnosis was 4 to 10 days earlier in Nutramigen®-fed and Enfamil®-fed animals relative to controls, and the mean age at disease diagnosis was significantly different between controls and Enfamil®-fed animals ( $p < 0.05$ , Table 1). Mean weight loss and mean blood glucose values at disease diagnosis were similar in afflicted animals of each group (Table 1). At 120 days, 60% (12/20) of control rats developed diabetes; in contrast, 100% of animals fed either type of infant formula prior to weaning developed diabetes (15/15:Enfamil®-fed; 19/19:Nutramigen®-fed) (Figure 1).

### Discussion

These data indicate that the early ingestion of commercially available cow's milk-based infant formula containing intact or hydrolyzed bovine protein was related to increased expression of diabetes in genetically susceptible BB/Wor rats. Daneman et al. (11) demonstrated that the incidence of diabetes in BB rats (BB/hooded colony, Hospital for Sick Children, Toronto; average incidence of diabetes, 54%) increased 2-fold if the experimental diet containing cow's milk protein was ingested pre-weaning, days 13-25, versus post-weaning (63-68% and 26-29%, respectively). However, Malkani et al. (13) fed BB/Wor rats experimental diets free of cow's milk protein

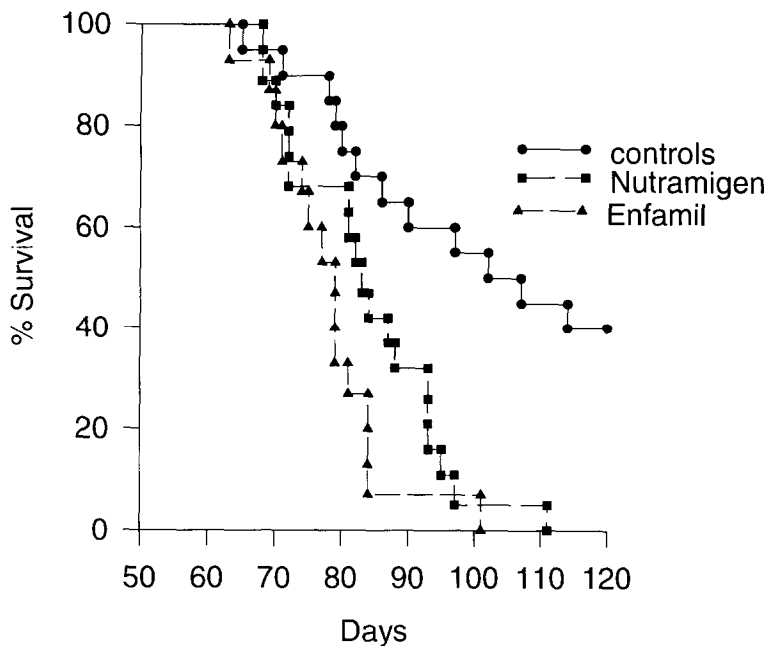


Fig. 1

Mortality data in diabetes-prone BB/Wor rats intubated with 1 to 2 ml commercially available infant formula from day 12 to day 25 of life. Control rats were sham-intubated from day 12 to day 25 of life. Rats were separated from dams at day 25, and all rats including dams were fed a milk-free rat chow ad libitum. The incidence of diabetes by 120 days of age was significantly higher in the formula-fed rats versus the controls ( $p < 0.05$ , chi-square statistic).

beginning at 21 days of life and did not observe a reduction in the incidence of diabetes. A potential confounding factor in the latter study is whether the young, suckling rats actually ingested the experimental diets prior to the maturation of the gut which occurs by day 22 of life (28,30).

In the present study, the novel approach of intubating, on a daily basis, cow's milk-based infant formula to very young rats (12 to 25 days of life) was utilized to insure ingestion of bovine products prior to gut closure. All animals ingesting cow's milk-based infant formulas pre-weaning developed diabetes by age 120 days. The incidence of diabetes in sham intubated animals not exposed to cow's milk products was 60%.

Since the intestinal passage of intact protein may factor in the etiology of IDDM, the timing of exposure to cow's milk products is crucial. In children, exclusive breast-feeding for the first 4 months of life or longer was associated with a significantly lower risk for developing diabetes than breast-feeding for 2 months or less (31). Furthermore, the introduction of dairy products during the first or second month of life was associated with a greater risk for diabetes compared to the risk if dairy products were introduced after the second month of life (31). Kostraba et al. (2) also reported that the age at introduction of cow's milk based substitutes was slightly lower in Black diabetic children compared to healthy, matched controls (3.9 and 8.5 weeks respectively,  $p = 0.07$ ).

Gimeno and DE Souza (7) reported that children breast-fed for < 7 days were more likely to develop IDDM compared to children breast-fed for a longer time. The children who received cow's milk products in the first seven days of life, when compared to those who did not, were more likely to develop IDDM (7). In breast-fed human infants, gut permeability to macromolecules was greatest in the first few weeks of life and decreased rapidly thereafter (32). Infants fed milk-based formulas from birth displayed evidence of significant macromolecule absorption for the first 3 months of life (33).

Although both cow's milk-based formulas utilized in the present report induced a significant increase in the incidence of diabetes in the BB/Wor rat, the mean age at disease diagnosis was significantly younger in the Enfamil®-fed rats versus control rats; whereas the mean age at onset did not differ between Nutramigen®-fed rats and control rats. Malkani et al. (13) noted that BB/Wor rats fed an experimental diet composed of Nutramigen® developed diabetes at only half the rate of rats fed a standard commercial rat chow containing intact protein. Nutramigen® is a hypoallergenic protein hydrolysate infant formula containing enzymatically hydrolyzed casein as the sole protein source. Enfamil®, based on cow's whey and nonfat cow's milk, contains numerous intact bovine proteins. These data indicate that enzymatic modification of bovine protein may attenuate the promotive effect of bovine products on diabetes frequency in BB rats.

In the present report, all animals ingesting Nutramigen® pre-weaning did, however, ultimately develop IDDM. Van Beresteijn and colleagues (34,35) have demonstrated that all commercially available hypoallergenic infant formulas tested (n=8) contained protein fragments which cross-reacted with the major proteins of cow's milk including bovine serum albumin,  $\beta$ -lactoglobulin and  $\beta$ -casein. These investigators did note, however, that the presence of specific immunoreactive peptides was highly variable among the formulas tested. Thus, although perhaps less immunoreactive than native casein, the enzymatically modified casein present in Nutramigen® may be associated with the development of diabetes in BB/Wor rats under certain experimental conditions. Cell-mediated immune responses to  $\beta$ -casein has been noted in individuals with recent-onset IDDM (5,19), and sequence homology between segments of bovine  $\beta$ -casein and several molecules expressed by pancreatic  $\beta$  cells, including the glucose transporter GLUT-2, may explain the role of  $\beta$ -casein in the pathogenesis of IDDM (5).

Due to conflicting reports in the literature, any association between the early ingestion of cow's milk products and IDDM is controversial and unresolved. Perhaps some of the inconsistencies in the literature are related to study design. Both genetics and the timing of the ingestion of bovine products are critical to the molecular mimicry model of IDDM. Our findings indicated that in genetically susceptible BB/Wor rats, intubation of cow's milk-based infant formulas to neonatal rats from 12 to 25 days of age (ie., prior to gut closure) was associated with the development of diabetes by 120 days of age. The incidence of diabetes using this research design was similar for rats fed infant formulas containing either intact or enzymatically hydrolyzed bovine proteins. Continued investigation to clearly delineate the role of cow's milk ingestion in the pathogenesis of IDDM is imperative since environmental agents can be modified to reduce risk of disease.

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