STOPPED AT THE BORDER

Professor Mikael Knip of the University of Helsinki on the DIABIMMUNE project that looks at surprisingly different rates of autoimmunity in children from Finland and neighbouring Russian Karelia

AN illustrative example of the rising incidence of immune-mediated disorders in modern society is that the number of new cases with Type 1 diabetes, one of the most common immune-mediated diseases, has been predicted to double from 2005 to 2020 among European children under five years of age. Finland, with the highest incidence of Type 1 diabetes in the world, has witnessed a five-fold increase in the incidence rate from the early 1950s up to now, i.e. in a time period of about 60 years. The current annual rate in Finland is 64 new cases per 100,000 children under the age of 15 years, whereas the corresponding average figure in Europe is less than 20. The rapid increase in incidence seen in most developed countries cannot be due to purely genetic reasons, but must reflect changes in lifestyle and living environment and their interactions with predisposing genes. There is a strong association between the incidence of immune-mediated diseases and improving the standard of living and hygiene. One of the steepest gradients in standard of living worldwide is present at the border between Russian Karelia and Finland, the latter having a eight times higher gross national product.

‘**What can we then expect to learn from the DIABIMMUNE study? One essential question which we wish to answer is whether there are specific microbes protecting Russian Karelian children from allergy and autoimmunity or whether the total microbial load in early life is the critical factor.**’

In earlier studies we have compared the incidence and frequency of a series of immune-mediated diseases, as well as the prevalence of antibodies, to a variety of microbes between Finnish and Russian Karelian children. All laboratory analyses have been performed based on a large biobank, including samples from altogether 5,700 unselected schoolchildren from Finland and Russian Karelia. We observed that the annual incidence rate of Type 1 diabetes among children under the age of 15 years was almost six times lower in Russian Karelia than in Finland (7.4 vs. 41.4/100,000 children) over a ten-year time period (1990-1999). In the same series, we noticed that Finnish

Professor Mikael Knip, PI of the DIABIMMUNE study

schoolchildren had coeliac disease five times more frequently than schoolchildren in Russian Karelia. Thyroid autoimmunity was also about six times more common among Finnish schoolchildren when compared to their peers in Russian Karelia. Allergen-specific IgE was measured as a marker of allergic sensitisation. The Finnish schoolchildren had significantly higher levels of birch and cat-specific IgE, suggesting increased allergic sensitisation to these common allergens in Finland. Altogether allergic sensitisation was detected in 22% of the children in Finland compared to 6% of the children in Russian Karelia. In the same group of subjects we compared the frequency of microbial infections during childhood between the two countries. *Helicobacter pylori, Toxoplasma gondii,* hepatitis A and enterovirus antibodies were all more frequent in Russian Karelia than in Finland, indicating that the Russian Karelian schoolchildren had experienced a considerably heavier microbial load by the age of 12 years, which was the mean age in the study population.

The scientific community in the Type 1 diabetes field has generated, over the last 30 years, data showing that the disease process eventually resulting in clinical diabetes starts months and years before the appearance of any symptoms. It has been estimated that if Type 1 diabetes become manifest in childhood, the asymptomatic disease process has started on an average about three years earlier, with a considerable individual variation from a few months up to more than ten years. We have observed that the overwhelming majority of those children who present with clinical diabetes before puberty, develop the first detectable signs of an on-going disease process before the age of three years – reflecting that the disease process is triggered early in life.

DIABIMMUNE

Based on our earlier observation among Finnish and Russian Karelian schoolchildren, we embarked in 2008 on the prospective EC-sponsored DIABIMMUNE study, recruiting newborn infants and young children in Finland, Estonia, and Russian Karelia. These three countries represent a living laboratory, since they are characterised by conspicuous differences in standard of living and hygiene, i.e. Russian Karelia has a relatively poor standard,

Handling of DIABIMMUNE samples in the Core Laboratory in Biomedicum, University of Helsinki, Helsinki, Finland

Estonia is a country in rapid transition, and Finland has a high standard of living and hygiene. In each country we have screened about 3,000 newborn infants for genetic susceptibility to autoimmunity and recruited three national cohorts each comprising about 330 newborn infants carrying increased predisposition to autoimmune diseases. We are monitoring these children up to the age of three years with sequential study centre visits at the age of three, six, 12, 18, 24, and 36 months. Biological samples including serum, RNA, peripheral blood mononuclear cells, nasal swabs, skin swabs and rectal swabs are obtained at each visit. In addition, the parents are asked to collect their child's stool sample once a month up to the age of three years. We have also recruited a cohort comprising about 1,500 three-year-old children in each participating country. These children are also analysed for genetic susceptibility to autoimmunity, but all children with parental consent are eligible for participation in the study, irrespective of their genotype. These children are also examined at the age of five years. Based on the three-year results we identify a group of cases characterised by positivity for diabetes-predictive autoantibodies, coeliac disease-associated autoantibodies or at least two allergen-specific IgEs out of eight analysed and a group of control children with no signs of autoimmunity or atopic sensitisation. These cases and controls have an additional study centre visit at the age of four years with a more extensive sampling of biological samples. The expanded

sampling procedure is repeated in the case and control children at the five-year visit.

What can we then expect to learn from the DIABIMMUNE study? One essential question which we wish to answer is whether there are specific microbes protecting Russian Karelian children from allergy and autoimmunity or whether the total microbial load in early life is the critical factor. Therefore we are comparing the frequency of various viral and bacterial infections based on objective measurements from the biological samples collected. One possible explanation to the lower frequency of immune-mediated diseases in Russian Karelia is that the Finnish children have an impaired immune regulation as a consequence of their reduced microbial exposure. Circulating regulatory cells appear to play a crucial role in suppressing allergic and autoimmune reactions. Our preliminary data from the DIABIMMUNE study indicate that six-month-old Estonian infants have more active regulatory cells than their Finnish peers. A similar phenomenon was observed when comparing three-year-old Finnish and Estonian children.

DIPP

Vitamin D deficiency has been implicated as a risk factor for Type 1 diabetes. The data available on the association between Type 1 diabetes and vitamin D is, however, contradictory. In the Finnish birth cohort study, DIPP, aimed at improving the prediction of Type 1 diabetes and at developing effective means for disease prevention, we have been unable to observe any association between the development of diabetes-predictive autoantibodies in the offspring

and the intake of vitamin D by the pregnant women or by the offspring. In a preliminary study based on four-year-old DIABIMMUNE participants in Finland and Estonia, we noticed that the Estonian children had clearly lower circulating concentrations of vitamin D than the Finnish children. More than half of the Estonian children had insufficient vitamin D concentrations, whereas only one out of 43 Finnish children was in the same situation. This observation does not support a critical role of vitamin D in the development of Type 1 diabetes, since the disease incidence is about three times lower in Estonia than in Finland.

Intestinal microbiota appears to be involved in the pathogenesis of Type 1 diabetes and other immune-mediated diseases. The disease incidence in NOD mice, an experimental model of autoimmune diabetes, does seem to depend on the environment, and there is data indicating that the rate is highest in a relatively germ-free environment, and that the intestinal microflora may affect its incidence via modulation of the host innate immune system. Preliminary findings from the DIPP study imply that the diversity of gut microbial composition is indeed diminished in children who later progress to Type 1 diabetes. Sensitive read-out of gut microbial variation, along with other genetic and environmental factors, may also help explain how the gut microbial composition affects host physiology.

A recent metagenomics analysis of stool samples from DIPP children, who developed diabetes-predictive autoantibodies and progressed to clinical Type 1 diabetes during prospective observation, and from matched non-diabetic control children, showed that genes involved in carbohydrate metabolism, adhesion, motility, phages, prophages, sulphur metabolism, and stress responses were more prevalent in cases, whereas genes with roles in DNA and protein metabolism, aerobic respiration, and amino acid synthesis were more common in controls. Mining 16S rRNA data from these datasets showed a higher proportion of butyrate-producing and mucin-degrading bacteria in controls compared to cases, while those bacteria that produce short chain fatty acids other than butyrate were more common in cases.

These data suggest that a consortium of lactate and butyrate-producing bacteria in a healthy gut induce a sufficient amount of mucin synthesis to maintain gut integrity. In contrast, non-butyrate-producing lactate-utilising bacteria prevent optimal mucin synthesis, as seen in autoimmune subjects. Those observations are supported by our very recent findings of a low abundance of butyrate and lactate-producing species and an increased abundance of the *Bacteroides* genus among children testing positive for two or more diabetes-predictive autoantibodies from two prospective studies when compared to autoantibody-negative control children. The recent observation by a Japanese group that indigenous *Clostridium* species are able to induce colonic regulatory T cells provide further evidence for an interaction between gut microflora and the immune system.

TRIGR

In a pilot study of the on-going international Trial to Reduce IDDM in the Genetically at Risk (TRIGR) we observed recently that weaning to an extensively hydrolysed casein formula reduced the cumulative incidence of diabetes-predictive autoantibodies to about half by the age of ten years in children with genetic disease susceptibility. Dutch studies have reported that weaning to a hydrolysed casein diet reduces autoimmune diabetes by 50% in diabetes-prone BB rats. That protective effect was associated with restoration of the impaired intestinal barrier function and a gut microflora characterised by stable *Lactobacilli* levels. We are in the process of embarking on a study assessing possible mechanisms mediating the protective effect against the appearance of diabetes-predictive autoantibodies conferred by a highly hydrolysed casein formula. In that study we randomise infants with increased genetic susceptibility to Type 1 diabetes to be weaned to an extensively hydrolysed casein formula or a regular cow's milk based formula. The mothers are encouraged to breast-feed as long as possible.

The intention is that the participating infants should be exposed to the study formula for at least 90 days before the age of nine months, allowing compliance with the current recommendation of exclusive breast-feeding up to the age of six months. The participating families will have study centre visits when their baby is three, six, nine and 12 months old. Intestinal permeability will be assessed at each visit with the lactulose/mannitol test and we will monitor the number and function of regulatory T-cells in the peripheral circulation. In addition, the parents will collect stool samples from their infant once a month starting from the age of two weeks up to the age of 12 months.

Possibilities

The examples from the DIABIMMUNE study above illustrate some of the possibilities provided by the extensive and versatile biobank created based on all the biological samples collected within that study. The application of modern –omics technologies (e.g. metabolomics, transcriptomics, epigenomics and metagenomics) will provide analytical challenges when starting to combine data from the various technologies, but such an approach will facilitate the generation of true new insights into the development of Type 1 diabetes and other immune-mediated diseases.

Finnish investigators recently introduced the biodiversity hypothesis, claiming that reduced contact of people with natural environmental features and biodiversity, including environmental microbiota, results in inadequate stimulation of the immune system, which favours the development of allergic and autoimmune diseases. Very recently the same group showed that decreased environmental biodiversity can be strongly associated with signs of allergy (Hanski *et al.* **Proc Natl Acad Sci USA.** 2012;109:8334). Such an association was observed both on the macroscale, represented by the reduced species richness of native flowering species and the low variation in land use types in the surroundings of the home of allergic individuals, and on the microscale, reflected by a decreased diversity of one class of bacteria (i.e. the gammaproteobacterial genus *Acinetobacter*) in the skin microbial community in those with allergy. These results indicate that exposure to natural environmental features may
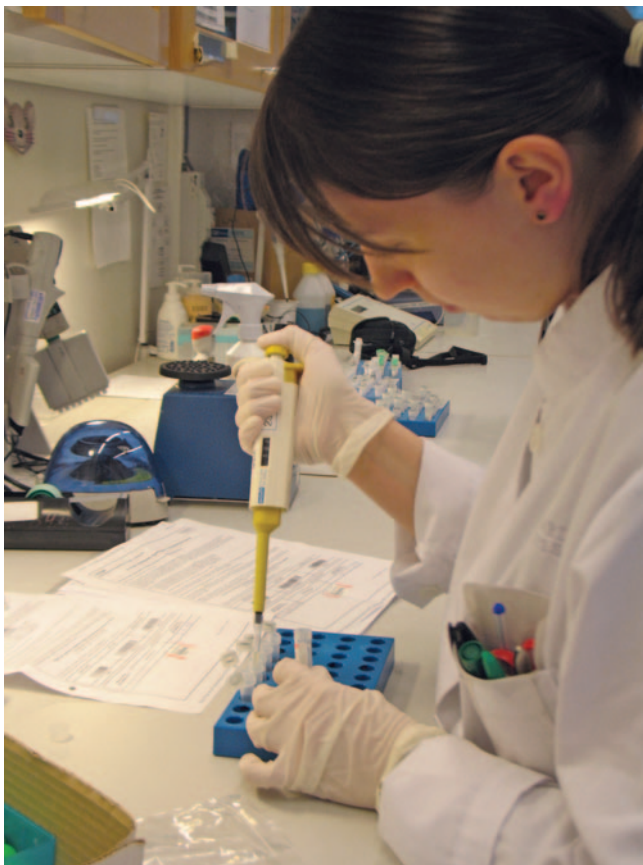
Analysis of DIABIMMUNE samples in the Core Laboratory in Biomedicum, University of Helsinki, Helsinki, Finland

enrich the commensal microflora and enhance its interaction with the immune system. One may ask whether a decreased environmental biodiversity might also contribute to the development of other immune-mediated diseases such as autoimmune disorders including Type 1 diabetes.

In the DIABIMMUNE study, the environmental biodiversity of the surroundings of each participant will be analysed with modern methods and that information will then be combined with all other data generated. Accordingly, the study design makes it possible to expand the testing of the hygiene hypothesis into testing the broader environmental biodiversity hypothesis. The study will for the first time combine information on environmental biodiversity, human microbiota including nasal, skin and intestinal microbial communities and the development of early signs of immune-mediated diseases in a prospective study of young children living in three countries with marked contrasts in the standard of living and hygiene.

The specific scientific objective is to compare the environmental biodiversity and nasal, skin and intestinal microbiota between young children, who present with progressive beta-cell autoimmunity and/or overt Type 1 diabetes during prospective observation, and matched control children in the three above-mentioned countries to identify which factors play a crucial role in the development of Type 1 diabetes. Environmental biodiversity is assessed based on the major land use types within a radius of 3km of the homes of the study subjects, type of housing,

presence of domestic animals and/or pets, and the quality of drinking water and milk. The commensal microbiota is assessed by analysing the nasal, skin and intestinal microbiota of the child by metagenomics. In addition, the microbiota in dust samples from the mattress of the child will be analysed. Other objectives include the comparison between young children who manifest signs of coeliac disease-associated autoimmunity and clinical coeliac disease and those developing signs of atopic sensitisation by measuring specific IgE to a series of common food and air-borne allergens with matched controls. This study provides a unique opportunity to test the biodiversity hypothesis. If that hypothesis can be confirmed, steps should be taken to increase the environmental biodiversity of growing children starting from at or even before birth.

Unique clinical setting

The data generated within the DIABIMMUNE study will be important in terms of increased knowledge of the process leading to Type 1 diabetes and other immune-mediated diseases, early disease prediction as well as for discovery of novel therapeutic avenues for disease prevention. Complex diseases such as Type 1 diabetes are characterised by long prodromal periods. Overt disease is manifested by expression of many pathological as well as compensatory processes aimed at maintaining the system homeostasis.

The DIABIMMUNE study focuses on infants and young children, which will afford investigations of early events and factors leading to Type 1 diabetes and other immune-mediated diseases. This globally unique clinical setting is matched by modern technologies to analyse environmental biodiversity and human microbiome. The role of human microbiota cannot be neglected when considering the genetic and environmental factors contributing to complex diseases. The human microbiome has emerged as a new territory for discovery of novel targets and treatments for complex diseases. The novelty of the DIABIMMUNE study is largely based on the combination of data generated by the parallel analysis of environmental biodiversity and human microbiome in individuals at risk for autoimmunity and/or allergy. The study potentially tackles new biology of high relevance to biomedical research and human health, being simultaneously an excellent example of interdisciplinary research.

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