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ORIGINAL ARTICLE

Maternal dietary n-6/n-3 fatty acid ratio affects type 1 diabetes development in the offspring of non-obese diabetic mice

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ABSTRACT **Environment factors, including maternal or infant dietary nutrition have been reported to have an influence on the pathogenesis of type 1 diabetes. In the present study, to investigate the effect of maternal or post-weaning offspring's nutrition, in particular the essential fatty acid ratio (n-6/n-3) on the development of type 1 diabetes, we prepared two kinds of chows with n-6/n-3 ratios of 3.0 (L) and 14.5 (H), and provided them to mothers of non-obese diabetic (NOD) mice during gestation and lactation and to the offspring after weaning. The n-6/n-3 ratios in breast milk and erythrocyte membrane of NOD offspring became nearly the same with that of the maternal diet at 2 weeks after birth. In the L chow-fed offspring from L chow-fed mother (LLL), levels of insulitis were higher than those in the H chow-fed offspring from H chow-fed mother (HHH) at 4 weeks of age, while the levels in the LLL offspring became lower than those in the HHH after 6 weeks. Early insulin autoantibody expressions were found from 2 to 6 weeks in the HHH offspring, but not in the LLL. The LLL offspring exhibited strong suppression of overt diabetes development in regard to the onset and accumulated incidence of diabetes compared to the HHH. The study with combined L and H chows during gestation, lactation in mother and in post-weaning offspring revealed that only the LLH chow significantly suppressed the development of diabetes with similar kinetics to LLL chow, although the other combinations may delay the onset of diabetes. The present findings suggest that n-6/n-3 ratio of the maternal diet during gestation and lactation rather than that of offspring after weaning strongly affects the development of overt diabetes in NOD mice.**

Key Words: essential fatty acid ratio, insulitis, maternal factor, NOD mouse, type1 diabetes

INTRODUCTION

Type 1 diabetes results from insulin deficiency, mostly due to the autoimmune-mediated destruction of the insulin-producing pancreatic islet β cells (Eisenbarth 1986; Atkinson and MacLaren 1994). It arises from incompletely understood interactions between β cells, the immune system, and the environment in genetically susceptible individuals (Tisch and McDevitt 1996). Among environmental factors in the development of type 1 diabetes, maternal environment has also been implicated (Dahlquist *et al*. 1999; Creacy *et al*. 2004). We previously demonstrated that some maternal factors modified the immune response to islets, which in turn may affect the pathogenic course from insulitis to overt diabetes in non-obese diabetic (NOD) mice, a type 1 diabetes model (Kagohashi *et al*. 2005a,b). Maternal diet is one of the important factors in the maternal environment, and while breastfeeding has been suggested to affect the incidence of type 1 diabetes, findings have been controversial and the underlying mechanisms involved remain unknown (Norris *et al*. 1996; Verge *et al* 1996; Kimpimaki *et al*. 2001; Atkinson and Gale 2003). A case-control study from Norway reported that children with diabetes were less likely to have been given cod liver oil during infancy than children without diabetes (Stene and Joner 2003). As cod liver oil contains both vitamin D and the n-3 fatty acids (i.e. eicosapentanoic acid [EPA] and docosahexaenoic acid [DHA]), it was not clear whether the protective factor in cod liver oil was vitamin D, the marine fatty acid or both. Although previous studies reported that children with diabetes were less likely to have taken vitamin D supplements in infancy than children without diabetes, similar investigations focusing on the intake of marine n-3 essential fatty acids (EFA) have not been conducted to resolve this question (Stene *et al*. 2000; Hibbeln *et al*. 2007). Studies have suggested that macrophage infiltration and inflammatory cytokine production are early events in the pathogenesis of type 1 diabetes (Rabinovitch 1994; Rabinovitch *et al*. 1995; Holm *et al*. 2006). Therefore, factors that either promote or block the impact of these early pathogenic inflammatory events may be critical for accelerating or inhibiting the development of type 1 diabetes. Previous studies have demonstrated a strong preventive effect of n-3 fatty acids on inflammatory response in animals and humans (Benhamou *et al*. 1995; Krishna Mohan and Das 2001; Simopoulos 2002; Norris *et al*. 2007; Washbum *et al*. 2007). An n-6/n-3 ratio of less than 4 is recommended to prevent autoimmune and chronic diseases in humans (Benhamou *et al*. 1995; Simopoulos 2002). However, to our knowledge, there has been no systematic experimental study that examined the effect of dietary EFA ratio, in particular the n-6/n-3 ratio, to prevent type 1 diabetes.

In this study, therefore, as a part of a series of studies on the effect of environmental factors on the life-long history of pathogenesis and clinical course of type 1 diabetes (Otani *et al*. 1991; Kagohashi *et al*. 2005a,b, 2006, 2007; Kagohashi and Otani 2010), we investigated the effect of maternal nutrition, in particular the n-6/n-3 ratio, on the development of type 1 diabetes in the offspring. We provided chows with different n-6/n-3 ratios to pregnant and lactating female NOD mice as well as post-weaning offspring and examined the effect on the development of insulitis and overt diabetes and found that n-6/n-3 ratio in maternal diet affects the development of type 1 diabetes in the offspring.

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MATERIALS AND METHODS

Mice and diets

NOD mice from 0 to 45 weeks old were used in this study, and were originally purchased from CLEA Japan (Tokyo, Japan). This study was approved by the Ethical Committee of Shimane University and all the experimental procedures were performed according to the institutional guidelines. The animals were maintained in the Institute of Experimental Animals at Shimane University. Amounts of chow and drinking water, which were given ad libitum, and the body weights were recorded every week before and after the onset of overt diabetes. The animals were kept in a room maintained at 24 ± 2°C with 60–70% relative humidity. The room was illuminated by artificial light from 8:00 to 20:00. Diets containing specific fatty acid composition were prepared. Based on the Nutrient Requirements of Laboratory Animals (National Academy of Science 1995), we prepared two types of chows; one with an n-6/n-3 ratio of 3.0 (L) and the other with an n-6/n-3 ratio of 14.5 (H) (Research Diet, New Brunswick, NJ, USA). L and H chows are identical in macronutrient constituents, except for the n-6/n-3 ratio (Table 1), and micronutrients were mostly in excess of the daily requirements, following standard processing. The experimental design for diets is shown in Figure 1. In the present study, NOD mothers were fed with the randomly assigned chow for the gestation period for longer than 4 weeks before being mated and becoming pregnant. According to the design, we provided H or L chow to pregnant mothers, lactating mothers after birth of their offspring, and to the offspring after weaning until the end of the experiment. Only female NOD offspring were used for the analyses because of the higher incidence of overt diabetes. Incidences of overt diabetes of female NOD in our facilities were 37% at 25 weeks of age and 70% at 40 weeks, while mice were fed in our facilities with a regular chow (MF, Oriental Yeast, Tokyo, Japan) with the n-6/n-3 ratio of 6.8 and slight differences in the macronutrient constituents from L or H (Table 1). The mice were deeply anesthetized by an intraperitoneal injection of 70 mg/kg pentobarbital sodium (Abbott, North Chicago, IL,USA), and then killed by cervical dislocation to obtain tissues for the analyses described below. Breast milk was harvested from the stomach of the sacrificed offspring.

Histological observation of insulitis

The pancreases from the female offspring at 0–12 weeks after birth were used for histological analysis. The pancreases were fixed in 10% formalin neutral buffer solution, and were embedded in paraffin. Serial sections were cut at 5 µm, stained with hematoxylin and eosin, and observed by light microscope using sections every 200 µm. We observed approximately 50 islets per pancreas, and

Table 1 Characteristics of diet formulas

	AIN-76		
	H	L	MF
Nutrients (%)			
Protein	20.3		23.6
Fat	5		5.3
Carbohydrate	66		65
Total calories (kcal/g)	3.9		3.6
n-6/n-3	14.5	3	6.8

AIN-76, A standard laboratory rat and mouse diet which was formulated and published by a committee of American Institute of Nutrition (AIN) members in 1976.

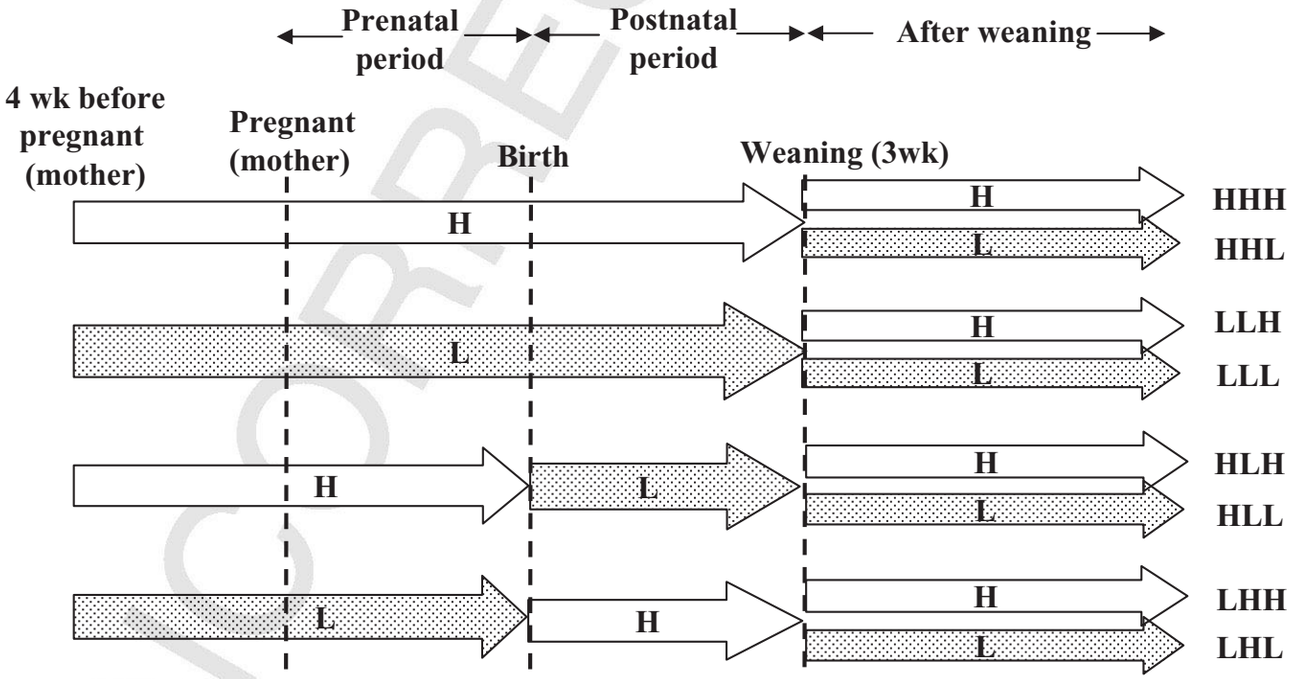


Fig. 1 Experimental design for diets. H, H chow with an n-6/n-3 ratio of 14.5; L, L chow with an n-6/n-3 ratio of 3.0.

investigated the incidence of insulitis with lymphocyte infiltration using a grading system, in which 1: <25%, 2: 25<<50%, 3: 50<<75% and 4: 75<<100% infiltrated area of each islet (Kagohashi *et al*. 2005a). Offspring that showed lymphocyte infiltration in more than one islet among 50 were defined as having insulitis regardless of insulitis level. Insulitis level was analyzed at 2–12 weeks after birth. We examined the islets from 5 to 10 offspring in each group each week.

Overt diabetes by checking urinary glucose level

Urinary glucose levels were checked with PRETEST (Wako, Osaka, Japan) once per week until onset of glucosuria, when mice were diagnosed as with overt diabetes.

Measurement of plasma and erythrocyte membrane fatty acids of offspring

Fatty acid composition was determined using a modification of the one-step analysis (Lepage and Roy 1986) as previously described for a good recovery for plasma and erythrocyte membrane fatty acid, rather than by the conventional Folch procedure. To 100 μL of plasma or erythrocyte membrane in phosphate buffered saline, 2.0 mL methanol-n-octane (4:1, v/v) containing 10 mg tricosanoic acid as an internal standard and 200 μL acetyl chloride were added. The mixture was incubated at 100°C for 60 min and cooled, then neutralized with 0.5N aqueous NaOH containing 10% sodium chloride. The neutralized mixture was shaken for 10 min at room temperature and centrifuged at 1800 × g for 5 min. The octane phase with the fatty acid methyl esters was directly subjected to gas chromatography. The gas chromatography separation was done on a Model 6850 (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and an automatic sampler Model 6850. A 30 mm × 0.25 mm capillary column (DB-WAX P/N 122-7032; J & W Scientific, CA, USA) was initially maintained at 100°C for 1 min, raised to 180°C at 20°C/min, then raised to 240°C at 2°C /min, and further raised to 260°C at 4°C/min and maintained for 5 min. Fatty acid composition was expressed as molecular percentage/mL plasma. Several fatty acid indexes were derived from the primary data: the total percentage of saturated fatty acids, which was calculated as the sum of the percentage of palmitic acid (16:0) and stearic acid (18:0); the total percentage of monounsaturated fatty acids, which was represented as the percentages of oleic acid (18:1); the total percentage of n-3 EFA, which was calculated as the sum of the percentages of α-linolenic acid (18:3 n-3), EPA(20:5 n-3), docosapentanoic acid (22:5 n-3, DPA) and DHA (22:6 n-3); and, the total percentages of n-6 PUFA, calculated as the sum of the percentages of linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6). The unsaturation index (USI) for each group was calculated by taking the molecular percentage of each fatty acid, and multiplying it by the number of double bonds in the fatty acids.

IAA assay

For insulin autoantibody (IAA) assay, blood was collected from NOD offspring, at 2–14 weeks after birth. IAA was measured with a 96-well filtration plate micro IAA assay. ^{125}I-insulin (Amersham Pharmacia Biotech, Piscataway, NJ, USA) of 20 000 cpm was incubated with 5 μL of serum with and without cold human insulin, respectively, for 3 days at 4°C in buffer A (20 mM Tris-HCl buffer pH7.4 containing 150 mM NaCl, 1% BSA, 0.15% Tween-20, and 0.1% sodium azide). Fifty μL of 50% protein A/8% protein G-sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) were added to the incubation in a MultiScreen-NOB 96-well filtration plate (Corning Incorporated Life Sciences, Acton, MA, USA) which was pre-coated with buffer A. The plate was shaken for 45 min at 4°C followed by two cycles of four washes each cycle with cold buffer B (the same buffer as buffer A except for 0.1% BSA). After washing, 40 μL of scintillation liquid (Microscint-20; Packard Instrument Company, Meriden, CT, USA) was added to each well and radioactivity determined directly in the 96-well plate with a TopCount (96-well plate β–counter; Packard) scintillation counter. The result was calculated based on the difference in counts per minute (delta cpm) between the well without cold insulin and the well with cold insulin and expressed as an index: index = (sample delta cpm-human negative control delta cpm)/(human positive control delta cpm-human negative control delta cpm). The limit of normal (0.010) was chosen by the analysis of IAA in non-diabetic strain mice.

Statistics

Student's *t*-test was used in statistical analyses, except for the degree of insulitis, and a level of $P < 0.05$ was regarded as significant. The degree of insulitis level was analyzed by Ridit analysis (Sermeus and Delesie 1996) and a level of $T > |1.96|$ ($T < -1.96$ or $1.96 < T$) was regarded as significant. Onset and incidence of overt diabetes was analyzed by Kaplan–Meier curves and log–rank test, and a level of $P < 0.05$ was regarded as significant.

RESULTS

Body weight

Food intakes did not significantly differ among age-matched groups during the study period. There were no significant differences in body weights among the groups before and after weaning until the onset of overt diabetes (data not shown).

Fatty acid composition in milk from dams, and plasma and erythrocyte membrane of the offspring

At 2 weeks after birth, the n-6/n-3 ratio in breast milk from H or L chow-fed dams became nearly the same as each of that of the H or L chow, respectively (Fig. 2A). In contrast, that in plasma of the offspring remained at the intermediate levels, and that in the erythrocyte membrane of the offspring from L chow-fed dams became nearly the same as that of the maternal L chow, while only intermediate level was reached by H chow (Fig. 2A). The n-6/n-3 ratio in the plasma of the offspring from mothers continuously fed with L chow became the same level with the diet at 4 weeks of age, while that in the plasma of the offspring from mothers continuously fed with H chow reached 8 or 8.5 at 2 weeks of age, but did not increase significantly thereafter (Fig. 2B).

Onset and severity of insulitis in the offspring

At 4 weeks after birth, insulitis had already occurred in the offspring of all groups, and interestingly insulitis was milder in the H-fed group than in the L-fed group (Fig. 3A for HHH vs. LLL, $T = -2.46 < -1.96$ by Ridit analysis, and data not shown). However, at 6 and 12 weeks, the severity of insulitis was significantly higher in the groups that were fed with H chow during gestation and/or lactation period (Fig. 3A for HHH vs. LLL, $T > 1.96$ by Ridit analysis both for 6 and 12 weeks, and data not shown).

Serum IAA levels in the offspring

At 2 and 4 weeks after birth, IAA was frequently detected in the offspring that were born from and lactated by dams provided with H chow and fed with H chow after birth (HHH), whereas IAA was detected at a slight-positive level in a limited number of offspring born from and lactated by dams provided with L chow and fed with

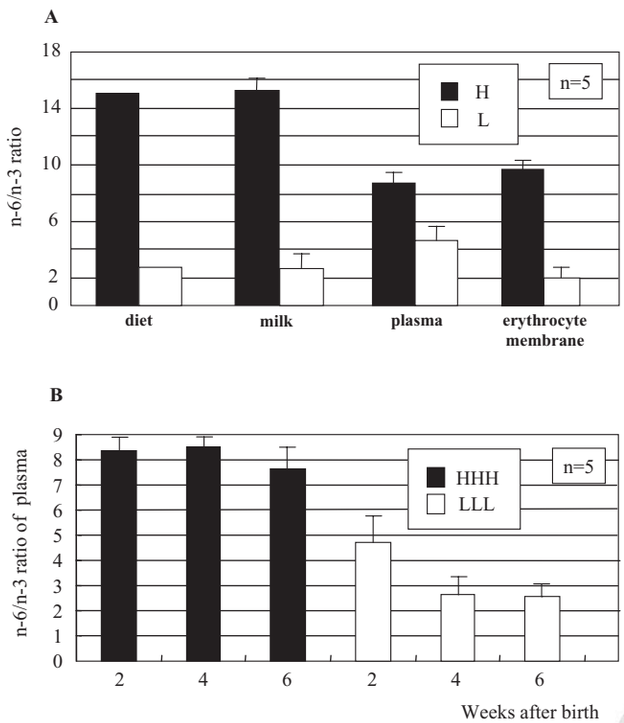


Fig. 2 n-6/n-3 ratios in the maternal chow, maternal milk, offspring plasma, and offspring erythrocyte membrane at 2 weeks of age (A), and postnatal changes in the n-6/n-3 ratio in the plasma of the offspring (B). (A) At 2 weeks after birth, the n-6/n-3 ratio in breast milk from H or L chow-fed dams became nearly the same as each of that of the H or L chow, respectively. However, that in plasma of the offspring remained at the intermediate levels, and that in the erythrocyte membrane of the offspring from L chow-fed dams became nearly the same as that of the maternal L chow, while only intermediate level was reached by H chow. (B) The n-6/n-3 ratio in the plasma of the offspring from L chow-fed mothers became the same level with the diet at 4 weeks of age, while that in the plasma of the offspring from H chow-fed mothers reached 8 or 8.5 at 2 weeks of age, but did not increase significantly thereafter. Bars represent standard deviations.

L chow after weaning (LLL) although there was not a statistically significant difference between the groups (Fig. 3B).

Onset and incidence of overt diabetes in the offspring

Overt diabetes was significantly and strongly suppressed in offspring born from dams provided with L chow and fed with L chow after weaning (LLL) compared with HHH offspring (Fig. 4). In these offspring, not only the ratio of overt diabetes was dramatically reduced, but also the onset was delayed compared with other groups (Fig. 4).

We further studied whether and how different combinations of L and H chows during gestation, lactation in the mother and in post-weaning offspring affect the development of overt diabetes. The results revealed that the only LLH chow significantly suppressed the development of diabetes with similar kinetics to LLL chow (Fig. 5A), while in the offspring from dams provided with H chow during gestation and/or lactation (HHL, HLH, HLL, LHH, LHL), overall incidence of overt diabetes reached approximately 70% and did not differ from that of historical data in our facilities, although involvement of L chow appeared to delay the onset of diabetes by 5 to 10 weeks compared with that in HHH group (Fig. 5A,B).

DISCUSSION

Recent epidemiological studies suggested that nutrition from a very young age, even from the prenatal period is important from the standpoint of prevention of type 1 diabetes (Stene *et al*. 2000; Stene and Joner 2003; Hummel *et al*. 2007; Norris *et al*. 2007; Lamb *et al*. 2008). Dietary intake of n-3 fatty acids was associated with reduced risk of islet autoimmunity in children at increased genetic risk for type 1 diabetes based on the longitudinal study of 1770 children (the mean age at follow-up was 6.2 years) at increased risk for type 1 diabetes (Norris *et al*. 2007). The present experimental study further demonstrated that n-6/n-3 ratio of 3.0 in the maternal diet when given during gestation and lactation periods, but not in the offspring diet after weaning, led to marked and sustained blockade of overt type 1 diabetes in the genetically susceptible NOD offspring. These effects were preceded by suppression of islet infiltration by lymphocytes (i.e. β-cell cytotoxic T-cells), and their islet toxicity. The present results suggest that the continuous supply of low n-6/n-3 ratio diet to mothers during both the gestation and lactation periods is critical for this preventive affect, because when the chow with the low n-6/n-3 ratio was applied to either the gestation or lactation period alone, the final accumulated incidence of disease in the group was not decreased.

Dietary n-6/n-3 fatty acid ratio affected the fatty acid composition in milk from NOD dams, and plasma and erythrocyte membrane of the offspring

While the observed effects were readily attributable to the food, they were not due to differences in nutrient composition, but due to the difference in the n-6/n-3 fatty acid ratio, as the chows used were of a single origin with equivalent energy profiles. When the n-6/n-3 ratio was changed in the chow for ICR and NOD mice, their serum fat levels and the fatty acid ratios in the blood cells changed to the same level with that in the chow after approximately 4 weeks (Kagohashi *et al*. 2006). In the present study, NOD mothers were fed with the assigned chow for longer than 4 weeks before being mated and becoming pregnant and therefore during gestation period, the n-6/n-3 ratio in the maternal serum remained at a steady level. The n-6/n-3 ratio in the mothers' milk was nearly the same with that of the diet 1 week after delivery, suggesting that dietary n-6/n-3 ratio was rapidly reflected in the milk (Kagohashi *et al*. 2007). EFA ratio in the human milk has also been drawing attention, and it has been reported that DHA and EPA are rich among EFA in the maternal milk (Uauy-Dagach and Mena 1995; Simopoulos 2002; Hibbeln *et al*. 2007). In the present study, milk from the mothers that were fed with either H or L chow showed almost the same level of n-6/n-3 ratios with those of chows, while blood plasma of the offspring did not necessarily reach the similar levels. Plasma n-6/n-3 ratio in the H-fed offspring reached approximately 8.5, much lower than that in the chow, at 2 weeks of age and remained at that level thereafter. In contrast, the n-6/n-3 ratio in the L-fed offspring reached 2.5 to 3, the same level with the chow, at 2–4 weeks of age. These findings suggest that an n-6/n-3 ratio in the maternal diet, in particular low values as in the present study, is fairly accurately reflected in those of the maternal serum and milk and therefore change in the n-6/n-3 ratio of the maternal diet during gestation and/or lactation periods would influence that in the body composition of the offspring.

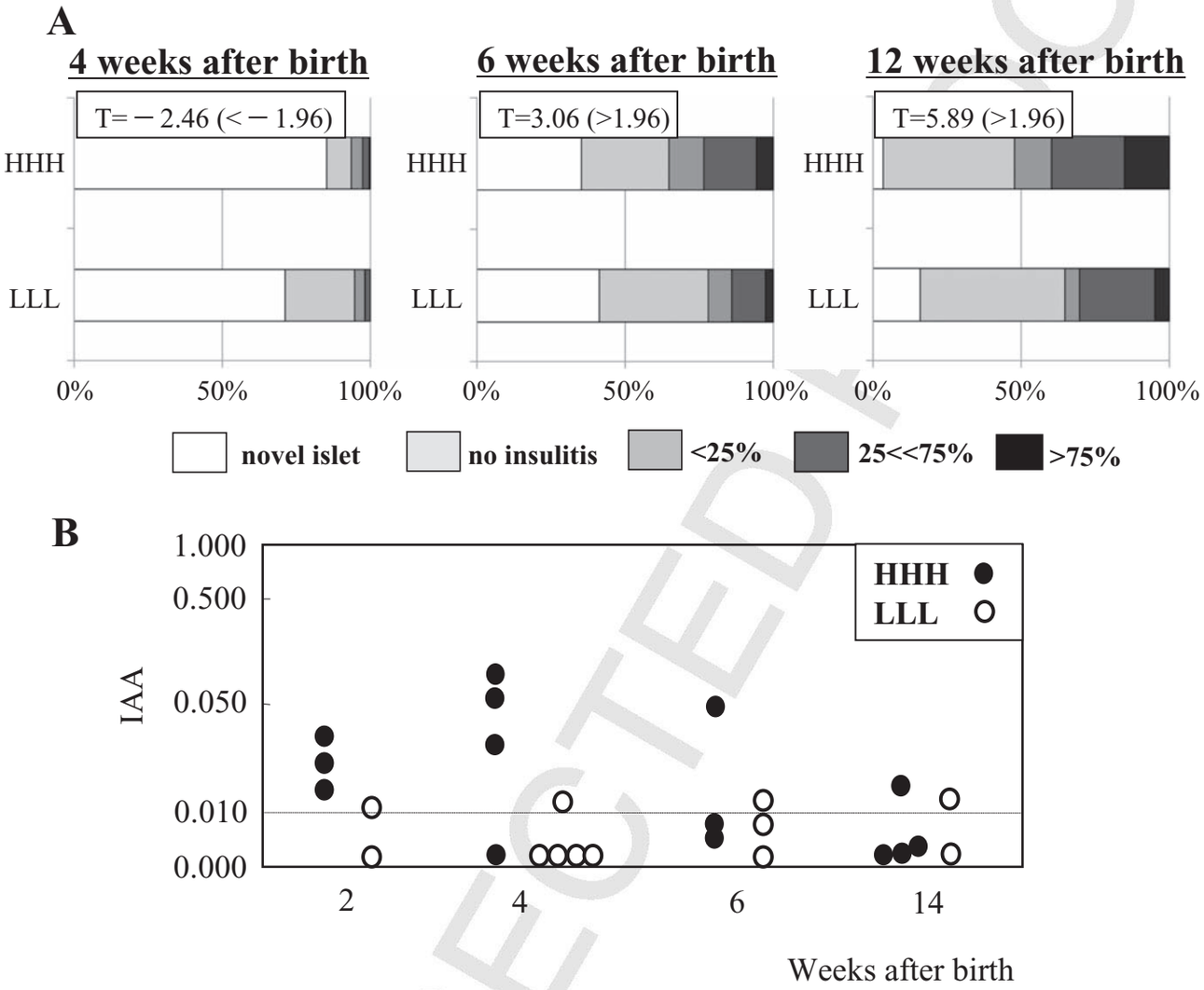


Fig. 3 Levels of insulitis (A) and serum insulin autoantibody (IAA) levels (B) in the offspring at different ages. (A) At 4 weeks after birth, insulitis had already occurred in the offspring both the offspring that were born from and lactated by H chow-fed dams and fed with H chow after weaning (HHH) and those born from and lactated by L chow-fed dams and fed with L chow after weaning (LLL). While the insulitis was milder in the HHH group than in the LLL group at 4 weeks of age, at 6 and 12 weeks, the severity of insulitis was significantly higher in the HHH offspring than in the LLL offspring. (B) At 2 and 4 weeks after birth, IAA was frequently detected in the HHH offspring, whereas it was detected at a slight-positive level in a limited number in the LLL offspring. In the LLL group, islets without insulitis were mostly those budding from the pancreatic duct (novel islets), whereas in the HHH group, all the islets without insulitis were located apart from the duct (no insulitis).

Effects of maternal n-6/n-3 fatty acid ratio on the development of type 1 diabetes in the NOD offspring

A case-control study reported that children with diabetes were less likely to have been given cod liver oil during infancy than children without diabetes (Stene and Joner 2003), and it was also reported that use of cod oil during pregnancy was associated with lower risk of type 1 diabetes in the offspring (Stene *et al*. 2000). These studies did not clarify which of n-6/n-3 fatty acid ratio or vitamin D, which are both included in cod oil, played the major role in the observed effects (Stene *et al*. 2000; Stene and Joner 2003).

In the present study, we examined the influence of nutritional n-6/n-3 ratio through the maternal diet during gestation, lactation periods, and the offspring diet after weaning. At 6 weeks onward, the NOD offspring fed with H chow before weaning through dams and L chow after weaning had significantly more advanced insulitis than those fed with L chow before and after the weaning. In our facilities, NOD female mice develop insulitis mostly from 4 weeks of age, and the incidence reaches 100% by 12 weeks (Kagohashi *et al*. 2005a,b). Those fed with L chow before weaning and H chow after weaning showed nearly the same level of insulitis with those fed with L chow before and after weaning. On the other hand, those fed with H chow continuously developed insulitis more strongly than those continuously fed with L chow. Curiously, at 4 weeks after birth, the level of insulitis was significantly lower in those fed with H chow continuously than in those fed with L chow continuously, despite that at 6 weeks onward, the levels reversed between

them. Although we have no clear explanation of these events, intriguingly we previously observed similar reversal of insulitis levels between two groups of NOD offspring that developed under different maternal environments (Kagohashi *et al*. 2005a,b). When pre-implantation stage NOD embryos were transferred into and were born from dams of ICR or DBA mice (NOD/ICR, NOD/DBA), they developed insulitis significantly earlier than NOD embryos transferred into NOD dams (NOD/NOD). However, eventually, the onset and incidence of overt diabetes were significantly later and lower in NOD/ICR and NOD/DBA than in NOD/NOD (Kagohashi *et al*. 2005a,b), suggesting the existence of different and interrelating phases during the course until the development of overt diabetes. Although the mechanism remains unknown, the present findings showed that when lactating dams were fed with L chow, although insulitis occurred in the offspring, its progress was inhibited. However, when lactating dams were fed with H chow, insulitis in the offspring could be only partially at most prevented even if they were fed with L chow after weaning. Another possible mechanism of these effects is that low dietary n-6/n-3 ratio may enhance prenatal/early-postnatal histogenesis of islets to increase the total β cell mass and/or postnatal islets neogenesis, the development of new islets from progenitor cells in the pancreas. It has been suggested that the development of new islets from progenitor cells occurs for curing diabetes (Pittenger *et al*. 2009). In this series of studies regarding the dietary EFA effects (Kagohashi *et al*. 2007; Kagohashi and Otani 2010), we changed the chow from H to L immediately after the onset of overt diabetes of NOD adult mice, and observed that, in the pancreas, most of the islets free from lymphocyte infiltration were those budding from the pancreatic duct, suggesting that these islets were formed by the islet neogenesis. In the present study, when L chow was provided either gestation or lactation period, the onset of overt diabetes tended to be delayed, however, the overall incidence did not significantly affected, suggesting the insulitis may be decelerated but not prevented. In contrast, when dams were fed with L chow through gestation and lactation periods, not only onset of overt diabetes was significantly delayed, but also the total incidence was markedly reduced.

Islet neogenesis is expected as another approach for curing diabetes (Pittenger *et al*. 2009), because neither islet transplantation (Couzin 2004) nor gene therapy (Morral 2004) has yet been established. In addition to islet neogenesis after maturation, the stronger effect of low n-6/n-3 ratio through prenatal and lactation periods in the present study might have caused embryos/offspring to enhance pancreatic histogenesis and islet neogenesis. Thus, the dietary n-6/n-3 ratio before weaning may not only prevent and/or decelerate of autoimmune destruction, but also possibly increase β cell supply to influence both the development of insulitis and incidence of overt diabetes.

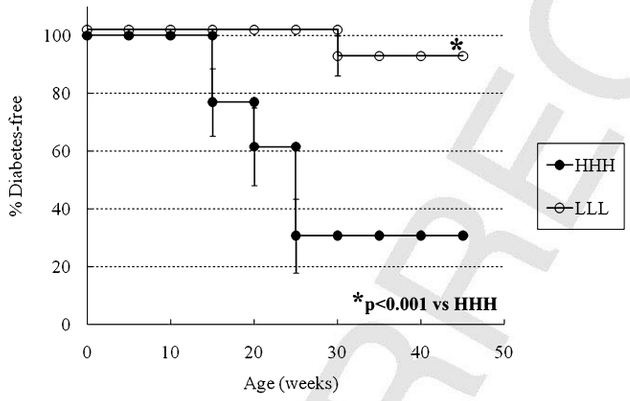


Fig. 4 Onset and incidence of overt diabetes in the HHH and LLL offspring. Overt diabetes was significantly and strongly suppressed in the offspring born from and lactated by dams provided with L chow and fed with L chow after weaning (LLL) than those continuously fed with H chow (HHH). In the LLL offspring, not only the ratio of overt diabetes was dramatically reduced, but also the onset was later than in the HHH offspring. Kaplan–Meier curves and the log–rank test were used for the analysis.

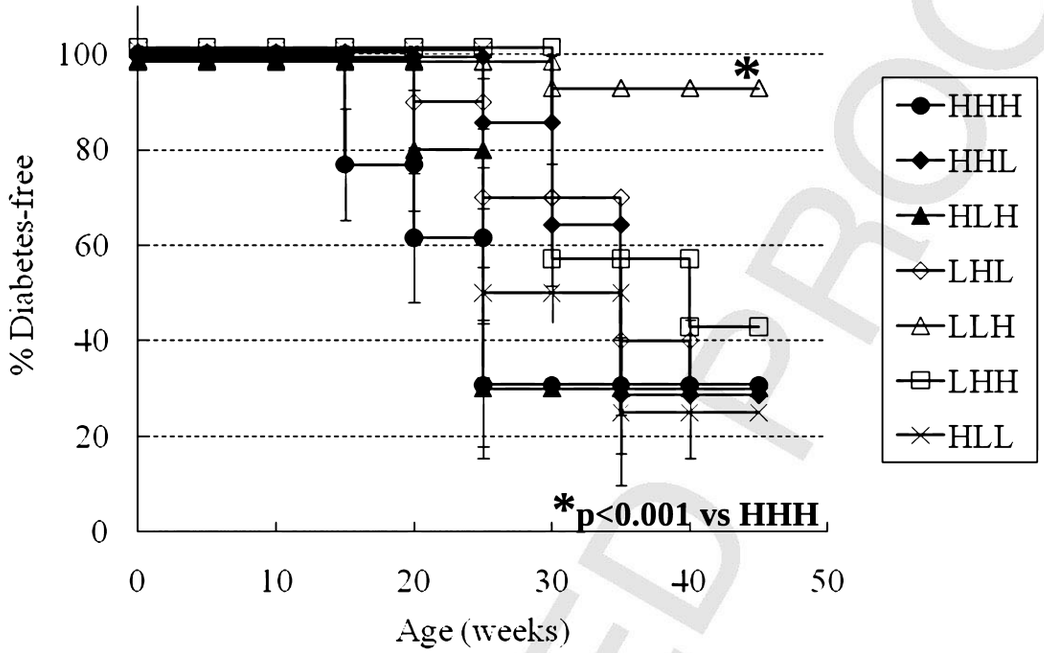
Maternal nutrition and induction of autoantibodies involved in the development of type 1 diabetes in the offspring

The relationship between maternal nutrition and autoimmunity in the development of type 1 diabetes has been investigated (Norris *et al*. 1996; Couper *et al*. 1999; Norris *et al*. 2007). When nutrition history was compared between 18 children with autoantibodies including IAA, GAD antibody, and IA-2 antibody and 153 without these autoantibodies among 253 children who had family member with type 1 diabetes and registered in Diabetes Autoimmunity Study in the Yong (DAISY) study, there was no significant difference in the intake of cow milk or products, or in the period of breastfeeding (Norris *et al*. 1996). Therefore, the authors concluded that there is no relationship between the early exposure to cow milk proteins and autoimmunity against pancreatic β cells (Norris *et al*. 1996). In a study on the effect of DHA (Fronczak *et al*. 2003), there was no association with incidence of type 1 diabetes, while fatty acid ratio was not examined either in this study. In a more recent study, Norris *et al*. (2007) examined in the DAISY study relationship between n-3 fatty acid intake by cod liver oil supplements and islet autoimmunity in children at increased genetic risk for type 1 diabetes, and found a reduced risk of islet autoimmunity by dietary n-3 fatty acid intake.

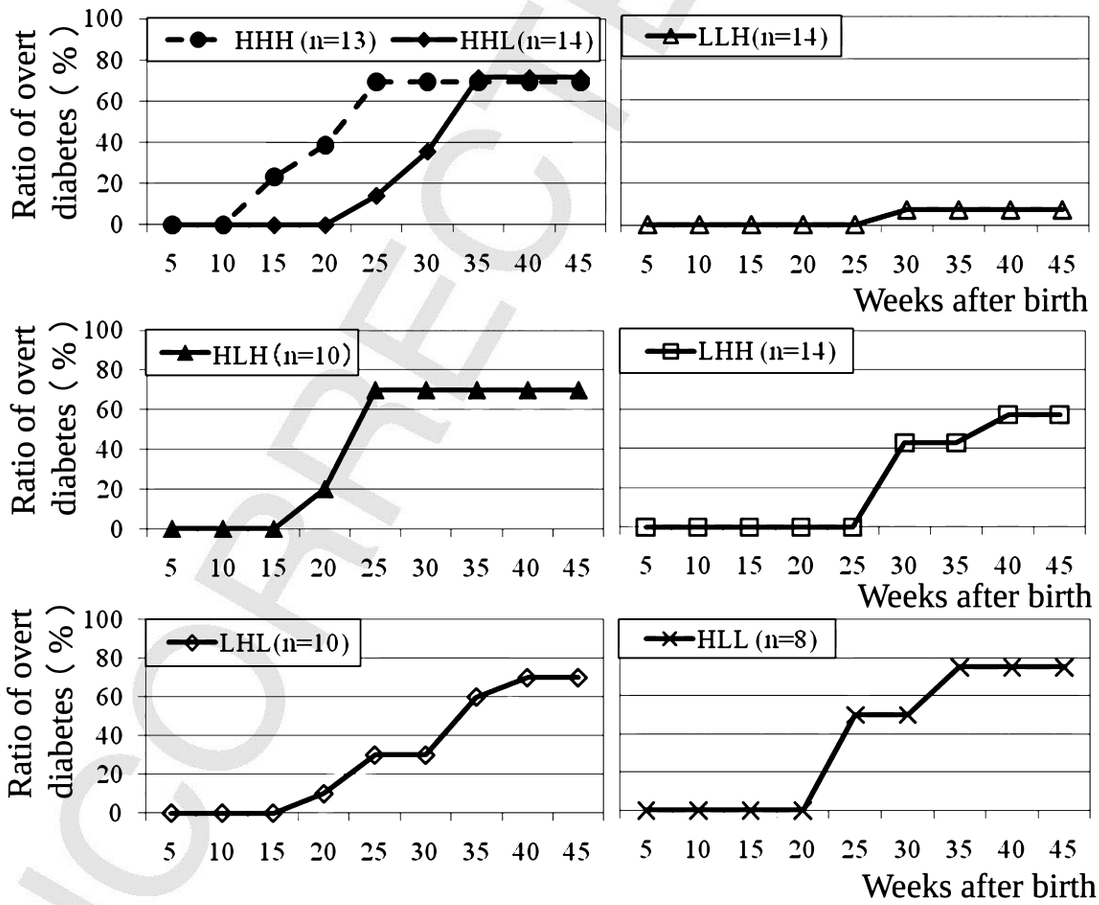
n-6/n-3 ratios in infant formula are usually similar to that in H chow in the present study, while that in human milk is much lower and closer to that in the present L chow. We therefore hypothesized that high n-6/n-3 ratios in the infant formula may be related with the increased incidence of type 1 diabetes. When NOD dams were fed with H chow through gestation and lactation periods in the present study, IAA tended to be detected at a higher frequency in the offspring in the early postnatal period. Thus, the present results suggested that increased n-6/n-3 ratio through maternal nutrition and breast milk may induce IAA in genetically predisposed individuals to cause autoimmune diabetes, while low n-6/n-3 ratio diet may prevent this from happening.

Fig. 5 Effects of combination of L and H chows on the onset and incidence of overt diabetes. The development of diabetes was significantly suppressed in the offspring born from and lactated by dams provided with L chow but fed with H chow after weaning (LLH) with similar kinetics to those fed with LLL chow (A). In the offspring from dams provided with H chow during gestation and/or lactation, the overall incidence of overt diabetes reached approximately 70%. However, in offspring that were fed with L chow more than one part out of three periods (i.e. maternal gestation, lactation, and the offspring's post-weaning growth) the onset tended to be delayed by 5 to 10 weeks compared with that in HHH group (A, B).

A



B



IAA has been suggested as a marker to predict the development of autoimmune diabetes both in humans and NOD mice (Liping *et al* 2000), while another study using NOD and NOR mice reported that temporary appearance of IAA did not correlate with the development of autoimmune diabetes (Abiru *et al*. 2001). Autoantibodies from mothers have been implicated in autoimmune diseases, such as systemic lupus erythematodes (Van Kerckhove 1990; Greeley *et al*. 2002; Herrath and Bach 2002). Greeley *et al*. (2002) reported that transition of maternal IAA to offspring may be involved in the development of diabetes in the offspring. However, it has been also reported that maternal immunity to insulin did not affect diabetes in NOD offspring (Koczwara *et al*. 2004). We previously reported that transition of maternal IAA to the fetus during late gestation did not appear to be directly related with the development of insulitis and further overt diabetes (Kagohashi *et al*. 2005b), while the present findings suggest that IAA in the offspring may be either directly transmitted from the mother or indirectly induced by maternal nutrition. Further detailed study is necessary to clarify the transition and/or induction of IAA and its significance in the development of autoimmune diabetes.

In conclusion, the present findings suggested that dietary n-6/n-3 EFA ratios during gestation, lactation and post-weaning periods affect the development of insulitis and incidence of overt type 1 diabetes.

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