

Susceptibility to nasal and oral tolerance induction to the major birch pollen allergen Bet v 1 is not dependent on the presence of the microflora

Andreas Repa^{a,c,1}, Hana Kozakova^{b,1}, Tomas Hudcovic^b, Renata Stepankova^b,
Tomas Hrnčir^b, Helena Tlaskalova-Hogenova^b,
Arnold Pollak^c, Ursula Wiedermann^{a,*}

^a Department of Specific Prophylaxis and Tropical Medicine, Center for Physiology, Pathophysiology & Immunology,
Medical University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria

^b Institute of Microbiology, Department of Immunology and Gnotobiology, Academy of Sciences of the Czech Republic,
CZ 54922 Nový Hrádek, Czech Republic

^c Department of Pediatrics and Adolescent Medicine, Division of General Pediatrics and Neonatology,
Medical University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Received 3 September 2007; received in revised form 27 November 2007; accepted 28 November 2007

Available online 31 December 2007

Abstract

The indigenous microflora plays an integrative role in the maintenance of immunological homeostasis. Several studies reported that immunological tolerance is dependent on microbial colonization of the gut. In the present study, we investigated whether the absence of the microflora influences the sensitization process to an allergen as well as the ability to develop mucosal tolerance in a mouse model of birch pollen allergy. Germ-free or conventional BALB/c mice were intranasally or intragastrically pre-treated with the major birch pollen allergen Bet v 1 prior to sensitization with this allergen. Both germ-free and conventional mice displayed comparable Th2 biased immune responses after allergic sensitization. Oral as well as intranasal tolerization led to suppression of allergen-specific serum antibodies (IgG1, IgE, IgA) as well as cytokine production by splenocytes (IL-5, IFN- γ) in both germ-free and conventional animals. Peyer's patches of germ-free animals were approximately 20 times smaller than in conventional animals, but the relative distribution of lymphocyte subpopulations was equal. We conclude that the absence of the microflora does not influence the ability to mount Th2 responses nor to establish tolerance towards the aeroallergen Bet v 1. Our findings may challenge the view that the commensal microflora is a key factor for breakdown of physiological tolerance and allergy development.

© 2008 Elsevier B.V. All rights reserved.

Keywords: Gnotobiotics; Mucosal immunity; Allergy; Immune tolerance; Experimental animal model

1. Introduction

The largest areas of the body exposed to the external environment are formed by the mucosal surfaces, thereby constituting a major site of contact with foreign material. The majority of antigens that are encountered by the mucosae of the gastrointestinal and respiratory tract consist of foods, harmless environmental antigens and microorganisms. The mucosa associated lymphoid

tissue needs to discriminate between pathogens towards which protective immunity has to be established and innocuous antigens that need to be tolerated. Immunological tolerance of mucosal tissues to harmless exogenous antigens is of great importance to avoid potentially detrimental immune responses against dietary proteins and the commensal microflora. In this respect, a failure or breakdown of mucosal tolerance may lead to the development of immunological disorders such as allergy or inflammatory bowel disease [1]. In animal models, immunological unresponsiveness after feeding of a defined antigen was first demonstrated using ovalbumin and referred to as “oral tolerance” [2]. As it was shown that immunosuppression is inducible via other mucosal routes (inhalative [3]

* Corresponding author. Tel.: +43 1 4049064890; fax: +43 1 4049064899.

E-mail address: ursula.wiedermann@meduniwien.ac.at (U. Wiedermann).

¹ These authors contributed equally to this work.

or intranasal [4]), it is now generally referred to as “mucosal tolerance”.

The mucosal surfaces are colonized by a vast variety of microbes. In particular, in the gut the commensal flora constitutes an important first line of defense against orally intruding pathogens by inhibiting their attachment and replication. In addition, the microflora directly promotes the development of the gut associated lymphoid tissue (GALT) by inducing lymphocyte influx to the mucosa and expansion of the Peyer’s patches (PP) with the formation of germinal centers and the production of secretory IgA [5]. Apart from boosting the anti-infectious capacities of the GALT, it has been proposed that also the capacity to induce oral tolerance is only fully developed under the influence of microbial colonization [6]. In this respect, it has been shown that animals reared under sterile conditions cannot be tolerized [7–9] or show a reduced capacity to maintain tolerance [10]. However, there are also reports showing that tolerance can be very well established under germ-free (GF) conditions [11,12]. Recent epidemiological and experimental studies [13] have indicated that the development of type I allergy may be linked to an altered microbial colonization of the gut. These studies are supported by investigations in GF mice showing that the capacity to establish tolerance towards an allergen can be only introduced after bacterial colonization [8,14].

We previously established a mouse model of type I allergy to birch pollen demonstrating that mucosal tolerance to the major birch pollen allergen Bet v 1 can be induced in naïve and sensitized conventional (CV) mice [15,16]. In the present study, we were interested to evaluate the role of the microflora for allergy development and tolerance induction. For this purpose GF and CV mice were orally or intranasally tolerized with Bet v 1 prior to sensitization. Our results indicate that the commensal microflora is not a pre-requisite for the development of an immunological environment capable of establishing mucosal tolerance.

2. Materials and methods

2.1. Animals and rearing conditions

Seven to eight week-old GF and CV inbred BALB/c mice were used for all experiments. GF mice ($n=7$ /group) were born and housed under sterile conditions and fed a sterile standard pellet diet (ST1, Inst. Physiol., Acad. Sci. Czech Republic, 59 kGy irradiated for 30 min) and sterile water ad libitum. CV mice were reared under standard conditions and fed the same but non-sterile

diet. All animals were kept in a room with a 12 h light–dark cycle at 22 °C. Faecal samples of GF animals were weekly evaluated for the presence of aerobic and anaerobic bacteria, moulds and yeast by standard microbiological methodology. 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)”.

2.2. Tolerization and immunization protocol (Fig. 1)

For tolerance induction, recombinant Bet v 1 (Biomay, Vienna, Austria, EU) was applied to GF and CV mice three times at 7-day intervals either intragastrically (i.g.), at a concentration of 100 µg/500 µl 3% NaHCO₃, or intranasally (i.n.) at a concentration of 10 µg/30 µl saline. Ten days later, all mice were sensitized three times at 2-week intervals by subcutaneous (s.c.) injection of 1 µg Bet v 1 absorbed to 2 mg Al(OH)₃ (Serva, Heidelberg, Germany, EU) in 100 µl phosphate buffered saline. Controls were sensitized without pre-treatment. Mice were sacrificed 7 days after the last immunization. Sera were prepared for analysis of allergen-specific antibodies and spleens were removed under sterile conditions for assessment of spleen cell proliferative responses and cytokine production.

2.3. Allergen-specific antibody responses

Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA as previously described [15]. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark, EU) were coated with Bet v 1 (2 µg/ml). Serum samples were diluted 1/1000 for IgG1, 1/100 for IgG2a and 1/10 for IgA. Rat anti-mouse IgG1, IgG2a and IgA antibodies (1 µg/ml, Pharmingen, San Diego, CA, USA) were applied, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson, Immuno Labs., West Grove, PA, USA) for detection. Antibody levels were reported as optical density units or arbitrary ELISA-units, which were calculated from a standard curve, obtained with a pool of standard sera of Bet v 1 sensitized mice. Allergen-specific IgE levels in sera were quantified by degranulation of rat basophil leukemia (RBL) cells as previously described [17]. Briefly, RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells per well) and

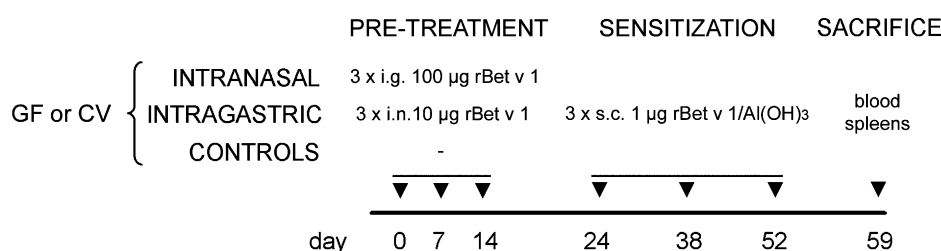


Fig. 1. Experimental design. Germ-free (GF) or conventional (CV) BALB/c mice were pre-treated i.g. or i.n. with recombinant (r) Bet v 1 or left untreated (controls) prior to subcutaneous (s.c.) sensitization with Bet v 1/Al(OH)₃. Spleens were removed for in vitro proliferation and cytokine assays. Blood samples were taken for measurement of Bet v 1 specific antibody levels.

passively sensitized by incubation with mouse sera (1/10) for 2 h. After washing, Bet v 1 (0.3 µg/ml) was added for 30 min at 37 °C for degranulation. Supernatants were incubated with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Sigma–Aldrich, St. Louis, MO, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader (λ_{ex} : 360 nm/ λ_{em} : 465 nm, Spectrafluor, Tecan, Austria). Results are reported as percentage of total β-hexosaminidase release from cells after disruption with 1% Triton X-100.

2.4. Cellular immune responses: spleen cell proliferation and cytokine production

Lymphocyte proliferation and production of cytokines were assessed as previously described [15]. For proliferative responses, 2×10^5 spleen cells/well were cultured in 96-well round-bottom plates (Costar, Cambridge, MA, USA) with or without Bet v 1 (2 µg/well) for 5 days and proliferation was measured by ^3H -thymidine incorporation. For in vitro cytokine production 5×10^6 spleen cells/500 µl were cultured in 48-well flat bottom plates with or without Bet v 1 (10 µg/well) and supernatants were taken after 48 h for measurement of interleukin (IL)-5 and interferon (IFN)-γ as previously described [16]. Cytokine levels were expressed as the percentage of cytokine levels produced by spleen cells of sensitized animals. Concanavalin A was used as a positive control in cell culture experiments (0.5 µg/well in proliferation assays, 2.5 µg/well in cytokine assays) to assess proliferation of cells at visual inspection (clonal proliferation of cells in light microscopy).

2.5. Peyer's patches of GF and CV mice lymphocyte subpopulations

2.5.1. Histology of Peyer's patches

Sections of ileum including Peyer's patches (PPs) from untreated GF and CV mice were excised, fixed in Carnoy's fluid for 30 min and transferred into 96% ethanol overnight, then via 100% ethanol (3 × 1 h) into methylsalicylate (4 h/overnight/4 h), benzene (3 × 10 min) and benzene/paraffin (30 min) to paraffin (2 × overnight). Tissue segments were sliced into 5-µm sections stained with haematoxylin–eosin and viewed under an Olympus BX 40 microscope equipped with a digital camera Olympus Camedia C-2000. Photographs were taken on proposal of Camedia Master 2.5 and DP-Soft (Olympus, Hamburg, Germany).

2.5.2. Flow cytometry analysis of Peyer's patch lymphocytes

Peyer's patches from untreated GF and CV mice ($n=5$) were carefully excised, disrupted in RPMI 1640 medium and filtered through a 70-µm cell strainer to obtain a single cell suspension. Cells were washed, counted and re-suspended in phosphate buffered saline containing 0.5% bovine serum albumin (Sigma, St. Louis, MO, USA) and 0.1% NaN₃, and placed in 96-well microtiter plates. For analysis of lymphocyte subpopulations, cells (10^6 cells/ml, 100 µl/well) were incubated with monoclonal antibodies (FITC anti-mouse CD3ε (145-2C11), FITC anti-mouse CD4 (L3T4), PE anti-mouse CD8a (Ly-2), PE

anti-mouse TCRγδ (GL3), PE anti-mouse CD45R/B220 (RA3-6B2), FITC anti-mouse DX5/CD49b (Ha1/29) for 30 min in the dark at 4 °C (all antibodies by Pharmingen). For the detection of CD4⁺Foxp3⁺ regulatory T cells, cell suspensions were intracellularly stained with PE anti-mouse Foxp3 (FJK-16s; eBioscience) according to the manufacturer's specifications. Analysis was performed using a FACS-Calibur[®] cytometer (Becton Dickinson, San José, CA, USA), acquiring 30,000 lymphocytes per sample, based on the forward and side light scatter properties of the cell preparations. Positive and negative controls have been included.

2.6. Statistical analysis

Statistical comparison was performed between i.n. and i.g. pre-treated mice of GF and CV animals and their respective sensitized control group using the non-parametric Kruskal–Wallis test followed by pair-wise comparison using the Mann–Whitney *U*-test. *P* values <0.05 were considered significant. All data were expressed as the mean ± standard error of the mean.

3. Results

3.1. Humoral immune responses in germ-free and conventional mice (Fig. 2)

Both GF and CV controls developed a Th2 biased immune response towards Bet v 1, as depicted by high allergen-specific IgG1 and IgE levels (Fig. 2A and C) versus low IgG2a antibody levels (Fig. 2B). Mucosal pre-treatment with Bet v 1 via the i.g. and i.n. routes led to significant suppression of in vitro IgE-dependent RBL cell degranulation (Fig. 2C) in GF and CV animals compared to the respective sensitized controls. Similarly, Bet v 1 specific serum IgG1 and IgA antibody levels were suppressed after i.g. or i.n. pre-treatment in both GF and CV animals (Fig. 2A and D). However, a significant reduction of antibody responses was generally achieved by the i.n. route. Bet v 1 specific IgG2a antibody levels were low and showed a tendency (n.s.) towards reduced levels in both GF and CV mice after i.n. pre-treatment (Fig. 2B).

3.2. Cellular immune responses in conventional and germ-free mice (Fig. 3)

Lymphoproliferative responses to Bet v 1 were suppressed in GF as well as CV mice pre-treated i.g. or i.n. with Bet v 1 compared to those of sensitized control animals, but the results did not reach statistical significance due to generally weak proliferative responses (stimulation indices: GF mice: controls 1.52 ± 0.4 , i.g. 1.19 ± 0.17 , i.n. 1.26 ± 0.18 ; CV mice: controls 1.85 ± 0.42 , i.g. 1.29 ± 0.43 , i.n. 1.19 ± 0.36). IL-5 levels upon stimulation of splenocytes with Bet v 1 were significantly reduced in both GF and CV mice particularly after i.n. pre-treatment (not significantly in CV after i.g. pre-treatment, –68.5%, $p=0.22$, Fig. 3A). Suppression of spleen cell INF-γ production (Fig. 3B) was more pronounced after i.n. compared to i.g. pre-treatment with Bet v 1, but did not reach statisti-

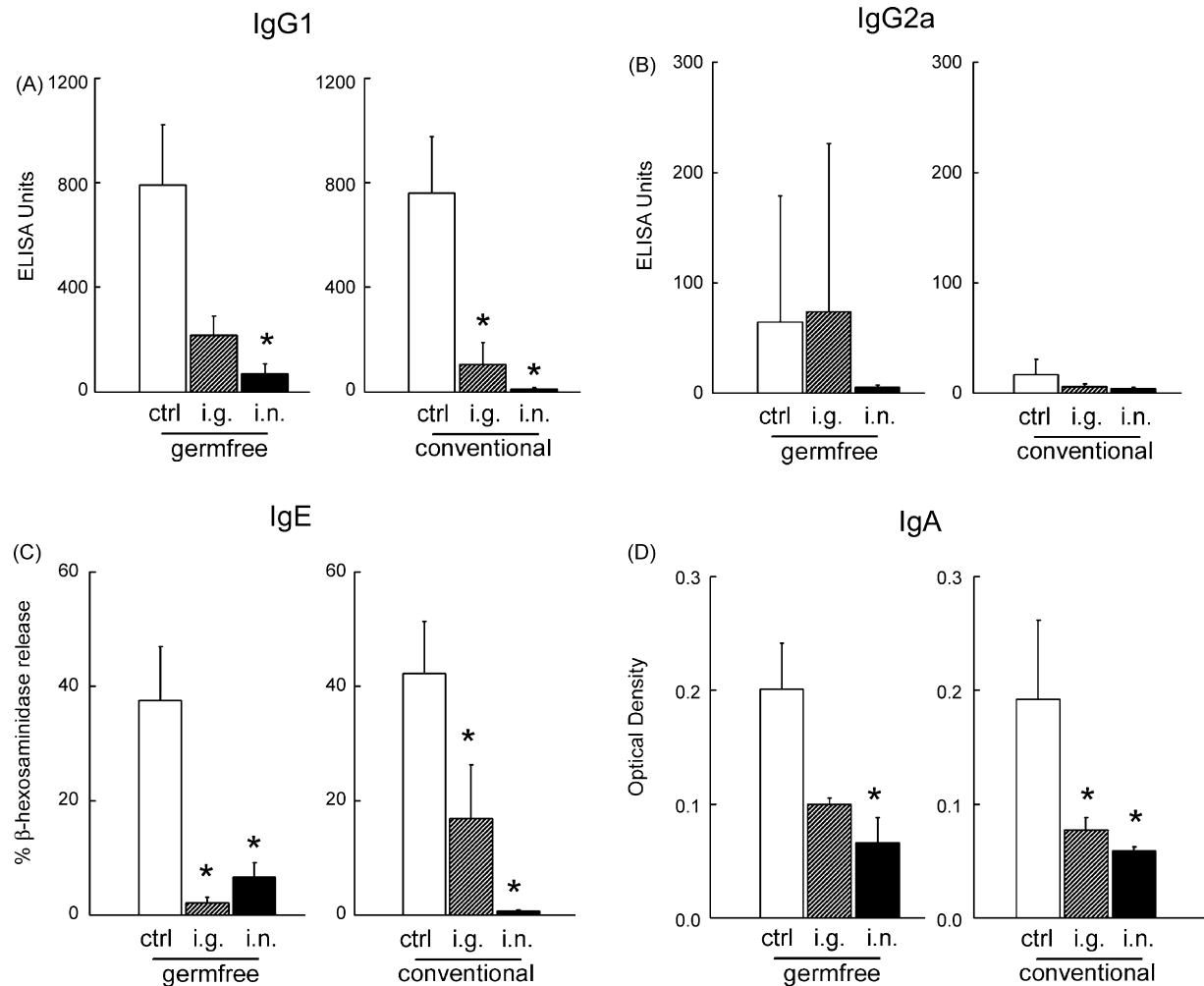


Fig. 2. Allergen-specific serum antibody responses. Bet v 1 specific serum antibody levels of germ-free (GF) and conventional (CV) animals were measured by ELISA (IgG1, IgG2a and IgA) or RBL cell degranulation assay (IgE). Error bars represent standard error of the mean. Ctrl = controls sensitized without pre-treatment, i.g. = intragastrically pre-treated with Bet v 1, i.n. = intranasally pre-treated with Bet v 1. I.n. and i.g. pre-treated mice of GF and CV animals were compared with their respective sensitized control group, *significant difference, $P < 0.05$, Mann–Whitney U after Kruskal–Wallis test.

cal significance in GF or CV mice. Polyclonal proliferation of spleen cells was confirmed upon stimulation with concanavalin A mitogen.

3.3. Peyer's patches in germ-free and conventional mice

3.3.1. Intestinal Peyer's patch histology of conventional and germ-free mice (Fig. 4)

Images represent sections of ileum with PPs which cross the muscularis mucosae forming domes that bulge into the lumen. PPs of GF mice (Fig. 4A) were at least 20 times smaller than those in CV mice (Fig. 4B) that were well developed with distinctive follicles.

3.3.2. Lymphocyte subpopulations in Peyer's patches (Table 1)

Surface marker expression on PP lymphocytes did not evidently differ between GF and CV mice (Table 1). The expression of CD3 in GF mice was 15% compared to 17% in CV mice. The expression of the B cell marker CD45R (B220) was found on

79% of PP cells in both GF and CV mice. CD3⁺CD4⁺ T helper lymphocytes were present in 10% in PPs of GF and 12% in PPs of CV mice. CD3⁺CD8⁺ positive cells were very low, 4% in PP of CV and 5% in GF mice. The percentage of regulatory CD4/Foxp3⁺ cells was comparable in GF and CV animals. Other cell populations such as NK cells (CD49b) and CD3⁺γδTcR⁺ were negligible/hardly detectable.

Table 1

FACS analysis of lymphocyte subpopulations in Peyer's patches (PP). Pooled PPs from untreated germ-free and conventional (CV) mice ($n = 5$) in percentage of total lymphocytes

Subpopulation	PP lymphocytes (%)	
	GF	CV
CD3 ⁺ CD4 ⁺	10	12
CD3 ⁺ CD8 ⁺	5	4
CD3 ⁺ γδTcR ⁺	1	2
B220/CD45 ⁺	79	79
CD4 ⁺ Foxp3 ⁺	3.0	3.1

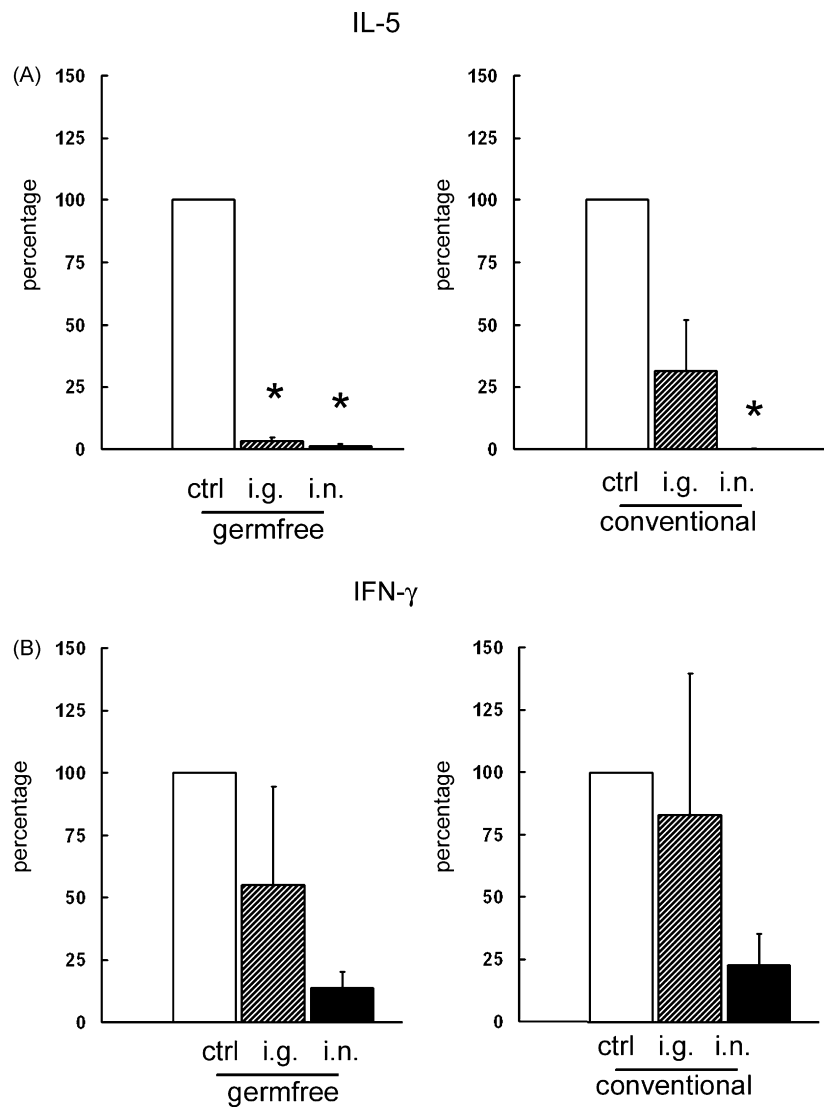


Fig. 3. Spleen cell cytokine production. Spleen cells of the different experimental groups were re-stimulated in vitro with recombinant (r) Bet v 1 for 48 h. IL-5 (A) and IFN- γ (B) levels were measured by ELISA in supernatants. Cytokine production was expressed as the mean percentage of production in sensitized controls (ctrl). Error bars represent standard error of the mean. I.n. and i.g. pre-treated mice of GF and CV animals were compared with their respective sensitized control group, *significant difference, $P < 0.05$, Mann-Whitney U after Kruskal-Wallis test.

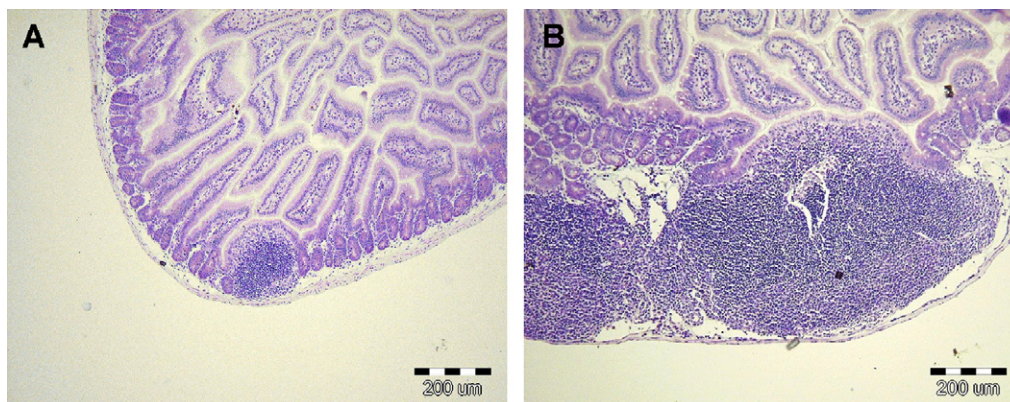


Fig. 4. Peyer's patches histology. Peyer's patches from ileum of untreated germ-free (A) and conventional (B) BALB/c mice were excised, fixed and stained with hematoxylin-eosin.

4. Discussion

It is generally accepted that the gut microflora has an important impact on mechanisms of immune defense and regulation [5]. In particular, with respect to harmless antigens it is believed that the microflora is involved in induction and maintenance of physiological tolerance, thereby preventing hyper-responsiveness and untoward immune responses leading to allergies and food enteropathies. Studies in GF animals aimed to directly investigate the influence of the microflora on immune responses as well as tolerance induction. However, the literature shows diverse and inconclusive results on the significance of the microflora for tolerance induction [7,9,11,12,14,18,19]. Our data from this study clearly show that mucosal (oral as well as nasal) tolerance induction can be equally induced in mice with or without the presence of a commensal bacterial flora. In CV mice, we have previously demonstrated that Bet v 1 acts as a potent tolerogen when mucosally applied prior or after sensitization [15,16,20]. Here, we show that the same suppressive effects of humoral and cellular responses were inducible under GF conditions. Both, nasal and oral application of Bet v 1 prior to sensitization led to marked reduction of allergen specific antibodies of all isotypes (Fig. 2), along with reduced IL-5 and IFN- γ levels (Fig. 3). Notably, in both GF and CV mice, the i.n. route seemed to be more effective compared to the oral route, which might in part be explained by a lack of antigen degradation by acidic and enzymatic processes. In contrast to other studies postulating that Th2 responses cannot be suppressed under GF [8] or CV [21,22] conditions, this is the first report showing that a general suppression of allergen specific immune responses can be achieved independent of the presence or absence of the commensal microflora. Furthermore, our data demonstrate that the degree of allergic sensitization in control animals did not differ between GF and CV animals (Figs. 2 and 3), arguing against previous observations that a lack of bacterial microflora is provoking an enhancement of allergic responses [8]. Along with recent epidemiological studies [13] it might therefore be argued that rather changes in the composition than the overall presence or absence of the microflora may have an impact on the development of allergies.

The contradictory results concerning oral tolerance obtained under GF conditions may largely reflect the complexity of mucosal tolerance, depending on several factors, such as the dose and nature of the antigen, genetic background and timing of antigen application. Wannemuehler et al. [9] were the first to show abrogation of oral tolerance in GF mice using a particulate antigen, i.e. sheep red blood cells. However, since particulate antigens display lower tolerogenic properties than soluble molecules [23], the antigenic characteristics might have played an important role for the outcome in this model. Along these lines, using the soluble antigen ovalbumin Moreau et al. [10,19] demonstrated that oral tolerance was inducible in the absence of a microflora, although immunosuppression was of shorter duration than under CV conditions. From our own previous studies in CV animals, we observed that also soluble antigens differ in their tolerogenic potency, showing that Bet

v 1 is of higher efficacy compared to ovalbumin and other allergens [24,25] leading to long-lasting immunosuppression [20]. Based on the fact that immunosuppression with Bet v 1 was successful under GF and CV conditions, it might well be that the impact of the commensal flora on tolerance is of minor importance when a strong tolerogen is used. Susceptibility to tolerance induction has been also related to the genetic background [23]. In this study, we used BALB/c mice, known to be prone to develop Th2 responses and being susceptible to mucosal tolerance induction [26]. Along these lines, tolerance induction using ovalbumin was successfully demonstrated in GF BALB/C mice [11,12], while a similar tolerizing protocol failed in NMRI mice [7].

Several investigators have studied the inductive and effector sites of mucosal tolerance induction within the gut associated lymphoid tissue. The importance of Peyer's patches (PPs) for oral tolerance induction has been differentially validated showing either a lack of tolerance induction in mice devoid of PPs or successful tolerance induction independent of the presence or functionality of PPs [27]. Furthermore, it was demonstrated that the inability of oral tolerance can be attributed to a smaller size and a reduced percentage of CD3⁺ cells in PPs under GF conditions [18]. In our hands, PPs of GF mice were also of smaller size and reduced number of total lymphocytes (Fig. 4). However, despite the smaller PPs size, the percentage of CD3⁺ cells did not differ compared to CV mice, as also described in GF rats [28]. Moreover, the detection of CD4⁺Foxp3⁺ cells in PPs of GF mice (Table 1) may indicate that these lymphoid organs were of sufficient functionality during the tolerizing process. Alternatively, the mesenteric lymph nodes rather than the PPs might be involved in successful oral tolerance induction [27] via induction of regulatory cells after oral antigen application. These cells have been shown to develop also in the absence of microbial colonization, as CD4⁺CD25⁺Foxp3⁺ cells were detected in mesenteric lymph nodes of GF mice [29].

With respect to intranasal tolerance induction, we have previously demonstrated in CV mice that also mucosal application of Bet v 1 is associated with induction of T regulatory cells and regulatory cytokines [20]. Since intranasal tolerance induction in GF animals was as efficiently induced as in CV mice, it is reasonable to assume that regulatory T cells were also operating under GF conditions. This assumption is further supported by the observation that the lymphoid tissue of the nasopharynx develops independently of microbial contact [30]. Further studies on regulatory T cells in different lymphoid compartments of GF and CV mice are currently undertaken.

Taken together, we demonstrated that the ability to establish nasal or oral tolerance or mount a Th2 response towards the aeroallergen Bet v 1 is not dependent on the presence of the commensal microflora. Our data may support the notion from experimental and epidemiological studies that an aberrant composition of the microflora [13,31] rather than the overall presence of the microflora may play a role in those settings where breakdown of tolerance with consequent development of allergic diseases occurs.

Acknowledgments

Supported by EU project QLK3-CT-2000-000340, Austrian Science Fund FWF 1814-SFB “mucosal tolerance induction”, grants 303/05/2249 and 303/06/0974 of the Czech Science Foundation, IAA500200710 and S5002005720 from the Grant Agency of the Academy of Sciences of the Czech Republic and by Institutional Research Concept AVOZ50200510.

References

- [1] Macdonald TT, Monteleone G. Immunity, inflammation, and allergy in the gut. *Science* 2005;307:1920–5.
- [2] Brandtzaeg P. History of oral tolerance and mucosal immunity. *Ann NY Acad Sci* 1996;778:1–27.
- [3] Holt PG, Batty JE, Turner KJ. Inhibition of specific IgE responses in mice by pre-exposure to inhaled antigen. *Immunology* 1981;42:409–17.
- [4] Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, et al. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J Exp Med* 1996;183:1561–7.
- [5] Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, et al. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunol Lett* 2004;93:97–108.
- [6] Cebra J, Han-Quiang J, Sterzl J, Tlaskalova H. The role of mucosal microbiota in the development and maintenance of the mucosal immune system. In: Ogra P, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee M, editors. *Mucosal Immunology*. San Diego: Academic Press; 1999. p. 267–80.
- [7] Rask C, Evertsson S, Telemo E, Wold AE. A full flora, but not monocolonization by *Escherichia coli* or lactobacilli, supports tolerogenic processing of a fed antigen. *Scand J Immunol* 2005;61:529–35.
- [8] Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997;159:1739–45.
- [9] Wannemuehler MJ, Kiyono H, Babb JL, Michalek SM, McGhee JR. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germ-free mice to sensitivity to oral tolerance induction. *J Immunol* 1982;129:959–65.
- [10] Moreau MC, Corthier G. Effect of the gastrointestinal microflora on induction and maintenance of oral tolerance to ovalbumin in C3H/HeJ mice. *Infect Immun* 1988;56:2766–8.
- [11] Furrie E, Turner M, Strobel S. The absence of gut flora has no effect on the induction of oral tolerance to ovalbumin. *Adv Exp Med Biol* 1995;371B:1239–41.
- [12] Walton KL, Galanko JA, Balfour Sartor R, Fisher NC. T cell-mediated oral tolerance is intact in germ-free mice. *Clin Exp Immunol* 2006;143:503–12.
- [13] Noverr MC, Huffnagle GB. The ‘microflora hypothesis’ of allergic diseases. *Clin Exp Allergy* 2005;35:1511–20.
- [14] Priault G, Fliss I, Pecquet S. Effect of probiotic bacteria on induction and maintenance of oral tolerance to beta-lactoglobulin in gnotobiotic mice. *Clin Diagn Lab Immunol* 2003;10:787–92.
- [15] Wiedermann U, Jahn-Schmid B, Bohle B, Repa A, Renz H, Kraft D, et al. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant bet v 1, the major birch pollen allergen, in a murine model of type I allergy. *J Allergy Clin Immunol* 1999;103:1202–10.
- [16] Winkler B, Baier K, Wagner S, Repa A, Eichler HG, Scheiner O, et al. Mucosal tolerance as therapy of type I allergy: intranasal application of recombinant Bet v 1, the major birch pollen allergen, leads to the suppression of allergic immune responses and airway inflammation in sensitized mice. *Clin Exp Allergy* 2002;32:30–6.
- [17] Repa A, Grangette C, Daniel C, Hochreiter R, Hoffmann-Sommergruber K, Thalhamer J, et al. Mucosal co-application of lactic acid bacteria and allergen induces counter-regulatory immune responses in a murine model of birch pollen allergy. *Vaccine* 2003;22:87–95.
- [18] Maeda Y, Noda S, Tanaka K, Sawamura S, Aiba Y, Ishikawa H, et al. The failure of oral tolerance induction is functionally coupled to the absence of T cells in Peyer’s patches under germ-free conditions. *Immunobiology* 2001;204:442–57.
- [19] Moreau MC, Gaboriau-Routhiau V. The absence of gut flora, the doses of antigen ingested and aging affect the long-term peripheral tolerance induced by ovalbumin feeding in mice. *Res Immunol* 1996;147:49–59.
- [20] Winkler B, Hufnagl K, Spittler A, Ploder M, Kallay E, Vrtala S, et al. The role of Foxp3⁺ T cells in long-term efficacy of prophylactic and therapeutic mucosal tolerance induction in mice. *Allergy* 2006;61:173–80.
- [21] Burstein HJ, Shea CM, Abbas AK. Aqueous antigens induce in vivo tolerance selectively in IL-2- and IFN-gamma-producing (Th1) cells. *J Immunol* 1992;148:3687–91.
- [22] Melamed D, Friedman A. In vivo tolerization of Th1 lymphocytes following a single feeding with ovalbumin: anergy in the absence of suppression. *Eur J Immunol* 1994;24:1974–81.
- [23] Chehade M, Mayer L. Oral tolerance and its relation to food hypersensitivities. *J Allergy Clin Immunol* 2005;115:3–12.
- [24] Hufnagl K, Wagner B, Winkler B, Baier K, Hochreiter R, Thalhamer J, et al. Induction of mucosal tolerance with recombinant Hev b 1 and recombinant Hev b 3 for prevention of latex allergy in BALB/c mice. *Clin Exp Immunol* 2003;133:170–6.
- [25] Wiedermann U, Jahn-Schmid B, Lindblad M, Rask C, Holmgren J, Kraft D, et al. Suppressive versus stimulatory effects of allergen/cholera toxin (CTB) conjugates depending on the nature of the allergen in a murine model of type I allergy. *Int Immunol* 1999;11:1717–24.
- [26] Herz U, Renz H, Wiedermann U. Animal models of type I allergy using recombinant allergens. *Methods* 2004;32:271–80.
- [27] Mowat AM. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 2003;3:331–41.
- [28] Stepankova R, Sinkora J, Hudcovic T, Kozakova H, Tlaskalova-Hogenova H. Differences in development of lymphocyte subpopulations from gut-associated lymphatic tissue (GALT) of germ-free and conventional rats: effect of aging. *Folia Microbiol (Praha)* 1998;43:531–4.
- [29] Ostman S, Rask C, Wold AE, Hultkrantz S, Telemo E. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol* 2006;36:2336–46.
- [30] Kiyono H, Fukuyama S. NALT- versus Peyer’s-patch-mediated mucosal immunity. *Nat Rev Immunol* 2004;4:699–710.
- [31] Björkstén B. Effects of intestinal microflora and the environment on the development of asthma and allergy. *Springer Semin Immunopathol* 2004;25(3/4):257–70.