

THE ROLE OF GUT MICROFLORA IN MUCOSAL TOLERANCE INDUCTION TO BIRCH POLLEN IN MOUSE ALLERGY MODEL

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SUMMARY

The "hygiene hypothesis" is based on the fact that limited bacterial load in early childhood increases the prevalence of allergic disorders in the population of developed countries.

Using germfree mice we studied the role of microbiota in the development of allergic sensitization and mucosal tolerance induction in a mouse model of type I allergy to birch pollen. In our setting, the absence of the microflora did not influence the capacity to establish tolerance via the oral or intranasal routes nor to establish an allergic immune response at the humoral or cellular levels. Our findings may challenge the common view that the commensal microflora is a key factor for the breakdown of physiological tolerance. Furthermore preliminary results from follow up experiments using mice mono-colonized with the probiotic bacterial strain *Lactobacillus plantarum* are discussed.

INTRODUCTION

Recent epidemiological studies have shown that more than 30% of the population of developed countries suffers from type I allergy, characterized by disorders such as rhinitis, conjunctivitis, asthma and eczema, with immediate and delayed type immune responses extending the duration of the diseases from hours to days after exposure. Epidemiological studies suggest the participation and important role of mucosal microbiota composition in increased prevalence of allergic dis-

eases in developed countries (*Björkstén et al., 1999*).

The rising prevalence of allergic disorders in western countries has been linked to the high hygienic standards associated with a reduced microbial stimulation of the mucosal immune system ('hygiene hypothesis'). In this context epidemiological studies connected infections with hepatitis A, *Helicobacter pylori*, and *Toxoplasma gondii* in inhabitants of the temperate climates, and geohelminths in those

living in endemic areas, to a reduced risk of atopic manifestations (*Sheikh and Strachan, 2004*). Besides genetic predisposition and environmental factors, the development of type I allergy seems to be influenced by alimentary habits, which affects the composition of the gut microflora (*Wells and Mercenier, 2008*). Recent studies on the pathogenesis of allergy in both man and experimental animals continue to show the importance of commensal bacteria in the gastrointestinal tract for stimulation and modification of the immune system (*Savilahti et al., 2008*). In this context, reduced microbial stimulation may lead to impairment of mucosal immune system maturation with a higher vulnerability to infections and more prevalent sensitization to allergens (reviewed by *Taskalova-Hogenova et al., 2004*).

The mechanisms of allergic sensitization as well as tolerance induction can be studied using suitable experimental animal models (*Herz et al., 2004*); even if the usefulness and optimization of models of allergic airway disease were viewed with some doubts (*Finkelman and Wills-Karp, 2008*). Advantages of mouse models are based on the availability of various strains of inbred mice with defined immunological and physiological properties of airways, including differences in susceptibility and features of allergy airway disease (*Finkelman and Wills-Karp, 2008*). However, animal models of allergy are not fully comparable with human allergic diseases.

Patterns of type I allergy can be attributed to allergen-specific IgE on mast cells and basophiles. A disturbed balance between Th1 and Th2 lymphocyte subpopulations or a lack regulatory T cells leads to elevated Th2 cytokine production needed for allergy induction. Interleukin (IL)-4, IL-5, IL-13 drive B-lymphocytes to the production

of antigen-specific IgE antibodies. IgE binds to its high-affinity receptor on mast cells (FcεRI) or basophiles. Degranulation of these cells occurs when antigen reacts with bound IgE antibodies and is accompanied by massive release of inflammatory mediators (serotonine, histamine, prostaglandins, adenosine) (*Mossman et al., 1986, Neurath et al., 2002, Van Bever et al., 2008*).

Mucosal tolerance is an antigen-specific suppression of immune responsiveness occurring after application of antigen to mucosal surfaces – intragastrical, intranasal, sublingual, intravaginal and intrarectal. The advantage of mucosal immunotherapy is non-invasive immunization leading to functional modulation of lymphoid cells on the mucosal compartment.

We have previously established a mouse model of type I allergy to birch pollen and its major allergen Bet v 1 (*Wiedermann et al., 1998*), displaying Bet v 1-specific IgE antibodies and positive skin test, and aimed at modulating the immune responses via the mucosal route. Therefore several mucosal adjuvants were tested for their capacity to modulate the allergic immune responses (*Wiedermann et al., 1998, 1999*). The administration of the major birch pollen allergen Bet v 1 via the intranasal and oral route was shown to suppress Th2-based immune responses in prophylactic and therapeutic settings (*Wiedermann et al., 1999, 2001, 2002, Winkler et al., 2002, Wiedermann, 2003, Hufnagl et al., 2008, Repa and Kozakova et al., 2008*).

Mouse gnotobiotic models are helpful to elucidate the pathogenic mechanisms of diseases as well as new preventive and therapeutic options. The role of microbiota in mucosal tolerance induction was studied by several authors (Table 1). While the first investigations using particulate antigens

Table 1: Mucosal tolerance induction to allergens in germ-free mice

Authors	Strain	Allergen/route	Humoral level	Cellular level
Wannemuehler et al., 1982	C3H/HeN BALB/c SWISS	Sheep red blood cells / intragastrical	IgM, IgG1, IgG2, IgA, not tolerized	Not investigated
Moreau and Corthier, 1988 Moreau and Gaboriau-Routhiau, 1996	C3H/HeJ	Ovalbumin / intragastrical	OVA specific IgG1 and IgE tolerized, but with reduced duration for IgG1	Not investigated
Furrie et al., 1995	BALB/c	Ovalbumin / intragastrical feed, serum transfer	IgG not tolerized either in CV or GF after serum transfer	DTH tolerized
Sudo et al., 1997	BALB/c	Ovalbumin / intragastrical	IgG1, IgE but not IgG2a tolerized	Th1 (IFN- γ) but not Th2 (IL-4) tolerized
Rask et al., 2005	NMRI	Ovalbumin / intragastrical feed, serum transfer	IgG, IgG1 not tolerized (data were not shown)	DTH suppressed in CV, only tendency in GF mice
Walton et al., 2006	BALB/c	Ovalbumin / intragastrical	not applicable (sensitization with an OVA peptide)	Proliferation and Th1 responses (IFN- γ) suppressed
Repa et al., 2008	BALB/c	Bet v 1 / intragastrical and intranasal	IgG1, IgA, IgG2a, IgE tolerized	Th1 (IFN- γ) and Th2 (IL-5) tolerized

indicated abrogation of tolerance in the absence of mucosal microbiota, most of the following findings including our

own using protein antigen indicate that the presence of intestinal microflora is not needed.

MATERIAL AND METHODS

Two-month-old BALB/c mice were used in our experiments. Germfree and *Lactobacillus plantarum*-monoassociated mice were kept under sterile conditions in separate plastic cages in Trexler-type isolators (Figure 1) and fed *ad libitum* sterile (59 kGy irradiated) standard pellet diet (ST1, Inst. Physiol., Acad. Sci. Czech Republic) and sterile water. Conventional mice kept in the conventional animal facility were fed the same but not sterile food. The animals were kept in a room with a

12 h light-dark cycle at 22°C. Recombinant (r) birch pollen allergen Bet v 1 (Biomay, Vienna, Austria) was used for tolerance induction as well as for allergic sensitization. For details, see Repa and Kozakova et al. (2008). Briefly, germfree and conventional mice were pre-treated before sensitization three times with 100 μ g allergen in 0.2 ml of 3 % NaHCO₃ using intragastric tubing (i.g.) (Figure 2) or three times with 10 μ g in 0.03 ml of saline intranasally (i.n.). Allergic sensitization



Figure 1: Germfree or *Lactobacillus plantarum*-monocolonized mice were reared in Trexler-type isolators in the Laboratory of Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic in Novy Hradek.

was done one week after pre-treatment three times s.c. with 1 μg r Bet v 1 adsorbed to 2 mg of aluminium hydroxide in 2-week intervals. The mice were sacrificed one week after last immunization. Peripheral blood was used for serum isolation. Spleen and mesenteric lymph nodes were aseptically removed and lymphocyte suspensions were prepared. Cells were cultivated in supplemented RPMI 1640 (Sigma, Germany) medium for 48 h. The levels of IL-5 and IFN- γ were determined by ELISA

in the medium of the cultivated cells. Allergen-specific antibody levels of IgE, IgG1, IgG2a and IgA were evaluated in serum by ELISA. All experiments were approved by the Animal Experimentation Ethics Committee of the Institute of Microbiology, Academy of Sciences of the Czech Republic and conducted in accordance with the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS No.: 123)".

RESULTS AND DISCUSSION: ALLERGIC SENSITIZATION AND MUCOSAL TOLERANCE INDUCTION TO BIRCH POLLEN IN GERMFREE MICE

To study of the role of the intestinal microflora in the development of allergic sensitization and induction of mu-

cosal tolerance to the main component of birch pollen was one of the goals of the European LABDEL project coordi-



Figure 2: Intra-gastrical administration of Bet v 1 using soft rubber tubing.

nated by Prof. Jerry Wells (for details see *Daniel and Repa et al., 2007*). We compared oral as well as intranasal treatment with Bet v 1 for the ability to induce tolerance to Bet v 1 (17 kD) before allergic sensitization in germfree and conventionally reared mice. For tolerance induction the mice were administered with high doses of Bet v 1 intra-gastrically (Figure 2) or intranasally. Allergen-specific humoral responses of IgE, IgG1, IgG2a antibodies to Bet v 1 are summarized in Table 2. Both intra-gastrical and intranasal pre-treatment with Bet v 1 before allergic sensitization induced significant reduc-

tion of Bet v 1-specific antibodies of all isotypes in both germfree and conventional mice. Similarly, we observed a decreased level of IL-5 in the supernatant of cultivated spleen lymphocytes from either intra-gastrically or intranasally Bet v 1-treated mice (*Repa and Kozakova et al., 2008*).

Based on our results we conclude that mucosal tolerance, which is known to be dependent on the dose and nature of the antigen, host genetic background, timing of treatment, and route of antigen application, does not seem to be dependent on the presence of microflora.

Table 2: Humoral immune responses expressed as allergen (Bet v 1)-specific serum antibody levels in conventional and germfree mice.

Antibodies		Conventional mice			Germfree mice		
Specific Igs	Units	Ctrl.	i.g.	i.n.	Ctrl.	i.g.	i.n.
IgE	Elisa units	206±150	37.1±1.5*	10.2±21	309±205	70.5±3	9.8±2.0
IgG1	Elisa units	513±307	60.5±75*	10.2±7.0*	593±415	167.6±112*	25.7±19.0*
IgG2a	Elisa units	16.6±14.3	6.3±1.5	4.6±1.3*	64.4±100	73.9±120	5.7±2.0
IgA	Opt. density	0.095±0.02	0.08±0.01*	0.056±0.02*	0.20±0.05	0.10±0.01	0.06±0.02*

Allergen-specific antibody levels of IgE, IgG1, IgG2a and IgA were detected by ELISA in blood sera after three s.c. immunizations with 1 µg r Bet v 1 adsorbed to 2 mg of aluminum hydroxide in 2-week-intervals (1) control mice (Ctrl), (2) mice pretreated before sensitization three times with 100 µg of r Bet v 1 in 0.2 ml of 3 % NaHCO₃ using intragastrical tubing (i.g.) or (3) mice pretreated before sensitization three times with 10 µg of r Bet v 1 in 0.03 ml of saline intranasally (i.n.).

Values are expressed as means ± SEM.

* $P < 0.05$ significantly different from controls; determined by the Mann-Whiney U after Kruskal-Wallis test.

EFFECT OF LACTOBACILLI ON ALLERGIC SENSITIZATION

Probiotics are dietary supplements containing potentially beneficial bacteria or yeasts. According to the currently adopted definition by FAO/WHO, probiotics are: 'Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host'. It is generally assumed that commensal and probiotic bacteria could induce mucosal tolerance to environmental antigens, especially to food and airborne allergens.

It has been shown that the composition of intestinal flora during the first year of life affects the occurrence of allergy: Infants who develop allergies have often disbalanced microflora with reduced diversity of bacteria (*Björkstén et al., 1999*). Based on this suggestion that the composition of the intestinal flora might play a role in the development of atopy/allergic diseases, the use of probiotics would be very attractive non-invasive approach in their prevention/therapy (*Tlaskalova-Hogenova et al., 2004*). There is a growing interest in probiotics such as lactic acid bacteria for their potency to modulate the Th1/Th2 balance, in addition to having

an immunomodulative effect through induction of Th1 bias (*Torii et al., 2007*). Yet we have only limited knowledge about the effects of colonization with the intestinal bacteria on the onset of physiological and pathological immune responses.

Lactic acid bacteria (LAB), strains of the genera *Lactobacillus* and *Bifidobacterium*, are the most widely used probiotic bacteria. LAB have been used in the food industry and food-fermentation processes for many years, because they are able to convert sugars (including lactose) and other carbohydrates into lactic acid. *Lactobacillus (Lb.) plantarum* dominates in fermented cabbage, olives, natural wines and beers and is a natural inhabitant of the gastrointestinal tract. Recently some *Lactobacilli* with probiotic effects (*Lactococcus lactis*, *Lactobacillus plantarum*) were genetically engineered to express various biologically important molecules (*Hanniffy et al., 2004*). It has been shown that mucosal application of genetically modified bacteria that produce allergens do shift the immune system to non-allergic immune

responses. Therefore the analyses of the role of recombinant intestinal bacteria in activation of both innate and adaptive immunity offers a promising approach to prevent the development of allergy (Wiedermann and Mercenier, 2007).

Lb. plantarum NCIMB8826, which we used in our studies, induces the production of IL-12 and IFN- γ (Repa et al., 2003). Its effects as well as effects of its recombinant form producing Bet v 1 were tested in a model of birch pollen allergy (Daniel and Repa et al., 2006). They found suppression of Th2 responses along with induction of local IgA after prophylactic but not therapeutic treatment. Recently, Piirainen et al. (2008) studied the effect of *Lactobacillus rhamnosus* GG on rBet v 1 and rMal d 1 specific IgA in the saliva of patients with birch pollen allergy. After a five-month treatment with *Lactobacillus rhamnosus* GG the patients developed an increased level of Bet v 1-

specific IgA in sera when compared with non-treated individuals. IgA, which is the most abundant immunoglobulin isotype in the body, protects the organism against intestinal toxins, respiratory and gastrointestinal mucosal pathogens. IgA also prevents local inflammation and systemic immune responses triggered by innocuous antigens and/or commensal flora (Mora and von Andrian, 2008).

To further elucidate the role of commensal bacteria in sensitization and establishment of mucosal tolerance to potential allergens, the use of gnotobiotic animal models (germfree or colonized with known microflora) is highly attractive. Our preliminary data with *Lactobacillus plantarum* NCIMB8826-monocolonized mice in birch pollen allergy model showed that *Lactobacillus plantarum* NCIMB8826 modifies Th2 cytokine responses and affects the production of IgA (unpublished data).

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