Old Herborn University Seminar Monograph

22. BIOLOGICAL CONSEQUENCES OF HOST-MICROBE INTERACTIONS

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Old Herborn University Seminar Monograph 22

ISBN 3-923022-33-6
ISSN 1431-6579

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Contents

Participating authors V

I. THE HYGIENE HYPOTHESIS AND MODULATION OF ALLERGY (*Ursula Wiedermann, Karin Hufnagl, Irma Schabussova, and Angelika Wagner*) 1

Summary 1
The hygiene hypothesis 1
New strategies for treatment against allergic diseases on the basis of the hygiene hypothesis 2
Concluding remarks 2
Literature 7

II. THE ROLE OF GUT MICROFLORA IN MUCOSAL TOLERANCE INDUCTION TO BIRCH POLLEN IN MOUSE ALLERGY MODEL (*Hana Kozakova, Andreas Repa, Martin Schwarzer, Helena Tlaskalova-Hogenova, Renata Stepankova, Tomas Hudcovic, and Ursula Wiedermann*) 11

Summary 11
Introduction 11
Material and methods 13
Results and discussion: Allergic sensitization and mucosal tolerance induction to birch pollen in germfree mice 14
Effect of lactobacilli on allergic sensitization 16
Acknowledgements 17
Literature 17

III. BREAST MILK-MEDIATED TRANSFER OF AN ANTIGEN INDUCES TOLERANCE AND PROTECTION FROM ALLERGIC ASTHMA (*Valérie Verhasselt, Valérie Milcent, Julie Cazareth, Akira Kanda, Sébastien Fleury, David Dombrowicz, Nicolas Glaichenhaus, and Valérie Julia*) 21

Summary..... 21
Introduction 21
Aim of the study 25
Methods 25
Results 31
Discussion 36
Acknowledgements 38
Literature 38

Contents (continued)

IV. IMMUNE REGULATION AND THE INFLUENCE OF MICROBES (*Sofia Östman*) 43

Summary 43
Introduction 43
Immunoregulatory mechanisms 44
Conclusion 50
Literature 50

V. IMMUNE MODULATION BY EXOSOMES (*Nina Almqvist, Susanne Hultkrantz, and Esbjörn Telemo*) 57

Summary 57
Exosome biology 57
Exosome function 60
IEC exoxomes and tolerance 61
Concluding remarks 63
Acknowledgements 63
Literature 64

VI. EARLY ANTIGEN EXPERIENCE SHAPES THE MUCOSAL T CELL REPERTOIRE (*Paul W. Bland, Amanda M. Williams, Renata Stepankova, Helena Tlaskalova-Hogenova, and Christopher S.J. Probert*) 67

Summary 67
Introduction 67
Discussion 68
Acknowledgements 72
Literature 73

VII. PROBIOTICS AND BACTERIAL COMPONENTS IN INTESTINAL INFLAMMATION THERAPY AND PREVENTION (*Miloslav Kverka, Klára Klimešová, Zuzana Zákostelská, Lenka Froľová, Tomáš Hudcovic, Tomáš Hrncir, Pavel Rossmann, and Helena Tlaskalová-Hogenová*) 75

Summary 75
Introduction 76
IBD pathogenesis 77
Probiotics and Bacterial components in intestinal inflammation therapy 82
Acknowledgements 84
Literature 85

Contents (continued)

VIII. ANTISECRETORY FACTOR AND ITS BIOLOGICAL ACTIVITIES (*Eva Jennische, Lars Å. Hanson, Hans-Arne Hansson, Ewa Johansson, Ivar Lönnroth, and Stefan Lange*) 93

Summary 93
Introduction 93
Results 94
Clinical Studies 98
Concluding remarks 101
Literature 101

IX. HOW PROBIOTICS *LACTOBACILLUS* GR-1 AND RC-14 IMPROVE UROGENITAL HEALTH IN WOMEN (*Gregor Reid and Andrew Bruce*) 105

Summary 105
Introduction 105
The origins of lactobacilli probiotics 106
Lactobacillus rhamnosus GR-1 and *Lactobacillus reuteri* RC-14 and mode of action 107
Next focus for clinical use 110
Conclusion 111
Acknowledgements 111
Literature 111

X. CHILDHOOD VACCINES AND INDUCTION OF ALLERGIC AND AUTOIMMUNE DISORDERS: FACTS AND FICTION (*Pearey L. Ogra*) 117

Summary 117
Introduction 117
Development of allergy or autoimmunity 119
Natural infection-induced immune response 121
Vaccine induced immune responses 124
Discussion and conclusions 127
Literature 129

XI. BIOLOGICAL CONSEQUENCES OF HOST-MICROBE INTERACTIONS: SUMMARY OF THE SEMINAR (*Lars Å. Hanson, and Helena Tlaskalová-Hogenová*) 135

Introduction: Microbiota and probiotics 135
Breastfeeding 136
Vaginal microbiota of the female genital tract 138
Microbiota in allergy and autoimmunity 139
Vaccination program and safety regulations 141

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THE HYGIENE HYPOTHESIS AND MODULATION OF ALLERGY

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SUMMARY

It has been suggested that a reduced or changed pattern of exposure to certain microorganisms has led to an unbalanced regulation of our immune system with consequently increased development of inflammatory diseases, such as allergic or autoimmune disorders. Studies on the basis of this so called "hygiene hypothesis" have concentrated on identifying microorganisms that may have the potential to re-establish a regulatory network important to prevent or counteract immune overreactions to innocuous antigens leading to immunopathology.

Using mouse models of type I allergy/asthma we tested several approaches to prevent or treat allergic immune responses, either by the use of mucosal adjuvants, the application of certain lactic acid bacteria as antigen delivery systems or by parasite inoculation. The goal of our research is to exploit the underlying mechanisms of immune modulation and to identify some of the microbial components with immunomodulatory properties, which may serve as novel treatment tools against inflammatory disorders, including allergic diseases.

THE HYGIENE HYPOTHESIS

Within the last decades a constant increase in allergic diseases has been recognized within the industrialized countries, leading to the fact that nowadays more than 25% of the population is affected by allergic diseases. This so called new epidemic of the westernized countries may be a result of a concerted action of genetic predisposition to immunological overreactions to innocuous antigens, increased pollution and enhanced exposure to allergenic molecules, as well as changes in nutrition. Furthermore, the hygiene hypothesis postulates that these epidemiological changes might be evoked by a reduced contact to certain microorganisms particularly early in life. The original description of the hygiene hypothesis by *Strachan* and colleagues (*Strachan*, 1989; *Strachan* et al., 1996), and *Matrichardi* et al. (1998) was based on the observation that in families with high numbers of siblings the risk to develop hay fever was less common. Similar observations were made in families living in farming environment (*Riedler* et al., 2001) or in subjects previously exposed to certain oro-faecal infections (*Matricardi* et al., 2000), or after vaccination with Bacillus Calmette-Guerin containing attenuated mycobacteria (*Aaby* et al., 2000). Moreover, changes in the composition

and homeostasis of the commensal gut flora have been linked to increased allergy development, as shown by a reduced number of lactic acid bacteria (LAB) and increased numbers of *Clostridia* and *Staphylococci* in the intestinal flora of atopic children (*Björkstén* et al., 1999; *Watanabe* et al., 2003).

Initially, it was suggested that a reduced production of particularly IL-12 and IFN-γ by Th1 immune cells due to a lack of bacterial infections may drive the excessive expansion of Th2 cells. However, this assumption was not compatible with the observation that industrialized countries have also experienced an increase in Th1 autoimmunity along with an increase in allergic diseases (*Kero* et al., 2001; *Simpson* et al., 2002). Another indication that a simple Th1/Th2 disbalance is insufficient to explain these epidemiological changes came from studies in developing countries, showing that helminth infections, such as with *Schistosoma mansoni*, which classically induces Th2 biased immune responses, are associated with a lower incidence of allergic disorders (*Yazdanbakhsh*, *Kremsner*, and *van Ree*, 2002). Recasting the hygiene hypothesis it was therefore suggested that the activity of suppressive regulatory cells, initiated by a certain pathogen burden, builds up a regulatory network to down regulate both Th1 and Th2 driven immunopathologies. This regulatory network might fail to develop in a high hygienic environment with low pathogen/adjuvant burden (*Maizels* and *Yazdanbakhsh*, 2003; *Maizels*, 2005). However, it does not seem adequate to postulate that active infections are necessary to achieve immunological homeostasis, as it may be sufficient that contact with only microbial products, such as bacterial endotoxins, can drive immunomodulatory responses via stimulation of the innate immune system, such as through Toll like receptors. Thus, exposure rather than live infection, probably at a very early stage in life, may be one of the most determining factors behind the hygiene hypothesis (*Rook*, 2009).

On the basis of these perceptions, new treatment approaches against allergy, and also autoimmunity, aim at using new adjuvant systems derived from specific microorganisms to counteract immunopathology by induction of regulatory/ immunosuppressive responses (Figure 1).

NEW STRATEGIES FOR TREATMENT AGAINST ALLERGIC DISEASES ON THE BASIS OF THE HYGIENE HYPOTHESIS

Animal models of type I allergy

Based on the fact that birch pollen and its major allergen Bet v1, belong to the most common airborne allergen in Europe we established a mouse model of birch pollen allergy. The established sensitization scheme is based on systemic immunization with recombinant Bet v 1 followed by an aerosol challenge with natural birch pollen extract. This sensitization protocol gives rise to allergen specific IgE and IgG1 antibodies in serum as well as production of Th-2 like cytokines (IL-4, IL-5, IL-13) in stimulated splenocytes. Among aerosol challenge, eosinophilic infiltration along with IL-5 and IL-13 leads to airway inflammation and airway hyperresponsiveness. These parameters represent similar features of human allergic asthma (*Wiedermann* et al., 1999a; 2001).

As it is known that the majority of allergic patients are sensitized against several allergens and these patients are particularly difficult to treat, we also

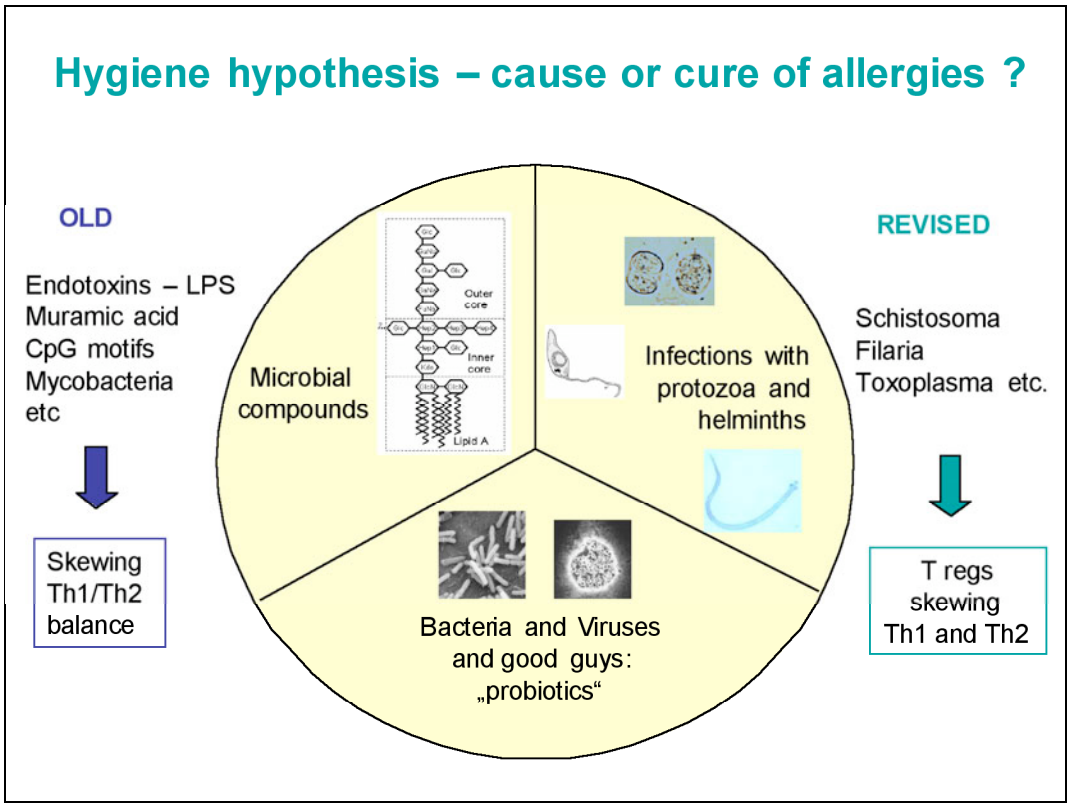

Figure 1: Originally the hygiene hypothesis was explained by induction of Th1 responses by certain bacteria or bacterial compounds to counter balance allergic Th2 responses. Nowadays, the induction of a regulatory network by different infectious agents is suggested to re-balance both Th1 and Th2 responses.

developed a mouse model of polysensitization to the major birch and grass pollen allergens, Bet v 1, Phl p 1 and Phl p 5. Polysensitization with these allergens led to allergic humoral and cellular Th2 immune responses as well as airway inflammation upon allergen-aerosol challenge. The fact that the immunodominant epitopes recognized by T cells from poly-sensitized mice were identical to some of the T cell epitopes in birch and grass pollen allergic patients indicated that our murine model of multiple allergen sensitivity shows similar immunological characteristics to those of human pollinosis (*Hufnagl* et al., 2005; 2008). These mouse models were used to study new treatment approaches against allergy on the basis of mucosal tolerance induction with or without the used of certain adjuvant systems.

Use of microbial compounds: Cholera B subunit as mucosal adjuvant/tolerogen

We have previously demonstrated that intranasal application of recombinant allergens (*Wiedermann* et al., 1999a; *Winkler* et al., 2002; 2006) or new allergen- constructs (*Hufnagl* et al., 2005; *Wild* et al., 2007) prior to sensitization prevents the development of allergic immune responses. However, it

has become obvious that tolerance induction in already sensitized mice, particularly in polysensitized mice, is more difficult to achieve. In order to enhance the tolerogenicity of mucosally applied antigens mucosal antigen delivery systems, such as the B subunit of cholera toxin (CTB), can be used. However, we have shown that the immunosuppressive properties of CTB are influenced by the nature of the coupled antigen as well as by the mode of conjugation: ovalbumin chemically coupled to CTB led to reduction, while Bet v 1 chemically coupled to CTB enhanced allergic immune responses (*Wiedermann* et al., 1999b). In order to improve the immunomodulatory property of CTB we recently genetically engineered a Bet v 1-CTB fusion molecule (*Bublin* et al., 2007). The clear advantage of a recombinant fusion molecule over a chemical conjugate is its homogeneity, since the molecular composition and position of the antigen do not vary and the amount of antigen is increased to five molecules per one molecule CTB pentamer. Intranasal treatment with this fusion molecule prior to sensitization with Bet v 1 led to significant reduction of allergen-specific IgE and in vitro IL-4 and IL-5 production, while humoral and cellular Th1-like responses were markedly enhanced. In the lung compartment a significant rise in IgA was detected after pre-treatment with the fusion molecule or CTB alone. Whether the immunomodulatory effects are only due to counter-regulatory Th-1 like immune responses and protective IgA or due to induction of regulatory cells and their products, as described by others (*Sun* et al., 2006), is currently under investigation. Moreover, the immunomodulatory properties of the Bet v 1-CTB fusion molecule in a therapeutic set up need further exploitation.

Supplementations with probiotics: studies on the right time point of intervention

Lactic acid bacteria (LAB) are non-invasive, non-pathogenic Gram-positive bacteria of which some are within certain foods and important for food processing and preservation, and others are members of the indigenous microflora. Alterations in the composition of the gut flora have been associated with an increase in inflammatory diseases, such as allergies, intestinal bowel diseases or autoimmune diseases (*Björkstén* et al., 2001; *Kalliomaki* et al., 2001; *Damaskos* amd *Kolios*, 2008). Clinical studies indicated that the number of LAB in the gut is different in atopic and non atopic children and that a lack of certain bacterial strains (e.g. lactobacilli, bifidobacteriae) precedes the development of allergic sensitization in infancy (*Björkstén* et al., 1999; *Sjogren* et al., 2009). Therefore, there has been increasing interest in supplementing the human diet with certain probiotics strains for allergy prevention (*Kalliomaki* et al., 2007; *Kukkonen* et al., 2007; *Marschan* et al., 2008). The overall finding of the different clinical trials was that the treatment efficacy, leading mainly to reduction of atopic eczema, depended on the respective strain as well as the time point of intervention.

In our recent studies on primary and secondary allergy prevention with probiotics, two strains, *Bifidobacterium longum* and *Lactobacillus paracasei*, were selected to study their immunomodulatory properties *in vivo* and *in vitro*. The two strains, which were shown to induce high levels of IL-10 in splenocytes upon in vitro stimulation, were intranasally applied at the time of sensitization and allergen challenge of adult mice. The treatment with either of the strains reduced allergen specific

humoral and cellular Th2 responses as well as airway inflammation. Moreover, probiotics treatment induced high levels of IgA antibodies in the respiratory mucosa, which might contribute to the local regulatory environment. Our results indicated that probiotic treatment during allergen exposure could constitute a form of seasonal treatment to ameliorate allergic responses during the allergen exposure (*Schabussova* et al, submitted).

With respect to the treatment efficacy, primary prevention, in particular intervention during pregnancy or early childhood, might however have considerable advantages compared to secondary prevention, as existing immune dysregulations are always more difficult to modulate. We have therefore studied different treatment windows in pregnant mothers during gestation and/or lactation and in the offspring during the neonatal phase (*Schabussova* et al, in manuscript). Preliminary data indicate that oral application of live probiotic bacteria to pregnant mice reduced eosinophilic airway inflammation and suppressed Th2 cytokine production in mesenteric lymph nodes and spleens of offspring upon allergic sensitization and allergen challenge. Interestingly, probiotic treatment only in the postnatal phase prior to sensitization did not - or only modestly - reduce allergic responses in the respective infant mice. These results, indicating that the efficacy of probiotics intervention is higher the earlier the intervention starts, are compatible with clinical data showing consistently that pre-and perinatal intervention, but not treatment in the first years of life, prevented allergic manifestations in infancy (*Rautava*, *Kalliomaki*, and *Isolauri*, 2002; *Kopp* et al., 2008; *Soh* et al., 2008). Further studies will be needed to elucidate the underlying mechanisms of immunosuppression by the selected probiotic strains during the prenatal period. Another important clinical aspect is to evaluate whether intervention with probiotics might have also unwanted suppressive effects on co-applied antigens, including common paediatric vaccines.

Use of probiotics as antigen delivery system

Within recent years lactic acid bacteria have also evoked interest as mucosal adjuvants and vaccine vehicles with immunomodulatory properties (*Schabussova* and *Wiedermann*, 2008). Due to their non-pathogenic status and their capacity to induce dendritic cell derived regulatory properties they are suggested as potent antigen delivery systems for humans (*Wells* and *Mercenier*, 2008). In particular, active delivery of recombinant molecules to mucosal surfaces by genetically modified LAB represents a novel vaccination approach. With respect to allergy treatment we recently evaluated the immunomodulatory properties of two LAB strains, *Lactococcus lactis* and *Lactobacillus plantarum*, showing that both strains are effective in shifting immune responses towards a Th1 profile *in vitro* (*Repa* et al., 2003). To evaluate their potential for modulation of allergic immune responses *in vivo*, recombinant strains producing the Bet v 1 allergen were constructed. Intranasal or intragastric pre-treatment with the Bet v 1-producing LAB led to significantly reduced allergen-specific IgE and increased IgG2a levels, indicating a shift to non-allergic Th1 responses (*Daniel* et al., 2006; 2007). With respect to local immune responses and airway inflammation, pre-treatment with Bet v 1- producing LAB and the control strains led to reduction of eosinophils and IL-5 in lung lavages, suggesting that the LAB strains themselves induce a counter regulatory milieu. In already sensitized mice mucosal application of

these recombinant strains did not sufficiently reduce allergic immune responses, which indicated that lactic acid bacteria inducing immunosuppressive cytokines, such as TGF-β or IL-10, rather than Th1-like cytokines might be more beneficial in therapeutic settings. The fact that the first human trial with a recombinant LAB strain has been completed (*Braat* et al., 2006) indicates that mucosal vaccination with LAB as antigen delivery system could be a successful treatment strategy against allergies in humans.

Infection with *Toxoplasma gondii* and prevention of allergy

According to the hygiene hypothesis, recent epidemiological studies have demonstrated an inverse relationship of certain oro-faecal and food borne infections, such as infection with hepatitis A, *Helicobacter pylori* or *Toxoplasma (T.) gondii*, and the development or manifestation of respiratory allergy (*Matricardi* et al., 2000). To test the immunomodulatory properties of *T. gondii*, a world wide prevalent, intracellular protozoan parasite (*Hill* and *Dubey*, 2002), on allergy development in detail, we recently established a mouse model of toxoplasma infection. *T. gondii* infection in BALB/c mice is somewhat comparable to the infection in humans, exhibiting an acute phase with high serum TNF-α, IL-6 and IFN-γ levels, followed by a chronic phase characterized by the presence of tissue cysts, particularly within the brain. We demonstrated that infection with *T. gondii* before or after allergic sensitisation with the major birch pollen allergen Bet v 1 reduced systemic and respiratory allergy (*Wagner* et al., 2009). According to the phase of infection different pathways of immunomodulation were initiated: during the acute infection upregulation of TLR 2, 4, 9 and 11 in splenocytes was associated with Th1 like immune responses along with transient production of IL-10, similarly as described by others (*Yarovinsky* et al., 2006). In the chronic phase, activation of IL-10 and TGF-β producing Fox p3+ regulatory T cells may have primarily accounted for the suppressive effects against allergic sensitization. Cell transfer experiments of T cells from infected mice supported this notion, as allergic sensitization could be prevented in the recipient mice. Similar findings were also described for other parasites (*Wilson* et al., 2005). Further studies are planned to analyze in depth the immunomodulatory properties of *T. gondii*, as well as of selected parasitic molecules in order to establish new preventive and therapeutic strategies against allergic diseases, including novel adjuvant systems for anti-allergy vaccines.

CONCLUDING REMARKS

Lessons learned from the hygiene hypothesis are that induction of regulatory T cells and the establishment of a regulatory network by certain microbes and/or infectious agents are essential to prevent or ameliorate immunological overreaction to innocuous antigens, including environmental as well as self-antigens. Exploring in detail the immunomodulatory repertoire of selected microorganisms at the molecular as well as immunological level may lead to the development of new treatment tools/adjuvant systems for prevention and therapy of different diseases based on immunological hyper-responsiveness, such as allergies and autoimmune diseases, which have nowadays become a great medical and socioeconomic problem in westernized societies.

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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 monocolonized 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 diseases 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 *Tlaskalova-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 (sensiti-zation 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 μg allergen in 0.2 ml of 3 % $NaHCO_3$ using intragastric tubing (i.g.) (Figure 2) or three times with 10 μg 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 mucosal tolerance to the main component of birch pollen was one of the goals of the European LABDEL project coordi-

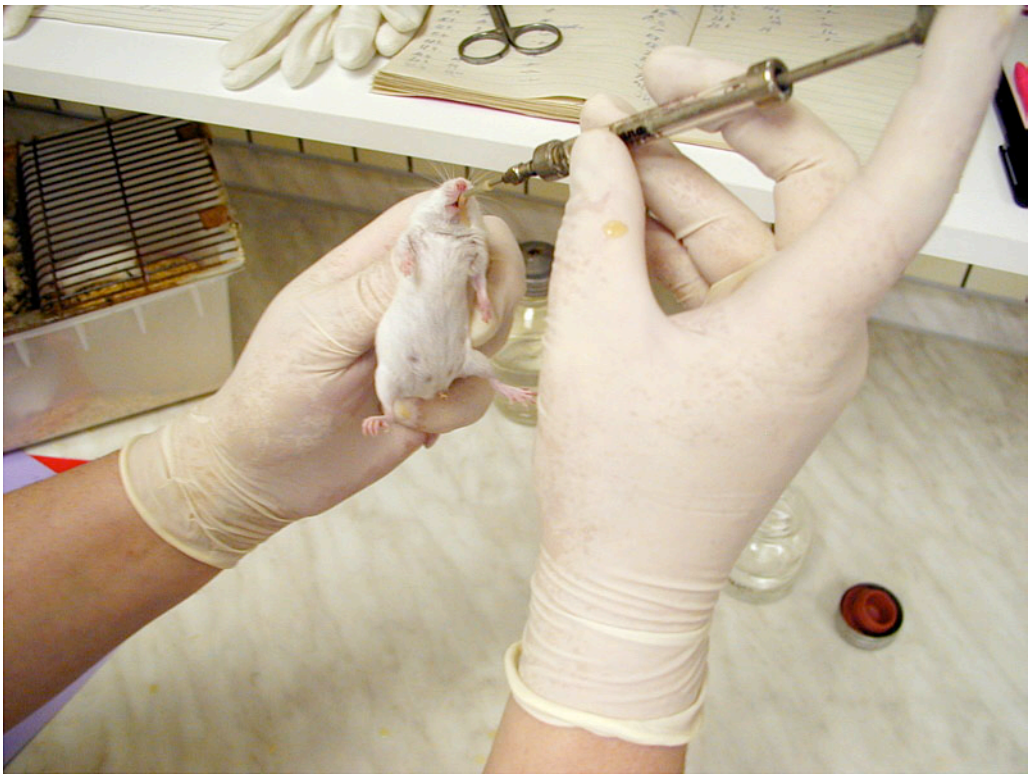

Figure 2: Intragastrical administration of Bet v 1 using soft rubber tubing.

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 intragastrically (Figure 2) or intranasally. Allergen-specific humoral responses of IgE, IgG1, IgG2a antibodies to Bet v 1 are summarized in Table 2. Both intragastrical and intranasal pretreatment with Bet v 1 before allergic sensitization induced significant reduction 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 intragastrically 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 % $NaHCO_3$ 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 (*Lactoccocus 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).

ACKNOWLEDGMENTS

This study was supported by EU project QLK3-CT-2000-000340, grants IAA500200710 and S5002005720 from the Grant Agency of the Academy of Sciences of the Czech Republic, grants 303/06/0974 and 303/08/0367 of the Czech Foundation of the Czech Republic and by Institutional Research Concept AVOZ50200510.

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BREAST MILK-MEDIATED TRANSFER OF AN ANTIGEN INDUCES TOLERANCE AND PROTECTION FROM ALLERGIC ASTHMA

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SUMMARY

Allergic asthma is a chronic disease characterized by airway obstruction in response to allergen exposure. It results from an inappropriate T helper (Th)-2 response to environmental airborne antigens and affects 300 million individuals. Its prevalence has dramatically increased in the recent decades most probably as a result from changes in environmental factors. Exposure to environmental antigens during infancy is critical for asthma development. Epidemiological studies on the relationship between breastfeeding, and allergic diseases have reached conflicting results. Here, we have investigated whether the exposure of lactating mice to an airborne allergen would impact asthma development in progeny. We found that airborne antigens are efficiently transferred from the mother to the neonate through milk and that tolerance induction does not require the transfer of immunoglobulins. Breastfeeding-induced tolerance relies on the presence of Transforming Growth Factor (TGF)-β during lactation, is mediated by regulatory $CD4^+$ T lymphocytes and depends on TGF-β signalling in T cells. In conclusion, breast milk-mediated transfer of an antigen to the neonate results in oral tolerance induction leading to antigen-specific protection from allergic airway disease. This study may pave the way for the design of new strategies to prevent the development of allergic diseases.

INTRODUCTION

Asthma and environmental factors

Asthma is a chronic respiratory disease characterized by reversible airway obstruction in response to specific and non specific stimuli. This lung inflammatory disease results from an inappropriate Th2 response against airborne antigens leading to pulmonary inflammation, airway eosinophilia, mucus hypersecretion and airway remodelling. The prevalence of asthma has increased steadily in the recent decades and is now one of the most common chronic diseases worldwide affecting around 300 million individuals (*Masoli* et al., 2004). The rapid increase in prevalence

of asthma is unlikely to result from genetic changes in populations but rather from changes in environmental factors such as exposure to pathogens, tobacco smoke, allergens, air pollution and changes in diet (*Devereux*, 2006; *Eder* et al., 2006). Furthermore, a large number of epidemiological studies have shown that exposure to environmental factors during infancy is critical for asthma development. Among these factors, exposure to airborne allergens may be of particular importance as allergen encounter is necessary for both sensitization in genetically prone individuals and for symptoms development in sensitized persons (*Holt* et al., 1999). This led to the hypothesis that reducing allergen exposure during infancy would lower the risk of being sensitized (*Arshad*, 2005; *Eder* et al., 2006; *Holt* and *Thomas*, 2005). This premise was tested in several allergen avoidance trials involving young children focusing on environmental control measures targeting a reduction in indoor allergen concentrations. Although allergen avoidance resulted in reduced symptoms in sensitized children, there was no convincing evidence that sensitization itself was reduced (*Arshad*, 2005; *Eder* et al., 2006; *Holt* and *Thomas*, 2005). In striking contrast, sensitization was actually increased in one study (*Woodcock* et al., 2004).

Breastfeeding and prevention of allergic disease

Breastfeeding is recognized as the main source of active and passive immunity in early life and is one of the most effective way of reducing death rate of children under five (*Brandtzaeg*, 2003; *Labbok* et al., 2004). Although the protective effect of breastfeeding on mortality is mainly observed in developing countries in which individuals are more exposed to infectious diseases, it is also beneficial for global health in developed countries. Many epidemiological studies have shown a protective effect of breastfeeding on asthma whether mothers were allergic or not (*Friedman* and *Zeiger*, 2005; *Gdalevich* et al., 2001; *Kull* et al., 2004; *van Odijk* et al., 2003). Although breast milk factors that are responsible for this protective effect have not yet been identified, it is noteworthy that breast milk contains high levels of immunosuppressive cytokines such as IL-10 and TGF-β (*Garofalo* et al., 1995; *Kalliomaki* et al., 1999; *Letterio* et al., 1994; *Oddy* et al., 2003; *Penttila* et al., 1998; *Saito* et al., 1993). Furthermore, epidemiological studies have shown a correlation between levels of TGF-β in breast milk and protection against wheeze and atopic dermatitis in breastfed children (*Kalliomaki* et al., 1999; *Oddy* et al., 2003). The presence of TGF-β in breast milk was also shown to prevent intestinal mucosa inflammation (*Penttila* et al., 2003) and to prevent allergy in allergic prone rat (*Penttila*, 2006).

Oral tolerance

In most cases, administration of antigen through oral route induces hypo-responsiveness to a subsequent challenge with the same antigen administered locally or systemically in an immunogenic form (*Mowat*, 2003; *Strobel* and *Mowat*, 2006). In animals, oral administration of autoantigen prevented and/or ameliorated various autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and type 1 diabetes (*Mayer* and *Shao*, 2004). Oral tolerance was also protective in several animal models of allergy (*Keller* et al., 2006; *Mucida* et al., 2005; *van Halteren* et al., 1997) and in humans in the case of food allergy (*Patriarca* et al., 2003). Although the mechanisms underlying oral tolerance have not been entirely elucidated (re-

viewed in *Macpherson* and *Smith*, 2006; *Mayer* and *Shao*, 2004; *Mowat*, 2003), it is now clear that $CD4^+$ T lymphocytes play a critical role in this process and that several subsets of $CD4^+$ T cells, including TGF-β-producing Th3 cells, IL-10-producing Tr1 cells and naturally occurring $CD4^+$ $CD25^+$ T regulatory cells, could be involved. The mechanisms underlying oral tolerance have been extensively studied in animals, and more recently in children with cow's milk allergy (*Karlsson* et al., 2004). Several studies have shown that mesenteric lymph node (LN) dendritic cells (DC's) play a role in oral tolerance by promoting the differentiation of antigen-specific $CD4^+$ T regulatory cells (*Macpherson* and *Smith*, 2006; *Mowat*, 2003; *Strobel* and *Mowat*, 2006; *Worbs* et al., 2006). These DC's, that are likely to belong to the myeloid subset, probably originate from the lamina propria where they have acquired orally-administered proteins from intestinal lumen (*Strobel* and *Mowat*, 2006). Plasmacytoid DC's may also be involved in oral tolerance induction as suggested by accumulating evidence showing a cross talk between myeloid and plasmacytoid DC's in particular in intestinal mucosa (*Kuwajima* et al., 2006; *Lou* et al., 2007; *Yrlid* et al., 2006). Epithelial cells are also likely to play an important role in oral tolerance by interacting with neighbouring DC's (*Rimoldi* et al., 2005).

Immune response and oral tolerance in neonate

Early studies pioneered by Sir Peter Medawar have suggested that neonates are immunologically immature and prone to tolerance induction. Thus, neonates injected at birth with allogeneic splenocytes become tolerant and accept transplant from an allogeneic donor when they are adult. In contrast with these studies, several authors have recently shown that neonatal exposure to antigen can prime T cell responses and that inducing oral tolerance is more difficult in neonates than in adults (*Adkins* et al., 2004; *Hanson*, 1981; *Miller* et al., 1994; *Singh* et al., 1996; *Strobel*, 2001). For example, oral administration of Myelin Basic Protein (MBP) to rat neonates increased susceptibility to Experimental Autoimmune Encephalomyelitis (EAE) (*Miller* et al., 1994). Likewise, oral administration of OVA to mouse neonates increased delayed type hypersensitivity (DTH) and antibody responses in mice (*Hanson*, 1981; *Strobel*, 2001).

Breast milk as a link between mother environment and breastfed child

Exposure of lactating mothers to pathogens impacts the immune status of the breastfed child. Breast milk contains antibodies that react to infectious agents that were present in the mother's environment. This is an ingenious mean to confer passive immunity to the breastfed child against infectious agents that he is likely to encounter shortly after birth (*Brandtzaeg*, 2003). In other studies, breast milk was shown to contain food protein such as OVA (egg), bovine β-lactoglobulin (cow's milk), gliadin (wheat) and Ara h1 and Ara h2 (peanut) (*Palmer* and *Makrides*, 2006). Therefore, it has been proposed that mothers' diet influences the immune response of the breastfed child towards dietary antigens. To test this hypothesis, lactating mothers were submitted or not to an eviction diet and the breastfed children were followed for development of allergic diseases (reviewed in *Palmer* and *Makrides*, 2006; *Sicherer*, 2002; *Zeiger*, 2003)). Unfortunately, consistency among these studies has been lacking (*Chandra* et al., 1989; *Hattevig* et al., 1989, 1999; *Herrmann* et al., 1996;

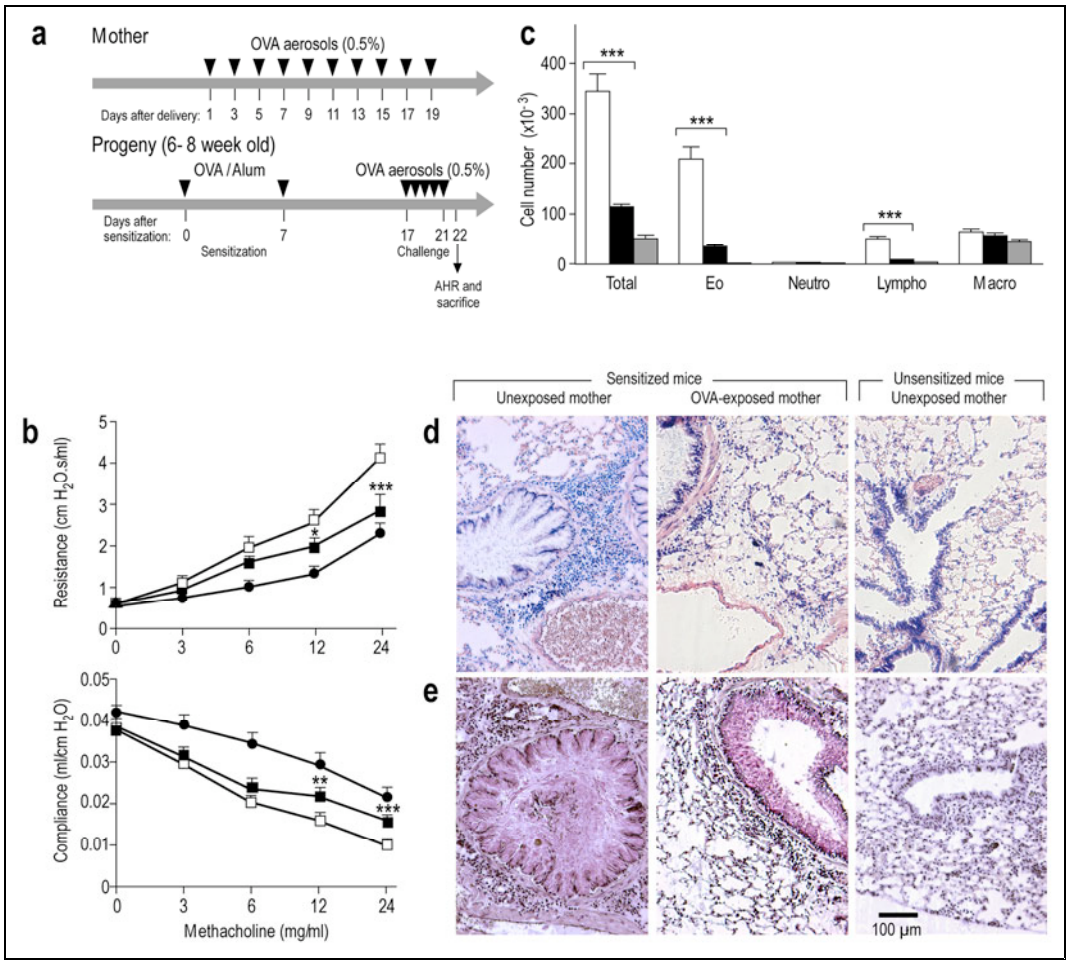

Figure 1: AHR and airway inflammation in mice breastfed on OVA-exposed mothers. (**a**) Experimental protocol. Lactating mothers were exposed or not to 0.5 % OVA aerosols during 20 min every other day from delivery until weaning. During aerosol exposure, pups were kept away from their mother. When 6-8 wk-old, offspring was sensitized with two injections of OVA in Alum, and challenged daily for 5 days with OVA aerosols. Mice were analyzed one day after the last aerosol. (**b**) AHR. Dynamic lung resistance and compliance were monitored in mice breastfed on OVA-exposed (filled squares) or unexposed mothers (empty squares) upon sensitization and challenge with OVA (n=6-7 mice per group in each experiment). Non-sensitized mice challenged with OVA (circles) were used as controls (n=3 in each experiment). Data are expressed as mean ± SEM of 2 independent experiments. **P*=0.03; ***P*=0.006 ****P*=0.0004. (**c**) Number and phenotype of BAL cells. BAL cells were analyzed by FACS in OVA-sensitized and challenged mice breastfed on OVA-exposed (black bars) and unexposed (empty bars) mothers. Non-sensitized mice challenged with OVA were used as controls (grey bars). Eosinophils, Eo; neutrophils, neutro; lymphocytes, lympho; macrophages, macro. Data are expressed as mean ± SEM of 5 experiments with n=6-8 mice per group for sensitized mice and of 2 experiments with n=5-6 mice for the non-sensitized group. ****P*<0.0001. (**d**, **e**) Histology of lung sections. Staining with May-Grünwald Giemsa (**d**) and PAS (**e**). Data show representative microscopic images at a 10-fold magnification.

Palmer and *Makrides*, 2006; *Sicherer*, 2002; *Zeiger*, 2003). Studies in rats and mice showed that oral administration of an antigen to the mother during lactation rendered the progeny tolerant towards that antigen as demonstrated by diminished immunoglobulins response and DTH (*Korotkova* et al., 2004; *Strobel*, 2001). Similar results were obtained in two studies in which an antigen was administered to the lactating mother through the parenteral route (*Fazekas de St Groth* et al., 1984; *Komatsu* et al., 1988).

AIM OF THE STUDY

The aim of this work was to understand how breastfeeding can confer protection towards asthma development. We formulated the hypothesis that an airborne antigen could be found in breast milk as described for dietary antigen and that the transfer of an airborne antigen to the neonate through breast milk could favour tolerance induction according to the numerous immunomodulatory factors presents in maternal milk.

METHODS

Mice

BALB/c mice and C57BL/6 mice were purchased from The Centre d'Elevage Janvier (France) and housed under SPF conditions. D0.11.10 TCR transgenic mice were provided from Dr. Fiona Powrie (University of Oxford). TGF-β DNRII (*Lucas* et al., 2000) were obtained from Dr. Lucas (NIH, USA), μMT and RAG-2-KO mice were obtained from the CDTA (France) and IL-10-deficient mice from Charles River (France). All non-transgenic mice used were on the BALB/c background unless indicated. TGF-β DNRII, μMT and IL-10-deficient mice were on the C57BL/6 background. In experiments using μMT and IL-10 deficient mice as foster mothers, adopted pups were wt BALB/c mice. In experiments in which TGF-βDNRII pups were adopted, mothers were wt C57BL/6.

Exposure of lactating mothers to antigen

Lactating mice were exposed or not to 0.5% OVA (Grade V, Sigma) aerosols for 20 min every other day starting 24 h after delivery until weaning using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13000 cm^3 box that served as the deposition chamber for the mice (Figure 1a). Aerosols were given in groups of a maximum of 5-10 mothers. During aerosol exposure, mothers were separated from their progeny. Alternatively, lactating mothers received either intra-nasal or intra-gastric administrations of 0.1 mg and 0.5 mg of OVA, respectively, from delivery until weaning. OVA endotoxin content was determined using the QCL1000 chromogenic LAL kit assay (Cambrex). LPS content of OVA was below 10 ng/mg of protein. When indicated, mothers were treated with 1 mg of anti-TGF-β mAb (1D11 clone, ATCC) or isotype (GL113, rat IgG1, DNAX) twice a week from delivery until weaning. Upon anti-TGF-β treatment, TGF-β2 content in milk was partially reduced (20.7 ± 4.0 to 12.8 ± 4.9 ng/ml in isotype-treated and anti-TGF-β-treated mothers respectively; mean of 3

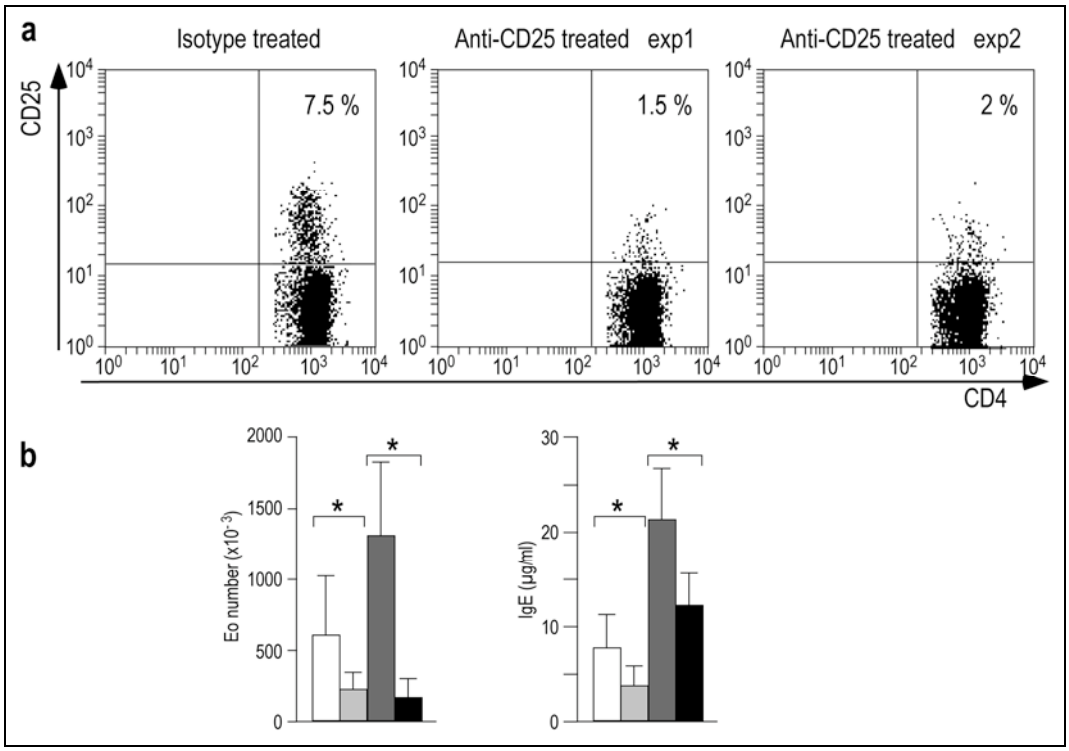

Figure 2: Role of $CD25^+$ cells in breastfeeding induced tolerance.
a: $CD25^+$ expression by $CD4^+$ T cells after anti-CD25 mAb administration. Spleens were collected one week after anti-CD25 mAb (PC61), or isotype (G113) administration. Cells were stained with anti-CD4, and anti-CD25 (7D4) mAbs and analyzed by FACS.
b: Breastfeeding-induced tolerance in anti-CD25 mAb~treated mice. 6-8 wk-old mice breastfed by OVA-exposed or unexposed mothers were treated with 0.5 mg of anti-CD25 mAb or control IgG 1 rat mAb. Mice were sensitized one week later and challenged with OVA. BAL eosinophil number and OVA-specific serum IgE levels are shown for isotype-treated mice breastfed by unexposed mothers (empty bars), or OVA-exposed mothers (light grey bars), anti-CD25-treated mice breastfed by unexposed mothers (dark grey bars) or OVA-exposed mothers (black bars). Data are expressed as mean + SD of values obtained in individual mouse for Eo and IgE and in pooled lung cells tor IL-13 secretion. n=5-7 mice per group, one representative experiment of two. *P=0.01.

independent experiments ± SD), and TGF-β-1 content in milk was reduced at least 6-fold (1.4±0.3 to 0.2±0.1 ng/ml; mean of 4 experiments ±SD). The administration of anti-TGF-β mAbs to lactating mothers also resulted in a partial depletion of TGF-β1 in newborn serum (56±13 to 26±12 ng/ml; mean of 3 experiments ± SD, n=14). TGF-β2 remained below the level of detection.

Induction of allergic asthma

Six to eight week old mice were used. Sensitization was performed by 2 i.p. injections of 10 µg of OVA in 2 mg of aluminium hydroxide (Alum) (Pierce) at day 0 and 7. From day 17 to day 21, mice were exposed to OVA (0.5%) aerosols for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia) connected to a 13000cm^3 box that served as the deposition chamber for the mice (Figure 1a). When indicated, mice were injected with 0.5 mg of anti-CD25 mAb (PC61 clone, ATCC) or with rat IgG1 (GL113 clone, DNAX) one week before sensitization

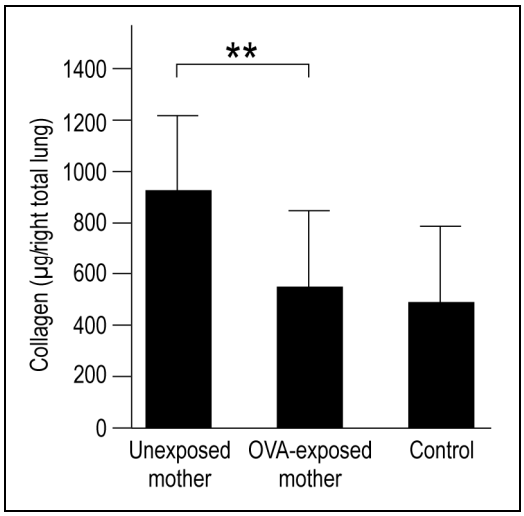

Figure 3: Collagen deposition in airways. Mice breastfed by OVA-exposed or unexposed mothers were sensitized with OVA in Alum and challenged with OVA aerosols (n=5-7 mice per group). Non-sensitized mice challenged with OVA aerosols were used as controls (n=3). One day after the last aerosol, lungs were collected and collagen content was assessed in right lungs. Data show means ± SD of two independent experiments. ***P*=0.005.

Treatment with anti-CD25 mAb resulted in a dramatic reduction in the frequency of $CD25^+$ $CD4^+$ T cells (1-2% versus 7.5%) (Figure 2a). In other experiments, mice were injected with 1 mg of anti-TGF-β mAb (1D11 clone, ATCC) or with rat IgG1 (GL113 clone, DNAX) one day before each sensitization. In some experiments, mice were sensitized with 10 µg LACK in 2 mg of Alum and further exposed to aerosols of 0.2% LACK as described (*Julia* et al., 2002). LACK was detoxified using an Endotrap column (Profos) according to the manufacturer's instructions to reach LPS amounts below 10 ng/mg of protein.

Airway hyperresponsiveness (AHR)

One day after the last aerosol, AHR was measured by invasive plethysmography (Emka Technologies) in response to inhaled methacholine (Sigma). For dynamic lung resistance and compliance, measurements were performed using a Flexivent apparatus (SCIREQ). Mice were anesthetized (5 ml/kg Dormitor 10 % (Medetomidine, Pfizer) - Imalgene 10% (Ketamine, Merial) tracheotomized, paralyzed (5 ml/kg Pavulon 1% (Pancuronium bromide, Organon) and immediately intubated with an 18-G catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of methacholine (0-24 mg/ml) were administered at the rate of 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 10 ms, via a nebulizer aerosol system with a 2.5-4 µm aerosol particle size generated by a nebulizer head (Aeroneb, Aerogen). Baseline resistance was restored before administering the subsequent doses of methacholine.

Analysis of BAL cells

Mice were bled and a canula was inserted into the trachea. Lungs were

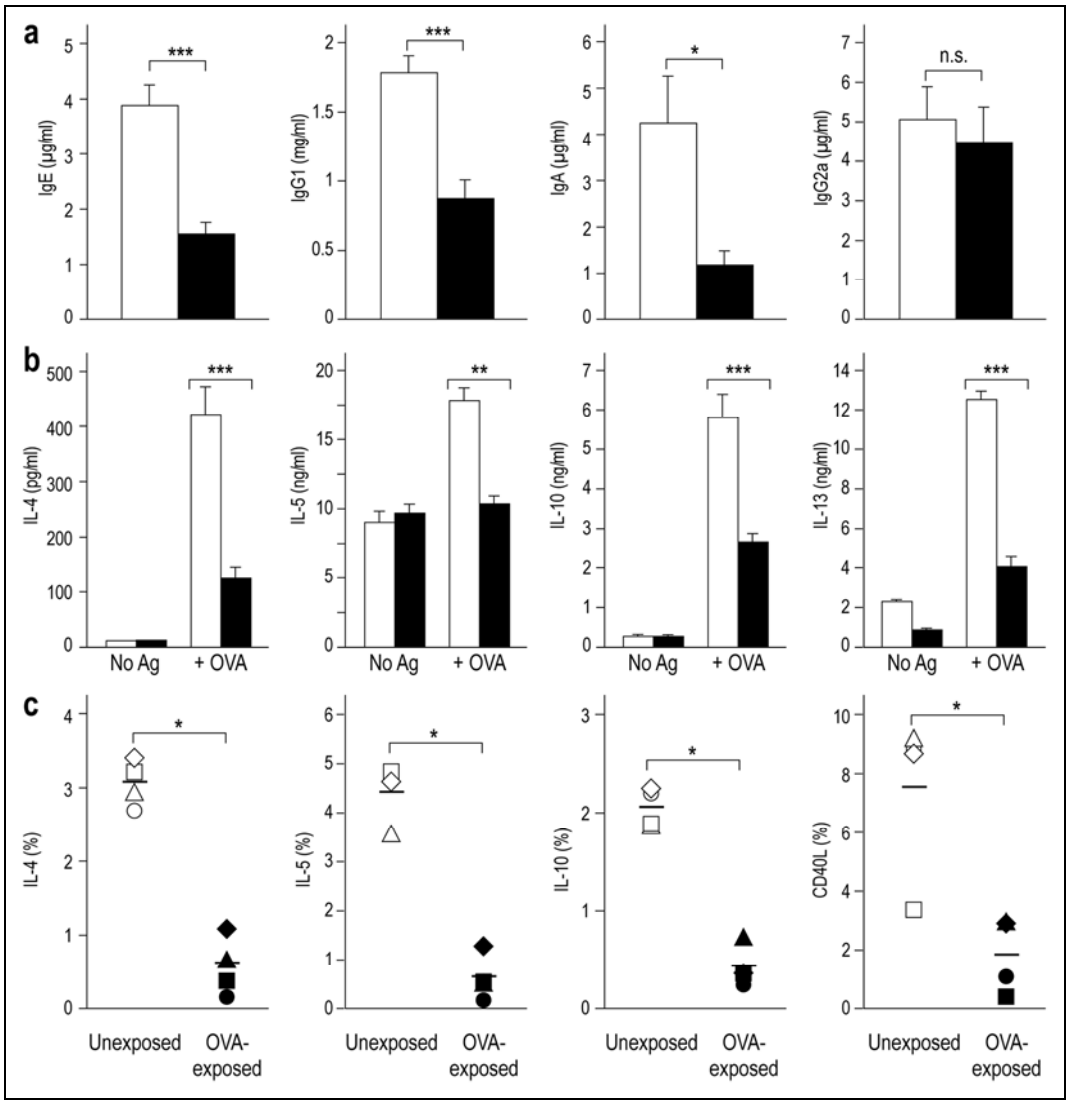

Figure 4: Immunoglobulin and T cell responses in mice breastfed on OVA-exposed mothers.
a: Serum levels of OVA-specific Ig. Sera from mice breastfed on OVA-exposed (filled bars) and unexposed (empty bars) mothers were analyzed for OVA-specific IgE, IgG1, IgA and IgG2a contents by ELISA. Histograms show the mean ± SEM of five independent experiments for IgE and IgG1 levels, and of two experiments for IgG2a and IgA levels with 6-8 mice per group. **P* = 0.01; ****P* <0.0001; ns *P*=0.4.
b: Cytokine secretion by lung cells. Lung cells of mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mothers were pooled in each group (n=6-8) and cultured in triplicate with or without 100 µg/ml of OVA. Supernatants were analyzed 72 h later for IL-4, IL-5, IL-13 and IL-10 contents by ELISA. Data are expressed as mean ± SEM of 5 independent experiments. ***P*=0.004; ****P*=0.0005.
c: Frequency of cytokine-secreting and OVA-specific lung $CD4^+$ T cells. Lung cells of mice breastfed on OVA-exposed (filled symbols) or unexposed mothers (empty symbols) were pooled in each group (n=5-7) and incubated with OVA and anti-CD28 mAb. Data show the frequency of IL-4-, IL-5- and IL-10-secreting cells and $CD40L^+$ cells after gating on $CD4^+$ T cells in four independent experiments.**P*=0.02

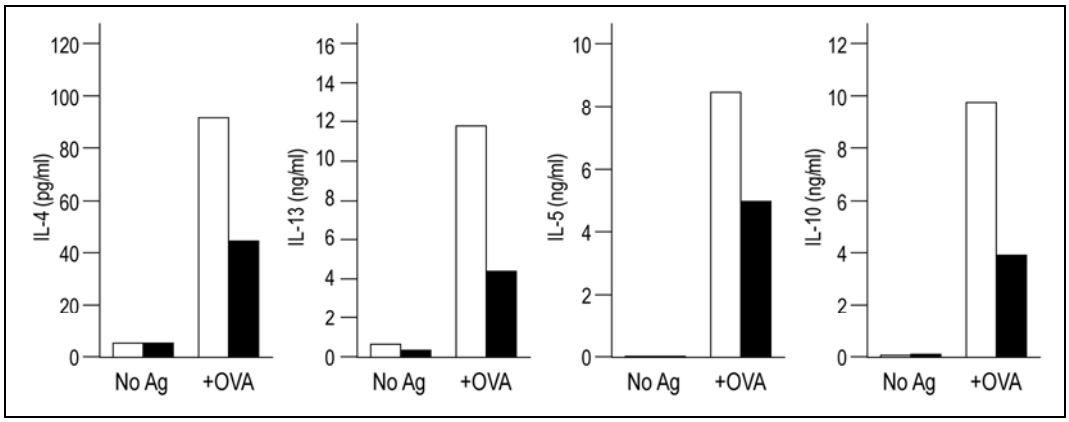

Figure 5: Cytokine secretion by mediastinal LN cells. Mice breastfed by OVA-exposed (filled bars) or unexposed (empty bars) mothers were sensitized and challenged with OVA. Pooled mediastinal LN cells were incubated in triplicate with or without OVA. Data show means of concentration of the indicated cytokine in supernatants of two independent experiments in which LN cells of each groups were pooled. n=5-7 mice per group.

washed 3 times with 1 ml of PBS. For differential BAL cell counts, cells were stained with mAb anti-CCR3 (R&D), anti-Gr1, anti-CD3 and anti-CD19 mAbs (Becton Dickinson**,** BD) and analyzed by FACS using a FACScalibur flow cytometer and Cellquest software. Eosinophils were defined as $CCR3^{+}$ $CD3^{-}CD19^{-}$, neutrophils as $Gr\text{-}1^{high}$ $CD3^{-}CD19^{-}$, lymphocytes as $CD3^{+}CD19^{+}$ and alveolar macrophages as large autofluorescent cells.

Serum antibody measurements

Serum OVA-specific or LACK-specific IgG1, IgG2a, IgA and IgE were measured by ELISA. For IgG1 quantification, antigen-coated Maxisorp plates (Nunc) were incubated with serial dilution of sera and biotinylated anti-IgG1 mAb (BD). For antigen-specific IgE, IgG2a, IgA, plates were first coated with the respective capture mAb (BD), and incubated with serum dilutions. Biotinylated-OVA or LACK antigen was then added. HRP-conjugated streptavidin (BD) and TMB (KPL) were used for detection.

Cytokine assays

One day after the last aerosol, lungs and mediastinal LN were removed separately, minced, and digested with collagenase I (Gibco) and DNAse (Roche) for 30 minutes at 37°C. Cell suspensions were filtered through a 70 µm cell strainer and depleted of red blood cells using red blood cell lysis buffer. Cells from each group were pooled and 4 X 10^6 lung and 1.5 X 10^6 LN cells were cultured in triplicate for 72 h in medium containing OVA (100µg/ml) or not in 48 well-plates, or 96 well-plates, respectively. Culture medium was RPMI 1640 (Gibco) containing 5% heat-inactivated FCS (Perbio), 50 µM β2-mercaptoethanol (Gibco), and penicillin/streptomycin (Gibco). Supernatants were analyzed by ELISA for IL-4, IL-5, and IL-10 using antibody pairs from BD and for IL-13 contents using kit from R&D Systems. Detection levels were 15 pg/ml (IL-4), 300 pg/ml (IL-5) and 150 pg/ml (IL-10 and IL-13). For intracellular staining, cells were incubated with 100 µg/ml OVA and 1 µg/ml of anti-CD28 (BD) for 6 h. Brefeldin A

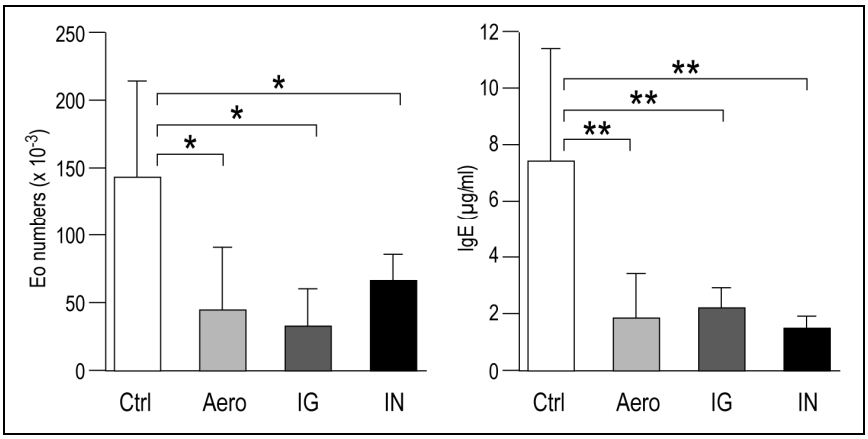

Figure 6: Eosinophil numbers in HAL and OVA-specific serum IgE in mice breastfed on mothers exposed to OVA via aerosol, intragastric or intranasal route. Newborns were breastfed by mothers that were left unexposed (empty bar), exposed to OVA by aerosols (light grey bar), intragastrie (dark grey bar) or intra-nasal administration (black bar). Once adult, mice were sensitized and challenged with OVA Data show means ± SD of values obtained in individual mice, one representative experiment of two. n=5-6 mice. **P* =0.03 and ***P* =0.004.

(5 µg/ml, Sigma) was added during the last 4 h. Cells were then stained with anti-CD4 mAb, fixed, permeabilized using cytofix/cytoperm reagent (BD), stained with anti-CD40L (BD) and anti-IL-4, IL-5 or IL-10 mAbs (BD) and analyzed by FACS. TGF-β1 and TGF-β2 amounts were determined in milk and sera using kits from R&D and Promega, respectively.

Histology

Left lungs were harvested and fixed with Immunohistofix and embedded in Immunohistowax (Infertiles). 4-µm sections were performed and stained with May Grünwald Giemsa or Periodic Acid of Schiff (Sigma).

Lung collagen content

Collagen lung content was determined by quantifying soluble collagen with the Sircoll Collagen assay kit (Biocolor), according to the manufacturer's instructions.

OVA content in milk

Four to six hours after OVA aerosol exposure of lactating mothers, breast milk was collected after ocytocin (Sigma) injection or from the stomach of 2 wk-old pups. Samples were spun down at 3500g for 10 min. Proteins from the aqueous phase were analyzed onto a 10% acrylamide SDS-PAGE followed by standard immunoblotting techniques. OVA was detected using a mouse mAb anti-OVA (Abcam), followed by a goat anti-mouse HRP-Ab (Jackson). Blots were developed using the super signal West femto kit (Pierce) and chemiluminescence was recorded using a luminescence image analyser LAS-3000 (Raytest, France). Quantification of captured images was performed using the Aida Image Analyzer software (Raytest).

Antigen-driven T cell activation in breastfed newborns

$CD4^+$ T cells were purified from DO11.10 TCR transgenic mice and 3 X 10^6 cells were injected into 2 wk-old BALB/c pups that were breastfed on OVA-exposed or unexposed BALB/c mothers. Pups were sacrificed 72 h later. Axillary and inguinal LN cells were analyzed by FACS after staining

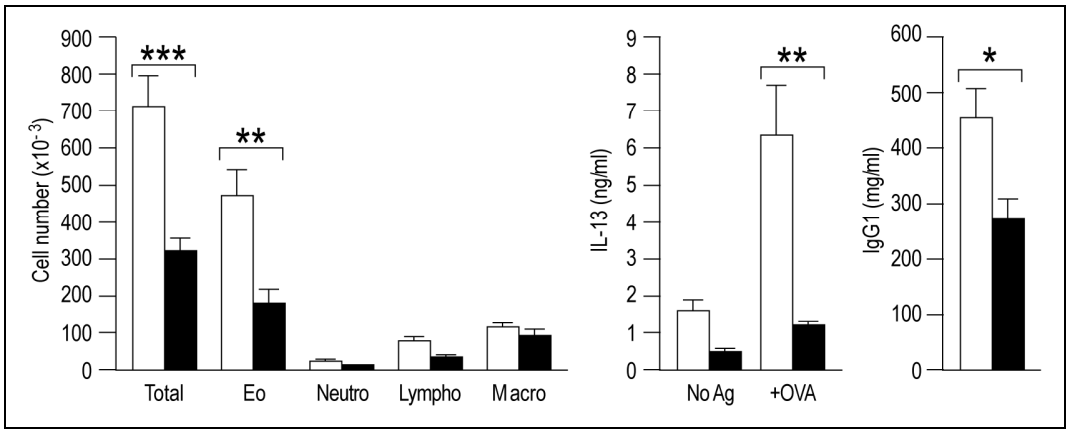

Figure 7: BAL cell numbers, IL-13 secretion and OVA-specific serum IgGI in C57BL/6 mice breastfed by OVA-exposed C57BL/6 mothers. C57BL/6 lactating mice were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adult, offspring was sensitized and challenged with OVA. Data are expressed as means ± SEM of value obtained in individual mice for Eo and IgG1 and in pooled lung cells for IL-13 secretion in three independent experiments with n=5-7 mice per group. OVA-specific IgE was not detectable in sera of C57BL/6 mice. **P*=0.01; ***P*= 0.002; ****P*= 0.0004.

with anti-CD4, anti-CD69, anti-CD44 and KJ1-26 mAbs (BD).

CD4 T cell transfer

Spleens from 6-8 wk old BALB/c mice that had been breastfed on OVA-exposed or unexposed mothers were collected. $CD4^+$ T cells were enriched by negative depletion using CD4 isolation kit (Dynal) and further sorted using a high-speed sorter VANTAGE $SETLO^+$ flow cytometer (BD) after staining with anti-CD4 mAb. Cells were pure to more than 98% as demonstrated by staining with anti-CD4 mAbs. $5x10^6$ purified $CD4^+$ T cells were injected i.v. into 6-8 wk old BALB/c naive recipients. Mice were sensitized and further challenged with OVA 24 h later.

Statistical analysis

In all experiments, statistical significance was assessed using a two-tail p-value calculated with Mann-Whitney non-parametric test. P-values were calculated by comparing mice breastfed on OVA-exposed mother to those breastfed on unexposed mothers.

RESULTS

We have assessed the impact of airborne antigen exposure of lactating mice on the development of allergic asthma in their progeny (Figure 1a). When adults, the offspring was sensitized, challenged with OVA and analyzed for allergic airway disease. As compared to mice breastfed on unexposed mothers, mice breastfed on OVA-exposed mothers exhibited decreased airway hyperreactivity (Figure 1b), reduced numbers of eosinophils in bronchoalveolar lavage (BAL) (Figure 1c), milder peribronchial and perivascular cellular infiltration and decreased mucus deposition in the airways (Figure 1d,e), lower collagen contents in lungs (Figure 3) and reduced levels of

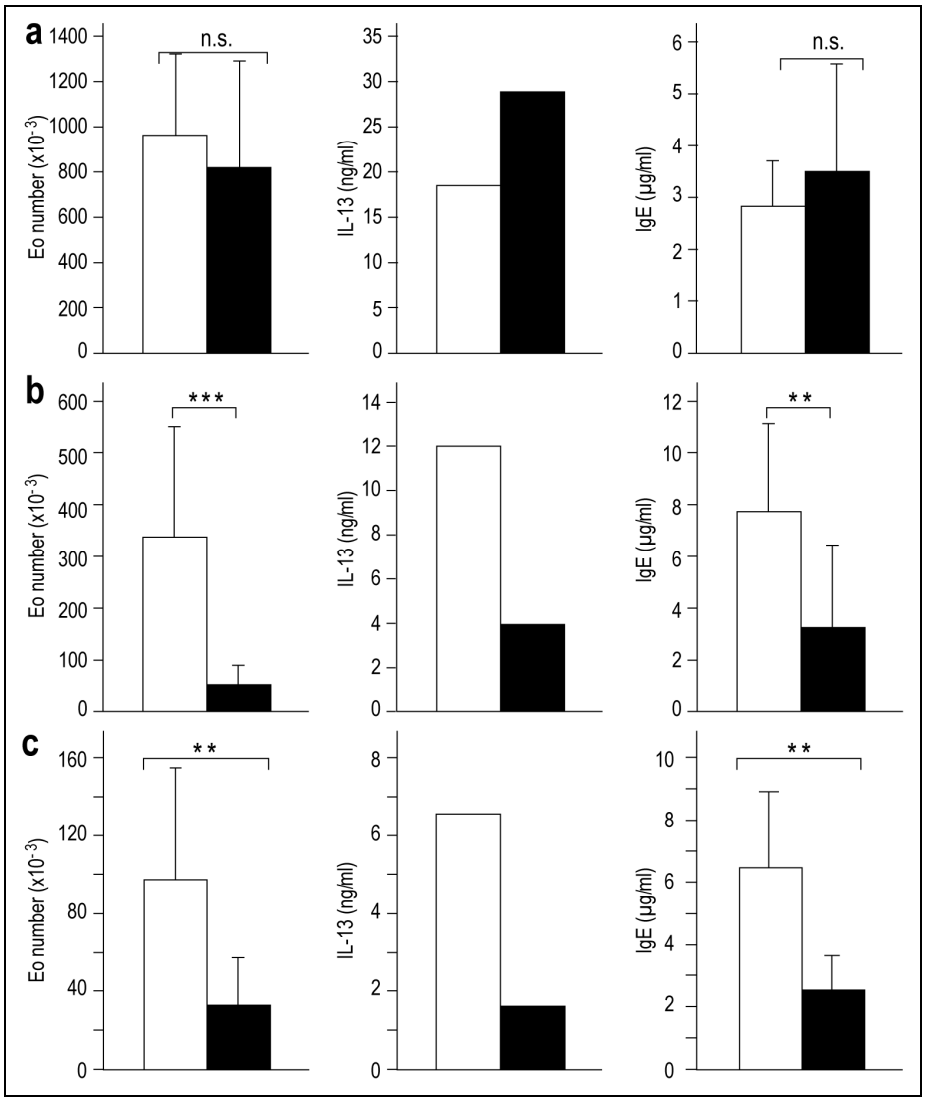

Figure 8: Breast milk factors involved in breastfeeding-induced tolerance.
a: Antigen-specificity of breastfeeding-induced protection. Mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mothers were sensitized and challenged with LACK. BAL cells were counted and analyzed by FACS. Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=7 mice per group in one representative experiment of two. ns: $P > 0.5$.
b: Eosinophil numbers in BAL, lung IL-13 secretion and OVA-specific serum IgE levels in mice breastfed on µMT foster-mothers. One day-old BALB/c newborns were breastfed on µMT foster-mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adults, mice were sensitized and challenged with OVA. Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=7-8 mice per group, one representative experiment of two. $^{**}P=0.009$; $^{***}P=0.0006$.
c: Eosinophil number in BAL, lung IL-13 secretion and OVA-specific serum IgE in mice fostered by RAG-2-KO mothers. BALB/c newborns were breastfed on RAG-2-KO foster mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Once adult, mice were sensitized and challenged with OVA Data show means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion in one representative experiment of two. n=5-7 mice. $^{**}P=0.008$ for Eo and $^{**}P=0.004$ for IgE.

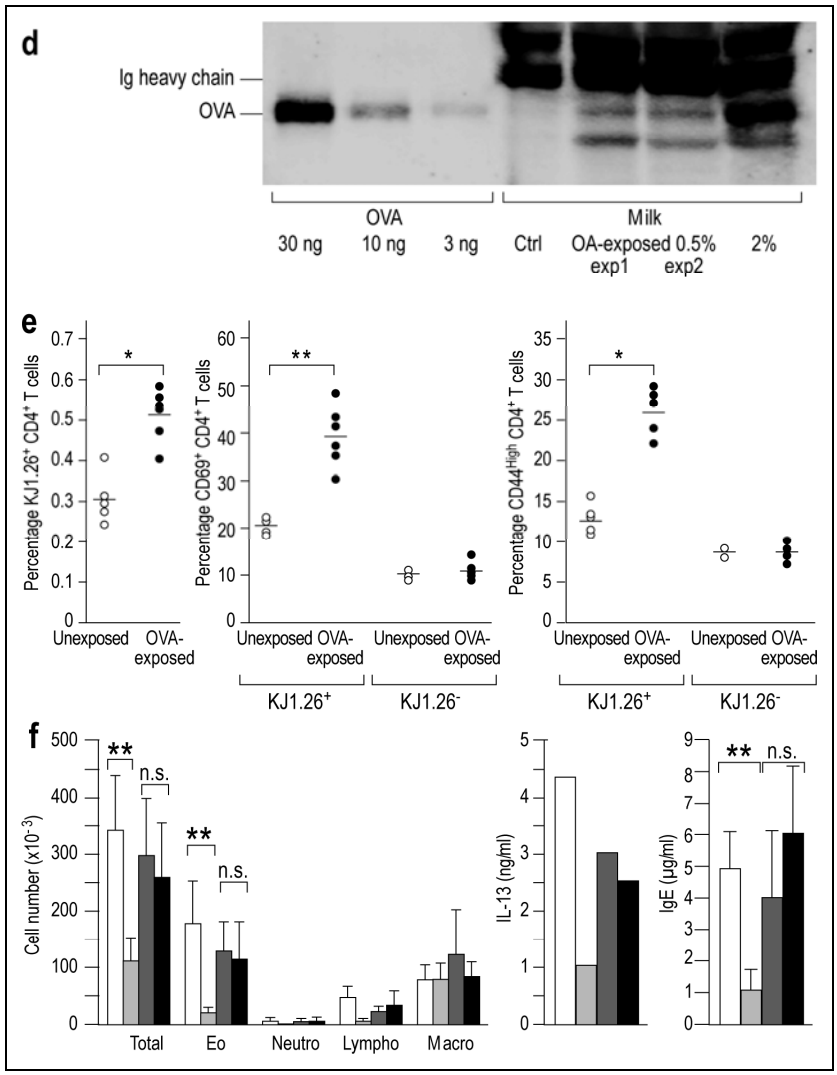

Figure 8 (continued): Breast milk factors involved in breastfeeding-induced tolerance.
d: OVA in breast milk. Lactating mothers were exposed to aerosols of the indicated concentration of OVA for 20 min. Breast milk was harvested 6 h later and analyzed for OVA content by Western Blot using a mouse anti-OVA mAb followed by an anti-mouse Ig mAb. One representative experiment of three.
e: Antigen-driven T cell activation in breastfed newborns. $CD4^+$ T cells from DO11.10 TCR transgenic mice (3 X 10^6 per mouse) were injected into 2 wk-old BALB/c pups that were breastfed on OVA-exposed (filled dots) or unexposed BALB/c mothers (empty dots). 72 h later, peripheral LN cells were analyzed by flow cytometry after staining with anti-CD4, anti-CD69, anti-CD44 and KJ1-26 mAb. Data show the results obtained in individual mice. n=4-6 in one representative experiment of three. **P*=0.01; ***P*=0.004.
f: Role of TGF-β during lactation in breastfeeding-induced tolerance. BAL cell numbers, lung IL-13 secretion and OVA-specific serum IgE levels in mice breastfed on mothers injected with anti-TGF-β mAb or with isotype rat IgG1. Newborns were breastfed on isotype-treated unexposed mother (empty bars), isotype-treated OVA-exposed mother (light grey bars), anti-TGF-β-treated unexposed mothers (dark grey bars) or anti-TGF-β-treated OVA-exposed mothers (black bars). Data are expressed as means ± SD of values obtained in individual mice for Eo and IgE and in pooled lung cells for IL-13 secretion. n=6-7 mice per group, one representative experiment of 2. ***P*=0.001; ns *P*>0.1.

serum OVA-specific IgE, IgG1 and IgA (Figure 4a). OVA-specific IgG2a levels were similar in both groups. Upon OVA restimulation, lung cells from mice breastfed on OVA-exposed mothers secreted reduced amounts of IL-4, IL-5, IL-10 and IL-13 as compared to cells from mice breastfed on unexposed mothers (Figure 4b). Similar results were obtained with mediastinal lymph node cells (Figure 5). The frequency of IL-4, IL-5 and IL-10-secreting lung $CD4^+$ T cells dropped in mice breastfed on OVA-exposed mothers as compared to control animals (Figure 4c). IFN-γ- and TGF-β-secreting cells were not detected. In addition, the frequency of OVA-specific $CD4^+$ T cells in mice breastfed on OVA-exposed mothers was reduced by 4-fold as demonstrated by the frequency of $CD40L^+CD4^+$ T cells upon OVA restimulation (*Frentsch* et al., 2005) (Figure 4c). Protection was also observed in BALB/c mice breastfed on mothers that have been exposed to OVA through the intranasal or the oral route and in C57BL/6 mice (Figures 6 and 7). Antigen-specificity was demonstrated by experiments in which mice breastfed on OVA-exposed mothers were sensitized and challenged with the *Leishmania* LACK antigen (*Julia* et al., 2002) (Figure 8a).

Antigen-specific protection could result from the transfer of immunoglobulins (Ig) (*Labbok* et al., 2004) and/or the antigen from the mother to the newborn through breast milk. To address this issue, wild type (wt) newborns were breastfed on either B cell-deficient μMT or lymphocyte-deficient RAG-2 KO foster-mothers. Mice breastfed on OVA-exposed μMT or RAG-2 KO foster-mothers exhibited reduced BAL eosinophilia, IL-13 secretion by lung cells, and serum OVA-specific IgE levels as compared to those breastfed on unexposed foster-mothers (Figure 8b,c). The levels of inhibition were similar to those observed in mice breastfed on wt mothers (Figure 1). Therefore, tolerance did not require the transfer of Ig from the mother to the newborn and was independent of the mother's lymphocyte compartment.

Western blotting analysis using anti-OVA mAbs showed two bands in milk of OVA-exposed mothers but not in unexposed animals, one at the level of OVA protein and one at a lower molecular weight that was likely to be a degradation product (Figure 8d). OVA concentration in milk of OVA-exposed mothers was in the same range as for dietary antigens in human milk (*Palmer* and *Makrides*, 2006), i.e. 180±20 ng/ml. Knowing that daily milk consumption by newborn mice is around 500 μl at day 10, mice breastfed on OVA-exposed mothers received about 100 ng of OVA daily.

To investigate whether milk-borne OVA was processed and presented to newborn lymphocytes, we injected OVA-specific TCR transgenic $KJ1\text{-}26^+$ $CD4^+$ T cells into 2 wk-old pups that were breastfed on OVA-exposed or unexposed mothers. Both the frequency of $KJ1\text{-}26^+$ $CD4^+$ T cells and the proportion of these cells that were $CD69^+$ or $CD44^{high}$ were higher in mice breastfed on OVA-exposed mothers as compared to those breastfed on unexposed mice (Figure 8e). Therefore, airborne OVA was transferred from the mother to the newborn through the milk and presented to $CD4^+$ T cells in the breastfed newborn.

Breast milk contains IL-10 and TGF-β that both exhibit immunosuppressive activities and favour tolerance induction (*Labbok* et al., 2004; *Letterio* et al., 1994; *Penttila* et al., 1998; *Saito* et al., 1993). However, mice breastfed on OVA-exposed IL-10-deficient mothers were protected from allergic

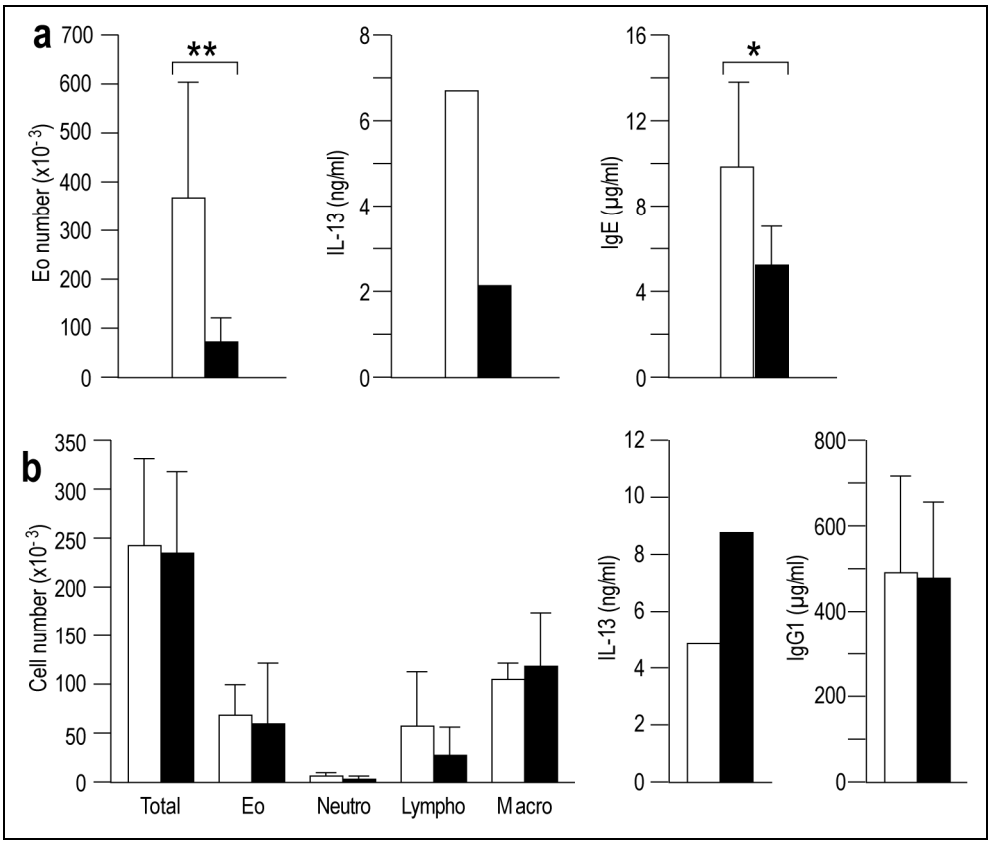

Figure 9: T_{reg} cells in mice breastfed on OVA-exposed mother
a: Tolerance transfer by $CD4^+$ T cells. Spleen $CD4^+$ T cells were purified from mice breastfed on OVA-exposed (filled bars) or unexposed (empty bars) mice and injected into adult naïve recipients that were further sensitized and challenged with OVA. Data are expressed as mean ± SD of values obtained in individual mice for Eo and IgE and in pooled lung for IL-13 secretion. n=6-8 mice per group, one representative experiment of three. ** P=0.001; * P=0.01.
b: BAL cell numbers, lung IL-13 secretion and OVA-specific IgG1 levels in TGF-DNRII mice breastfed on wt mice. TGF-DNRII mice were breastfed on C57BL/6 wt foster-mothers that were exposed (filled bars) or not (empty bars) to OVA aerosols. Data show means ± SD of values obtained in individual mice for Eo and IgG1 and in pooled lung cells for IL-13 secretion. OVA-specific IgE were not detectable in sera of TGF-DNRII mice. n=5-7 mice per group, one representative experiment of two.

airway inflammation as efficiently than those breastfed on OVA-exposed wt mothers further suggesting that protection could occur in the absence of IL-10 in milk (data not shown). Since TGF-β-deficient mice die prematurely, we assessed the role of milk borne TGF-β by injecting anti-TGF-β mAb to lactating mothers. Anti-TGF-β mAb treatment did not result in growth retardation. While mice breastfed on OVA-exposed isotypic control mAb-treated mothers were protected against allergic airway inflammation, mice breastfed on OVA-exposed TGF-β-depleted mothers developed OVA-induced airway inflammation as assessed by BAL eosinophilia, IL-13 secretion by lung cells and OVA-specific IgE serum level (Figure 8f). Therefore, both the reduced airway inflammation and lower antigen-specific Th2 re-

sponse exhibited by mice breastfed on OVA-exposed mothers required the presence of TGF-β during lactation. This result is in agreement with a previous study that showed that oral tolerance induction towards a dietary antigen in formula-fed rats was achieved when it was administered together with exogenous TGF-β (*Penttila*, 2006).

To assess whether protection from allergic airway disease relied on the presence of regulatory T cells (T_{reg}), we injected $CD4^+$ T cells from mice breastfed on OVA-exposed mothers to adult naïve mice. As compared to mice injected with control $CD4^+$ T cells, animals injected with $CD4^+$ T cells from mice breastfed on OVA-exposed mothers exhibited reduced BAL eosinophil numbers, IL-13 secretion by lung cells and serum levels of OVA-specific IgE (Figure 9a). These data suggested that a mechanism of active immune suppression by $CD4^+$ T cells was responsible for the breastfeeding-induced tolerance.

$CD25^+$ T_{reg} cells are involved in the regulation of allergic disease (*Robinson* et al., 2004). To assess whether $CD25^+$ T_{reg} cells were necessary for breastfeeding-induced protection, mice breastfed on OVA-exposed or unexposed mothers were injected with anti-CD25 or isotypic control mAb once adults and sensitized and challenged with OVA. As previously reported (*Lewkowich* et al., 2005), this treatment resulted in increased allergic airway inflammation in mice breastfed on unexposed mothers (Figure 2b). In this setting, the levels of inhibition of airway eosinophilia and OVA-specific IgE induced by mother exposure to OVA were similar in mice treated by anti-CD25 mAb and in those treated by rat IgG1: 87 versus 61% inhibition for eosinophilia; 47% versus 53% inhibition for OVA-specific IgE, (Figure 2b). Therefore, while $CD4^+$ T_{reg} cells are involved in breastfeeding-induced protection from allergic airway disease, $CD4^+$ $CD25^+$ T cells are not required.

To assess whether breastfeeding-induced protection required TGF-β signalling in T cells, we used TGF-β DNRII mutant mice in which T cells do not respond to TGF-β (*Lucas* et al., 2000). TGF-β DNRII newborns were breastfed on wt mothers that were exposed or not to OVA aerosols. Exposure of foster mothers to OVA did not induce protection in TGF-β DNRII mutant mice (Figure 9b). Therefore, the reduced airway inflammation and antigen-specific T cell responses observed in mice breastfed on OVA-exposed mothers were dependent on TGF-β signalling in T cells. We next investigated whether TGF-β was required for protection when mice were adults. Mice breastfed on OVA-exposed mothers were treated with either anti-TGF-β or isotypic control mAb one day before sensitization, challenged with OVA aerosols and analyzed for allergic airway inflammation. Neutralization of TGF-β before sensitization did not prevent breastfeeding-induced protection demonstrating that TGF-β was no longer required once T_{reg} cells have already been induced during the neonatal period (Figure 10).

DISCUSSION

We have demonstrated that an airborne antigen can be transferred from lactating mice to their progeny through breast milk eventually resulting in antigen-specific tolerance and prevention from asthma.

Breast milk contains dietary antigens (*Palmer* and *Makrides*, 2006) but

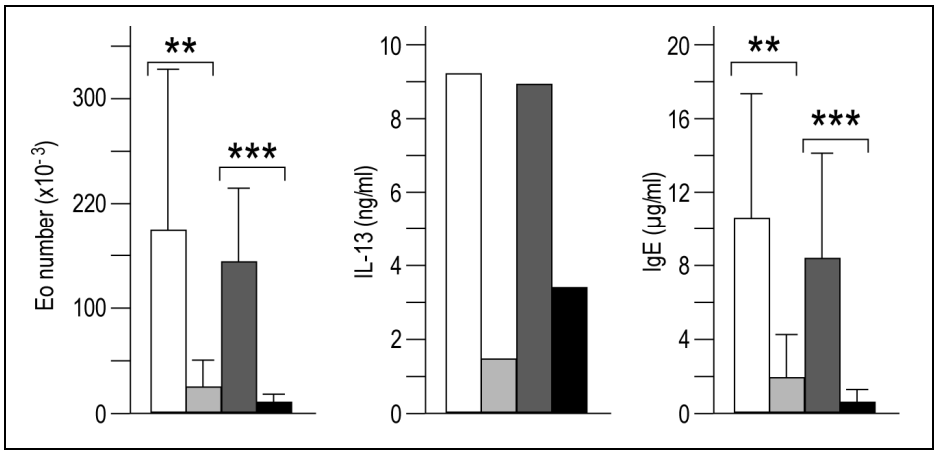

Figure 10: Breastfeeding-induced tolerance in anti-TGF-β mAb-treated offspring.
6-8 wk-old mice breastfed by OVA-exposed or unexposed mothers were treated with 1 mg of anti-TGF-β or isotype mAb before sensitization to OVA, and challenged with OVA. BAL eosinophil numbers, IL-13 secretion by lung T cells and OVA-specific serum IgE levels are shown for isotype-treated mice breastfed by unexposed mothers (empty bars), or OVA-exposed mothers (light grey bars), anti-TGF-β-treated mice breastfed by unexposed mothers (dark grey bars) or OVA-exposed mothers (black bars). Data are expressed as mean ± SD of values obtained in individual mice for Eo and IgE and in pooled lung cells for IL-13 secretion. n=6-7 mice per group, one representative experiment of two. ***P*=0.004 ****P*=0.0006.

the presence of airborne antigen has not yet been assessed. Antigen distribution after aerosol administration has been previously assessed using radiolabelled ^{125}I-BSA or ^{125}I-OVA (*Holt* et al., 1981; *Willoughby* and *Willoughby*, 1977). Both studies demonstrated that 2-4% of antigen was found in the lung and 65-80% in the digestive tract 1-2 hr after aerosol exposure. Therefore, although some airborne antigens penetrate into the distal alveoli, the bulk of inhaled antigen is found in the gut. Indeed, inhaled antigens are either trapped in the nasal passage and swallowed or deposited to the lung and cleared via the mucociliary escalator to the digestive tract. Therefore, the presence of airborne OVA in milk most likely results from the transfer of OVA from the airways to the mammary gland mainly through the gut and for a small proportion through the alveolar-capillary barrier of the lung (*Bensch* et al., 1967; *Braley* et al., 1978).

While the oral administration of an antigen to adult rodents results in tolerance induction (*Faria* and *Weiner*, 2005), inducing oral tolerance in neonates is far more difficult (*Adkins* et al., 2004; *Hanson*, 1981; *Miller* et al., 1994; *Strobel* and *Ferguson*, 1984). Neonates are biased for Th2 responses as compared to adults (*Adkins* et al., 2004). This is in apparent contrast with our data showing that the transfer of an antigen from the mother to the newborn via the milk induces tolerance towards a Th2-mediated disease. Breastfeeding-induced tolerance may rely on the chronic administration of an antigen at low dose, a setting known to promote tolerance induction *(Apostolou and von Boehmer*, 2004; *Faria* et al., 2003) together with the presence of milk-borne TGF-β. It remains to be determined whether tolerance is also observed when lactating mothers are allergic.

Epidemiological studies on the relationship between breastfeeding and the

development of allergic diseases have reached conflicting results whether the atopic status of the mothers was taken into account or not (*Friedman* and *Zeiger*, 2005; *Gdalevich* et al., 2001; *Guilbert* et al., 2007; *Kramer* et al., 2007; *van Odijk* et al., 2003). However, maternal airborne allergen exposure and antigen content in milk were not recorded in these studies. Our work may confer a rationale for new epidemiological studies assessing the presence of airborne antigens in human milk and the prevalence of allergic diseases in children breastfed on mothers exposed to airborne allergens. This report gives new insights into the mechanisms underlying tolerance induction in neonates and pinpoints maternal influence through breast milk antigen transfer as a critical factor in this process.

ACKNOWLEDGMENTS

We thank Frank Aguila for his help for figures, and Nicolas Guy and the animal facility staff for their excellent animal care. This work was supported by a grant from the Fondation pour la Recherche Médicale (to VJ) and by a grant from the EU (DC-THERA) (to NG). VV was sponsored by fellowships from the FRM, the Juvenile Diabetes Research Foundation (USA) and the Fond National de la Recherche Scientifique (Belgium).

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IMMUNE REGULATION AND THE INFLUENCE OF MICROBES

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SUMMARY

Allergies, inflammatory bowel disease and celiac disease are common and increasing in Western societies. According to the hygiene hypothesis, they are caused by insufficient microbial exposure. This review will go through vital immunoregulatory mechanisms and discuss how these mechanisms may be influenced by microbes and microbial products.

INTRODUCTION

Allergy is the most common chronic disease amongst children in the Westernized parts of the world and affects as much as every third child. The increase in allergic diseases has become evident during the last twenty years and correlates with an increased living standard and excessive hygiene in the society. Accordingly, the hygiene hypothesis state that an exaggerated hygienic lifestyle give rise to an incorrect composition of the gut flora which leads to a defective maturation of the immune system which in turn give rise to immunoregulatory diseases such as allergy (*Wold*, 1998).

The reason to why some individuals suffer from diseases related to inadequate immune regulation, such as allergy, is not completely known. However, oral tolerance is a vital mechanism as it prevents untoward immune responses against food or airborne antigens, which get stuck in the upper airways and are subsequently swallowed, and thus prevents allergic reactions and inflammatory conditions in the gut. Oral tolerance is introduced by feeding of an antigen and result in development of regulatory T cells (Treg) and antigen-specific tolerance. Regulatory T cells suppress other immune cells and play a decisive role in maintaining the balance within the immune system. Regulatory T cells exist as natural Treg (nTreg) that are induced during thymic development and as induced Treg (iTreg), which arise from naïve cells and for instance during development of oral tolerance.

The gut flora is the major stimulus for the immune system and the absence of a gut flora result in an immature immune system, especially in the gut and also to a decreased ability of oral tolerance induction (*Moreau* and *Corthier*, 1988; *Moreau* and *Gaboriau-Routhiau*, 1996; *Sudo* et al., 1997). How microbial exposure can protect against allergy development remains elusive and no particular microbe(s) that better than others protect against development of allergy has yet been identified (*Adlerberth* et al., 2007). A complex microbiota, or rapid strain turnover, may be of importance, as a low com-

plexity of the comensal gut flora at one week of age predicted high risk of allergy development in a subsample of the ALLERGYFLORA cohort (*Wang* et al., 2008).

IMMUNO REGULATORY MECHANISMS

Mucosal tolerance

A large part of the immune system is localised to the gastrointestinal tract and it's also where the majority of all antigens encounter our body. We are readily exposed to foreign antigens from the food but also from airborne antigens that get stuck in airway mucus and transported to the throat and swallowed. It's crucial for the immune system to avoid aggressive immune responses such as allergic reactions towards these harmless substances.

Oral administration of soluble protein antigens result in the development of mucosal (oral) tolerance i.e. a state of immunological unresponsiveness. Even if extensive research has been performed during the last decades the mechanism or mechanisms behind mucosal tolerance remain unsolved. However, the work has revealed that almost all states of immunological immune responses can be suppressed by different regimens of oral Antigen administration. This includes *in vivo* responses such as DTH responses (*Miller* and *Hanson*, 1979; *Mowat* et al., 1982), formation of different Ig isotypes (*Vaz* et al., 1977; *Ngan* and *Kind*, 1978), changes in the clearance rate of Antigens from the circulation (*Hanson* et al., 1979) as well as *in vitro* assays such as lymphocyte proliferation (*Titus* and *Chiller*, 1981; *Hanson* and *Miller*, 1982), specific plaque forming cells and production of certain cytokines. Two main effectors mechanisms have been considered in oral tolerance and the outcome is generally thought to depend on the feeding strategies. The dogma state that feeding a low dose of Antigen results in the generation of regulatory T cells, whereas a high Antigen dose would result in clonal deletion and/or clonal anergy.

Tolerogenic antigen presenting cells

The antigen presenting cells residing both in the intestinal lamina propria and in the mesenteric lymph nodes have distinct tolerogenic properties and their antigen presenting ability favours development of regulatory T cells (*Chirdo* et al., 2005). The vitamin A metabolite retinoic acid (RA) has been discovered to be responsible for the up-regulation of the α4β7 integrin and CCR9, permitting newly formed T cells to accumulate preferentially in the GALT. The $CD103^+$ gut associated DCs express relatively high levels of retinal dehydrogenases, the enzymes required for the irreversible generation of RA from vitamin A (retinal). Inhibiting these enzymes reduced the expression of the α4β7 integrin on T cells and resulted in their depletion from the intestinal lamina propria (*Iwata* et al., 2004). These results explained why T cells stimulated by antigen on gut-associated DCs return to GALT.

New studies (*Benson* et al., 2007; *Coombes* et al., 2007; *Sun* et al., 2007) support this idea by showing that $CD103^+$ DCs in GALT are specially equipped for converting antigen-specific T cells into $Foxp3^+$ Treg cells in an RA- and TGF-β–dependent manner. The RA-enhanced conversion process also leads to the up-regulation of α4β7 integrin and CCR9 permitting the newly formed Treg cells to accumulate preferentially in GALT. Finally, RA

can reduce the negative impact of co-stimulation on the TGF-β–dependent conversion of T cells into Foxp3$^+$ Treg cells.

Tolerogenic processing

Studies performed by the group of Stephan Strobel, revealed that a tolerogenic moiety is present in the circulation of mice that were fed OVA and that induced tolerance upon transfer into naïve recipients (*Strobel* et al., 1983). The tolerogenic processing was also shown to be different from physiochemical alteration of the protein, since neither the native or chemically altered protein could induce tolerance in naïve recipients (*Bruce* and *Ferguson*, 1986). At that point the features of the tolerogenic moiety i.e. serum factor, were left unsolved. However E. Telemo and his colleagues could for the first time identify the serum factor and it was shown to consist of a supra-molecular structure that sedimented at 70.000 x g. The structures sized 40-50 nm, showed similarity to exosomes that are released from B cells and DCs and due to their tolerogenic function were named tolerosomes (*Karlsson* et al., 2001).

Tolerogenic processing involve sampling of luminal antigens by the small intestinal epithelial cells, co-localisation of antigenic peptides with MHC II molecules in vesicular structures of the intestinal epithelial cells, production of multivesicular structures by invagination of the vesicular membrane and finally the release of MHC class II expressing small vesicular structures on the basolatheral side of the intestinal epithelial cells (*Zimmer* et al., 2000). This tolerogene trafficking is of vital importance for the oral tolerance mechanisms and studies has shown that germfree animals (*Moreau* and *Corthier*, 1988; *Moreau* and *Gaboriau-Routhiau*, 1996; *Sudo* et al., 1997) and severe combined immunodeficiency (SCID) mice (*Ostman* et al., 2005), that both have an immature gut and lack of MHC class II expression in the intestinal epithelium, have a defective tolerogen trafficking and impaired oral tolerance.

Regulatory T cells

Naturally occurring CD4$^+$CD25$^+$ regulatory T cells

Nishizuka and Sakakura were the first to show that T cells are involved in the control of autoreactions, by demonstrating that mice, thymectomized at day tree of life developed spontaneous organ specific autoimmunity (*Nishizuka* and *Sakakura*, 1969). Then, 30 years later, Sakaguchi and colleagues demonstrated that the adoptive transfer of T cells, depleted of the IL-2 receptor a-chain (CD25) expressing cells, induced multiorgan autoimmunity in immunodeficient recipient animals (*Sakaguchi* et al., 1995), thus positioning regulatory T cells as part of the natural and essential tolerance system acting to control autoreactions.

The fundamental need for functional nTregs become evident in humans suffering from IPEX, which is a fatal disorder characterized by immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance and caused by mutations of the human gene FOXP3, the ortholog of the gene mutated in scurfy mice forkhead box p3 (Foxp3). Recent findings have demonstrated an important role of the forkhead transcription factor FoxP3, for development and regulatory function of nTreg (*Fontenot* et al., 2003; *Hori* et al., 2003; *Setoguchi* et al., 2005).

Induced CD4$^+$ T regulatory cells

Feeding of protein antigens induce a state of immunological tolerance i.e. oral tolerance and Weiner and colleagues were the first to describe that oral feeding of myelin basic protein induced a distinct subset of Ag specific

T cells (Th3) that secreted IL-4 and tumour growth factor-beta (TGF-β) and prevented development of EAE (*Chen* et al., 1994). In addition, Roncarolo and colleges reported that repetitive stimulation of naïve T cells, from OVA TCR-transgenic mice, with OVA and IL-10 resulted in T cell clones with a unique cytokine profile that were distinct from Th0, Th1 or Th2 cells (*Groux* et al., 1997). These Tr1 cells produce IL-10 and some IL-5 and interferon-γ (IFN-γ), with or without TGF-β, but with little or no IL-2 and IL-4 production and show immunosuppressive properties *in vivo*. In contrast to nTregs, induced Tregs have been shown to be specific for antigens not present in the thymus such as food antigens, bacterial flora antigens (*Cong* et al., 2002; *McGuirk* et al., 2002; *Satoguina* et al., 2002) and some self-antigens such as insulin (*Chen* et al., 2003) or altered self-peptides (*Wildbaum* et al., 2002). These cells arise from naïve precursor cells and achieve regulatory function only after proper induction in the presence of endogenous local factors, such as cytokines.

Suppressive mechanisms of regulatory T cells

nTregs are the most well studied Tregs and despite intense research the mechanisms behind the suppressive properties of nTreg remain unclear. nTreg can suppress cells from both the innate and adaptive immune system (*Maloy* et al., 2003; *Fehervari* and *Sakaguchi*, 2004) require TCR and IL-2 stimulation to achieve suppressive function (*Nelson*, 2004). In contrast to nTreg, iTregs mediates their suppressive function primarily by cytokine dependent mechanisms. Tr1 mediated suppression depend on IL-10 and debatably on TGF-β and suppress the proliferation and cytokine production of naive $CD4^+CD25^-$ T, Th1 and Th2 cells. Th3 cells are dependent on TGF-β and mediate suppression of Th1 and Th2 cells and can also stimulate plasma cells to secrete IgA.

Microbial interactions with the immune system

Newborn children are brought from the sterile uterus to an environment that is crowded with microbes. Colonization of the newborn intestinal mucosa and epithelium of the skin starts immediately after birth. Several studies have provided information of the host-microbe interaction and have revealed a mutual beneficial relationship. The bacteria benefit from the stable habitat that is rich in energy sources from the food we eat. Bacteria in the normal gut flora have been shown to provide the mammalian host with essential nutrients, defence against pathogens and contribution to the intestinal architecture and development of the immune system.

Intestinal microorganisms

The mammalian microflora is largely composed of bacteria but other organisms such as, protozoa and fungi are also present. The colon harbours the majority of bacteria (10^{10}-10^{12} organisms per gram or ml of luminal content), which can comprise 400 different species. Anaerobes predominate in the lower intestine, particularly bacteroides, bifidobacteria, fusobacteria and peptostreptococci, while aerobes and facultative aerobes including enterobacteria and lactobacilli are more rare. The distal portion of the small intestine, the ileum, has less dense colonization (10^8 per gram) and limited colonization is observed in the stomach and the proximal small intestine (10^3-10^5 per gram), consisting mainly of acid-tolerant lactobacilli and streptococci.

The intestine of a newborn is rich in oxygen and therefore, facultative aerobes, such as *Escherichia coli* and

other enterobacteria, enterococci and staphylococci are the first colonizers of the human intestine. The oxygen is gradually consumed and the aerobe bacteria receive competition from the thus favoured anaerobic bacteria, including bifidobacteria, bacteriodes, clostridia and lactobacilli that start to colonize. The microbiota of infants is rapidly developed during the first week and remains unstable for the first year of life, becoming more stable later on. The colonization is largely influenced by the maternal microbiota, environmental factors and by infant feeding patterns. Alterations in the established microflora, such as turnover of individual strains occur constantly and are influenced by the presence of microbes in the environment, intestinal infections and exposure to antibiotic. Accordingly, the turnover of individual bacterial strains is much higher in developing countries, as compared to developed countries (*Nowrouzian* et al., 2003). Moreover, colonization with bacterial strains, such as *E. coli*, enterococci and lactobacilli is less frequent and occurs later in life of children in westernized countries as compared with children in developing countries or former socialist countries in East Europe (*Bennet* et al., 1991; *Sepp* et al., 1997; *Adlerberth* et al., 1998).

The hygiene hypothesis

Certain diseases are on the increase in Western countries. This has been clearly demonstrated for allergies (*Williams* et al., 1994; *von Mutius* et al., 1994), inflammatory bowel diseases (*Langholz* et al., 1991; *Munkholm* et al., 1992; *Farrokhyar* et al., 2001) and autoimmune disorders such as insulin-dependent type I diabetes and multiple sclerosis (*Rosati* et al., 1988; *Patterson* et al., 1996; *Parslow* et al., 1997; *Pundziute-Lycka* et al., 2002). The reasons for the increase in these diseases are not known, but they may all be related to the increased hygienic lifestyle in the modern Western society. Good housing standard, small families (*von Mutius* et al,, 1994) have all been linked to the high risk of developing allergies, while exposure to early day-care (*Kramer* et al., 1999; *McKinney* et al., 2000), pets (*Hesselmar* et al., 1999) or a live-stock farming environment (*Braun-Fahrlander* et al., 1999) all protect against allergies. In 1989, David Strachan formulated the hygiene hypothesis (*Strachan*, 1989) that was based on previous studies. He proposed that microbial stimulation was required in order to educate the immune system properly and that a decreased microbial exposure would lead to failure of such stimulation and development of allergic disease.

There is also an inverse correlation with previous infections with hepatitis A, Toxoplasma gondii or Helicobacter pylori and a decrease in the risk of developing allergy (*Matricardi*, 1997; *Matricardi* et al., 2000). One suggested explanation for the observed protection against allergy is that interaction with certain microorgansims causes immune deviation, thereby skewing immune responses away from the neonatal Th2, bias towards Th1 cell responses (*Busse* and *Lemanske*, 2001) However, this explanation cannot account the observation that infections with helminths, that are known to induce Th2 cytokines, are connected with protection from asthma (*van den Biggelaar* et al., 2004) or that humans with an immunodeficiency that affect Th1 cell cytokine pathways do not have an increased incidence of allergies (*Lammas* et al., 2000). The observed increase in allergic diseases, seen during the last decade has been accompanied by similar increase in autoimmune diseases that are considered to be Th1 biased (*Kero*

et al., 2001; *Stene* and *Nafstad*, 2001) again arguing against a general Th1 skewing by a westernised life style.

Several studies aim to link microbial infections with various autoimmune disorders, however, no epidemiological, statistically relevant associations have been observed so far. In opposition, several experimental studies support the hygiene hypothesis. For example, injection with coxsackievirus cannot only enhance (*Horwitz* et al.m 2000) but also prevent disease in none obese diabetic (NOD) mice (Tracy, Drescher et al. 2002). Furthermore, IFN-γ and TNF-α have protective effects in EAE or diabetes models (*Jacob* et al., 1990; *Christen* et al., 2001). Inflammation caused by viruses, bacteria and especially by parasitic worms can shift the T cell balance towards a more immunosuppressive state that would favour induction of regulatory T cells. Indeed, studies have provided evidence for regulatory T cells with specificity for the pathogens to occur in Leishmania major (*Mendez* et al., 2004), HSV (*Toka* et al., 2004) and Friend retrovirus (murine leukaemia virus) infections (*Dittmer* et al., 2004). Infection might also cause hyperactivation of autoaggressive lymphocytes, which may lead to activation induced cell death and diminish the systemic load of autoreactive T cells (*Christen* et al., 2001; *Qin* et al., 2004). Infection at a site away from the autoimmune reaction might keep autoaggressive cells from reaching the site of autoimmune destruction and prevent disease (*Christen* et al., 2001).

Infections result in an immune response that is partly specific (T cell clones specific for pathogenic peptides and high affinity neutralizing antibodies specific for surface epitopes) and partly non-specific (class switch recombination of natural antibody specificities, resulting from bystander help by specific T cell clones) and could cause an alteration in the T cell repertoire. In addition, cells from both the innate and adaptive immune system can recognize microbial structures, as shown by the finding of several TLRs expressed on T cells (*Caramalho* et al., 2003), thus indicating that the immune system has evolved to be highly susceptible for microbial stimulation. A cleaner environment results in a reduced and more stable microflora and fewer infections, which seem to disturb the immune homeostasis. One possible explanation for this could be that encounters with microbes helps to maintain the Treg population via an IL-2 dependent mechanism (*Maloy* and *Powrie*, 2005). Certain comensal bacteria has been proposed to aid in the hosts health and has resulted in recent years development of probiotics i.e. bacterial preparations that impart clinically verified beneficial effects on the health of the host when consumed orally(*Salminen* et al., 1998). Most probiotics are currently either lactic acid bacteria or bifidobacteria. The probiotic action includes competitive exclusion of pathogens, effects on the composition of the microbiota as well as modulation of the intestinal and systemic immune responses.

Immune cell activation by intestinal microbes

The intestinal microbes are separated from the underlying tissue by a single epithelial cell layer. Mucus produced by specialized cells in the epithelium i.e. goblet cells and epithelial cell derived defensins, supports the mucosal barrier and diminish the entry of microbes. Even so, certain bacterial species can penetrate this barrier to reach into the tissue and other bacteria make their entry via the M-cells in the PP. Recent studies have also shown that lamina propria DCs can make a way

through tight junctions and pick up luminal bacteria(*Rescigno* et al., 2001). The majority of comensal bacteria that pass over the mucosal barrier are rapidly killed by macrophages but they can survive for several days inside DCs (*Macpherson* and *Harris*, 2004). DCs that have been loaded with comensal bacteria in the Peyer´s patches or lamina propria migrate only as far as to the mesenteric lymph nodes (*Macpherson* and *Harris*, 2004) and priming by live comensals is presumably restricted to the mucosal immune system. This *in vivo* priming induces IgA production that in turn, aggravates penetration of additional comensals by means of exclusion. However, bacterial degradation products contaminate the systemic circulation and act as stimulators of the peripheral immune system.

Natural adjuvants

Several animal studies have suggested a role for microbial stimuli or so called natural adjuvants, like LPS or the B subunit of CT, for the effective induction of mucosal tolerance (*Michalek* et al., 1982; *Wannemuehler* et al., 1982; *Khoury* et al., 1990; *Rask* et al., 2000; *Bregenholt* et al., 2003). Epidermological studies have shown that there is an association with high levels of endotoxin in the sleeping mattress and protection against allergy development (*Braun-Fahrlander* et al., 2002). It was also found in a subsample of the ALLERGYFLORA cohort that infants colonized in the first week(s) of life with *Staphylococcus aureus* (*S. aureus*) had lower risk of developing food allergy than other children (*Lundell* et al., 2007). There was also a correlation between early colonization with enterotoxin producing *S. aureus* and the expansion of putative Tregs ($CD4^+CD25^+CTLA\text{-}4^+$) in the blood of 4 months old children in this cohort (*Karlsson* et al*.,* unpublished).

S. aureus is foremost a skin bacterium, but we it has become a quite common inhabitant of the neonatal gut in Swedish infants (*Lindberg* et al., 2000; *Lindberg* et al., 2004), probably as a result of decreased competition from "classical" faecal bacteria whose circulation has decreased strongly in today's highly hygienic hospitals and homes. Of *S. aureus* strains colonizing the neonatal gut, approximately 45% have the capacity to produce a toxin with superantigenic function. *S. aureus* enterotoxins have previously been incriminated in the pathogenesis of eczema, as eczematous skin lesions are often colonized by *S. aureus*, and since *S. aureus* colonization aggravates the lesion. However, data from animal experiments indicate that exposure to *S. aureus* superantigen may in fact favour development and functional activity of Treg. Thus, repeated i.v. injections of SE to mice result in development of T cells with regulatory function (*Sundstedt* et al., 1997; *Feunou* et al., 2003) and increased *in vitro* suppressive ability of isolated regulatory T cells (*Grundström* et al., 2003) as well as increased serum levels of the cytokine IL-10 (*Sundstedt* et al., 1997) which down regulates T cell activation and IFN-γ production . $CD4^+$ T cells from mice injected with SEA are about 3-fold more potent suppressors of SEA-induced T cell proliferation and IL-2 production compared to natural $CD4^+CD25^+$ regulatory T cells from untreated mice (*Grundström* et al., 2003).

CONCLUSION

Allergies are the most common chronic diseases amongst children in the Western society and affect as much as every third child. Many people believe the underlying reason for the increase in allergic diseases is due to an incorrect stimulation of the immune system during early infancy. Strategies to improve the development and maturity of the infant immune system by exposure to bacteria or bacterial products such as superantigens during early infancy is a possible way for allergy prevention and if this would succeed, the positive health effect would be enormous.

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IMMUNE MODULATION BY EXOSOMES

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SUMMARY

Exosomes are small membrane vesicles ranging in size from 40-90 nm secreted by a number of different cells such as dendritic cells, B- and T cells, mast cells and intestinal epithelial cells. Exosomes have also been isolated from several body fluids such as bronchoalveolar lavage (BAL), urine, serum and breast milk. Exosomes are believed to function as communicators between cells, carrying a message of both activation and suppression of the immune response. They are also believed to be utilised as a transport for retroviruses and to be responsible for the intracellular membrane exchange and the spread of prions. The different functions of exosomes are most likely dependent on their origin since the composition and expression of surface markers of the exosomes vary with the type of cell they are secreted from. Recent studies indicate that exosome formation is a highly regulated process but the mechanisms behind exosome biogenesis are still far from being completely understood. There is no doubt though that these small vesicles play an important part in the spread of immunological information and immune regulation. This review aims to adress some of the many functions of exosomes with emphasis on the role of intestinal epithelial cell (IEC) derived exosomes in tolerance induction.

EXOSOME BIOLOGY

Exosomes are small, 40-90 nm membrane vesicles of endocytic origin that are secreted by a variety of cells in culture. They were described for the first time in 1981 as microvesicules containing 5´-nucleotidase activity secreted by neoplastic cell lines (*Trams* et al., 1981). A few years later two independent groups reported secretion of small vesicles of endocytic origin by cultured reticulocytes. Using electron microscopy they observed these small vesicles in the late endosomes which by fusion with the cell membrane released the vesicles extracellularly. The supposed function of the exosome in this study was to remove the transferrin receptor from the cell surface (*Harding* et al., 1983; *Pan* et al., 1985). A decade later, in 1996, exosomes were for the first time shown to have an immunological function. Antigen pulsed B cells secreted exosomes originating from multivesicular bodies which activated antigen specific T cells (*Raposo* et al., 1996). Today we know that exosomes can be secreted by a variety of cells in culture and can be isolated *in vivo* from

body fluids such as serum (*Caby* et al., 2005; *Janiszewski* et al., 2004), bronchoaveolar lavage (*Admyre* et al., 2003) and urine (*Pisitkun* et al., 2004). So far intestinal epithelial cells (*Karlsson* et al., 2001; *Van Niel* et al., 2001), T- and B-lymphocytes, dendritic cells, macrophages, reticulocytes, mast cells and platelets have been shown to produce exosomes (*Thery* et al., 2002). Exosomes are believed to function as communicators between cells, carrying an antigen specific message resulting in either activation or suppression of the immune response. The significance of the message that the exosomes carry seem to depend on what cell they originate from and the state of the cell during exosome formation.

The process of exosome formation starts with invagination of the cell membrane and formation of endosomes. Invagination and inward budding of the membrane of late endosome then forms the exosome. Upon fusion with the cell membrane these multivesicular endosomes release exosomes extracellularly (Figure 1). So far two different mechanisms has been suggested which supports the idea that exosome formation and release is a highly regulated process. The first one is the identification of the endosomal sorting complex required for transport (ESCRT) in association with exosomes (*Williams* and *Urbe*, 2007), the second was just recently identified as ceramide-triggered budding (*Trajkovic* et al., 2008). The ESCRT sorts ubiquinated proteins for transport in the endosomal network, however not all proteins found in exosomes are ubiquitinated. So far no "exosome-specific" marker has been identified hence they are characterised on morphological and biochemical criteria. Exosomes are commonly defined as small membrane bound vesicles originating from the cell surface and processed/modified intracellularly resulting in a multi-vesicular compartment, which is emptied to the extra cellular space, thus releasing the exosomes (Figure 1). Due to their size exosomes can only be visualized in electron microscope. Figure 2 shows exosomes isolated from serum stained with ICAM-1 (*Ostman* et al., 2005).

Due to the formation pathway of exosomes, the molecules found on their surface are typically of endocytic/lysosomal origin e.g., CD9, CD63 and CD81 (*Escola* et al., 1998). These molecules belong to a family of proteins called tetraspanins, which have been suggested to be involved in cell adhesion, activation, proliferation and antigen presentation. Exosomes from antigen presenting cells express MHC class I and II together with co-stimulatory molecules like CD54, CD80 and CD86, which explains their capacity to activate T cells (*Escola* et al., 1998; *Lamparski* et al*., 2002)*. The enrichment of these co-stimulatory molecules on the exosome seems to depend on the maturation state of the antigen presenting cell (APC), indicating that they would also stimulate the immune system in different ways (*Segura* et al., 2005). Exosomes from intestinal epithelial cells (IECs) also express MHC class II along with e.g., CD63, CD81 and A33 which is a marker specific for IECs (*Karlsson* et al., 2001; *Van Niel* et al., 2001). IEC exosomes can be immunostimulatory, both as suppressors and activators of the immune system (*Karlsson* et al., 2001; *Van Niel* et al., 2003). Mast cells secret exosomes expressing MHC class II, CD86, LFA-1 and ICAM-1 which mediates Mast cell-dependent B and T cell activation (*Skokos* et al., 2001). Recently it has also been reported that mast cell derived exosomes contain mRNA which can be transferred between cells (*Valadi* et al., 2007). Depending on what cell they originate

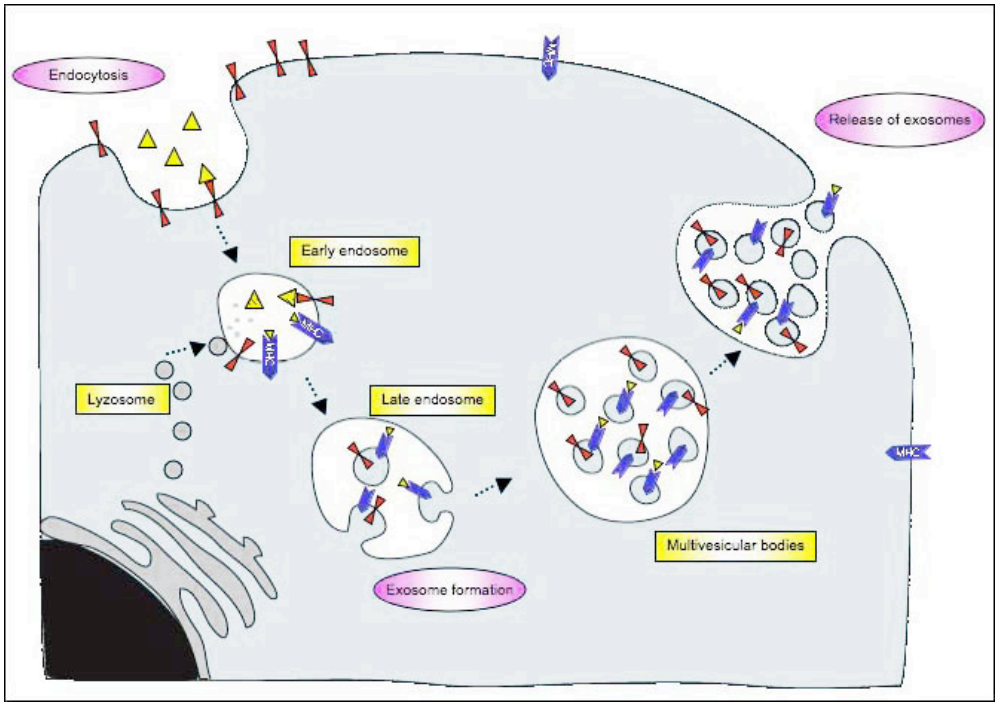

Figure 1: The process of exosome formation starts with invagination of the cell membrane and formation of endosomes, invagination and budding of the membrane of late endosome then forms exosomes. Upon fusion with the cell membrane these multivesicular endosomes release exosomes extracellularly.

from, exosomes express different surface markers and have different lipid composition (*Laulagnier* et al., 2004), hence they are likely to have different functions.

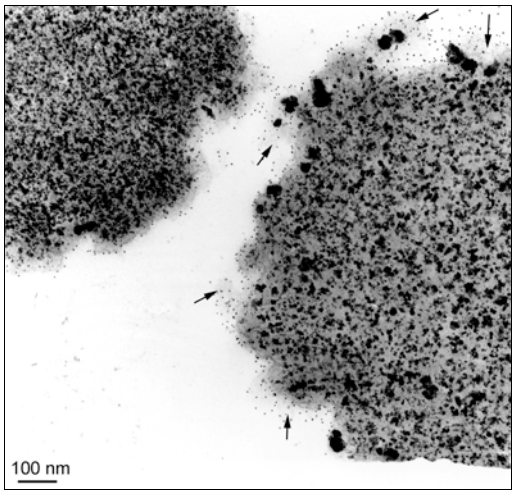

Figure 2: Electron microscope picture of exoxomes.

EXOSOME FUNCTION

Exosomes have been studied most extensively in cancer therapy due to their T-cell stimulatory capacity. One of the pathogenic problems with cancer is the unresponsiveness of the immune system and one way to overcome this is to give the patient autologous APC's primed with tumour antigen. However it has been shown that also the exosomes, derived from these primed DC's, most effectively trigger a tumour specific T cells response. The advantage exosomes have over the whole cell in this case is their size; they spread through the system more efficiently then an activated dendritic cell, but still carry the same message. They can also be collected at one occasion and stored frozen for multiple dosing. Studies show that exosomes loaded with tumour antigens can stimulate CD4+ and CD8+ T cells. For example, exosomes isolated from *in vitro* cultured dendritic cells, pre-pulsed with tumour antigen, induced rejection of established tumours (*Amigorena* 1998; *Hsu* et al., 2003; *Zitvogel* et al., 1998). Depending on the maturation state of the secreting dendritic cell, the exosomes released from the latter appear to trigger the immune response in different ways. It has been shown that ICAM-1, which is more abundant on exosomes from mature DCs, is crucial for naïve T cell priming by exosomes (*Segura* et al., 2005). Another recent study shows that exosomes from IL-10 treated DCs are capable to suppress inflammation and collagen-induced arthritis (*Kim* et al., 2005). In conclusion, the current literature shows that the outcome of an immune response to DC derived exosomes depend on the state of the originating DC. Moreover, along with the type of markers on the surface, the amount of exosomes secreted from dendritic cells at different maturation state seems to differ (*Segura* et al., 2005).

As in the case of DC derived exosomes, the exosomes secreted from IEC can both suppress as well as activate the immune system. We have shown that IEC exosomes can transfer antigen specific tolerance (*Karlsson* et al., 2001) while another group showed that IEC exosomes prime for an aggressive immune response rather than tolerance (*Van Niel* et al., 2003). The conclusion of these experiments is that IECs are capable of producing exosomes that can initiate an immune response, but the outcome of such response may differ in different experimental settings.

We have also shown that exosomes isolated from serum shortly after an antigen feed have the capacity to transfer tolerance to recipients and protect against allergic airway sensitization (*Almqvist* et al., 2008). Moreover, it has recently been shown that exosomes from B cells, isolated and cultured from human PBMC, can present allergen peptides and activate allergen specific T cells to proliferate and produce Th2 cytokines (*Admyre* et al., 2007). Taken together these findings suggests that exosomes from different sources may play a role in the development of asthma and allergy in at least two ways; either as a failure to induce effective tolerance or as enhancers of an already established allergic response. This makes exosomes highly interesting as therapeutic targets in anti-allergy treatment.

Exosomes are also believed to be utilised as a transport for retroviruses (*Pelchen-Matthews* et al., 2004). Gould et al suggests 'The Trojan exosome hypothesis' which states that retroviruses use the pre-existing nonviral exosome biogenesis pathway for the formation of infectious particles (*Gould*

et al., 2003). The exosome-like vesicles would contain virus particles undetectable to the host's own immune system. It has been shown that macrophages infected with HIV release HIV particles displaying, to a certain extent, similar molecules as exosomes (*Nguyen* et al., 2003). In addition HIV virons assemble in the MVBs of macrophages, which is the site where exosomes are formed (*Kramer* et al., 2005). Another study has shown that HIV infected immature DC release exosomes that can transfer HIV to CD4+ T cells. They also show that the exosome-associated HIV was 10 fold more infectious then free virus particles (*Wiley* et al., 2006). Using exosomes as a transport is an excellent strategy to escape the host defence. The virus is protected inside a membrane bound vesicle that is readily taken up and processed by a number of different cells unaware of its infectious content, just like a true 'Trojan Horse'.

Furthermore there is evidence suggesting that exosomes are contributing to the intracellular membrane exchange and the spread of prions (*Fevrier* et al., 2004; *Vella* et al., 2007). These studies show that infectious prion proteins, abnormally folded prion proteins (PrPs), scrapie (PrPsc) are associated with exosomes. Furthermore the exosomes had the capacity to transfer the PrPsc to uninfected cells and transform normal PrPs into scrapie PrPsc. Exosomes enriched in PrP have also been isolated from sheep cerebral spinal fluid and is suggested by the authors to be a way of detecting abnormal forms of the prion (*Vella* et al., 2008).

IEC EXOSOMES AND TOLERANCE

Oral administration of an antigen gives rise to a generation of $CD4^+$ T cells that down regulate the immune response and induce tolerance to the antigen (*Chen* et al., 1994; *Groux* et al., 1997; *Karlsson* et al., 1999). The mechanisms behind oral tolerance and the induction of these regulatory CD4+ T cells remain largely unknown. We have shown that one possible route for tolerance is via exosomes produced by intestinal epithelial cells (tolerosomes) (*Karlsson* et al., 2000, 2001, 2002). The initial step in this process is active sampling by the small intestinal epithelial cells of the luminal content at the mucosal surface. The antigen is processed and peptides are loaded on MHC class II molecules, which are constitutively present in IECs (*Lin* et al., 2005). Exosomes, carrying MHC class II-peptide complexes, are formed and released at the basolateral side of the IEC (*Karlsson* 2001). We believe that these exosomes are transported across the endothelium, enters the circulation and quite rapidly reaches the liver. This is supported by the fact that we can isolate exosomes from serum expressing the A33 molecule (*Ostman* et al., 2005). The liver is important for oral tolerance induction since it is the major draining site of the intestinal circulation. It contains APC's that effectively clears particulate matter of similar size as the exosomes (≈40nm) from the blood (*Matsuno* et al., 1996; *Willekens* et al., 2005). In addition it has previously been reported that portal drainage through the liver is a prerequisite for establishing this form of tolerance (*Callery* et al., 1989; *Yang* et al., 1994). The environment in the liver is naturally very tolerogenic with high levels of TGF-β and IL-10 (*Knolle* and *Gerken*, 2000), and the message sent as a consequence of the processing and presentation of antigen-loaded exosomes by liver APC's is therefore

likely to be one of tolerance induction. The presentation by APC's to naïve T cells, resulting in an increased population of induced antigen-specific regulatory T cells, is likely to occur in the liver or in the liver draining lymph nodes, due to migrating APC's. This theory is supported by a previous study, which shows that after oral administration of an antigen there is an induction of regulatory T cells in the liver draining lymph node (*Hultkrantz* et al., 2005). The same study shows that the celiac lymph nodes draining the liver rapidly become engaged in the response to fed antigens and that the T-cells become activated within 6h and later develop into a distinct antigen specific T-cell population with a regulatory phenotype and a suppressive function (*Hultkrantz* et al., 2005). These results have been confirmed in a recent study which also shows that regulatory, Foxp3 expressing, T cells are induced in the liver draining lymph nodes after feeding an antigen orally (*Siewert* et al., 2008). This strengthens the idea of a central role for the celiac lymph node (CLN) in tolerance induced by feeding an antigen, and suggests that CLN function as a "boot camp for regulatory T cells". The possible fate of exosomes/tolerosomes and their involvement in tolerance induction is overviewed in Figure 3.

As previously mentioned we have shown that exosomes can be isolated from serum 1h after an antigen feed and transfer antigen specific tolerance when injected into naïve recipients (*Almqvist* et al., 2008; *Karlsson* et al., 2001). The recipient animals were protected against both Th1 and Th2 dominated responses. Moreover we have shown that exosome-mediated tolerance is MHC class II dependent and requires an intact immune system in the fed donor (*Ostman* et al., 2005). Germ-free mice lack MHC II-expression in the small intestinal epithelium, which results in the formation of non-informative exosomes that without MHC class II lack antigen presenting capacity, and failure to induce regulatory T cells after oral antigen administration (*Rask* et al., 2005). A full flora generally provides the required stimuli for the maturation of the intestinal immune system and the intestinal epithelial cells, but it is not known which individual bacteria or bacterial products that delivers the necessary signals. A collaborating group investigated whether neonatal mucosal exposure to SEA could influence the capacity to develop oral tolerance and reduce sensitisation and allergy. Their results show that SEA pretreated mice are more efficiently tolerised by OVA feeding. This suggests that strong T cell activation in infancy promotes the development of oral tolerance (*Lönnqvist* et al., unpublished data). We have examined the role of mucosal exposure to *S. aureus* enterotoxin A regarding the capacity of tolerogenic processing by the intestinal epithelium in adult mice. Our results indicates that the *S. aureus* enterotoxin A potentiates the development of oral tolerance, and we show for the first time that this effect can be transferred to naive recipient mice by the adoptive transfer of serum. Our results suggest that the exosome fraction produced by SEA-exposed epithelium more efficiently modulates the immune system into a tolerogenic response to a fed antigen (*Hultkrantz et al*., unpublished data). In conclusion, bacterial stimuli are important both for the tolerogenic processing and the development of oral tolerance.

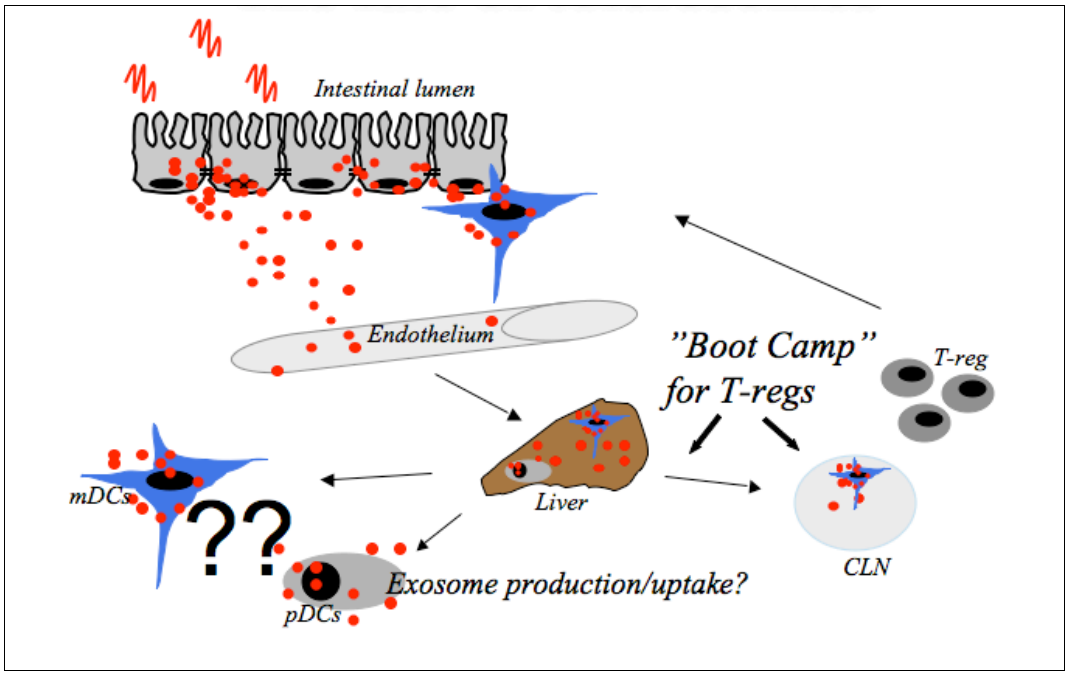

Figure 3: The fate of tolerosomes. The Intestinal epithelial cells release exosomes at the basolateral side. The exosomes are transported across the endothelium and travels with the blood to the liver were they are taken up and processed by local APCs. The APCs present the exosome-message in a tolerogenic milieu resulting in elevted numbers of regulatory T cells.

CONCLUDING REMARKS

It is clear that the functions of exosomes are many and their complete biological role is yet to be understood. What can be concluded so far is that exosomes are important players for passing on immunological information between cells and thus take active part in immune regulation. They are released by many different cell types including professional APC's and the epithelial cells lining the major mucosal interface to the environment, and found in various body fluids. Exosomes can be produced and manipulated *in vitro* and safely administered to patients. This makes them highly interesting as therapeutic targets e.g. as vaccine vehicles in cancer. Their tolerogenic capacity could further be exploited in autoimmune and allergic diseases.

ACKNOWLEDGEMENTS

This work has been supported by the Swedish Medical Research Council (Grant nr. 20066096) and Ernst Collianders foundation.

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EARLY ANTIGEN EXPERIENCE SHAPES THE MUCOSAL T CELL REPERTOIRE

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SUMMARY

It is becoming ever more apparent that influences very early in life mould the individual and immune responsiveness is no exception. This short review summarizes the evidence for mechanisms which are likely to have an impact during foetal and neonatal life on the responsiveness of mucosal T cells in the intestine of the adult. These neonatal mechanisms are important because they influence disease susceptibility in later life. It is concluded that the commensal microflora has the major impact on mucosal T cell development, but that there are also influences from food antigens and from developmental aspects of the antigen presenting machinery. Finally – or first – maternal antigen experience probably has a long-lasting influence on the mucosal T cell repertoire.

INTRODUCTION

Despite the obvious importance of the mucosal immune system of the intestine during the first vulnerable days and weeks of life, we understand remarkably little about how the environment – be it the relatively protected foetal environment, or the subsequent exposure to commensals and pathogens after birth – affect our ability to survive this susceptible period or regulate responses to challenges by food and microbial antigens later in life.

If we understand these early mechanisms, we can then design strategies for intervention – either prophylactic through neonatal vaccination, or therapeutic through targeted pharmaceutics. Given the huge interest over recent years in the Hygiene Hypothesis and in possible links between environment and early immune development and the increasing prevalence of immune hypersensitivity diseases – asthma, food allergies – an understanding of cellular and molecular mechanisms during immune development may guide recommendations for social change and dietary habits.

This brief review has three aims: to provide a synthesis of the current state of the art in the area; to stimulate new work in this important area; and to put forward a hypothesis for discussion.

The first statement that must be made is that there are considerable inter-species variations in immune development during foetal and neonatal life, so that although we know some-

thing about relevant parameters in rodents and man, and to some extent in the pig, the strategies for developing an effective mucosal immune system have been driven through evolution by environmental pressures. Assumptions cannot be made across species and models for the human system – and the questions asked of these models – must be appropriate to the system under study. Factors such as placental structure; permeability of the neonatal epithelial barrier; length of gestation; and environmental niche add largely unknown levels of complexity when we start to define factors shaping the immune repertoire.

In our own studies, we are interested primarily in the mechanisms involved in regulating the antigen specificity repertoire of human mucosal T cells. To some extent we are able to make descriptive studies of gut mucosal T cell phenotype and receptor sequence, but even this is difficult because of obvious and necessary ethical constraints. To draw mechanistic conclusions from these observations challenges probabilities because of low numbers of observations. We have therefore chosen the mouse as a model system in which to probe more mechanistic questions about the nature of the environmental antigens which might impact neonatal T cell development and T cell receptor (TCR) repertoire development.

DISCUSSION

Although the gut of the young neonate is protected by exogenous innate factors in the mother's milk and by endogenous innate cells and antimicrobial peptides (*Newburg* and *Walker*, 2007), full protection against microbial challenge depends on the appropriate development of the adaptive immune system, in particular effector, regulatory and memory T cells. In the adult animal, these gut T cells take on a compartmentalization, with some naïve cells found within the B cell follicular structures in both small and large intestine, but with the majority of gut T cells residing in the mucosal lamina propria (LPL) and epithelial (IEL) compartment, with a mostly activated or memory phenotype. While IEL comprise a complex mixture of phenotypes, with both α/β and γ/δ T cell receptors (TCR) represented and with variable proportions of cells bearing α/β and α/α CD8 co-receptors (*Hayday*, 2001), lamina propria T cells are mostly α/β TCR $CD4^+$. While these are mostly of effector/memory phenotype, they also contain significant numbers of regulatory T cells, likely to be involved in suppressing responses to autoantigens and inappropriate responses to dietary and commensal antigens (*Izcue* and *Powrie*, 2008).

These effector/memory T cells protect the adult gut from pathogens. They are activated by interaction with antigen-bearing dendritic cells in the draining mesenteric lymph nodes. This causes a change in membrane phenotype from the CD62L (L-selectin)$^+$, $CCR7^+$, LFA-1^+, $CD45RA^+$ naïve phenotype to the $CD62L^{lo\ /\ neg}$, $CCR9^+$, $\alpha4\beta7^+$ or $\alpha E\beta7^+$, $CD45RO^+$ activated/memory phenotype. These preferentially home to mucosal sites through $\alpha4\beta7$ integrin-MadCAM-1 interactions and, within the intestine, are likely to seed the initial site of antigen challenge through receptors for tissue-specific chemokines (CCR9 inducing homing to CCL25 in the small intestine and CCL10 to CCL28 in the large intestine [*Johansson-Lindbom*

and *Agace*, 2007]). The intestine-homing properties of these effector/memory cells are imprinted by the gut-derived dendritic cells, through mechanisms involving IL-4 and vitamin A-derived retinoic acid (*Iwata* et al., 2004; *Elgueta* et al., 2008).

The diversity of antigenic epitopes, which can be recognised by the antigen receptors of the T cell pool within an individual, is generated through a combination of developmental processes that occur predominantly (but see below) in the thymus. Thus, the germline-encoded V, D and J segments of the TCR genes recombine randomly during development to yield highly diverse mRNA sequences encoding the three complementarily determining regions (CDR) of the V domain of α, β, γ or δ TCR chains. The diversity is particularly focussed on the central CDR3 regions of β and δ chains through recombination with D gene segments (which do not exist within α or γ chain genes) and through the addition of N and P nucleotides at the VD and DJ junctional regions. Through these random events there is a high probability that the antigen specificity of T cell clones within the numerically stable adult pool will be highly diverse and that the pool will, therefore, be polyclonal in specificity. In the peripheral adult naïve T cell pool this is, in fact, the case and this permits responsiveness by an individual to a wide range of antigens. During adult life, however, it is likely that, through antigen exposure, the tissue-seeded segments of the T cell pool will undergo clonal expansion through generation of memory cells to useful, pathogenic, specificities, and possible clonal deletion through induced apoptosis of self-reacting clones. The circulating, naïve segment, however, will remain polyclonal, retaining the ability to respond to a wide range of pathogenic epitopes. With age, then, one would predict that the T cell pool in tissues experiencing environmental antigen would become biased and oligoclonal.

Before the detailed mechanisms of activation and homing of T cells to the gut were defined, it was predicted that the TCR repertoire of gut mucosal T cells would be polyclonal in response to the wide array of antigens within the gut environment. However, numerous studies over the past few years have demonstrated in rodents and in man that the adult gut T cell repertoire is, in fact, remarkably restricted (*Blumberg* et al., 1993; *Holtmeier* et al., 1997; *Regnault* et al., 1996) reviewed by *Probert* et al. (2007a). Localized homing of particular clones seems unlikely as clones with shared specificity have been described in different regions of the intestine (*Gross* et al., 1994), so it is probable that equivalent seeding of clones takes place after activation in the nodes, followed by local expansion in the tissues. Thus, it is clear that at least from puberty onwards human gut intraepithelial and lamina propria, αβ and γδ T cells are oligoclonal.

The question is: How early is this oligoclonality established and what drives the restriction of the TCR repertoire?

In the human foetus, T cells develop in the epithelium and lamina propria of the gut during the second trimester of gestation and increase between weeks 11 and 19 of gestation (*Spencer* et al., 1986). They express an essentially polyclonal Vβ TCR repertoire (*Thomas* et al., 1996; *Koningsberger* et al., 1997). These cells have an activated phenotype, but do not express α4β7 integrin (*Howie* et al., 1998), so it seems unlikely that they are activated in the periphery and home to the intestine, as in the adult, although they could be activated in situ in response to maternally-derived antigens

(but see dendritic cells, below). More recently, a population of $CD3^{-}7^{+}$ cells has been described as early as seven weeks gestation in human foetal gut, which could represent precursors of T cells and, moreover, these cells have the potential to develop ex vivo into $CD3^{+}$ cells (*Gunther* et al., 2005). Thus, human foetal gut T cells could be generated in situ and potentially provide a polyclonal effector population intact to deal with postnatal challenge, although the mechanisms of their in situ activation remains unknown and distinction between these T cell precursors and immature NK cells was not made. This does suggest, however, that the postnatal human gut is already primed with effector T cells with polyclonal receptors, but that these have not been driven by bacterial antigens other than those experienced by and processed by the mother. The observation of pre-Tα chain (pTα) expression in the foetal gut (*Howie* et al., 1998) suggests that these precursors may be rearranging their receptors extrathymically.

In the immediate postnatal period, the human infant gut (small and large intestine) is populated by T cells with polyclonal Vβ receptors and there are multiple populations of cells with phenotypes resembling thymic precursors: $CD3^{+}4^{+}$; $CD3^{+}8^{+}$; $CD3^{+}4^{+}8^{+}$; $CD3^{+}4^{-}8^{-}$, together with expression of pTα, TdT and Rag, suggesting that all the machinery for extrathymic rearrangement of TCR genes is present in the mucosa after birth as well as in the foetus (*Williams* et al., 2004). The "thymic precursor" phenotypes persist in some individuals up to nine months of age. The T cell precursor phenotype - $CD3^{-}7^{+}$ - described by *Gunther* et al. (2005) is also present in these postnatal samples and the co-expression of CD2 suggests that these are closer to T cell lineage than NK lineage. At least in the relatively small group that we investigated, our evidence suggests that by 18 months of age the rearrangement machinery becomes diluted and the TCR repertoire (determined by sequencing) becomes more oligoclonal, approaching the adult gut picture. However, although restriction of the repertoire was noted in two individuals between 6-month samples, and 9-month samples, there was variation between samples and some samples at 18 months were still relatively polyclonal (*Williams* et al., 2004). Of further interest, in one six-day-old individual with a sterile duplication cyst parallel to the ileum, but with no luminal continuity, the repertoire was polyclonal in both sections with no shared clones, indicating antigen-independent homing to different regions of the gut. Also, in one child of 56 months who had a non-functioning colon from birth, the repertoire was not different from other individuals' colons, with a significant restriction of clonality, indicating that developing colonic flora per se was not required to restrict the repertoire – although there was no information available on development of small intestinal flora.

The evidence in human foetus and infant, then, suggests that T cell precursors are seeded into the intestine before birth by non-MadCAM-1-dependent mechanisms; that these precursors can develop into functional $CD3^{+}$ T cells with rearranged receptors and a polyclonal repertoire by birth; that in situ rearrangement continues after birth; and that these endogenously-derived T cells with a polyclonal receptor are gradually replaced over the first 18 months of life by $\alpha4\beta7^{+}$ T cells activated in the periphery to the growing intestinal challenge; that these immigrant T cells develop a restricted repertoire by expansion of clones to dominant enteric antigens.

But are these dominant antigens from the commensal flora or from

food? In order to answer this, we must turn to studies in rodent models.

In comparison to humans, T cell development in the foetal mouse gut takes place relatively late in gestation. From approximately day 18 of gestation, $CD3^+$ cells are seen associated with Peyer's patch anlagen (*Adachi* et al., 1997; *Yoshida* et al., 2001), but nothing is known of the foetal intraepithelial and lamina propria T cell compartments. We have recently characterised the postnatal mouse intestine at three time points: pre-weaning; peri-weaning; and post-weaning, in parallel in germfree and SPF mice in an attempt to separate the effects of diet (weaning) and colonization by commensal flora on intestinal T cell development (*Williams* et al., 2006). In summary, we have shown that a few days after birth, the SPF gut is populated by significant numbers of $CD3^+$ T cells, about one-third of which expresses $\alpha4\beta7$ integrin, and as the numbers of these cells increases in small and large intestine, the proportion of $\alpha4\beta7^+$ cells increases also. The germfree gut, however, contains very few T cells at birth and numbers do not increase significantly through weaning, but all germfree gut T cells are $\alpha4\beta7^+$ throughout. Significantly, immediately after birth germfree and SPF guts contain similar numbers of $CD3^+$, $\alpha4\beta7^+$ T cells. These are presumably T cells that have seeded the gut during foetal life. In the absence of flora, this foetus-derived population expresses L-selectin, a marker of naivety, and the population does not change postnatally. However, in SPF mice, the foetus-derived cells do not express L-selectin, suggesting they are antigen-experienced, and they are gradually overlaid by further antigen-experienced T cells. Administration of a commensal flora to germfree mice causes an influx of $CD3^+\beta7$ T cells into the small intestine after weaning, but these cells express L-selectin and so are presumably not activated to commensal antigens. This may indicate that the combination of food and incomplete microflora leads to homing of cells to the mucosa, but that these are not conventionally activated in the absence of an unknown microflora species. Mucosal $CD11c^+$ dendritic cell maturation was defective in the germfree intestine, with development delayed until just before weaning. This could explain the reduced activation status of those T cells infiltrating the colonized germfree intestine.

These studies suggest that, although foetal intestinal T cell development is delayed in the mouse compared to the human, mucosal T cells are transferred from the foetus to the neonate in the same way. If these cells are transferred from foetal to neonatal gut to protect the neonate from pathogenic antigens, it seems likely that the repertoire of these early cells is established during foetal life under the influence of maternal antigen experience – in those neonates derived from germfree dams, the starting population of gut T cells has a naïve phenotype. However, the protective nature of this population of early gut T cells is an assumption. Without functional and further phenotypic data, these cells could equally be regulatory cells, helping to shape the neonatal T cell repertoire, based on maternal antigen experience. Adoptive transfer of gut cells from germfree or SPF foetuses into TCR transgenic recipients will address this question.

With regard to the intestinal TCR repertoire, as in humans, the adult gut T cell repertoire of mouse and rats is oligoclonal (*Regnault* et al., 1996; *Edwards* et al., 2008). Interestingly, the recent comprehensive study of TCR V-segment usage in the adult rat by *Edwards* et al. (2008), analyzing clonality in all 22 Vβ families, compares the

mucosal and peripheral repertoires and clearly demonstrates restriction of the mucosal repertoire together co-existing with a polyclonal peripheral repertoire in the same individuals. Both of these studies, on inbred mice and rats, show that although the mucosal T cell repertoire is restricted in all adult individuals, the repertoire differs significantly between different inbred individuals in the same cage fed the same diet. Although this may reflect different stable flora in different individuals, it seems more likely that this represents the end stage of many activation and clonal expansion events during the lifetime development of a stable flora. The effects on the adult repertoire of introduction of a normal flora have been investigated in IEL in rats and show restriction of the germfree polyclonal repertoire after introduction of a flora at birth or weaning (*Helgeland* et al., 1996, 2004).

In the first study of the intestinal T cell TCR repertoire during the neonatal period in germfree and SPF mice (*Probert* et al., 2007b), we have analyzed Vβ clonality by spectratyping and sequencing and have confirmed concordance of the data generated by the two methods. In both the small intestine and colon of SPF mice the data show that the repertoire (total gut T cells, IEL and LPL) is polyclonal before weaning and restricted with expanded clones after weaning. In the germfree mice, there are very few clones at five days of age and thus the repertoire appears oligoclonal. By weaning, the germfree repertoire in both small intestine and colon is restricted, but showed some evidence of polyclonality in the large intestine from weaning onwards. As the SPF repertoire becomes oligoclonal at weaning, it is possibly influenced by diet, developing microflora, or both, but as food at weaning did not appear to have a marked effect on the repertoire, we suggest that the major effect on the repertoire is bacteria. On the other hand, in the absence of bacteria the few examples of polyclonality observed after weaning are presumably driven by food antigens. The contraction of the repertoire in small and large intestine occurred almost simultaneously and we observed identical clones in large and small intestine in post-wean samples, suggesting that effector/memory clones activated to bacterial antigens from one region of the intestine are then seeded throughout the intestine. With regard to our phenotyping studies, one would predict that the small population of neonatal gut $CD3^{+}\beta7^{+}$ cells derived from the foetus is oligoclonal in the germfree mouse, but may be polyclonal or shielded by a larger polyclonal population of cells in the SPF gut.

In summary, we hypothesise that the major influence on the shape of the adult gut T cell repertoire is the development of the commensal flora, either alone, or itself influenced by growing dietary complexity. This will, in turn, be influenced by those aspects of development of the individual that will affect presentation of antigen. However, it seems certain that the final repertoire of effector/memory T cells in the adult gut will, like so many other aspects of biology, be influenced by history – antigenic in this case – of the mother.

ACKNOWLEDGEMENTS

The original work quoted in this review was funded by BBSRC grant No. 7/D13513; Wellcome Trust Biomedical Research Collaboration Grant No.

AL069896; Vetenskapsrådet Grant No. K2002-06X-14233; Swedish Fund for Strategic Research Mucosal Immunology & Vaccine Research Centre of Gothenburg University; and the Grant Agency of the Czech Republic (Grant No. 303/06/0974).

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PROBIOTICS AND BACTERIAL COMPONENTS IN INTESTINAL INFLAMMATION THERAPY AND PREVENTION

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SUMMARY

Crohn's disease (CD) and ulcerative Colitis (UC), the two major forms of inflammatory bowel disease (IBD), are both severe chronic inflammatory disorders. Although the exact etiology and pathogenesis of both forms of IBD have yet to be completely understood, it is widely accepted that they result from a continuous microbial antigenic stimulation of pathogenic immune response in genetically predisposed individuals. Genome-wide association studies identified several defects in genes responsible for mucosal barrier function, bacterial sensing and killing and for the regulation of the inflammatory response. The changes in microbiota composition or abilities (epithelial adhesion and invasion) are supposed to be the trigger of the inflammation. These findings are further supported by conclusions of studies in both humans and experimental animals. Those studies showed that impaired host reaction to commensal microbiota, or their abundant presence in the subepithelial layer, leads to the pathological stimulation of the mucosal immune system.

Several clinical studies as well as some interventional studies on animal models demonstrated that antibiotics, probiotics and bacterial components are useful in maintaining disease remission and in disease prevention. This effect is partially due to the changes in microbiota composition and partially due to imunomodulation.

During many years of co-evolution with humans, the microbiota, indigenous as well as pathogenic, have acquired immunomodulatory mechanisms to bypass our mechanisms of protective immunity. Our aim is to isolate the immunomodulatory components from bacteria and use them in IBD therapy and prevention. Compared to use of live bacteria, this approach seems to be safer and easily applicable in practice. The differences in immunomodulatory properties of these components also suggest the need of individualized therapy. This review will focus on IBD pathogenesis and the possibility of influencing it by therapy with bacterial components.

INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis, are both severe relapsing inflammatory diseases of the intestine, each associated with a typical phenotype. Ulcerative colitis is characterized by diffuse continuous mucosal inflammation that extends proximally from the rectum to a varying degree. Although it is located only in the large intestine, in some patients, the terminal ileum is also affected (so-called backwash ileitis). Patients typically suffer from bloody diarrhea, abdominal pain, rectal bleeding and malnutrition. Crohn's disease is characterized by segmental and transmural inflammation, fistulas and granulomas in any part of the gastrointestinal tract, most commonly in terminal ileum. Crohn's disease leads to strictures, abscesses and fistulas and the clinical manifestation depends mainly on the disease localization. The IBD usually starts to manifest in the second and third decades of life and the majority of affected individuals progress to chronic relapsing disease (*Baumgart* and *Sandborn*, 2007).

The IBD is a systemic disorder and in almost half of the patients other organs are affected by the disease as the extraintestinal disease manifestation or disease (therapy) complications.

These manifestations and complications typically affect the musculoskeletal system (peripheral arthritis, ankylosing spondylitis and osteoporosis), skin (erythema nodosum, pyoderma gangrenosum and fistulas), eye (iritis and uveitis) and biliary system (primary sclerotising cholangiopathy and gall stones) (*Rothfuss* et al., 2006).

The highest prevalence of IBD is traditionally in North America, northern Europe and the United Kingdom, with averages ranging from 100 to 300 cases per 100 000 (*Ehlin* et al., 2003; *Loftus* et al, 2004). Recent epidemiological studies suggest that the increase in disease incidence has probably reached its plateau in these countries, however, there is a strong increase in IBD incidence in regions with traditionally low IBD prevalence (e.g. Asia and Latin America) (*Linares de la Cal* et al., 1999; *Yang* et al, 2000; *Lee* et al., 2000; *Yang* et al., 2008).

Although the etiopathogenesis of the IBD remains obscure, IBD is thought to be a result of uncontrolled inflammatory response to indigenous intestinal microbiota in genetically predisposed individuals. The genetic predisposition is associated with genes related to host bacteria interaction, suggesting the crucial importance of intestinal microbiota in IBD pathogenesis. General intestinal dysbiosis, presence of some pathogenic bacteria or even enhanced virulence of certain commensal bacteria (*Chiodini et al.*, 1989; *Darfeuille-Michaud* et al., 1998; *Swidsinski* et al., 2002; *Seksik* et al., 2003; *Frank* et al., 2007; *Rabizadeh* et al., 2007) were all proposed to be the trigger of the IBD. The importance of intestinal microecology is further supported by the finding that manipulating intestinal microbiota using probiotics and antibiotics is effective in IBD therapy. (*Guslandi* et al., 2000; *Kruis* et al., 2004; *Rahimi* et al., 2007)

Some strains of probiotic bacteria also possess immunomodulatory properties, so their effect in IBD therapy could be mediated by modulation of the mucosal immune system or intestinal barrier function, rather than by intestinal ecology changes. (*Damaskos* and *Kolios*, 2008) Therefore, we could use bacterial lysates or isolated bacterial components to mimic the therapeutic

effect that probiotics have. Use of sterile bacterial components with immunomodulatory properties seems to be a safer and more practical approach than the use of live bacteria.

IBD PATHOGENESIS

The luminal antigens can initiate the pathogenetic inflammatory cascade in the gut only after four conditions are met. First, the host's mucosal immune system must be genetically susceptible to recognize the antigens from indigenous microbiota and misinterpret them as potentially harmful. Second, the antigen must reach the gastrointestinal tract. Third, the antigen must pass through the intestinal barrier to reach the immunocompetent cells in the mucosa. And finally, the regulatory mechanisms of mucosal immune system must fail to control the inflammation.

Genetics

Family aggregation of IBD is a well known phenomenon; the life-time risk of developing IBD for first-degree relatives of a CD proband is 5 % and that of an UC proband 1.6% in white non-Jewish Euro-American population (8 % and 5.2% for Jews). The concordance in monozygotic twins is 28% and 16% for CD and UC respectively, and 4% for dizygotic twins for each disease (*Halfvarson* et al., 2003; *Halme* et al., 2006). On one hand, these data clearly indicate that genetic factors definitely contribute to IBD, but also that the environmental and developmental factors are more important in disease pathogenesis.

Genome-wide association studies for IBD susceptibility genes performed in the last few years have reported many genes that contribute to disease susceptibility. Interestingly, most of these newly identified genes are related to the epithelial barrier permeability, bacteria sensing and killing, and regulation of the immune system. (*Duerr* et al., 2006; *Rioux* et al., 2007) All these findings fit well into the current concept of IBD pathogenesis and stress the importance of host-microbiota interaction.

Microbiota

Humans are colonized by huge number of microbes; microbial cells form 90% (10^{14}) of all cells in adult humans (*Savage*, 1977). This colonization starts during the birth and continue during the first few years of life. After this period, the composition of the individual microbiota seems to be relatively stable. Although there are some representatives from archaea and eukarya, as well as viruses and bacteriophages, most members of gut microorganisms belong to the domain bacteria. (*Eckburg* et al., 2003; *Breitbart* et al., 2003; *Curtis* and *Sloan*, 2004). Over 98% of all gut bacteria in mammals belong to the two divisions of Bacteria - Firmicutes and Bacteroidetes. (*Eckburg* et al., 2005). Although this uniformity probably reflects the specific conditions in the gut, the differences on a species level is striking, as it resembles an individual fingerprint. The diversity of individual microbiota is caused by many variables such as dietary habits, use of pharmaceuticals, health status and by microbial exposure of both mother and child during the child's perinatal and infant period. Introduction of probiotic bacteria during this period could result in long term colonisation and could have impact on health later in adult life (*Kalliomäki* et al., 2001; *Lodinová-Zádníková* et al., 2003).

Although the microbiota composition has a distinctive pattern in every individual, under physiological conditions it serves important biological functions for all hosts. The microbiota prevent colonization with pathogens, providing important nutrients (vitamins and short-chain fatty acids), and modulate intestinal barrier maturation and development of the immune system (*Chapman* 2001; *Hooper* et al., 2002; *Tlaskalová-Hogenová* et al., 2004). Luminal microbiota also positively influence the development and preservation of oral tolerance in a strain-dependent manner (*Gaboriau-Routhiau and Moreau*, 1996; *Moreau and Gaboriau-Routhiau*, 1996; *Prioult* et al., 2003).

The close link of intestinal inflammation to microbiota was proposed many years ago and later proven with animal models of IBD, which showed that intestinal inflammation is much milder or even fails to develop, if animals are reared under germ-free conditions (*Sellon* et al., 1998; *Hudcovic* et al., 2001; *Stepankova* et al., 2007). These findings led to closer investigation of microbiota composition in IBD patients. Many studies initially focused on searching an individual pathogen responsible for IBD. The pathological similarity of Johne's disease in cattle to Crohn's disease in humans led to a proposition that *Mycobacterium avium* subsp. paratuberculosis (MAP) is the causative agent of IBD. This hypothesis was supported by finding of MAP in inflamed tissue of CD patients (*Chiodini*, 1984; *Sanderson* et al., 1992; *Fidler* et al., 1994). Although there were reservations about these studies, recent meta-analysis confirmed specific association of MAP with CD (*Feller* et al., 2007). The controlled trials for the therapy of CD with antimycobacterial drugs failed, so the question how this bacterium could influence the IBD pathogenesis remains unclear (*Goodgame* et al., 2001; *Selby* et al., 2007).

Several ecological studies of gut microbiota showed that there is a difference in microbiota composition in IBD patients compared to healthy individuals. This dysbiosis or increase in some bacterial group was proposed to cause or at least perpetuate the intestinal inflammation in IBD. These studies also identified some candidate bacteria that could be responsible for the IBD development in susceptible individuals (*Seksik* et al., 2003; *Sokol* et al., 2006). All these studies must be interpreted with caution, because the real trigger might be only transient and changes we are detecting are just secondary.

There are also changes in mucosa-associated bacteria, suggesting the increased ability of bacteria to adhere to mucosa or some other changes in the bacterial metabolism could be responsible for IBD triggering (*Darfeuille-Michaud* et al., 1998; *Swidsinski* et al., 2002) To date, the question remains open whether these bacteria are introduced into the gut from the outside environment, or whether this new ability is introduced to intestinal microbial society by horizontal gene transfer or by an other, yet unknown, signal.

Although we do not completely understand the natural relations between intestinal microbiota, the success of antibiotics and probiotics in IBD therapy clearly shows, that manipulation with intestinal microecology might be the future treatment of at least some forms of IBD.

Intestinal barrier function

Intestinal barrier prevents viable enteric bacteria from excessive interaction with lamina propria immune cells. This barrier is formed by several components including mucus layer, epithelial cells, tight junction, mucosa associ-

ated lymphoid tissue, SIgA and it is an incredibly dynamic and actively regulated apparatus.

Several studies reported that the intestinal permeability is increased in inflamed as well as in noninflamed IBD mucosa of patients and even in first degree relatives of CD patients (*Jenkins* et al., 1988, *Katz* et al., 1989). These findings clearly demonstrate the importance of the intestinal barrier function, and its genetic control, in IBD pathogenesis. Increased intestinal permeability also has been shown useful in the prediction of relapse in asymptomatic CD patients (*Wyatt* et al., 1993; *D'Incà* et al., 1999).

Several mechanisms might be involved in increased gut permeability. First, there is a defect in mucous production in IBD patients (*Buisine* et al., 2001). Mucus forms a rather thick layer (approximately 100 μm in jejunum and over 800 μm in colon) on the gut epithelium (*Atuma* et al., 2001). This layer acts as a mechanical and antimicrobial barrier protecting the underlying epithelium. In healthy individuals, this layer contains high concentrations of secreted IgA, lysozyme and other antimicrobial components keeping the epithelial surface free of bacteria.

Defensins, antimicrobial peptides produced by the Paneth cells in the base of the Lieberkühn's crypts, are concentrated in the mucus layer protecting vulnerable epithelium from invasive bacteria, yet allowing the presence of harmless enteric microbiota (*Meyer-Hoffert et al.*, 2008). The production of defensins is, however, significantly decreased in terminal ileum of CD patients, which may result in aberrant ileum colonisation causing the inflammation (*Wehkamp* et al., 2004; *Wehkamp* et al., 2007).

Another mechanism, capable to increase the intestinal permeability is a downregulation of tight (ZO-1 and occludin) and addherens (E-cadherin and α-catenin) junctions' proteins in the epithelium of IBD patients. The degree of this downregulation positively correlates with degree of inflammation, showing the importance of these proteins for intestinal epithelium integrity. (*Gassler* et al., 2001).

Another explanation for the increased intestinal permeability in IBD is increase of extracellular matrix degrading endopeptidases - matrix metalloproteinases (MMP). These enzymes were found upregulated in the inflamed gut tissue of IBD patients causing mucosal degradation and ulceration (*Heuschkel* et al., 2000). They are produced by activated gut myofibroblasts, macrophages and resident plasma cells in the presence of proinflammatory cytokines IL-1β and TNF-α (*Okuno* et al., 2002; *Gordon* et al., 2008).

Although the defect in intestinal barrier function could be the initial defect in IBD pathogenesis, the production of TNF-α and IFN-γ secondary to the inflammation perpetuates the increased intestinal permeability by reorganizing the tight junction, causing further leakage of luminal content to submucosa (*Ma* et al., 2005; *Wang* et al., 2005). This way, the vicious circle of inflammation is created.

Mucosal immune system and its regulation

In healthy subjects, there is an immunological tolerance to intestinal flora from autologous but not heterologous intestine. This tolerance is, however, broken during intestinal inflammation (*Duchmann* et al., 1995).

There is an increase of activated macrophages and dendritic cells (DCs) and changes in cytokine production in the intestinal mucosa of IBD patients. The cytokine profiles are, however, unique for each form of IBD. While proinflammatory cytokines TNF-α, IL-

1β and IL-6 are increased in both forms, the increase of IFN-γ, IL-12, IL-17, IL-23 and IL-27 are specific for CD and increase of IL-5 and IL-13 for UC (*Fujino* et al., 2003; *Fuss* et al., 2004; *Schmidt* et al., 2005). On the other hand, the deficiency in IL-10 and TGF-β signaling in the intestine might contribute to the development of IBD, because both cytokines are important in directing naïve T cell maturation to a regulatory pathway (*Hahm* et al., 2001). Moreover, local delivery of IL-10 with genetically engeneered bacteria, *Lactococus lactis* producing IL-10, shown good results in experimental colitis therapy (*Steidler* et al., 2000).

Even under normal physiological condition, the microbes in the intestine make contact with the host's immune system without inducing inflammation. It has been shown, that DCs in the lamina propria actively sample the gut lumen, but also there is active intake of IgA coated bacteria to the Peyer's patches through the M cells (*Rescigno* et al., 2001; *Mantis* et al., 2002).

There is also evidence that live commensal bacteria are shuttled by DC through the Payer's patches to mesenterial lymph nodes causing specific mucosal, but not systemic IgA response (*Macpherson* and *Uhr*, 2004).

All these observations shows that at least some intestinal microbes make contact with the mucosal immune system, but also raise the question of why this contact does not cause the inflammation as the loss of the intestinal barrier does. The answer is not known but it is thought, that the amount of antigen is too small to trigger the inflammation or it is well "guarded" by the unresponsive innate immunity cells. Once there is a big load of bacteria in the mucosa due to the barrier failure or the cells are more susceptible to the inflammatory response due to the defect in bacteria sensing, the mucosal immune cells overcome their unresponsivenes and the inflammation starts. Three immune mechanisms are involved in this process: defect in microbe sensing by the resident mucosal cells, the accumulation of the effector cell in the mucosa and a loss of the local tolerogenic signals.

Microbe sensing by the resident mucosal cells

Luminal antigens are continuously sampled by intestinal epithelium as well as by cells of innate immunity such as DCs using several evolutionarily conserved and structurally related receptors, pattern recognition receptors (PRR). These receptors could be membrane-bound (e.g. Toll like receptor (TLR) 1, 2, 4, 5, 6, 10 or membrane bound CD14), residing in the cytoplasm (e.g. nucleotide-binding oligomerization domain containing (NOD) 2 protein, TLR 3, 7, 8 and 9) or even released from the cell (e.g. mannan-binding lectin or soluble CD14). These receptors are recognizing conserved structural motives on microbiota or microbe-associated molecular patterns such as lipopolysaccharide, peptidoglycan, lipoteichoic acide, single- and double-stranded RNA and methylated DNA (CpG-motives).

Interestingly, epithelial cells are also expressing PRRs and are able to be activated in response to microbes and produce cytokines. This way, the epithelium could deliver the inflammatory signals to underlying cells in lamina propria. In normal gut, the epithelial cells are activated by invading pathogens yet maintain the tolerance to resident bacteria (*Duchmann* et al., 1995).

Antigen-presenting cells (APCs; e.g. DCs and macrophages) are found in a resting (inactive) state in lamina propria and Peyer's patches of normal gut. In this state the cells do not respond to

bacterial stimuli and pro-inflammatory cytokines (*Smythies* et al., 2005). They became activated with conserved structural motives, they migrate to the local lymph nodes and activate naive T cells. They also contribute to the T cell activation with production of IL-6, IL-12, IL-23 and TGF-β (*Drakes* et al., 2005). Interestingly, the expression of some of some PRR is dysregulated in the intestines of CD and UC patients. This may result in easier triggering of the inflammatory cascade in these individuals (*Frolova* et al., 2008). This is further stressed by the fact, that the IL-$10^{-/-}$ mice are resistant to spontaneous colitis, if they are lacking MyD88 - important adaptor protein in TLR signaling. (*Rakoff-Nahoum* et al., 2006).

Accumulation of the effector cell in the mucosa

Pro-inflammatory molecules, responsible for tissue damage during the inflammation, are produced by effector cells that have accumulated to high numbers in the inflamed mucosa. There are two main mechanisms responsible for the accumulation of effector cells in the inflamed intestine: enhanced recruitment due to the upregulation of adhesion molecules or chemokines, and by increased cell recruitment and prolonged survival caused by decreased cellular apoptosis.

High levels of inflammatory cytokines (e.g. TNF-α, IL-1β and IL-6) in the mucosa of IBD patients increase the expression of chemokines and adhesion molecules (on endothelium as well as on circulating CD^+ cells) causing circulating leucocytes to adhere to endothelium and enter to the inflamed mucosa (*Burgio* et al., 1995; *García de Tena* et al., 2006). The presence of TNF-α and IL-6 in the inflamed mucosa also mediates T-cell resistance against apoptosis causing their accumulation and further tissue damage (*Atreya* et al., 2000; reviewed in *Neurath* et al., 2001).

Loss of the local tolerogenic signals

While innate immunity is activated in both forms of IBD, the T cell response differs. In CD, there is upregulation of T_H1 (characterized by IFN-γ) and T_H17 (characterized by IL-17) pathways. The T_H1 response is inicated by IL-12 and T_H17 response is enhanced by the presence of IL-6, TGF-β and IL-23. These cytokines are produced by activated local APCs and other innate immunity cells upon bacterial colonization (*Becker* et al., 2003; *Kamada* et al., 2005). On the other hand, the UC is characterised by atypical T_H2 response, with increase IL-5 and IL-13 produced by natural killer T (NK-T) cells. These NK-T cells are stimulated by APCs bearing nonclassical MHC class molecule CD1d, which is specialised in presenting the lipids (*Fuss* et al., 2004).

In normal gut, the inflammatory response of effector T cells is regulated by T regulatory cells. To date, there are three types of T regulatory cells identified in humans. Naturally occurring T regulatory cells (T_{REG}) originate in thymus and typically express transcription factor forkhead box P3 (FoxP3) and high levels of CD25. These cells regulate the effector cells with cell-cell contact. The other two types, regulatory T cell type 1 (T_R1) and T helper type 3 (T_H3), originates in intestine and react to luminal antigens with production of IL-10 and TGF-β on bacteria or food proteins respectively. The function of T regulatory cells is under close control of APCs and local cytokine milieu as recently reviewed by *Belkaid* and *Oldenhove* (2008). Interestingly, the anti-inflammatory T_{REG} as well as pro-inflammatory T_H17 cells could be induced locally in the intestinal mucosa

from naïve T cells by TGF-β. The balance between these two functionally opposite subsets of T cells is kept with IL-6 and IL-21, because these cytokines promote the differentiation to T_H17 by blocking the expression of FoxP3 in the naïve T cells (*Fantini* et al., 2007).

PROBIOTICS AND BACTERIAL COMPONENTS IN INTESTINAL INFLAMMATION THERAPY

Recent advances in our understanding of IBD pathogenesis and mucosal immune response regulation led to a proposal of novel strategies in intestinal inflammation therapy.

As mentioned before, the indigenous microbiota is crucial for induction and perpetuation of intestinal inflammation and manipulation with microbiota composition with probiotics and antibiotics is possible approach in IBD therapy. However, each probiotic strain have unique effect on immune system, therefore deeper insight into the particular microbe-host interaction and careful choice of particular strain for particular function might be needed for successful therapy (*Maassen* et al., 2000).

Each strain could also have several mechanisms of action, interfering with one or more steps of IBD pathogenesis. Live probiotic bacteria could change composition of intestinal microbiota resulting in changes in host's sensitivity to inflammation. These changes could be mediated by simple competition with other microbes for limited resources and limited number of receptors or they could produce antimicrobial peptides or even increase the host antibody production against other microbes (*Kaila* et al., 1992; *Liévin* et al., 2000; *Hütt* et al., 2006). Moreover, probiotic *E. coli* Nissle 1917 and *Lactobacillus casei* DN-114 001 have inhibitory effect on adhesion and invasion of adherent invasive *E. coli* isolated from patients with Crohn's disease (*Boudeau* et al., 2003; *Ingrassia* et al., 2005). The mechanism of this action could also be explained by recent findings, that *L. acidophilus* secrete molecule(s) capable of downregulating expression of genes involved in attachment of enterohemorrhagic *E. coli* to gut mucosa (*Medellin-Peña* et al., 2007). This indicates that we might be able to convert adherent bacteria into non-adherent simply by administering this bacterial component to the gut. Furthermore, treatment with *L. casei* or mixture of probiotic bacteria VSL#3 results in an improvement of intestinal barrier integrity and thus prevents enteric antigens from excessive stimulation of lamina propria immune cells (*Madsen* et al., 2001; *Llopis* et al., 2005).

Several probiotics have shown immunomodulatory properties on basically all levels of regulation, including downregulation of the PRRs expression, NF-κB signaling and pro-inflammatory cytokine production (*Sturm* et al., 2005; *Matsumoto* et al., 2005; *Grabig* et al., 2006; *Sougioultzis* et al., 2006). Interestingly, some of these effects could be achieved by soluble factor produced by these bacteria or their lysates. Lysate of *Lactobacillus brevis* and *Streptococcus thermophilus* could induce apoptosis in immune cells, which could reverse the insensitivity to apoptosis of lamina propria immune cells in IBD patients (*Di Marzio* et al., 2001). Oral administration of lysate prepared from normal intestinal flora containing anaerobes reduces the severity of acute experimental colitis in mice which suggests that there might be some bacterial components with special

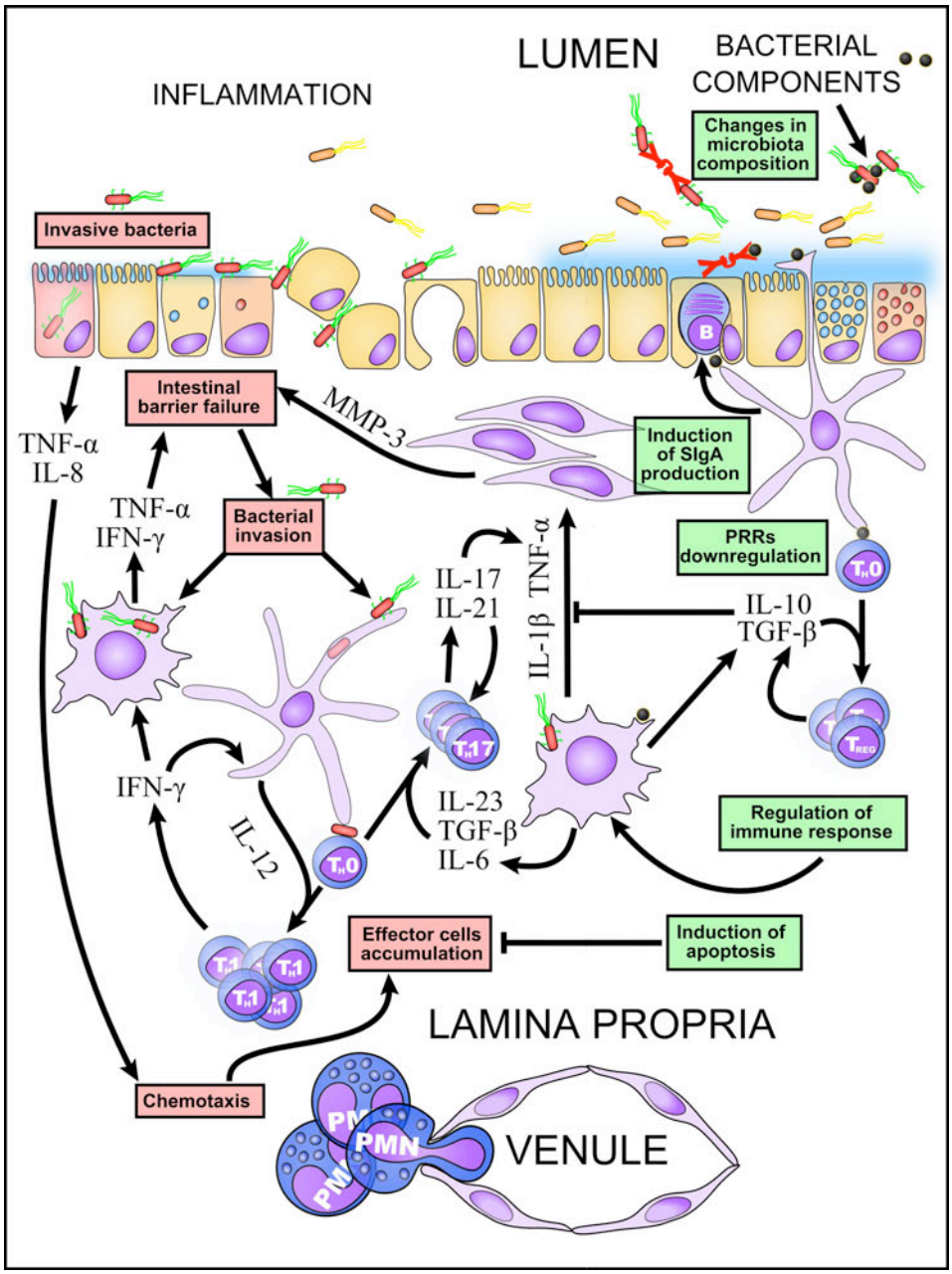

Figure 1: Simplified diagram of IBD pathogenesis summarizing the possible mechanisms by which bacterial components could beneficially influence the natural course of intestinal inflammation.

anti-inflammatory properties among commensals (*Verdù* et al., 2000).

Interestingly, also some well known pathogens could decrease the immune response by dampening inflammatory signals or inactivating immune cells which help them to evade host's immune system and establish infection or persistence (*Ruckdeschel* et al., 1997; *van der Velden* et al., 2008;

reviewed in *Sansonetti* and *Di Santo*, 2007). It would be therefore interesting to isolate the active component and to exploit it in the therapy of IBD with these components.

Several recent studies showed that TLR-9 ligand, an immunostimulatory bacterial oligonucleotide (CpG-ODN), ameliorates experimental colitis and decreases the production of proinflammatory cytokines by human colonic mucosa, but this effect is present only in certain CpG-ODN (*Rachmilewitz* et al., 2002; *Rachmilewitz* et al., 2006). Experiments with isolated bacterial DNA showed that intestinal epithelial cells respond to pathogenic bacterial DNA by increasing surface localization of TLR9 and production of IL-8, but remain unresponsive to DNA isolated from commensal or probiotic bacteria (*Ewaschuk* et al., 2007) In dextran-sulfate sodium (DSS) induced colitis, exposure to CpG-ODN during acute inflammation was found to exacerbate the disease, whereas preexposition proved to be protective (*Obermeier* et al., 2003). These results could explain the observed positive correlation between IBD and domestic hygiene in infancy, and they also suggest that CpG-ODN is a promising candidate for the maintenance therapy, but not for the therapy of active disease.

Another interesting approach is to think about bacteria that have been proposed as the IBD triggers as targets for vaccination. If we will identify the causative bacteria, than the protective immune response against this bacteria could prevent the IBD in otherwise susceptible individuals. Although *B distasonis* and mycobacteria were both proposed to be involveded in induction and perpetuation of intestinal inflammation, we found that introduction of *B. distasonis* lysate, its DNA or mycobacterial heat shock proteins by gavage led to decreased sensitivity of mouse to DSS colitis (*Kverka* et al., unpublished). We still do not know whether we induce the oral tolerance to indigenous microbiota or protective immunity against some closely related potential pathogen causing the inflammation, but this therapy promotes changes in cytokine production in the intestine. Recent findings on how could bacterial components beneficially influence the natural course of intestinal inflammation are summarized in figure 1.

It is important to mention that the reaction of the immune system to the addition of the bacterial component depends on the actual tuning of mucosal immune system. As shown on animal models the introduction of the bacterial component into the inflamed condition could lead to opposite effect.

Although there are still mysteries about the role of microbe-host interactions in IBD pathogenesis, our deeper understanding of these underlying mechanisms is important for new strategies in IBD therapy. It seems that the use of immunomodulatory properties of microbiota could be fundament for that purpose.

ACKNOWLEDGEMENTS

This work was supported by grants IBD-0159 from Broad Medical Foundation; A5020205 and S500200572 from the Academy of Sciences of Czech Republic; 310/03/H147 and 303/06/0974 from the Czech Science Foundation; The Danone Institute; and Institutional Research Concept Grant AV0Z50200510.

LITERATURE

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ANTISECRETORY FACTOR AND ITS BIOLOGICAL ACTIVITIES

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SUMMARY

The Antisecretory Factor, AF is a phylogenetically ancient, multifunctional protein that mediates clinical protection against various forms of diarrhoeal diseases and intestinal inflammation. The cDNA of AF has been cloned and sequenced, and AF antisecretory activity can be mapped to a peptide located between position 35 and 50 of the full length AF protein sequence. AF is present in all mammalian tissues investigated so far, and is secreted into blood, bile and breast milk. Many of the effects of AF observed clinically as well as experimentally seem to be nerve-mediated.

Dietary intake of Specially Processed Cereals, (SPC-flakes®), increases endogenous AF synthesis in man. Thus, positive effects of treatment with SPC-flakes® have been shown in patients suffering from inflammatory bowel disease and Ménière's disease. Intake of SPC-flakes® also prevented mastitis in lactating women. In a controlled study treatment with AF-rich egg yolk, B221® (Salovum®) was shown to reduce diarrhoeal disease in infants and children in a developing country. Recently, we have demonstrated that AF-16, i.e. a peptide representing the antisecretory/anti-inflammatory AF domain is capable of preventing neurological malfunctions by reducing an experimentally induced raise of the intracranial pressure. Together, these results advocate that the AF concept can be used clinically both by stimulation of the endogenous synthesis via intake of SPC-flakes®, and also by administration of the AF-16 peptide.

INTRODUCTION

Fluid, ions and nutrients in the gut are transported across or between the epithelial linings as the results of a most intricate action of the enteric nervous system, which include cooperation of chemoreceptors, mechanoreceptors, and thermoreceptors. Intestinal motility is also regulated by neuronal activity (*Booth*, 1992; *Cook,* 1994), which, in turn, is significantly influenced by a multitude of gastrointestinal peptides, capable of inhibiting as well as stimulating the transports of ions, nutrients and water (*Hansen and Skadhauge*, 1995; *Vagne-Descroix* et al., 1991).

By using Cholera Toxin, CT, as the intestinal secretagogue in a rat diarrhoea model we discovered an endogenous factor, a protein, of central im-

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portance for the intestinal resistance to various forms of experimental diarrhoeal diseases, but also inflammatory diseases. This protein was named Antisecretory Factor, AF. The endogenous synthesis of AF increases in several tissues after intestinal challenge with enterotoxins such as cholera toxin, *Escherichia coli* LT and *Clostridium difficile* toxin A. These findings initiated a thorough characterization of AF and evaluation of its biological actions in experimental and clinical studies (reviewed in *Lange and Lönnroth*, 2001). The present publication points out various aspects in human medicine where functional capacities of AF play an important role.

RESULTS

Chemical characterization of the AF protein and AF peptides

Extracts from rodent and pig intestinal mucosa and pituitary gland were originally used for the purification and characterization of AF. The procedures used included isoelectric focusing, gel filtration, and affinity chromatography in agarose gels. The antisecretory potency of the purified products was determined in rat jejunal ligated loops subjected to CT challenge and suitable products were used for production of rabbit polyclonal antibodies. After screening a cDNA library from human pituitary glands, cloning of AF was performed (*Johansson* et al., 1995). Sequencing of the full-length protein revealed that human AF cDNA consisted of 1309 base pairs, of which 1131 compromised the coding sequence. The human AF protein demonstrated a molecular weight of 41 kDa with an isoelectric point of 4.9. Treatment of the full-length AF with trypsin resulted in smaller peptide fragments with preserved antisecretory and anti-inflammatory effects. The shortest peptide fragment with a preserved effect was an eight amino acid long sequence, ^{35}IVCHSKTR^{42}, located in the N-terminal part of the protein (*Johansson* et al., 1997 a and b).

AF has later been shown to be a component of the proteosome and it is therefore also named S5a (*Ferell* et al., 1996). Expression of AF has been demonstrated in mammals as well as in non-mammals, and it can definitely be concluded that AF is a unique protein mediating a multitude of biological functions.

We produced polyclonal rabbit AF antibodies, and determined their attachments to different portions of the AF-molecule (Figure 1). Western blot and 2D gels showed that all of these antisera detected a single protein with a similar molecular mass and pI, while immunohistochemistry performed on various tissues resulted in an epitope-specific sub-cellular staining pattern (Jennische et al., 2006). Antisera with affinity to epitopes in the N-terminal part of the AF protein, which represent the antisecretory activity, demonstrated a more restricted localisation than antisera with an affinity to the C-terminal part that include the ubiquitin binding sites. We suggest that AF can exist in several conformational variants, probably related to functional importance which might be related to differences in redox state and/or pH in the various cellular compartments. Furthermore, chromosome 1, 19 and 23 in the human genome contain genes with a potential AF code. In mouse only one AF-gene, called rpn10, has been sequenced entirely (*Kawahara* et al., 2000).

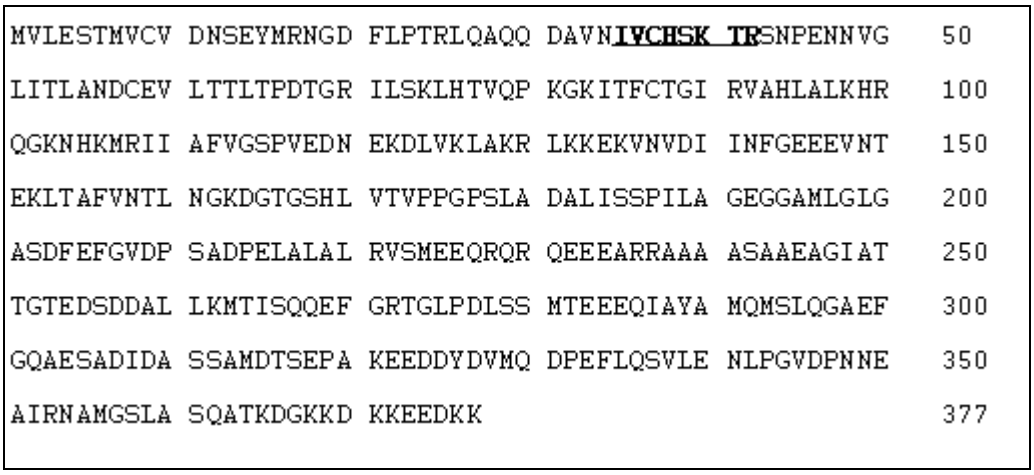

Figure 1: The amino acid sequence of AF. The antisecretory and anti-inflammatory sequence is marked with bold letters.

AF cellular distribution and expression

Immunohistochemistry and mRNA *in situ* hybridization were used to enable differentiation between AF synthesis (*in situ* hybridization) and AF uptake and storage (immunohistochemistry). AF was demonstrated in the epithelial lining of the nasal mucosa, the trachea, the bronchial tree, in the alveolar type II cells, but not in the type I or squamous alveolar cells. This suggests a role for AF in the regulation of a pulmonary surfactant. AF was distinctly present in all of the surface epithelium of the gastrointestinal canal, but also in mononuclear cells located in the lamina propria. AF probably participates in urine production since AF expression was evident in the thick ascending limb of Henle. The adenohypophysis displayed AF-positive cells, while no stained cells were seen in the pars intermedia or in the neurohypophysis (*Lange* et al., 1999). AF expression in all tissues so far examined has been restricted to a most specific and defined cell population, in most cases compatible with a postulated role of AF as a regulator of water and ion transport processes (Figure 2).

AF effects determined by *in vitro* methods

A two-chamber system separated by isolated membranes of Deiter's cells was used for studies of AF influence on the passage of Gamma-Amino-Butyric Acid (^{3}H-GABA) or ^{36}Cl- between the two compartments. AF was demonstrated to inhibit ^{3}H-GABA diffusion across the membrane in a dose dependent fashion (*Lange* et al., 1985), but we also demonstrated that 10^{-13} M of AF suppressed permeation of $^{36}Cl^{-}$ (*Lange* et al., 1987). All results consistently demonstrated an interaction between AF/^{3}H-GABA/^{36}Cl- suggesting a multiphase AF action on the membrane most probably mediated by a direct effect of AF on the Cl^{-} channels not under GABA regulation. We have extended these studies by investigating whether AF can modulate neuronal synaptic transmission in a model system using brain slices from adult rats. Extracellular recordings were performed in the CA1 region of the hippocampus, and

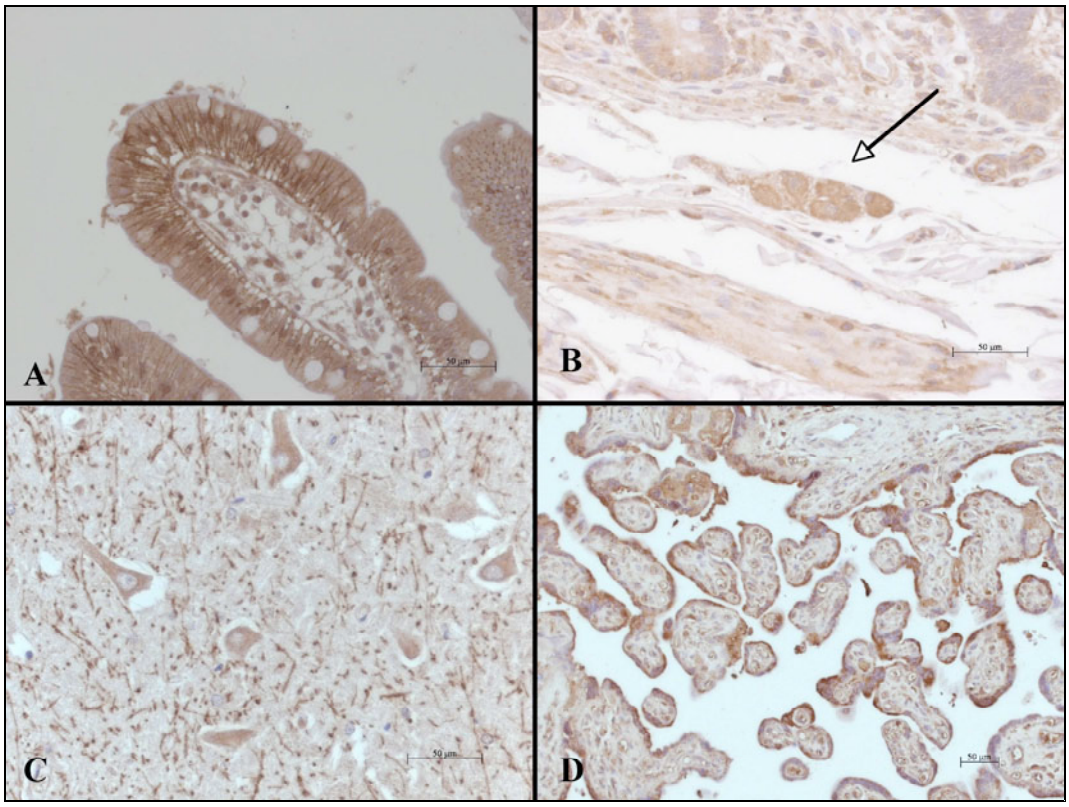

Figure 2: Paraffin sections from some human tissues processed to demonstrate AF-immunoreactivity.
Positive signal brown, nuclei are counterstained blue with hematoxylin. Bars = 50 µm
A. Small intestine. The epithelium and immune cells in lamina propria are stained. B. Nerve cells in Meissner's plexus (arrow) are stained. C. CNS. Positive staining is seen in neuronal bodies and processes. D. Placenta. The syncytiotrophoblast layer is stained.

addition of AF was followed by a 40% suppression of the $GABA_A$-mediated synaptic transmission. No influence on glutamatergic transmission could, however, be registered. A suppression of hippocampal $GABA_A$-mediated transmission (*Kim* et al., 2005) was also seen after treatment with cholera toxin per os or by feeding the animals an AF-inducing feed (SPC-flakes®). These results strongly suggest that AF acts as a neuromodulator, but also indicate that AF regulates intestinal secretion and inflammation via a neuronal gut-brain loop system.

AF effects determined by *in vivo* experimental methods

A. *Intestinal inflammation*

Clostridium difficile is the most frequently identified pathogen in patients with antibiotic-associated diarrhoea and colitis. Its pathogenicity is mediated via the two enterotoxins toxin A and toxin B, respectively (*Bartlett*, 1994). Toxin A induces increased AF synthesis (*Torres* et al., 1991), but we have also shown that AF production is stimulated in patients suffering from acute colitis of unknown aetiology (*Torres* et al., 1993). Toxin A seemed relevant to use for studies of the anti-inflammatory

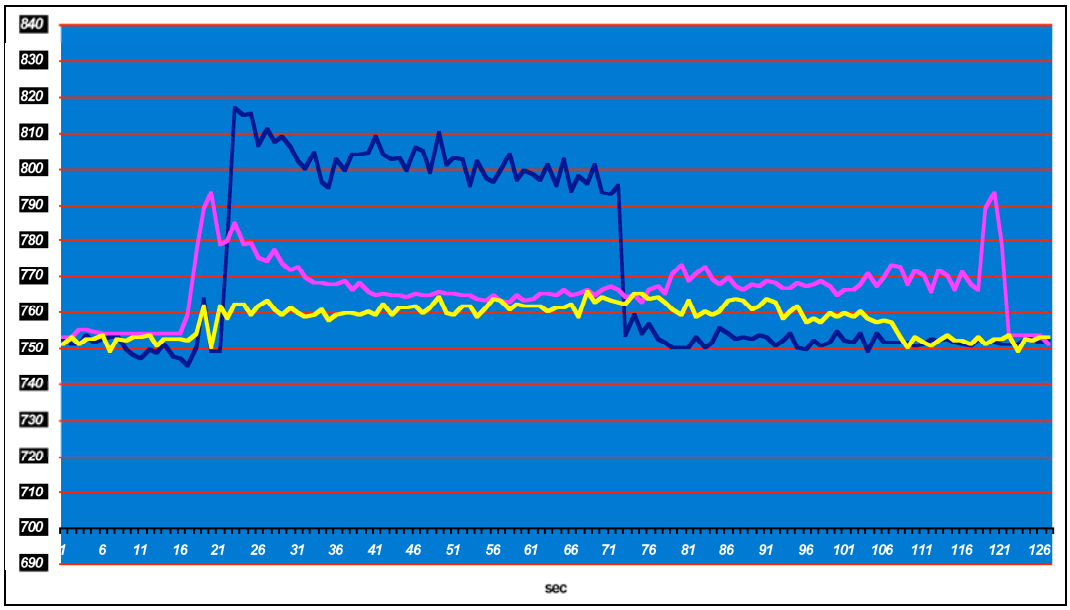

Figure 3. Intracranial pressure (ICP) in anaesthetized rats. The blue line visualizes the strongly raised ICP, approaching 65 mm Hg, in a rat with Herpes simplex encephalitis 6 days after intranasal instillation of Herpes simplex virus type 1. Such a high ICP is likely to prevent blood circulation and to cause herniation, i.e. persistent severe brain damage. The red line reveals that the ICP is decreasing to tolerable levels, 10-15 mm Hg, after intranasal instillation of the peptide AF-16, thereby preventing the otherwise unavoidable brain injury. For comparison, the ICP in a normal, uninfected rat is illustrated by the yellow line, fluctuating between 4 to 8 mm Hg.

effects of AF, and the results revealed a strong anti-inflammatory effect along with a diminished bleeding in the small intestinal submucosa as demonstrated by histological examination (*Johansson* et al., 1997a).

B. *AF and the intestinal capillary permeability*

CT was used for challenge in rat intestinal ligated loops, followed by an intravenous injection of the azo dye Evans blue. The results demonstrated CT-induced extravasation of the dye in the upper parts of the villi, but not in the crypts. This extravasation of the transcapillary albumin flux (due to the binding between Evans blue and albumin) was reduced back to normal after intravenous AF administration (*Lange* et al., 1998).

C. *AF and intracranial pressure*

Elevated intracranial pressure (ICP) is an aggravating factor in infectious encephalitis, and high ICP levels may be fatal or cause persistent neurological and psychiatric malfunctions. The means of pharmacological treatments are limited. We used the peptide AF-16, comprising 16 of the amino acids located to the amino terminal part of the endogenous AF protein, for regulating the raised ICP in rats with experimental herpes simplex encephalitis (Figure 3). Intranasal instillation of the AF-16 peptide counteracted the increment of ICP, while it at the same time abrogated the neurological morbidity and the mortality in a dose-dependent manner. AF-16 achieved this effect most probably by restoring the hampered outflow of cerebrospinal fluid along the olfactory nerve bundles through the cribriform

plate to the nasal lymphatic vessels. No effects on viral replication or antigen distribution within the CNS tissue were found. Thus, AF-16 abolished the prevalence of symptoms, ICP elevation, neurological malfunctions and deaths (*Jennische* et al., 2008). Further studies of AF-16 in situations with brain inflammation and elevated ICP seem warranted.

CLINICAL STUDIES

AF activity in patients suffering from diarrhoea, studies in Mexico and Pakistan

AF was found to rapidly increase after onset of diarrhoea in Mexican patients (*Torres* et al., 1993), the highest increment registered in patients suffering from infection with *Gardia lamblia*. However, in patients not suffering from diarrhoea, circulating AF was low irrespective of colonization or not with enteropathogens. This finding suggests that intestinal hypersecretion rather than the mere presence of intestinal pathogens is activating the endogenous AF production.

High levels of AF were determined in the breast milk of Pakistani women, probably reflecting a high prevalence of enteric pathogens in the environment. Swedish women demonstrated significantly lower AF milk levels (*Hanson* et al., 2000; *Hanson* et al., 2008; *Svensson* et al., 2004). The high AF level in milk probably provides protection against diarrhoeal diseases in the suckling child. Demographic studies of the AF levels in milk in developing countries are presently performed in our laboratory.

Use of the AF concept in human diseases

In clinical studies we have used two forms of AF-therapy:

1. Intake of SPC-flakes® which stimulates to increased endogenous synthesis of AF.
2. Salovum® (B221®) which consists of egg yolk with a high content of AF (*Lange* et al., 1994).

AF-inducing food

Intake of SPC-flakes®, in healthy volunteers convincingly demonstrated an increase in endogenous plasma AF activity. The most significant AF increase was attained after 3-4 daily meals with a total administration of 1 gram of SPC-flakes® per kg body weight (*Lange* et al., 2003). Furthermore, a 12-16 days long period of intake of SPC-flakes® is necessary for obtaining a significant AF plasma concentration, commonly followed by a positive clinical outcome. However, an increased AF response is achieved after only 2-3 days after a second period of intake of SPC-flakes®. Thus, there exists some sort of a "biological memory" for the human capability of AF synthesis, and the first period of intake of SPC-flakes®, seems to be responsible for "priming" of the secondary, enhanced AF response. A sensitive ELISA has been developed which permits studies of dose response relationships and follow up of compliance to AF-inducing diet (*Johansson* et al. manuscript in preparation).

AF-inducing food and Inflammatory Bowel Diseases (IBD)

We performed a double blind placebo controlled clinical trial using SPC-flakes® in a group of patients suffering from *ulcerative colitis* and *Crohn´s disease*. All medication was kept unchanged during the 4 week long diet period. The clinical outcome of the disease was significantly improved by the diet of SPC-flakes®, and a positive correlation to plasma AF activity

($p<0.001$) was also demonstrated (*Björck* et al., 2000).

AF and chronic diarrhoea due to intestinal resections

The clinical effects of SPC-flakes® during a fourteen day long period were tested in patients suffering from chronic diarrhoea due to extensive intestinal resections. Subjects with signs of active intestinal inflammation were excluded from the study. Only two out of six patients responded with increased plasma AF activity at the end of the test period, and both of these patients had a remaining small intestinal length of at least 300 cm, i.e. longer than the other four patients. Thus, an increase in plasma AF activity by dietary means requires a remaining small intestine of a certain length. This finding indicates that the small intestine is the main source of active AF (*Lange* et al., 2003).

AF administrated to patients suffering from severe *colitis ulcerosa*

In a double blind placebo controlled study Salovum® was given as a supplement to patients with acute onset of severe colitis ulcerosa. The histological and clinical laboratory outcome was studied. In mid rectum biopsies from patients treated with AF there was a less severe inflammatory reaction than in biopsies from patients treated with placebo. There was also a lowering in the inflammatory blood parameters erythrocyte sedimentation rate and C-reactive protein. The results demonstrate that Salovum® mediates an anti-inflammatory effect in cases of acute onset of *colitis ulcerosa* (*Eriksson* et al., 2003a).

AF and patients with Crohn's disease

A 38-year-old patient with a 20 year history of severe and progressive Crohn's colitis, combined with a continuously declining response to conventional pharmacological medication was selected for complementary treatment according to the AF concept. Thus, initially he was supplemented with Salovum®, followed by intake SPC-flakes® in order to increase endogenous AF synthesis. The clinical, endoscopic, biochemical and histological outcome was rapidly improved (*Eriksson* et al. 2003b). We have also demonstrated such a persistent positive effect of AF treatment in other patients with Crohn's disease refractory to conventional medical treatment (to be published). The pathophysiological reasons behind these exceptionally positive effects of AF treatment in patients suffering from Crohn's disease remains to be elucidated.

AF and severe Ménière's disease

The pathogenesis behind Ménière's disease is unknown, but impaired production and/or transport of endolymph seems to be of central importance. The clinical symptoms of this disease include attacks of fluctuating sensorineural hearing loss, rotatory vertigo, tinnitus and feeling of fullness in the affected ear. The influence of increased AF activity in patients with incapacitating Ménierè's disease was studied for a 14-30 day long period in an open pilot study. Intake of SPC-flakes® was followed by increased AF levels in 80 per cent of the cases. The attacks of rotatory vertigo were reduced in 54 per cent of the patients**,** and in 12.5 per cent the hearing was normalized and vertigo completely cured. Patients who experienced a reduction of the vertigo attacks had significantly higher final AF-levels ($p<0.01$) than those with no effects of the treatment (*Hanner* et al. 2003, 2004). Consequently, we suggest that AF might play an important part in the regulation of endolymph turnover,

thereby improving the clinical outcome of Ménière's disease. This hypothesis is further supported by immunohistochemical demonstration of AF in the epithelial lining of the endolymphatic space in the inner ear (*Hanner* et al 2004).

A double blind, placebo-controlled study was thereafter performed in 51 patients with long-standing and well-documented Ménière's disease. The patients were randomized to intake of SPC-flakes® or control cereals for 3 months, and they were examined by otoneurological methods before and after the treatment. The results show that vertigo decreased significantly in the group treated with SPC-flakes®, while no influence on the hearing capacity was registered (*Hanner* et al. 2008, in preparation). We conclude that in patients suffering from Ménière's disease intake of SPC-flakes® may improve their clinical performance especially concerning the vertigo component, and therefore could be initially recommended for treatment of this disease.

AF and diarrhoea induced by carcinoid tumours

In Sweden 50-60 new cases of carcinoid tumours are diagnosed each year (*Wilander* et al., 1989). These tumours are clinically associated with severe diarrhoea, and often there is a persistent hypersecretory state in the small intestine despite adequate surgical and medical therapy. A diet of SPC-flakes® combined with Salovum®, was therefore offered to carcinoid patients with residual intestinal hypersecretion. In an initial, open part of the study all of the patients received a four week long period with Salovum®, followed by a double blind cross-over period with SPC-flakes® or control cereals for 6 weeks each. A significant decrease of bowel movements in response to Salovum®, was registered, combined with a further reduction of bowel movements during the SPC-flakes® period. It seems that patients with carcinoid tumours may improve clinically following use of the AF concept, since significant reduction of bowel movements was registered in response to both forms of AF-therapy (*Laurenius et al*. 2003).

AF and mastitis.

The influence on mastitis of AF activity in milk was studied in lactating women during and after a 5 week long period of SPC-flakes® /placebo intake in a double blind study. The frequency of acute mastitis in the group receiving SPC-flakes® was significantly reduced when compared with the control group. The clinical outcome was also reflected by the high AF levels in milk in the SPC-flakes® group. We can conclude that intake of SPC-flakes® in lactating women increase AF in milk, and this increase is followed by significant protection against clinically manifest, acute mastitis (*Svensson et al.* 2004). The diminished inflammation in the lactating mammary gland might also protect against transfer of HIV-1 from the mother to her breast fed offspring, but also against diarrhoeal disease via the passively transferred AF. We are presently testing these hypotheses.

Subclinical mastitis is a condition which has been linked to an increased risk of HIV-1 transfer from the mother to her breastfed infant. In a Pakistani study of milk samples from 107 mothers with or without subclinical mastitis, measured as the Na/K ratio in the milk we did not see any difference between mothers receiving AF-stimulating SPC-flakes® or placebo cereals (*Jalil et al*., to be published).

AF and acute and prolonged paediatric diarrhoea.

Salovum®, was used for treatment of acute (<7 days) or prolonged (>7 days) diarrhoea in children 6-24 months of age in a double blind randomized study in Pakistan. The children (N=240) were randomly given 2 g Salovum®, or placebo every 5 h for 3 days, in addition to an oral rehydration salt solution. Patients receiving Salovum®, and suffering from acute diarrhoea improved with a successful clinical outcome in 83% of the cases, while in the placebo group 54% of the patients improved similarly within 3 days. The children suffering from prolonged diarrhoea treated with Salovum®, improved clinically in 91% of the cases within 3 days, compared to the 63% improvement registered in the placebo-treated group. Salovum®, seems to significantly improve the clinical condition of children suffering from diarrhoea, caused by a broad range of undefined pathogens (*Zaman et al.,* 2007).

CONCLUDING REMARKS

The AF protein regulates transports of water and ions across various forms of biological membranes, and is capable of mediating and modulating a multitude of biological reactions.

Our studies show that a peptide situated between position 35 and 50 in the AF protein sequence has potent anti-inflammatory and anti-secretory actions, and consequently stands out as an interesting candidate for future drug development.

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HOW PROBIOTICS *LACTOBACILLUS* GR-1 AND RC-14 IMPROVE UROGENITAL HEALTH IN WOMEN

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SUMMARY

The urogenital tract of females is important for reproduction as well as urine excretion. The anatomical nature of the area, so close to the anal skin and open to the exterior, makes it particularly susceptible to microbial colonization and infection. In addition to innate immune factors, mucins and epithelial barrier function, the indigenous microbiota, especially lactobacilli, help protect the niche from disease. A capsule product containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 is the world's most documented probiotic designed for women and shown in clinical studies to repopulate the vagina, displace pathogens and reduce the risk of infection. The mechanisms of actions continue to be uncovered but include modulating host immunity, altering the micro-environment to be less receptive to pathogens through production of acid, biosurfactants, hydrogen peroxide and signalling compounds, and dislodging pathogen biofilms. With urogenital infections inflicting an estimated one billion women each year, the use of probiotic lactobacilli to augment or replace antimicrobial agents represents an important addition to the options available for women.

INTRODUCTION

The health of the female urogenital tract is like any other part of the body, it falls under the World Health Organization's 1946 definition: "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity." In today's technologically and medically advanced society, this definition is clearly out of date, if only for the fact that it is almost impossible for a human to have 'complete physical, mental and social well-being'. Indeed, so much attention has been paid to disease and so little to health, that there are few indicators of health per se.

The 'health' of the urogenital tract of females is influenced by a number of factors so much so that it is rarely 'healthy'. For example, at any given time large numbers of women have bacterial vaginosis (BV), a condition not believed to represent health, due to elevated pH, dominance of pathogens and potential pathogens, modulated immune profiles, and an increased risk of acquiring sexually transmitted infections, urinary tract infection (UTI)

and having preterm labour (*Allsworth* and *Peipert*, 2007; *Cauci* et al., 2003; *Chaim* et al., 1997; *Cherpes* et al., 2003; *Klebanoff* et al., 2004; *Sewankambo* et al., 1997; *Sharami* et al., 2007). Vulvovaginal candidiasis (VVC) and UTI are common in females, and the menstrual cycle often causes pain, discomfort and mental and physical disruption that are not consistent with optimal well-being (*Sobel*, 2007; *Sobel* and *Chaim*, 1996). Thus, the 'health' of the urogenital tract is a relative term and discussing the ability of probiotic lactobacilli to 'improve' urogenital health must be viewed in perspective. For the record, probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2001). This definition therefore requires that products be tested in humans and shown against placebo or standard therapy to provide measurable benefits.

THE ORIGINS OF LACTOBACILLI PROBIOTICS

The basis for application of lactobacilli to the urogenital tract comes from studies performed at least 35 years ago which showed that women who had recurrent urogenital infections had severely depleted lactobacilli counts in their vagina, while healthy women were densely colonized by these indigenous bacteria (*Bruce* et al., 1973). This association was not well understood at the time, nor was the actual composition of the vaginal microbiota, the species of *Lactobacillus* that dominated, or the nature of the benefits accrued by these bacteria. Nevertheless, Bruce and subsequently Reid, pursued this line of enquiry with a view to understanding the role of lactobacilli in female health, and in devising novel strategies to restore and retain urogenital health.

The hypothesis was that lactobacilli strains could be identified and administered to the vagina to improve health and reduce the risk of infection.

In order to select appropriate strains for this challenge, one must understand what species are present in the natural state, how they function to counter infection, then make sure that they can be scaled up for human studies and potential commercialization (to make them available to as many people as possible). This selection process is by no means an easy step. In the 1980s when this research began, and even until recently, it had been assumed that lactobacilli needed to adhere to the vaginal surface and inhibit the growth of pathogens (*Reid* et al., 1997). This was based upon pathogen studies showing that infection was often causes by adherence to cells and subsequent growth and production of virulence properties.

In fact, as we now appreciate, adherence of pathogens is not a pre-requisite for infection, and completely inhibiting their adhesion is all but impossible. In many cases of where a female is 'healthy', pathogenic bacteria or yeast can be found in the vagina (*Devillard* et al., 2004; *Hyman* et al., 2005). Likewise, the need to inhibit growth or kill pathogens is based upon chemotherapeutic concepts where the aim is to wipe out pathogens. As we now appreciate, this is not always a necessary outcome in curing infections of the urogenital tract. Pathogens can be displaced, disarmed through inhibition of expression of virulence factors, or eradicated by priming of the immune system (*O'Garra* et al., 2004; *Laugh-*

ton et al., 2006; *Saunders* et al., 2007). Thus, the selection of interventional strains is somewhat complex.

A common approach by the few companies with lactobacilli products ostensibly of use for females, has been to select a *Lactobacillus acidophilus* strain in the belief that this species was the most commonly found in the healthy vagina. Or, for a strain to produce hydrogen peroxide (H_2O_2) as the main antagonistic compound (*Al-Mushrif* et al., 1998). However, it is now clear that *L. acidophilus* is not the most common species in the vagina, it is *L. iners*, an organism that is fastidious (*Burton* et al., 2003) and problematic to upscale for commercial use. In addition, while H_2O_2 appears to have a role in disease prevention, based upon epidemiological observations that strains expressing it are more common in healthy women, there are other factors that clearly play a role in disease control.

Another common misconception promoted by some companies is that the more lactobacilli delivered in a product, the better the effect. Thus, some clinically untested products contain six or more strains of lactobacilli and bifidobacteria (chosen for reasons not disclosed) and claim to deliver tens of billions of viable cells. There are several concerns with this approach, not the least of which is lack of clinical data, mechanisms of action, properties of the strains, and viable counts at end of shelf-life. The vaginal milieu is not designed to harbour tens of billions of bacteria. On the contrary, the vagina and the organisms present modulate the microbial count, at least through quorum sensing, as well as other means yet to be fully understood. The organisms invariably form biofilms, and send out and receive signals to determine the extent to which they can multiply. This method of self-controlling an environment is necessary, in essence so that the species do not run out of nutrients or fall foul to detection and eradication by the immune system (*Quadri* et al., 2002; *Swidzinski* et al., 2005). Thus, one rarely finds more than 10^9 cfu/g in the vagina (*Delaney* and *Onderdonk*, 2001) and to add $20x10^9$ in a product makes little sense, unless the viable count drop-off is rapid, or the hope is that one of the strains might have an effect.

A further problem with multi-strain applications is that no apparent due note has been made of the potential for one strain to counter the activity of another. Studies have shown that some strains induce a particular immune response, and others induce the opposite effect (*Diaz-Ropero* et al., 2007; *Fink* et al., 2007).

LACTOBACILLUS RHAMNOSUS GR-1 AND *LACTOBACILLUS REUTERI* RC-14 AND MODE OF ACTION

In selecting *Lactobacillus rhamnosus* GR-1 (originally *L. casei* subsp. *rhamnosus*) and *Lactobacillus reuteri* RC-14 (originally published as *L. acidophilus* then re-defined as *L. fermentum*), a stepwise process was involved.

Initially, the GR-1 strain was selected for its antagonistic properties against uropathogens and for its ability to adhere to uroepithelial cells (*Chan* et al., 1984; *Reid* et al., 1987). In addition, its ability to coaggregate with uropathogens was believed to be useful in potentially blocking the ascension of these organisms to other areas of the vagina and into the bladder (*Reid* et al., 1988). The strain was able to be propagated into a form that could be deliv-

ered to humans, either in a milk product (*Gardiner* et al., 2002; *Reid* et al., 2001b) or dried capsule (*Reid* et al., 2001a). Subsequently, *Lactobacillus* GR-1 has been shown to modulate host immunity in a way that enhances antimicrobial activity yet down-regulate the potent inflammatory processes associated with discharge and symptoms and signs of infection (*Kim* et al., 2006; *Lorea Baroja* et al., 2007; *Kirjavainen* et al., 2008). The strain produces by-products, likely acid, that kill bacteria and viruses rapidly including HIV (*Reid* et al., 2006), quantities of lactic acid that stress uropathogenic *E. coli* cell surfaces (*Cadieux* and *Reid*, 2008), and quorum sensing molecules that can also play a role in interference with pathogenesis (*Elwood* et al., 2008). The organism was not found to produce H_2O_2 when originally tested (*Tomeczek* et al., 1992), but recently, using TMB agar it has been shown to be a producer (Schellenberg, J. unpublished). In short, this organism has a number of properties suitable to vaginal and distal urethral activity and restoration of health to this region.

The reason for adding a second strain to the probiotic formulation was to have better activity against Gram-positive pathogens. The *Lactobacillus* GR-1 strain has been shown to have bacteriocin-like properties against *E. coli* (*McGroarty* and *Reid*, 1988), but when used in a small pilot human study, there was evidence that enterococci were not displaced (*Bruce* and *Reid*, 1988). Enterococci are becoming more and more recognized as urinary pathogens, as well as being part of a disrupted flora associated with BV and infection (*Kelly* et al., 2003; *Jahic* et al., 2006). Furthermore, Group B streptococci are problematic in the vagina of women about to give birth, as they can infect and potentially kill the newborn (*Bayo* et al., 2002).

The screening for a second strain focused primarily on inhibition of growth of Gram-positive cocci. This resulted in selection of *L. fermentum* B-54 (*Reid* et al., 1987) to accompany strain GR-1 in clinical studies. The combination showed great promise based upon a series of clinical studies, three of which showed reduced incidence of UTI recurrences (*Bruce* et al., 1992; *Reid* et al., 1992, 1995). This formulation would have been retained but for the 1996 discovery of biosurfactants produced by lactobacilli (*Velraeds* et al., 1996). These compounds altered the microenvironmental surface tension and produced adverse conditions for the adhesion of a wide range of pathogens (*Velraeds* et al., 1998). Studies using polymer substrates showed that even low numbers of lactobacilli could significantly reduce pathogen colonization, biofilms and also displace these organisms (*Reid* and *Tieszer*, 1993, 1995; *Reid* et al., 1995b). The most potent activity was found in *Lactobacillus* RC-14, and therefore it replaced B-54 in the combination with strain GR-1 with a view to determining if these two strains were clinically compatible and able to improve vaginal health. Conjointly, studies were performed on RC-14 and various proteins and peptides were discovered which played a role in the strains anti-Gram positive coccal activity (*Heinemann* et al., 2000; *Howard* et al., 2000; *Reid* et al.2002; *Laughton* et al., 2006), as well as against E. coli virulence expression (*Medellin-Pena* et al., 2007). Most recently, we have shown that the mode of action of RC-14 is not reuterin (*Cadieux* et al., 2008), the antibiotic described as being critical for *L. reuteri* probiotic activity (*Dobrogosz*, 1998).

The concept of delivering lactobacilli for urogenital health had historically involved direct implantation into the vagina. However, recognizing that

pathogens ascend into the vagina from the woman's own intestine, and these organisms then infect the host, we hypothesized that lactobacilli also originated from the woman's own gut, and thus probiotics could be delivered to the vagina by oral intake. The first step in proving this concept was to show that *Lactobacillus* GR-1 and RC-14 could pass through the intestine. This was shown by *Gardiner* et al. (2002) who recovered the strains in faeces of volunteers who ingested the organisms suspended in milk. In order to produce a product that could be more widely used and therefore had longer shelf-life, a capsule was produced by Chr. Hansen (Horsholm, Denmark) with these strains in dried form. Using a technology that improved shelf-stability and increased passage of the organisms beyond the stomach, the capsules were then used in a series of clinical trials.

Upon a successful proof-of-concept study (*Reid* et al., 2001a) and independent verification of the results (*Morelli* et al., 2004), the strain combination was shown to reduce pathogenic bacteria and yeast ascension into the vagina from the rectum (*Reid* et al., 2003), and produce a more consistently normal lactobacilli-dominated vaginal microbiota (*Reid* et al., 2001a, 2003, 2004), with a dosage of one billion or more bacteria (*Reid* et al., 2001a). The vaginal counts of lactobacilli increased, including indigenous strains, indicating that the treatment itself encouraged recovery of the host's microbiota. These studies fulfilled the definition of a probiotic and have led to the strains being the first and most documented probiotics for urogenital health in the world.

The potential for probiotic lactobacilli to reduce the risk of preterm labour is based upon displacement of an aberrant microbiota and interference with the inflammatory pathway that leads to cyclooxygenase (COX)-2 and prostaglandin production. Having shown that *Lactobacillus* GR-1 and RC-14 can reach the vagina after oral intake, the next step was to determine if this could restore the vaginal microbiota in pregnant women with BV. In a study of 22 pregnant women with BV given the probiotics once daily for 30 days, the vaginal pH returned to normal in 73% and no safety issues arose (*Oleszczuk* et al., 2008). Further studies are needed to confirm the findings, but *in vitro* experiments strongly support the potential for these strains to lower the risk of preterm labour. Using trophoblast and placental cells in tissue culture, we have shown that *L. rhamnosus* GR-1 can significantly down-regulate COX-2 and TNF-α and up-regulate the protective prostaglandin dehydrogenase (*Yeganegi* et al., 2008).

The disruption of the vaginal microbiota can have other consequences, such as chronic vulvovaginitis. In a pilot study in Russia where vulvovaginitis is common amongst young girls, the use of *Lactobacillu*s GR-1 and RC-14 daily for one month in four 7-10 year olds and nine 11-19 year olds was found to restore the vagina to having no clinical signs of infection (*Uvarova* et al., 2007).

Another concern is the potential for BV to increase the risk of squamous metaplasia. In other Russian study, 30 women of reproductive potential aged 18 to 40 with diagnosed intraepithelial squamous cell cervical lesions were studied. Histology revealed mild (CIN 1/condyloma) intraepithelial lesions in 24 patients and severe (CIN2-3) intraepithelial lesions in 6 patients. All study patients had Human Papilloma Virus infection confirmed by RT-PCR: high oncogenic risk viruses in 17 patients, low oncogenic risk viruses in 7 patients, and both high and low oncogenic risk

HPV in 6 patients. The control group was comprised of 20 healthy women of reproductive age. Following once daily oral administration of two capsules of *Lactobacillus* GR-1 and RC-14 for 15 days, no BV was found and there was a significant reduction in pathogen counts in the vagina (*Minkina*, 2007).

NEXT FOCUS FOR CLINICAL USE

In recent times, several studies have been performed to explore the breadth of usefulness of these probiotic strains. Given that antibiotics and antifungals are widely used to treat urogenital infection, and that side effects and drug resistance rates are increasing, it was hypothesized that *Lactobacillus* GR-1 and RC-14 could augment the cure of infections through their ability to displace pathogens, modulate immunity, and reduce drug side effects.

Three studies have now shown support for this hypothesis. In the first, 106 patients diagnosed with BV were randomized to receive metronidazole and either lactobacilli or placebo. Thirty-day follow-up showed significantly improved cure of BV with probiotic supplementation (*Anukam* et al., 2006a). The precise mechanisms were not investigated in the trial, but subsequently, it has been shown that the lactobacilli are able to resist metronidazole and even grow in its presence (Anukam and Reid, 2008). A second study, this time performed on 64 women in Brazil, showed almost identical findings, whereby use of *Lactobacillus* GR-1 and RC-14 improved cure of BV in patients treated with 2g tinidazole (88% versus 50%) (*Ruiz Martinez* et al., 2009b). This is the first series of studies to show augmentation of antimicrobial cure, and it provides hope that the longevity of antibiotics and antifungals usefulness may be extended by adding probiotics. Most importantly, given the adverse effects of urogenital health on quality of life (*Ellis* and *Verma*, 2000; *Lowe* and *Ryan-Wenger*, 2003), any supplemental or alternative approaches must be welcomed.

Regulatory agencies do not extend the approval or food or dietary supplements to applications other than oral. Thus, the insertion of a *Lactobacillus* capsule into the vagina constitutes a drug therapy. Previous studies showed the potential for intravaginal use of lactobacilli (*Reid* et al., 1995; *Cadieux* et al., 2002), so in order to determine if *Lactobacillus* GR-1 and RC-14 have the ability to cure BV, a study of 40 women was undertaken in which they inserted lactobacilli capsules vaginally for five days. At 30-day follow-up, the cure rate with the probiotics was superior to using metronidazole on its own (*Anukam* et al., 2006b). A larger trial is warranted, but the signs are very encouraging that probiotic lactobacilli have the potential in at least this application, to replace antibiotics to cure an infection.

A misconception with lactobacilli in the urogenital tract is that they should be used to treat VVC, because this infection is caused by loss of lactobacilli. In fact, this is not the case, and lactobacilli are often present when VVC arises, and there is little evidence to indicate that lactobacilli alone can cure VVC. Nevertheless, we explored the question of whether *Lactobacillus* GR-1 and RC-14 could augment the cure of VVC by antifungal. In a randomized, placebo controlled study of 68 women diagnosed with VVC, a single dose of fluconazole (150mg) was administered supplemented every

morning for the following four weeks with either two placebo or two probiotic capsules containing *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14. At four weeks, the probiotic treated group showed significantly less vaginal burning and itching (11.8% versus 32.4%; p=.04), lower presence of yeast detected by culture method (14.7% versus 38.2%; p=.03), and less vaginal discharge (14.7% versus 44.1%; p=.008) (*Ruiz Martinez* et al., 2009a). The mechanisms of action have to be determined, but is not likely associated with H_2O_2 (*Ruiz Martinez* et al., 2008), and may comprise increased displacement of the yeast by the lactobacilli as shown *in vitro* (*Velraeds* et al., 1998; *Koehler* and *Reid*, 2006), reduced re-ascension of yeast from the rectum to vagina (*Reid* et al., 2003), or up-regulation of antifungal host defences (*Kirjavainen* et al., 2008).

CONCLUSION

The use of lactobacilli for urogenital health in women requires careful consideration of the strains to be used, the properties they confer, how they are manufactured, stored and delivered, and clinical evidence must be obtained that they are beneficial before they should be termed probiotic. The success of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 demonstrates for the first time that this approach to restoration and retention of a healthy vaginal microbiota is possible. As more genomic and functional data become available on these strains, we will better understand how they work, and under which circumstances. Other strains will also become available, possibly selected for different scientific or clinical reasons. As long as women are the beneficiaries of such probiotic regimens, we will have achieved a laudatory goal as scientists.

ACKNOWLEDGEMENTS

Our research is supported by NSERC, AFMnet, and the Ontario Neurotrauma Foundation.

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CHILDHOOD VACCINES AND INDUCTION OF ALLERGIC AND AUTOIMMUNE DISORDERS: FACTS AND FICTION

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SUMMARY

During the past century, there has been either a total elimination or a dramatic decline in the global prevalence of many common, sometimes fatal human infectious diseases. Although a number of societal changes have contributed to such a decline, the introduction of specific vaccines appears to be the single most important approach underlying the disappearance of many serious childhood infections. Interestingly, however, this period has also been characterized by the increasing evidence of many other pathologic states, including respiratory and intestinal allergic or autoimmune diseases and other immunologically mediated disease processes. The later part of the 20th century has also seen emergence of many new disease states, such as HIV, autism, and antibiotic resistant microorganisms.

Because of the apparent temporal association observed between introduction and large-scale use of vaccines against infectious diseases and the increasing prevalence of allergic and autoimmune diseases, the use of childhood vaccines has been subject of intense global discussion and concerns. Such concerns and discussions have been fuelled by anecdotal reports of serious occasional side effects, followed by other recent observational studies, and the rapid dissemination of their results through modern mass media. This review summarizes the available evidence for the role of natural infections, or their available vaccine counterparts in the mechanisms of protection against or the pathogenesis of allergic or autoimmune disorders in man.

Available carefully controlled studies have suggested that some vaccines will have infrequent and rarely even serious reactions. Some vaccines have also been associated with the development of transient allergic or autoimmune reactivity. However, these observations have also documented that childhood vaccines do not cause, and are not responsible for the recent surge in the incidence of some allergic or autoimmune disease. Childhood vaccines are highly effective and safe and continue to represent the best available approach to prevention of many serious infectious diseases of man.

INTRODUCTION

For the contemporary medical practitioner who completed medical training after the late 1970's, and the general public who were born after the late

Table 1: Impact of childhood vaccines: 2008•

Disease	% Reduction in morbidity-mortality post vaccine introduction
Diphteria, measles, polio rubella Congenital rubella syndrome, smallpox	>99
Mumps, tetanus, pertussis	>90
Hepatitis A	87
Hepatitis B	80
Haemophilus type B	>99
Streptococcus pneumoniae	34-25

• Roush et al., 2007

1960's, it is difficult to identify with or fathom the emotional impact of the high morbidity and mortality that was the rule for many infectious diseases in the North American Continent and Europe until about 50 years ago, and still continues to be a major societal problem in many parts of the world.

Most individuals today have never seen a case of smallpox, plague, measles, poliomyelitis, congenital rubella, mumps, diphtheria, tetanus, or meningitis due to *Hemophilus influenzae* type B. These diseases have been either eradicated or effectively controlled in most parts of the developed world. Currently efforts are underway to effect their control or elimination throughout the rest of the world. The possible reasons underlying the decline of these childhood diseases include; improvement in socio-economic conditions; introduction of sanitation and public hygiene; improvement in nutrition and introduction of dietary supplements; changes in the ecology and environment; and introduction of antimicrobials, chemotherapeutic and chemoprophylactic agents. However, the single most important reason for the decline of infectious diseases is the introduction of vaccines against specific childhood infections. Smallpox has been eliminated globally, the morbidity and mortality of *Hemophilus influenzae* type B, diphtheria, measles, polio, rubella, has declined by >99%, mumps, tetanus, pertussis by >90%, hepatitis A and B by 80-87%, and of *Streptococcus pneumoniae* by 25-34% (Table 1). In addition to their impact on disease prevention, the introduction of childhood vaccines have significantly improved other societal functions, including school, social and employment-related work attendance. It is estimated that in the United States alone, the introduction of vaccines have prevented >33,000 deaths annually, saved over 10 billion dollars in direct costs in each birth cohort, and resulted in over 33 billion dollars in savings by prevention of disability and loss of productivity (*Roush* and *Murphy*, 2007; *Stratton* et al., 1999).

Despite these phenomenal societal gains, many justifiable concerns have been raised about the role of modern day immunization practices, largely because of the somewhat parallel increase in the incidence of many allergic and autoimmune diseases. In order to address these issues in some detail, this review will attempt to consider available evidence for the role of naturally acquired infections in the mechanism

of protection against, or the pathogenesis of allergic and autoimmune disease. Based on this evidence, it will then be attempted to determine if childhood immunization against such infectious diseases do similarly contribute to the development or outcome of such autoimmune or allergic disease states.

DEVELOPMENT OF ALLERGY OR AUTOIMMUNITY

Concurrent with the improvement of socioeconomic conditions, and the introduction of community sanitation, and improvements in nutrition between the 19th and 20th centuries in many societies, there has also been a discernable increase in the incidence of disease states such as asthma, eczema, urticaria, angioedema, food allergies, systemic lupus erythematosis, rheumatoid arthritis, diabetes mellitus, inflammatory bowel disease and other autoimmune or immunologically mediated diseases (*Bach*, 2002). Autoimmune reactions represent auto reactive B and T cell responses directed toward self-antigens or neoantigens generated in the host. Many microbial antigens exhibit mimicry for certain host tissue (self) antigens. On the other hand, allergic reactions represent immune responses to non-self antigens such as vaccine antigen(s), or other components in the vaccine preparations which may elicit specific IgE, immune complexes, delayed type or cytotoxic T cell mediated hypersensitivity responses. Finally, expression of host tissue damage may be directly related to tissue replication of infectious vaccine antigens, and direct cytotoxicity, independent of host immune response (*Kamradt* and *Mitchison*, 2001). The incidence of allergic disorders (asthma, food allergy, and atopy) has manifested a dramatic increase during the past 5 decades especially in the technologically advanced Western hemisphere and other developed parts of the world. Similarly, the incidence of several autoimmune diseases has increased mostly over the past few years. These include diabetes mellitus, multiple sclerosis, rheumatoid arthritis, systemic lupus and other autoimmune disorders. These diseases have been seen with increasing frequency in age groups often selected for national immunization programs. It is estimated that about 5-8% (14-22 million) of the population in the United States is affected by one or more autoimmune disorder. The prevalence of allergic disorders is estimated to be even higher in some parts of the world (*National Institutes of Health*, 2000). Autoimmune disorders can involve any human body site. At least 15 distinct clinical disorders have well defined evidence of autoimmune immunologic reactivity. A number of autoimmune diseases have been reproduced in experimental animal models. These include experimental allergic encephalitis (EAE), rheumatoid arthritis, myocarditis, diabetes mellitus, and inflammatory bowel disease. Recent data have demonstrated that autoimmune disorders affect women disproportionately more than men. It is estimated that over 78.8% (6-7 million) subjects with autoimmune disorders in the United States are female. The reason for high prevalence in women remains to be defined. However, experimental data have suggested that sex hormones may significantly amplify autoimmune responses and, possibly other forms of hypersensitivity. Recently, it has been observed that sex hormones, especially oestrogen, progesterone, and testosterone determine the outcome of Th1 vs. Th2 type T cell responses by their abil-

Table 2: Impact of natural infections on development of protection against or induction of allergy

Development of protection against allergy (immunity)	Induction of allergic disease (pathology)
BCG	Bordetella pertussis
Bifidobacterium lactis	**Chlamydia pneumoniae**
Chlamydia pneumoniae	**Chlamydia trachomatis**
Chlamydia trachomatis	Mycoplasma pneumoniae
Lactic acid bacteria	Staphylococcus aureus
Lactobacillus rhamnosus	**Influenza A virus**
Listeria monocytogenes	Metapneumovirus
Mycobacterium tuberculosis	Rhinovirus
Hepatitis A virus	**Respiratoir syncytiaal virus (RSV)**
Influenza A virus	Anisakis simplex
Respiratoir syncytiaal virus (RSV)	Ascaris sp.
Heligmosomoides polygyrus	Fasciola hepatica
Hookworm sp.	**Nippostrongylus brasiliensis**
Nippostrongylus brasiliensis	**Strongyloides stercoralis**
Strongyloides stercoralis	**Strongyloides venezuelensis**
Strongyloides venezuelensis	Toxocara sp.

Bold: Associated with both protection and pathology

ity to interact directly with specific receptors on immunocompetent cells (*Rose*, 2002; *Fairweather* and *Rose*, 2004).

Autoimmune disorders and allergies tend to cluster in families, suggesting an important genetic predisposition to the expression of disease. Studies in monozygotic twins have also identified the role of environmental factors, including infections, in the development of immunologically mediated disease processes (*Shoenfeld* et al., 2002). Although the mechanisms that govern the development of disease states are quite distinct and separate, some allergic and autoimmune diseases may exhibit common underlying mechanisms of expression. This opinion has been based on the observation of appearance of asthma and autoimmune conditions in the same patients, and the presence of autoantibodies in some allergic patients (*Rottem* and *Shoenfeld*, 2003). It has been proposed that altered T cell, mast cell, cytokine response and common genetic determinants may exist both for autoimmune as well as allergic diseases. Mast cells and their inflammatory cytokines, activation of protein kinase by such cytokines and T cell receptors now may represent areas of common disease susceptibility within the immune system for diseases such as asthma and autoimmunity (*Rottem* and *Shoenfeld*, 2003).

The influence of natural or induced infections, and vaccine induced immunologic responses on the outcome of allergic or autoimmune diseases is reviewed below.

NATURAL INFECTION-INDUCED IMMUNE RESPONSES

Allergic disorders

Role in protection

The pathogenesis of common allergic disorders involve development of allergen specific immune responses triggered by CD4+ (Th2) T cells, resulting in the expression of several immunoregulatory cytokines, including IL-4, IL-5, IL-9, IL-13. Such cytokines induce production of specific IgE and other immunoglobulin isotypes by B cells, recruitment of eosinophils, other inflammatory cells, and mucus production. Such a cascade eventually leads to IgE mediated degranulation and release of cytokines, chemokines and other pharmacologic mediators (including histamine, eotoxin) from mast cells and eosinophils, immune complex mediated cellular activation, local inflammation, development of smooth muscle contractibility, and eventually clinical expression of allergic symptoms (*Herz* et al., 2000).

Although genetic susceptibility is an essential component of the development of allergy, it is clear that a variety of environmental factors play a critical role in the mechanisms of protection against or the pathogenesis of clinical disease. The role played by different infectious agents in the outcome of allergic disease is outlined in Table 2. Many infectious agents which induce a potent Th1 type T cell response are associated with significant protection from allergic disorders. These include infection with *Mycobacterium tuberculosis*, *Mycobacterium bovis*, BCG, several probiotics, and some parasitic agents and viruses (*Trujillo* and *Erb*, 2003). Children immunized with BCG as neonates, and women with active tuberculosis prior to age 20 years, appear to have significantly lower incidence of asthma, atopic disease, and airway disease (*Marks* et al., 2003; *da Cunha* et al., 2004). BCG or immunization with inactivated BCG vaccine also seems to reduce allergen specific IgG and IgM serum antibody responses in mice and guinea pigs (*Trujillo* and *Erb*, 2003).

In additional studies, exposure to *Chlamydia trachomatis*, *Listeria*, several lactic acid bacteria and other probiotics have also been shown to suppress development of allergic responses by Th1 induced suppression of Th2 response. Suppression of allergic responses by IL-10 and TGF-β cytokines secondary to the induction of CDIIC+ cells independent of Th1 type of T cell response has also been proposed in animal models of airway hypersensitivity (*Sayers* et al., 2004; *Han* et al., 2004; *Repa* et al., 2003; *Matricardi* et al., 2000).

Limited evidence is available to suggest that infection with some helminthic agents is associated with lower incidence of atopic disease. These include infestation with *Schistosoma*, and hookworm. Infections in mouse model with *Strongyloides stercoralis* or *Nippostrongyloides brasiliensis* have been shown to suppress pulmonary allergic responses (*Wang* et al., 2001; *Wohllenben* et al., 2004). Clinical trials with live or killed commensals and probiotics have also provided interesting evidence to suggest suppression of allergic disease expression, including atopic dermatitis after maternal prenatal use of *Lactobacillus rhamnosus*, or intradermal application of killed mylobacterial vaccine, or feeding of *Lactobacillus* or *Bifidobacteria* species in infants (*Helin* et al., 2002; *Drachenberg* et al., 2001).

Infection with certain viruses such as hepatitis A virus appear to provide some protection against atopic diseases, based on an increase in the correlation

between levels of hepatitis A antibodies and reduced incidence of allergy. Additional data have suggested that viruses such as RSV, influenza A decrease the development of airway eosinophilia after airway challenge with allergens in experimental animal models. The anti-allergic effect appears to be associated with induction of prompt Th1 response by the virus in such situations (*Matricardi* et al, 2000; *Walzl* et al, 2000; *Bach*, 2002).

Role in Pathogenesis

Based on several recent studies, it is clear that infection induced Th1 responses do not necessarily protect against the development of allergy, but may in fact exacerbate its evolution (Table 2). Studies with *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Staphylococcus*, and *Bordetella pertussis* showed to enhance allergic inflammation in the bronchopulmonary tree. These effects may be mediated by induction of potent Th2 responses or via exotoxin (superantigen) mediated cytokine release (*Lieberman* et al, 2003; *Blasi*, 2004; *Hardy* et al., 2002; *Ennis* et al., 2004).

Most helminths induce very prominent Th2 responses and such responses should promote expression of allergy. Infections with certain helminths result in suppression of oral tolerance to allergens and enhancement of Th2 responses. This may explain increased allergic manifestation in children infected with ascaris, toxicara and other parasitic agents listed in Table 2. Antihelminthic treatment in such children results in prompt improvement in the clinical symptoms of asthma and urticaria (*Hurst* et al., 2001; *Demirci* et al., 2003; *Audicana* et al., 2002).

Although anti-allergic effects have been observed with RSV proteins and influenza A viruses in animal models, numerous publications have suggested that respiratory viruses are more commonly associated with airway hyperreactivity and respiratory allergy in man. In particular, RSV, rhinovirus, influenza virus, parainfluenza virus and metapneumovirus have been shown to induce wheezing in infants and children, and may pose a very significant risk for childhood asthma. Several mechanisms have been shown to contribute to viral induced allergic disease exacerbations. These include development of viral specific IgE response, induction of IL-13, and direct Th1 mediated airway hyperactivity. Recent data have also suggested that abnormal innate immune responses especially to RSV infection may be an important determinant of the severity of RSV disease, regardless of Th1 vs. Th2 T cell responses. In particular, activation of pulmonary dendritic cells, TLR activation, induction of IL-8 by lower airway epithelial cells (recruitment of neutrophils in airway), induction of IL-17 (increased mucous production) have been observed in association with viral induced respiratory allergic disease in human and experimental animal studies (*Grunewald* et al., 2002; *Welliver* et al., 2007; *Ogra*, 2004; *Garofalo* et al., 1999).

Autoimmunity

This information has been reviewed quite extensively in several recent publications (*Kamradt* et al., 2005; *Fairweather* and *Rose*, 2004; *Sandborg*, 2002; *Mackay* et al., 2008).

Role in protection

It is well known that induction of autoreactive T and B lymphocytes is an integral, albeit limited, component of the normal human lymphoid cell repertoire. However, under physiologic conditions such cells are quiescent or exhibit only minimal functional activity. It has been suggested that autoimmune

Table 3: Association of human infections with different autoimmune diseases

Disease	Possible aetiologic agent
Guillain-Barré Syndrome	CMV, EBV, **Campylobacter sp.**
Multiple sclerosis	EBV, measles, HBV, measles
Acute Disseminated Encepahlomyelitis (ADEM)	measles
Immune complex diseases: dermatomyositis polyarteritis nodosa (PAN), nephritis	**HBV and HBsAg carriers**
Myasthenia gravis	HSV, HCV
Lymphoproliferative disease	**EBV**
Rheumatoid arthritis	E. coli, M. tuberculosis, EBV, HBV, Proteus, Serratia
Arthritis	**B. burgdorferi**
Myocarditis	Coxsackie B virus, CMV, Chlamydia
Rheumatic fever	Group A Streptococcus
Systemic lupus erythematosus (SLE)	EBV
Insulin dependent diabetes mellitus (IDDM) type 1	Coxsackie B virus, rubella, CMV, mumps
Schizophrenia, thyroiditis, vasculitis syndromes	Parvovirus B-19

Bold: Strong evidence

disease-incidence may be a mirror image of the incidence of certain infections, especially, mycobacterial, helminthic and certain viruses (*Bach*, 2002). A number of recent epidemiologic studies have raised the possibility that some infections acquired during the first year of life are associated with reduced risk of diabetes mellitus, inflammatory bowel disease or multiple sclerosis later in life (Table 3). More importantly, studies carried out in non obese diabetic (NOD) mice and other rodents infected with *Salmonella*, *Schistoma mansoni*, BCG, mycobacteria, lactobacilli, *Heligmosomoides polysyrus*, and other helminths have demonstrated dramatic protective effects against development of diabetes in diabetic mice, and in other animal models against other autoimmune diseases such as EAE, and experimentally induced IBD and arthritis (*Madsen* et al., 1999; *Zaccone* et al., 2003; *Van der Kleij* et al., 2002; *Sharif* et al., 2001; *Elliott* et al., 2004). It appears that many such cell-associated bacterial and parasitic infections offer protection against autoimmunity by activation of CD4+ T cells, NKT cell expression of IL-10, and induction of CD25+ regulatory T (Treg) cells (*Madsen* et al., 1999; *Kamradt* et al., 2005).

Several recent studies have extended the observation in animal models to the treatment of autoimmune disorders in man. Probiotics have been employed for the treatment of inflammatory bowel disease, including ulcerative colitis or Crohn's disease. The use of probiotics appears to be beneficial in the management of pouchitis in patients with ulcerative colitis. Treatment with BCG has been attempted, but without success in the prevention or therapeutic management of diabetes mellitus, BCG therapy has been found to reduce magnetic resonance imaging

activity (MRI) in a few patients with multiple sclerosis. However, its therapeutic implications are not clear at this time (*Ristori* et al., 1999; *Gionchetti* et al., 2003; *Allen* et al., 1999). Other investigators have employed helminths in the therapeutic management of inflammatory bowel disease (*Sewell* et al., 2003). Oral administration of eggs of *Trichuris suis* in patients with Crohn's disease appears to be beneficial in decreasing the severity of the disease (*Summers* et al., 2005).

Role in pathogenesis

Although the data summarized above strongly suggest a beneficial role of many bacterial agents especially commensals and several helminths on the outcome of autoimmunity, equally impressive data are available to suggest that many autoimmune diseases are also triggered by infections (Table 3). Certain bacteria, namely group A streptococcus and Borellia have been characteristically associated with clinical disease such as rheumatic fever, and post infectious encephalitis, and lyme arthritis respectively (*Kamradt* et al., 2005; *Fairweather* and *Rose*, 2004). Studies in animal models have shown that certain transgenic mice do not develop EAE, spontaneous arthritis, or inflammatory bowel disease under pathogen-free conditions. However, such mice will develop diseases when bred under conventional microflora (*Kamradt* et al., 2005).

The most impressive association between infection and autoimmune disease has been observed with viruses. Studies with Theilers virus in mice have shown that intracerebral inoculation induces autoreactive T-cell responses against myelin antigens, possibly due to release of hidden or otherwise sequestered autoantigens (*Benoist* and *Mathis*, 2001; *Bach*, 2002). Other viruses clearly associated with the development of autoimmune disease processes include Coxsackie virus B4 (diabetes in transgenic mice), and rubella virus (congenital rubella and diabetes mellitus). Epstein-Barr virus infection may represent another possible candidate for viral induced autoimmune disease processes, in view of its potent immunoregulatory influence on B cell development and proliferation. The precise mechanisms underlying the development of autoimmunity after natural or induced infections remain to be defined. It has been proposed that both antigen-dependent as well as antigen-independent mechanisms are involved in the development of autoimmunity in genetically predisposed individuals (*Bach*, 2002).

Based on the information summarized above, it is suggested that any infection can be associated with the evolution of autoimmune immunologic reactivity. However, only a few agents have been clearly shown to be associated with the development of distinct immunologic markers of autoimmunity and expression of clinical disease. On the other hand, epidemiologic data collected over the past 4 decades have also suggested a strong relationship between a number of other human infections and the subsequent development of clinical autoimmune disease process as outlined in Table 3.

VACCINE INDUCED IMMUNE RESPONSES

Allergic disorders

Role in protection

Although the current discussions on vaccination related issues have largely focused on adverse effects of vaccine antigens or other vaccine components, there is interesting historical and some recent evidence to suggest that child-

hood vaccination may in fact provide protection against allergies (*Ennis* et al., 2005; *Gruber* et al., 2001a; 2001b; 2003). In a large epidemiologic study in Japan, conversion to tuberculin skin reactivity after natural infection or immunization with BCG was associated with significantly decreased incidence of asthma, hay fever, eczema and other atopic manifestations compared to subjects who continued to remain tuberculin negative. Tuberculin positive status appears to suppress the expression of IgE mediated atopic disease (*Shirakawa* et al., 1997).

Role in pathogenesis

Immediate type allergic hypersensitivity reactions to childhood vaccines occur with varying frequency with different vaccines in use at this time. Hypersensitivity reaction may be directed against the organism-specific vaccine antigens, other constituents necessary for the stability, adjuvants or other soluble vehicles included for delivery of the vaccine. These include gelatine, egg proteins, formaldehyde, chick proteins, thimerosol, aluminum, conjugating proteins, phenoxyethanol, antimicrobials, yeast, latex, and possible adventitious agents and tissue culture components in which the vaccine antigens are propagated (*Heidary* and *Cohen*, 2005; *Kelso*, 2007).

Solitary or multiple case reports of IgE mediated reactions have been observed with many childhood vaccines. These include both non-fatal as well as serious and rarely (2 cases) fatal anaphylactic reactions to diphtheria-tetanus booster, hypsensitivity reactions to HBV (about 1:1000,000 doses administered), hemophilus type B, reactivity to diphtheria conjugate proteins (4 cases), influenza vaccine (1 in 4 million doses), Japanese encephalitis (many late onset cases), pneumococcal conjugate (1 case), rabies (several late onset cases), tetanus (several cases), varicella (about 3 in 1 million doses), yellow fever (many cases).

Most of the hypersensitivity reactions reported to date with childhood vaccines seem to be associated with the nonvaccine antigen constituents such as gelatine (MMR, varicella, Japanese encephalitis), eggs (influenza, yellow fever), chicken proteins (yellow fever), aluminum (DPT, HIB, hepatitis A, pneumococcus), thimerosol (DT, HIB, HBV, typhoid, influenza, Japanese encephalitis) (*Kelso*, 2007). Many of these constituents have now been removed from more recent vaccine formulations. And, important lessons have been learned from prior use of some of these constituents. For example, development of painful and pruritic nodular lesions at the site of aluminum containing vaccines is the most frequent manifestation of hypersensitivity reaction in such vaccinees. Thimerosol is a common allergen but the clinical relevance of thimerosol allergy is relatively low. However, with the initiation of mass vaccination campaigns, the incidence of allergy in one setting increased from 6% in 1980 to 86% in 2001, after administration of thimerosol containing tick borne encephalitis vaccine. However, since 2001, most childhood vaccines employed in the United States are thimerosol free.

The development of hypersensitivity reactions to specific vaccine microbial antigens has also been reported, but is extremely rare. In one carefully conducted study, levels of IgE specific for tetanus toxoid were found to be significantly elevated in atopic subjects after immunization with DT vaccine. However, the levels declined to baseline levels over a 12-month period. Such increase in specific IgE did not promote allergic sensitization to allergens and did not promote atopic disease (*Dannemann* et al., 1996). With

other case-reports of hypersensitivity to vaccines, it is often not possible to separate reactivity to non-antigen components from specific microbial vaccine antigens.

Autoimmunity

Role in protection

As pointed out earlier, many naturally acquired bacterial and helminthic infections confer significant protection against development of autoimmunity. However, it remains to be seen if immunologic reactivity induced by vaccines, especially when it mirrors the reactivity observed after natural infection, can also offer similar protection. During early 1960's mass immunization campaigns with smallpox vaccine, an interesting bystander observation demonstrated that the incidence of diabetes mellitus declined precipitously during the peak of immunization (*Classen* and *Classen*, 1996; 1999). Studies have also shown elimination or decline in the incidence of subacute sclerosing panencephalitis after the introduction of measles vaccine in this country (*Campbell* et al., 2007). Data on other childhood vaccines relative to any possible protective role against autoimmunity are currently not available, although some studies have suggested that the timing of immunization and induced immunomodulation may prevent development of diabetes in murine models (*Classen*, 1996).

Role in pathogenesis

Several childhood infectious diseases have now been effectively controlled or eradicated in many parts of the world. At the same time, certain autoimmune disease states have shown a significant increase in their incidence and prevalence, often in the same regions where the childhood infection infections have been controlled through vaccination. Current medical literature and mass media is full of claims and counter claims regarding their possible relationship. One website designed for autoimmune diseases, claims that at least 60 such diseases ranging from alopecia areata to Wegener's granulomatosis are directly related to the use of vaccines in childhood.

Despite these claims, only a few well-defined autoimmune disease processes have been reported, albeit very rarely after childhood immunization (*Wrath* et al., 2003; *Schattner*, 2005; *Aron-Maor* and *Schoenfeld*, 2004; *Offit* and *Hackett*, 2003). For example, an earlier rabies vaccine prepared from rabies-infected brain was found to result in the development of acute disseminated encephalomyelitis in 0.1% of vaccinees (*Stuart* and *Krikorian*, 1928). Possible incidence of autoimmune diseases associated with other childhood vaccines is estimated to be as follows. Thrombocytopenic purpura, with measles vaccine alone (1:6000), with rubella vaccine alone (1:3000), with MMR (1:30,000); Guillian-Barré syndrome after swine influenza vaccine (1:100,000), other influenza vaccines (1:100,000). Acute demyelinating encephalomyelitis (ADEM) with measles vaccine is about 1:100,000 (*Chen* et al., 2001; *Zilber* et al., 1983). Arthritis has been reported after immunization with an outer surface protein A (ospA) of *Borella burgdorferi* vaccine in man, as well as in animal model after induced challenge with lyme spirochette (*Rose* et al., 2001; *Christopherson* et al., 2003).

In addition to the documented autoimmune effects summarized above, a large number of published reports have attributed vaccination to the development of multiple sclerosis, diabetes mellitus, autism, sudden infant death syndrome (SIDS), induction of immune dysfunction, chronic fatigue syndrome and other neurodevelop-

mental disorders. However, a number of large multicenter studies and several immunization safety reviews undertaken by the Institute of Medicine, National Academy of Science USA have consistently failed to demonstrate any link for the association of vaccines such as hepatitis B and MMR, and others, with autism, immune dysfunction, demyelinating neurologic diseases, multiple sclerosis, SIDS or diabetes mellitus (*Drutz*, 2007; *Schattner*, 2005).

DISCUSSION AND CONCLUSIONS

The information discussed in the preceding sections of this review suggests that acquisition of natural infections in childhood is an essential component of the maturation and development of immune response and induction of a diverse spectrum of immunoregulatory mechanisms. Acquisition of natural infections may be protective or pathogenic in the evolution of autoimmune or allergic diseases, based on underlying genetic susceptibility, and the nature of infecting organisms. Different infectious agents may exert strikingly different influences on the mechanism of protection against or the pathogenesis of autoimmune vs. allergic disease processes. Different agents may also exert strikingly different influences on the expression of disease. In general natural infections with helminths and mycobacterium are more protective and less pathogenic, while some cell associated viral infections may contribute more to the pathogenesis and development of autoimmune diseases. In addition to genetic susceptibility, other factors which influence the outcome of infections include, temporal pattern of the acquisition of the infection, age at the time of infection, route, localization and antigen burden, severity of infection, and modulation of the immune response by innate immune mechanisms. The principal immune mechanisms proposed to be responsible for development of infection-induced autoimmune reactivity include molecular mimicry, where antigenic epitopes of microorganisms closely resemble self-antigens. As a result, induction of immune responses to microbial antigens cross reacts with self-antigens and may induce autoimmunity. Other possible mechanisms relate to the bystander effects secondary to the tissue damage as a consequence of active infection, and exposure of otherwise sequestered (self) antigens. Several non-specific mechanisms may involve activation of the innate immune system, which is necessary and sometimes essential for development of adaptive B and T cell immune responses specific for each infectious agent. Abnormal or lack of intrinsic activation of innate immunity may result in loss of immunologic tolerance and development of abnormal T cell regulation for Th1 vs. Th2 type of cellular responses, which predispose to the eventual expression of physiologic, or abnormal (allergic or autoimmune immunologic) phenotypes (*Ogra* and *Welliver*, 2008). Thus, it appears that the development of transient and varying degree of autoimmune reactivity is a consistent feature of most naturally acquired infections.

The introduction of many new vaccines associated with emergence of increased frequency of allergic and autoimmune disorders in our society has lead to the current concerns about the risk of vaccine-induced immunologically mediated diseases. In retrospect, it is remarkable that a large number of successful and highly effective vaccines were developed and used

Table 4: Induction of different cytokine profiles by environmental factors and their disease association*

	Increased Th1 responses	Increased Th2 responses
Environmental factors	Normal mucosal bacterial flora	Diet and processed foods in developed world
	Inflammatory cytokines	Cow's milk and formula
	Breast feeding	Antibiotics
	Infectious agents	Infectious agents
Disease associations	Autoimmune thyroiditis	Leishmania; Toxoplasma
	Exp. autoimmune uveoretinitis	Candida; mycobacteria (M. tuberculosis; M. leprae)
	Crohn's disease	HIV, atopic dermatitis
	EAE; MS; IDDM	Asthma; allergic rhinitis

*Ogra and Welliver, 2008

extensively for childhood immunization long before the acquisition of our current knowledge about the immune system in early childhood. It has been now demonstrated that prenatal and postnatal periods in general favour Th2 T cell cytokine profile, and the profile shifts toward a Th1 type response by preschool age. The shift toward Th1 T cell response is largely driven by common childhood infections, and there is a delayed or incomplete shift toward Th1 responses in atopic subjects. Increased Th1 T cell responses have been associated with autoimmune thyroiditis, EAE, MS, diabetes mellitus and Crohn's disease. On the other hand, increased Th2 T cell response have been associated with diseases due to *Toxoplasma*, *Leishmania*, *Candida*, HIV, mycobacteria, as well as in atopic and allergic disorders such as asthma, atopic dermatitis, and allergic rhinitis (Table 4) (*Ogra* and *Welliver*, 2008).

It is clear now that most primary immunizations are given during the period in childhood, which favours Th2 responses. By 6 months of age, most children will have received as many as 16 different microbial vaccines. Because of the elimination of smallpox, poliomyelitis, and significant decline in mortality and morbidity associated with other vaccine preventable infectious diseases, concerns have now shifted toward the relative risk of childhood vaccines, rather than the dangers of natural infection, which still prevail in many parts of the world. The information summarized above provides evidence for the safety and effectiveness of existing vaccines in the prevention of several serious infectious diseases. However, it is also now recognized that no vaccine developed to date is completely safe and or completely effective in each and every immunized individual. Some vaccinees may have an adverse reaction, and some may not be fully protected. Some existing vaccines have been clearly associated with the development of transient autoimmune disorders such as thrombocytopenic purpura, and Guillain-Barré syndrome. However, such side effects are relatively rare (1 in 30,000 to 1 in 1,000,000). There are other immunologic alterations observed after

childhood immunization. These include increased IgE responses to tetanus and diphtheria toxoids, increased class switch for mRNA for IgE after MMR immunization, possible immune enhancement with prior priming for Japanese encephalitis, spontaneous reversion to virulent phenotypes after oral administration of live attenuated (Sabin) poliovaccine, and development of vaccine associated paralytic polio in some (1 in 10,000 to 1 in 1,000,000 doses of OPV administered) in vaccinated subjects, and development of intussusception after administration of an earlier rhesus reassortant rotavirus vaccine.

The biological significance of the many immunologic alterations observed after immunization must be assessed in the context of associated clinical symptoms. For example, it may not be surprising to elicit laboratory markers of immunologic hypersensitivity or autoimmunity after immunization in a manner similar to natural infection. However, such immunologic reactivity does not necessarily reflect development of established allergic or autoimmune disease. The human immune system is well endowed with other compensatory mechanisms to prevent development of such diseases. However, it is important to monitor the induction of such laboratory findings with existing as well as with next generation of vaccines which may contain, different or more potent adjuvants, other carrier proteins, or other unique antigenic epitopes, which may mimic pathogenic processes associated with the development of natural disease.

It is neither advisable, nor possible at this time, to completely eliminate pathogenic or disease-producing microorganisms from our environment. The goal of future development of new and continued usage of existing vaccines is to induce an optimum degree of protection against disease with the lowest possible rate of adverse side effects, including the development of allergic and autoimmune disease processes. Until we are able to develop highly effective vaccines with no side effects whatsoever, the rationale for the use of existing vaccines should consider each specific situation, the burden of natural disease, its global magnitude, morbidity and mortality; epidemiologic nature of the disease relative to endemic and epidemic spread, relative risks associated with natural disease; long term effects of the vaccine, and the societal acceptance of the vaccine in a changing global population base.

Finally, it must be emphasized that the recent increase in the incidence of many allergic and autoimmune diseases may have more to do with the overwhelming environmental alterations and changes in global ecology than with the prevention of childhood infectious diseases by immunization practices.

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BIOLOGICAL CONSEQUENCES OF HOST-MICROBE INTERACTIONS: SUMMARY OF THE SEMINAR

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INTRODUCTION: MICROBIOTA AND PROBIOTICS

Each organism lives in continuous interaction with its environment. This interaction is of great importance but at the same time it could be life-threatening. The largest interface between the organism and its environment is represented by surfaces covered by epithelial cells. Mucosae represent in humans about 300 m^2 while skin covers approximately 2 m^2 of the human body. Starting from first hours after the delivery from the sterile uterine environment the interaction of the macroorganism with microorganisms begins. Physiologically occurring interaction with bacteria leads to colonization of epithelial surfaces and this co-existence is usually beneficial for the host. A complex, open ecosystem formed by resident bacteria and transiently present microbes interacting with the macroorganism is founded. The number of bacteria colonizing mucosal and skin surfaces exceeds the number of cells forming human body. However, under some conditions the interaction with "endogenous" microbes can be harmful for the host. Beneficial or harmful effects of commensal bacteria and their components in pathogenetic mechanisms of various complex and multigenic diseases have been recently recognized. Animal models of human diseases reared in defined gnotobiotic conditions (e.g. germfree animals) are helping to elucidate the aetiology of these frequently occurring disorders. An improved understanding of commensal bacteria – host interactions employing germfree animal models with selective colonization strategies combined with modern molecular biological techniques analysing the microbiota composition is bringing new insights into the mechanisms of several inflammatory autoimmune and neoplastic diseases.

Increased interest in an influence of intestinal microflora in human and animal health resulted in attempts of improving optimally its composition by using probiotics (most frequently bacteria of lactic fermentation). Probiotics, live microorganisms acting beneficially on host health were shown to influence favourably development and the stability to microflora, inhibit colonization by pathogens, influence the mucosal barrier by their trophic effect on intestinal epithelium and stimulate innate and adaptive components on the immune system. Orally administered probiotic bacteria are expected to survive passage through stomach and colonize intestinal mucosal surfaces even if it were only for a short period. It was shown that colonization of newborns with probiotic *E. coli* 083 stimulated

local and systemic humoral and cellular immune responses and reduced the number of pathogens. Moreover it was shown that repeated oral application of a non-pathogenic *E. coli* strain in the early postnatal period prevented the incidence of allergies as confirmed by a long-term (10 and 20 years) study. Understanding the regulation of mucosal immune responses to commensal microflora may be the key for the targeted manipulation of microflora composition and successful intervention in a wide range of chronic diseases.

There are more than 4000 hits on the Internet concerning the various outcomes of using probiotics. Numerous strains of microbes have been tested, including recombinant bacteria. Very variable results have been reached. There are claims of success i.e. against rotavirus infections, reducing diarrhoea by one day in infants. The effect was better in rats. It was stated on one hand that critical reviews of the literature only left orchitis as a condition, which can be efficiently treated by probiotics. It was claimed that the extensive literature on other conditions did not give support for proven effects against other conditions. This was debated by others who claimed that several other diseases can be treated with probiotics, like irritable bowel disease and atopic dermatitis. Rotavirus infections can be shortened with probiotics. It was mentioned that just for Lactobacilli more than 20 strains have been tested.

More probiotic effects have been noted in mice models than in man. The effects of probiotics on tumours were studied. It was found that germfree animals get adenomas, whereas conventional animals get carcinomas. Microbes and microbial enzymes were said to be without significance for carcinogenesis, e.g. β-glucuronidase. This is also true for nitrosamine. An animal model, with the animals carrying a tumour, was mentioned in which Bifidobacteria were given together with an anti-tumour drug. The tumour took up the microbes and the drug followed the bacteria; as a consequence the tumour contained substantial amounts of the anti-tumour drug.

BREASTFEEDING

Human milk is said to contain some 100.000 components. Of these we have knowledge of about just a few, and also that information remains incomplete. Breastfeeding should start immediately after birth. Actually, if the baby directly after delivery is put onto the mother's abdomen, it will start crawling up towards her breast and massage it to start the milk to flow. Thus breastfeeding is initiated. Within an hour just about all babies have performed this task, which also adds to the bonding between mother and offspring. But in many societies the baby is taken away from the mother and in some cultures it may instead be given various fluids and foods, which may be heavily contaminated, especially in poor communities, bringing a high risk of infections in early life, contributing to high infant mortality. Also it may derange the early bacterial colonization so that the neonate not only will be colonized with the mother's microbes, but also those from the hospital staff and others. This may expose the neonate to microbes of higher virulence and microbes to which the mother is not providing protection via her transplacentally transferred IgG antibodies.

A recent study from Ghana showed that infant mortality was 12% higher if the baby was not breastfed until one

day after delivery and 20% higher if it was not breastfed until day 2-3, compared to breastfeeding within one hour.

The milk secretory IgA (SIgA) antibodies may be specifically protective since they, thanks to the enteromammaric link, are directed against the intestinal microflora of the mother.

Normally this is the microflora the offspring is exposed to during normal delivery. The microbial exposure starts already in the birth canal. However, Caesarean section is becoming more and more common and impairs the normal contamination of the neonate with the mother's microflora. From South America up to 30-40% of deliveries are now reported to be section deliveries. It has not been properly studied what this means for the early microbial colonization of neonates. It has recently been made likely that the M cells covering the Peyer's patches have receptors binding SIgA. This might in the newborn result in milk SIgA antibodies, which are taken up together with the microbes against which they are directed. Protection would be provided, but also enhancement of mucosal immune responses in the infant.

Human milk has numerous additional defence components, e.g. lactoferrin, which on one hand can kill microbes and on the other turn off proinflammatory host responses, presumably because they are costly to the host by causing loss of appetite, tiredness etc, all symptoms most undesirable in early life. The blocking of inflammation occurs because the large amounts of milk lactoferrin is taken up by leucocytes and block their NFκB. Thus, they cannot produce proinflammatory cytokines.The major milk protein, α-lactalbumin, has recently been shown to have the capacity together with adequate co-factors to kill tumour cells. Human milk contains about 5-7% carbohydrates. They are not taken up by the infant, but remain in the gut where they function by being analogues to the structures used as receptors by the microorganisms, which try to invade the host.

The potential capacity of the major human milk protein α-lactalbumin to take a shape that makes it capable of killing tumour cells was also discussed. The structure with this capacity appears as HAMLET (*H*uman *A*lpha Lactalbumin *M*ade *L*ethal to *T*umour Cells) after being exposed to the low pH in the stomach together with exposure to oleic acid.

There are some suggestions that breastfeeding may enhance certain vaccine responses, e.g. against *Haemophilus influenzae* type b. The mechanism is unknown.

A very impressive study of the effect on IQ by breastfeeding showed a gain of 11 points, presumably indicating uptake of structures from milk favourable for brain development.

The question was brought up of when gut closure, preventing uptake of large molecules, takes place. It was indicated that in man this occurs before birth, in contrast to what is seen in certain other species, like mice and rats. On the other hand there are suggestions of uptake both of cells and large proteins later. There are several studies showing uptake in various species. In man one might consider that these studies should be repeated in the light of the fact that the receptors on the M cells covering the Peyer's patches are capable of uptake of SIgA, but not other immunoglobulins. On the other hand this special mechanism also results in an uptake of the SIgA-bound antigens. The great majority of the milk SIgA remains in the gut, preventing microbes from entering the gut mucosa. Other species have different mecha-

nisms for their mucosal defence and protection of the offspring via the milk.

Banks for human milk were discussed. They are no longer used in USA, because of the risk of giving one mother's milk to the baby of another mother. The risk of infection was regarded as a major problem, especially the risk of transfer of HIV-virus. CMV can be transferred to infants via the milk also from their own mothers, but symptoms do not follow. In Europe human milk banks remain in use and are regarded as safe. There is limited information about how much biological function of milk proteins are lost by heating. This varies of course with what form and extent of heating which is used and this seems not to have been carefully studied.

Human milk has been tried for treatment of inflammatory diseases, but there are no controlled studies. Commercial cow's milk comes in 80% from pregnant cows and contains much oestrogen, which might be carcinogenic. The potential risk of this oestrogen content of the milk fat, e.g. for causing breast cancer, has not been investigated. The finding of pesticides in human milk is a problem, which has not been properly taken care of.

By now there are 3 major evidence based studies of the short and long term effects of breastfeeding, one from the WHO, one from Holland and one from the US Dept of Health. They conclude that breastfeeding provides significant protection against the acute conditions sudden infant death syndrome (SIDS), necrotizing enterocolitis (NEC), acute gastroenteritis, pneumonia and otitis media. Long-term therapeutic, preventive, or reducing effects are noted on blood pressure, obesity, cardiovascular disease, acute lymphatic and myelogenous leukaemia, diabetes type 1 and 2, coeliac disease, serum cholesterol, asthma and eczema, if there is heredity. Furthermore, breastfeeding mothers experience less risk for breast cancer and rheumatoid arthritis.

VAGINAL MICROBIOTA OF THE FEMALE GENITAL TRACT

As an example of luminal habitation was discussed the female genital tract microbiota. The microbiota of the vagina is in contrast to those of other sites of the body regulated by oestrogen. As shown by Gram-staining of wet smears it appears as if lactobacilli may not be firmly attached to the epithelial cells, but rather are located in the mucus. When imbalance of the microbiota appears (bacterial vaginosis, BV), characterized by high numbers of anaerobic bacteria (at least 1000-fold increase), *Gardnerella*-like bacteria are firmly attached to the cells. This induces increased levels of IL-1 and sometimes IL-8 without signs of inflammation, showing that possibly the total number of bacteria or the increased number of attached bacteria to cell surfaces is triggering a higher level of "host-awareness". In fact, we have found a correlation between the number of CFU in the vagina and cervical IL-1 in asymptomatic women.

Also, there is a commensal symbiosis between various bacterial species. For example it has been shown that *P. revotella bivia*, a typical BV-associated species may enhance the growth of both *G. vaginalis* and *Peptostreptococcus anaerobius* by producing ammonia and amino acids, respectively (*in vitro* experiments). We have observed that there are significant correlations between certain bacteria such as *G. vaginalis* and *Atopobium vaginae*, *F. nucleatum* and *S. anginosus* and may

imply that some accumulated metabolites favours such symbiosis.

As an example of influence on the luminal habitation Dr. Mattsby-Baltzer briefly presented their investigation on the prevalence of *Lactobacillus*-dominated biota (LBD) in healthy fertile women of three age cohorts and relation to contraceptive methods. The study was performed on 313 women scheduled for cervical screening. It was found that women in the age cohort 20-29 years had a LDB in approximately 90%. The frequency was reduced with age to ca 70% at 30-39 and 50% at 40-49 years of age. The use of oral contraceptives resulted in almost no difference between the three age groups, a high level of LBD was present in all (83-90%). The use of copper intra-uterine device (IUD) disturbed the LBD, since the frequency decreased to 40% in the age group 30-39 years of age (no IUD users in the 20-29 year group). The frequency among women using hormone-releasing IUDs was 50% in the age group 30-39 years and appeared to increase to 70% in the age group 40-49. The most deviating LDB frequencies were thus observed between non-users and IUD-users in the 30-39 age group, or oral contraceptive-users and hormone-releasing IUD-users in the 40-49 age groups. The two latter ones were increased with respect to LDB.

It was concluded that decreasing oestrogen levels with age, contributes to a lower frequency of LBD. However, use of IUDs and oral contraceptives strongly influences the vaginal microbiota.

MICROBIOTA IN ALLERGY AND AUTOIMMUNITY

It is generally accepted that gut microflora has an important impact on mechanisms of immune regulation. It is believed that the microflora is involved in induction and maintenance of physiological tolerance, preventing hyper-responsiveness leading to allergies and food enteropathies. The literary data on studies in germfree animals show diverse results on the significance of the microflora for tolerance induction. Data presented in the seminar show that mucosal tolerance induction can be equally induced in experimental model of pollen allergy, in mouse with or without the presence of commensal bacterial flora: Oral as well as intranasal tolerization led to suppression of allergen specific serum antibodies as well as cytokine production by splenocytes in both germfree and conventional animals. We therefore concluded 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.

We propose that dysfunction of the immune system associated with the gut and other mucosal surfaces is a prerequisite for impairment of physiologically developing regulatory mechanisms. The balance in intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the mucosae, by qualitative or quantitative changes in the composition of mucosal microbiota, or by inadequately functioning components of the innate or adaptive immune system occurring in cases of dysregulated mechanisms of mucosal immunity, or in immunodeficiencies. An expression of pathologically increased immunological activity may induce inflammatory processes of a different character, depending on that type and mediators of inflammation. Thus, numerous chronic diseases may occur as a result of disturbances of mu-

cosal barrier function or of changes in mechanisms regulating mucosal immunity. The main characteristics of chronic, "idiopathic", inflammatory, and autoimmune diseases are tissue destruction and functional impairment as a consequence of immunologically mediated mechanisms that are principally the same as those functioning against dangerous (pathogenic) infections. One of the most attractive explanations for inflammatory and autoimmune phenomena has centred on various infections as natural event capable of initiating the process in genetically predisposed individuals. We propose that not only pathogenic microorganisms but also components of normal microflora could participate in the triggering and development of inflammatory and autoimmune processes.

We used colitis, induced by dextran sulfate sodium (DSS) feeding of mice, to study the immunological factors involved in the pathogenic mechanisms of chemically triggered intestinal inflammation.

These experimental models were used also to analyze the role of commensal bacteria and innate immunity in the development of intestinal inflammation. Using the DSS-induced model of intestinal inflammation, we have shown that, as in conventionally reared, immunocompetent Balb/c mice, mice with severe combined immunodeficiency (SCID) developed profound inflammatory changes in colonic mucosa. Balb/c and SCID mice reared in germfree conditions developed only minor signs of mucosal inflammation. Interestingly, conventionally reared SCID mice, lacking T and B cells, developed intestinal inflammation similar to the inflammation that developed in immunocompetent Balb/c mice. This finding suggests that under physiological conditions, innate immunity components are able to regulate (keep in balance) the interaction of macroorganisms with commensal bacteria, and, after chemically induced breakdown of mucosal barrier, commensal bacteria could induce severe forms of intestinal inflammation in the absence of components of adaptive immunity (T and B cells).

Experiments performed in gnotobiotic models suggest that the composition of gut microbiota plays a decisive role in the pathogenetic mechanism of intestinal inflammation. Exogenous application of commensal organisms (probiotics) exerting beneficial effects on host health has recently been shown to have protective and therapeutic effects on diarrhoeal disease, including IBD, and to reduce the risk of infections and allergies. Oral application of probiotic bacteria is associated with an alleviation of intestinal inflammation and normalization of increased intestinal permeability, together with promotion of intestinal barrier functions.

Components of commensal bacteria were tested in experimentally (DSS) induced intestinal inflammation to study the effects of their oral application. DSS colitis in immunocompetent Balb/c mice was mitigated by oral administration of the lysate and fractions of some *Bacteroides* strains. Similarly oral application of heat shock proteins alleviated intestinal inflammation induced by DSS treatment. This protective effect was accompanied by stabilization of intestinal microflora composition and decrease of proinflamatory cytokine production.

A not yet solved crucial question is why we see increased prevalence of autoimmune and allergic diseases during the last 30 years. Many hypotheses have been proposed to explain the increasing tendency in occurrence of inflammatory and neoplastic diseases: Less exposure to microbes, decreased

diversity of the microflora, changes in food intake and food quality, etc. The skewing of the immune system noted in early life is considered of aetiological significance. The argumentation about autoimmunity indicated that autoimmune diseases can be mediated via TH1 as well as TH2 mechanisms. The crucial role of T-regulatory cells and the role of microflora in their development and differentiation were pointed out. Since infections can induce autoimmunity, at least certain autoimmune diseases may be contagious. The example was mentioned about the discovery of the Gm factor. It was found that infections with cross-reacting herpes viruses gave rise to the Gm factor, which had found its clinical use as a test of autoimmune disease in the form of rheumatoid arthritis.

VACCINATION PROGRAM AND SAFETY/REGULATIONS

A rotavirus vaccine is now produced in Austria and further vaccines are being developed. For instance in Belgium a vaccine is being worked on, containing Lactococci producing IL-10. There are many other vaccines being considered for development, now in a broader way than previously. Thus probiotic effects are sought, and new adjuvants are tested.

Existing vaccines are being improved and new areas are considered like effects on the microflora. The role of undernutrition of the host is better analysed and existing vaccines are improved.

The control of drugs and biologicals is fundamental for our safety. It is a complex issue.

Bacteria, which have grown for 4 hours compared to 40 hours, may be quite different. Sthe same is true for live compared to dead bacteria. Thus determining safety of a bacterial product becomes complicated. Vaccines come on the market easier than probiotic products. The presence of gross contaminants is a major issue.

The requirements in US for regulation of vaccines and probiotics were presented in rather strict details. The corresponding rules for biologicals like probiotics seemed to be more lax. The same is true for the regulatory demands within the European Union. The rules are continuously being adjusted and it is expected that the rules within EU will become similar to those now in use in USA. Lobbying by large companies is common. Clinical trials seem to be simpler approved in the EU than in the USA.