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EFFECTS OF PROBIOTIC BACTERIA ON SYMPTOMS AND ON IMMUNOLOGIC RESPONSES IN INFANTS WITH ATOPIC DERMATITIS

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To Ronny and Melinda

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1. ABBREVIATIONS

AD	Atopic dermatitis
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Antigen-presenting cell
AT	Alpha-1 antitrypsin
cfu	Colony-forming unit
CI	Confidence interval
CM	Cow's milk
CMA	Cow's Milk Allergy
CRP	C- reactive protein
DBPCFC	Double-blind placebo-controlled food challenge
DC	Dendritic cell
ECP	Eosinophil cationic protein
EHF	Extensively hydrolysed formula
ELISA	Enzyme-linked immunosorbent assay
FAE	Follicle-associated epithelium
GALT	Gut-associated lymphoid tissue
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule
IEL	Intra-epithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LFA	Lymphocyte function-associated antigen
LGG	*Lactobacillus* GG
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M cell	Microfold cell
MAD-CAM1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility class
MIX	Mixture of probiotics
MLN	Mesenteric lymph node
mRNA	Messenger ribonucleic acid
NK cell	Natural killer cell
PBMC	Peripheral blood mononuclear cell
PP	Peyer's patch
ra	Receptor antagonist
s	Soluble
SCORAD	Severity Scoring of Atopic Dermatitis
SPT	Skin prick test
TCR	T-cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
VCAM-1	Vascular cell adhesion molecule-1

2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by their Roman numerals (I-IV):

I **Viljanen M**, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, and Kuitunen M. Probiotics in the treatment of atopic eczema/dermatitis syndrome in infants: a double blind placebo controlled trial. Allergy 60:494-500, 2005.

II Pohjavuori E*, **Viljanen M***, Korpela R, Kuitunen M, Tiittanen M, Vaarala O, and Savilahti E. Lactobacillus GG effect in increasing IFN-γ production in infants with cow's milk allergy. J Allergy Clin Immunol 114:131-6, 2004.

III **Viljanen M***, Pohjavuori E*, Haahtela T, Korpela R, Kuitunen M, Sarnesto A, Vaarala O, and Savilahti E. Induction of inflammation as a possible mechanism of probiotic effect in atopic eczema/dermatitis syndrome.
J Allergy Clin Immunol 115;1254-9, 2005.

IV **Viljanen M**, Kuitunen M, Haahtela T, Juntunen-Backman K, Korpela R, and Savilahti E. Probiotic effects on faecal inflammatory markers and on faecal IgA in food allergic atopic eczema/dermatitis syndrome infants. Pediatr Allergy Immunol 16:65-71, 2005.

* These authors have contributed equally.

Reprinted here with the permission of the publishers. In addition, some previously unpublished data are presented.

3. ABSTRACT

Imbalance of Th1/Th2 immune responses and defects in production of suppressive cytokines are characteristics of atopic dermatitis (AD) and cow's milk allergy (CMA). Epidemiological data suggest that changes in gut microbiota are associated with emergence of allergies. These changes are proposed to be counter-balanced by probiotics. Previous studies suggest that probiotic bacteria alleviate symptoms of AD in infants. The immunomodulating mechanisms of probiotics are, however, poorly understood.

The present study investigates whether *Lactobacillus* GG or a combination of probiotics alleviates AD symptoms and also explores the immunological effects of these preparations in infants with AD and CMA.

In this study, 230 infants with AD and suspected CMA received, in a randomised double-blinded manner concomitant with elimination diet and skin treatment, *Lactobacillus* GG (LGG), a mixture of four probiotic strains (MIX), or placebo for 4 weeks. IgE-association was determined by skin prick tests and antigen-specific IgE antibody determinations. Four weeks after this treatment, CMA was diagnosed with a double-blind placebo-controlled milk challenge in 120 infants. Alleviation of AD symptoms was assessed by reduction in SCORAD index. In addition, we evaluated probiotic effects on production of cytokines of stimulated PBMCs, on plasma levels of cytokines and inflammatory markers, on faecal IgA, and on the intestinal inflammation markers.

In infants with IgE-associated AD, the LGG group showed a greater reduction in SCORAD than did the placebo group, -26.1 vs –19.8 ($p=0.036$), from baseline to 4 weeks after treatment. The MIX treatment did not differ from placebo. In the whole study population or in CMA, the probiotics demonstrated no effect on AD. Before the treatment, secretion of IFN-γ by PBMC was significantly

lower in infants with CMA than in infants without CMA (p = 0.016). Among the infants who received LGG, secretion of IFN-γ increased in those with IgE-associated AD (p = 0.017) and in those with CMA (p = 0.006), when compared to the placebo group. Treatment with LGG induced higher CRP levels (geom. mean 0.83 μg/mL vs 0.42 μg/mL; p=0.021) and higher plasma IL-6 levels (mean 3.4 pg/mL vs 0.9 pg/mL; p=0.036) in IgE-associated AD infants, compared to placebo. In the same subgroup, post-challenge faecal IgA was elevated in the LGG group, compared to the placebo group (geom. mean 0.74 g/L vs 0.44 g/L; p=0.012). In AD, post-treatment faecal IgA tended to be increased after probiotic treatments, compared to the placebo (p=0.064). In AD and in CMA, plasma levels of IL-10 increased during the MIX treatment (p=0.016, and p=0.004), and IL-4 secretion of PBMCs increased significantly in infants with CMA (p = 0.034).

In conclusion, among infants with IgE-associated AD, LGG treatment alleviated AD symptoms, compared to the placebo group. In the same subgroup, LGG treatment provided enhancement in Th1 signals. IL-6 and CRP levels in plasma were also increased after LGG treatment, suggesting activation of the innate immunity. After challenge, faecal IgA was elevated in the LGG group, compared to the placebo group. Interestingly, those infants with the greatest alleviation of AD symptoms more often showed an increase in IFN-γ secretion, elevated IL-6 levels in plasma, or increased faecal IgA levels after treatment, compared to those who showed a smaller alleviation of AD symptoms. This association suggests that these factors mediate the clinical effect of LGG in IgE-associated AD. In CMA, LGG induced up-regulation of Th1 response, and faecal IgA tended to be increased in the AD group after probiotics, with no concomitant alleviation of symptoms, suggesting that the effect of probiotics is modified by host-related factors. During the MIX treatment, plasma levels of IL-10 in AD and in CMA increased, with enhancement of Th2 type cytokine in CMA. These differences in immunologic responses in comparison to the LGG may explain the negligible clinical effect of the MIX.

4. REVIEW OF THE LITTERATURE

4.1 GUT IMMUNE SYSTEM

The gut associated-immune system is probably the largest and the most complex part of the immune system. It performs a very delicate task, as it defends mucosal membranes from invasive pathogens, and at the same time accepts dietary antigens and commensal bacteria without reactivity. Effective immune responses are needed for defence, as most offending infections enter the human body via mucosal sites. By contrast, active immunity against necessary substances would be harmful, for instance, hypersensitivity reactions against food antigens or normal intestinal microbiota, causing food allergies or intestinal inflammatory diseases. Thus, a normal healthy person instead develops local and systemic immunologic tolerance: oral tolerance (reviewed by Mowat 2003).

4.1.1 Anatomy and function of the gut immune system

The mucosal barrier in the gut consists of cellular and non-cellular components. Proteolytic enzymes together with acidity in the stomach and proximal intestine break down ingested proteins into peptides, and this destroys immunogenic epitopes. Other protective factors are gastrointestinal peristalsis and mucus that lines epithelium and prevents antigens to contact it. If antigens make contact with epithelium, they meet gut-associated lymphoid tissue (GALT). GALT consists of various immune cells found in the epithelium and lamina propria, and organized tissues that are Peyer's patches (PP), mesenteric lymph nodes (MLNs), and isolated lymphoid follicles (reviewed by Chehade and Mayer 2005; Mayer 2003; Mowat 2003).

PPs are lymphoid aggregates localized in the submucosa of the small intestine. They consist of B-cell follicles surrounded by a small number of T cells. The lymphoid area is separated from the gut lumen by columnar epithelial cells called follicle-associated epithelium (FAE). It contains specialized epithelial cells, microfold cells (M cells), that take up particulate, but not soluble antigens, and transport it to so-called professional antigen-presenting cells (APCs) either in the

epithelium or sub-epithelial dome region of PP. Professional APCs, for example macrophages, dendritic cells (DCs), and B cells, are cells that can express major histocompatibility class (MHC or human leukocyte antigen, HLA, in humans) II molecules and also costimulatory adhesion molecules. APCs move to T cell areas or B cell follicles, where interaction with T or B cells occurs. B cells undergo immunoglobulin (Ig)A switching under the control of local factors from APCs and T cells, including transforming growth factor (TGF)-β1 and interleukin (IL)-10, as well as by cellular signals. Lymphocytes that are activated in PPs exit through efferent lymphatics to MLNs. There they differentiate further and migrate to the circulation when L-selectin expression is lost. From the circulation they accumulate in the mucosa, by expression of $\alpha_4\beta_7$ integrin that interacts with its ligand mucosal addressin cell adhesion molecule-1 (MAD-CAM1) (reviewed by Bellanti et al. 2004; Brandtzaeg 2001; Chehade and Mayer 2005; Mowat 2003).

4.1.2 T cells

Two-thirds of T lymphocytes are CD4+ T cells, which recognize exogenous antigens on class II MHC molecules. After migration to the mucosa, they remain in the lamina propria, and are localized evenly in the villus-crypt area. The remaining one-third are CD8+ T cells, which recognize endogenous antigens presented on class I MHC molecules, and after entering the mucosa prefer the epithelium. T cells with a 'memory' phenotype, indicating previous contact with antigen, are major constituent of the epithelium and lamina propria. Lamina propria CD4+ T cells play an important role in local immune regulation. They normally do not respond to T cell-receptor (TCR) mediated signals, but in humans, accessory molecules can induce them to proliferate. Expression levels of costimulatory molecules on APCs affect differentiation of naïve T cells. Interaction of the T cell CD28 receptor with the B7.1 molecule favours T-helper (Th)1 type development, and with B7.2, Th2 development. CD8+ lamina propria T cells may show cytotoxic T-lymphocyte activity. Antigen-stimulated T cells in the lamina propria may be true effector cells that aid in B cell

production of IgA. Lamina propria T cells may also act as regulatory T cells and take part in development of tolerance to environmental antigens (Bellanti et al. 2004; Brandtzaeg 2001; Mowat 2003).

Intraepithelial lymphocytes (IELs) are a particular population of T cells, as their features differ from those of the lamina propria and systemic lymphoid tissues. The major proportion of IELs in the human intestine is of the CD8+ cell subpopulation, and fewer are of the CD4+ subpopulation (reviewed by MacDonald 2003). Most IELs express TCRα/β, which suggests that they are memory T cells (reviewed by Brandtzaeg et al. 1989).

4.1.2.1 Th1/Th2 dichotomy and regulatory T cells

T helper type 1 (Th1) and type 2 (Th2) cells represent two different subsets of CD4+ cells with different types of immune responses and cytokine patterns (Table 1). Th1 cells produce interferon (IFN)-γ, and tumour necrosis factor (TNF)-β. Th2 cells produce IL-4, IL-5, IL-9, and IL-25. Both subsets produce granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-2, IL-3, IL-10, and IL-13. In humans, the dichotomy in cytokine profiles is not completely clear. Th1 cells evoke cell-mediated immunity, induce T cells and macrophages, and B cell production of opsonizing- and complement-fixing antibodies. Th2 cells promote induction of allergic responses in the absence of IFN-γ by evoking strong antibody responses, including IgE, and inducing eosinophil differentiation and activation (reviewed by Romagnani 2000; Borish and Steinke 2003). Th1/Th2 cells and mechanisms involved in their polarisation are described briefly in Figure 1.

Regulatory T cells may affect immune responses. Th3 cells produce TGF-β cytokine, plus various amounts of IL-4 and IL-10. Increased TGF-β levels have been associated with induction of oral tolerance (Zemann et al. 2003). TGF-β1 inhibits activity of antigen-presenting cells, which inhibits induction of immune responses (Arkwright et al. 2001); it also promotes IgA production by switching immunoglobulin isotype in human B cells (van Vlasselaer et al. 1992). T

regulatory 1 cells secrete IL-10, which down regulates antigen specific immune responses, and in animal model prevents development of colitis. CD4+CD25+ regulatory T cells are derived from the thymus. In addition to ability to prevent auto-reactivity, these cells have been associated with oral tolerance induction in a murine model after ovalbumin ingestion, by expressing an increased number of CD4+CD25+ cells with secretion of cytotoxic T lymphocyte antigen-4, TGF-β, and IL-10. Immunosuppression by CD4+CD25+ cells is suggested to be mediated by cell-surface bound TGF-β. This type of active suppressive mechanism may elicit 'bystander suppression': Suppressive cytokines released to the local microenvironment may suppress ongoing immune responses to other antigens nearby (reviewed by Chehade and Mayer 2005; Mowat 2003; Strobel 2002).

Table 1. T-helper cell subsets, and cytokines they produce. (Modified from Alam and Gorska 2003; Borish and Steinke 2003; Chehade and Mayer 2005)

Th cell subset	**Cytokines produced**
Th0	IL-2
Th1	IFN-γ, TNF-β GM-CSF, TNF-α, IL-2, IL-3, IL-10, IL-13
Th2	IL-4, IL-5, IL-9, IL-25 GM-CSF, TNF-α, IL-2, IL-3, IL-10, IL-13
Th3	TGF-β, IL-4, IL-10
Tr1	IL-10
CD4+CD25+T reg cells	TGF-β, IL-10

Abbreviations: GM-CSF, Granulocyte-macrophage colony-stimulating factor; IFN, Interferon; IL, Interleukin; TGF, Transforming growth factor; TNF, Tumour necrosis factor

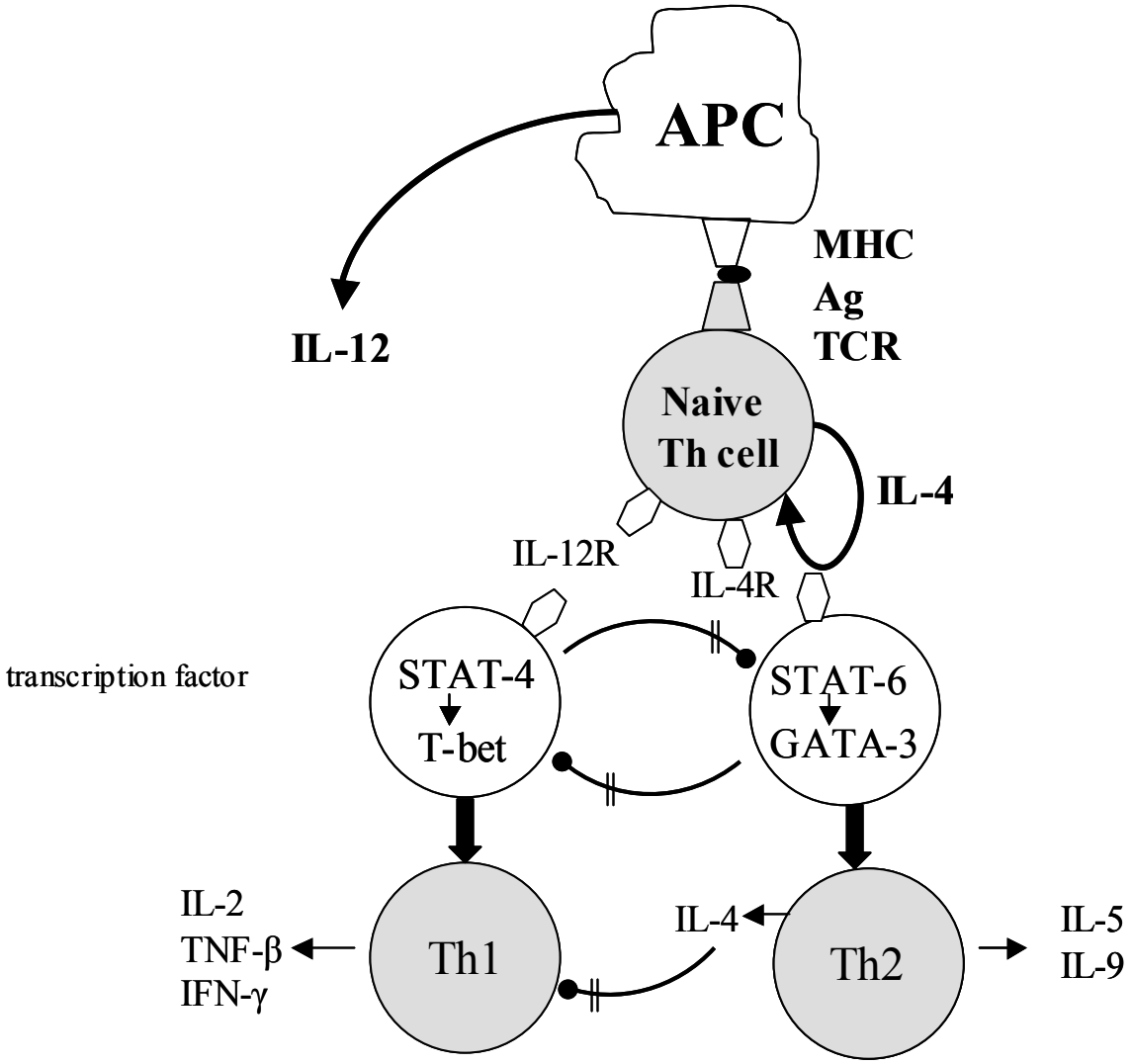


Figure 1. Mechanisms involved in polarisation of Th1 and Th2 cells (Modified from Romagnani 2004).

Abbreviations: GATA, transcriptional factor binding to nucleotide sequence element GATA; IFN, Interferon; IL, Interleukin; T-bet, T-box transcription factor; TNF, Tumour necrosis factor; STAT, Signal transducer and activator of transcription

4.1.3 B-cells

APCs present antigens in PP to T and B cells. B cells are characterised by having immunoglobulins as antigen receptors, naïve B cells expressing IgM and IgD. When B cells act like APCs, antigens

bind to membrane immunoglobulins, then B cells process them and present them to T cells on class II MHC grooves. The intimate interaction of T and B cells allows signal transmission via CD40 ligand-CD40 coreceptor, leading to initiation of immunoglobulin class switching. T cells modulate B cells by secreting cytokines (Alam and Gorska 2003). IgA class switching occurs under the effect of TGF-β1, and IL-10 (Mowat 2003), while IL-4 induces IgE and IgG secretion (Pene et al. 1988). From PPs, activated B-cells exit via regional lymph nodes into the circulation, and enter the lamina propria, where they mature into IgA-producing plasma cells (Mowat 2003).

The major production of intestinal immunoglobulin is secretory (s)IgA. In lamina propria, 30 to 40% of plasma cells secrete IgA, while in the jejunum 80% secrete IgA, 18% IgM, and only 3% IgG. Few plasma cells produce IgD and IgE. Two subclasses of IgA are secreted: IgA1 and IgA2. The majority of IgA secreted is dimeric, 100% of IgA2 has J chains, and 88% of IgA1. Via the J chain, the secretory component binds dimeric IgA specifically to crypt epithelial cells, and is then actively transported to the gut lumen (reviewed by MacDonald 2003). Secretory IgA does not bind complement, and thus does not initiate a complement-dependent inflammation process. It inhibits colonisation and invasion by bacteria. IgA may also inactivate viruses in epithelial cells. SIgA is also proposed to make complexes with antigens, which then are trapped in the mucus and removed from the body (Brandtzaeg et al. 1999; Mayer 2003).

4.1.4 Epithelial cells in the gut

The mucosal surface is covered with a single layer of epithelial cells. Tight junctions of the epithelial layer prevent small peptides from passing through and allow only ions to pass. In neonates, in inflamed and allergic states, permeability seems to be increased. Epithelial cells take up dietary antigens by endocytosis, and molecules that remain undigested enter the extracellular space by exocytosis. Intestinal epithelial cells may act as unprofessional APCs. They express MHC class II molecules and present antigens to primed T cells. They normally activate CD8+ suppressor T

cells, thereby playing a role in local immune suppression. They secrete pro-inflammatory chemokines and cytokines after pathogen stimulus. This activation can be changed or inhibited by contacts between epithelial cells and commensal bacteria (Chehade and Mayer 2005; Mayer 2003; Mowat 2003). Epithelial cells take up and transport sIgA to the gut lumen via a membrane-bound receptor. The secretory component which they produce protects sIgA from luminal proteases (reviewed by Mayer 2003; Perdue 1999). Epithelial cytokines that mediate signals to immune cells are IL-1α, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNF-α, and TGF β1-β3 (reviewed by Perdue 1999).

4.1.5 Dendritic cells

DCs that are found in PPs and the lamina propria are an important feature of the gut immune system. DCs bear MHC II molecules, which enable them to make contact with TCR and to activate naïve T cells to become memory/effector cells (Brandtzaeg 2001). After activation by ligation of co-stimulatory molecule, DCs produce IL-10 in PPs. In the spleen under the same conditions, DCs produce IL-12. Parallel to this, PP DCs participate in polarisation of antigen-specific T cells, causing them to secrete Th2 cytokines and IL-10 (Mowat 2003).

The lamina propria under the epithelium contains a large number of DCs, which presumably pick up dietary antigens and present them to T cells. IL-10-producing DCs are found in the lamina propria, and these are believed to play an important role in induction of oral tolerance. In the presence of pathogen bacteria, lamina propria DCs may pick up antigens from the lumen by migrating into the epithelial layer and extending their dendritic processes into the gut lumen. After sampling the environment, DCs return to the lamina propria, and participate in initiation of protective immunity. After entering MLNs, DCs meet naïve T cells, and this process may be enhanced by inflammatory stimuli from lipopolysaccharides (LPS) (Mowat 2003).

4.1.6 Cytokines

Cytokines are secreted proteins that control growth, differentiation, and activation of various immune cells. They regulate the type of immune responses and control trafficking of cells. The cytokines produced largely determine whether the subsequent response is cytotoxic, humoral, cell-mediated, or allergic. Each cytokine possesses different functions depending on the type of cells that produce it, its target, and its phase of immune responses (reviewed by Borish and Steinke 2003). The following sections briefly describe those cytokines studied.

IL-2 and high affinity IL-2R secretion are induced when T cells are stimulated by antigens simultaneously with accessory signals and the interaction of B7 with CD28. The binding of IL-2 to IL-2R induces clonal T cell proliferation. IL-2 is a T cell growth factor, and activator of natural killer (NK) cells, B cells, cytotoxic T cells, and macrophages (Borish and Steinke 2003).

IL-4, which is secreted by Th2 cells, eosinophils, basophils, and mast cells, induces MHC class II, B7, CD40, surface IgM, and low-affinity IgE receptor expression of B cells, thereby promoting antigen presentation. IL-4 also promotes immunoglobulin isotype switching of IgM to IgE. It promotes differentiation of Th0 cells to the Th2 subtype, and is a major allergy-associated cytokine. On macrophages it plays a role in proinflammatory effects by inducing MHC molecules and inducing low-affinity IgE receptor expression. IL-4 plays a role in down-regulation of antibody-dependent cellular cytotoxicity, in production of NO, IL-1, IL-6, and TNF-α, and in expression of Fc receptors. A further anti-inflammatory effect is induction of IL-1 receptor antagonist (ra) synthesis (Borish and Steinke 2003).

IL-5 is secreted by Th2 cells, mast cells, naïve T cells, and also by eosinophils. The major function of IL-5 is associated with eosinophils: It promotes chemotaxis of eosinophils, activates mature eosinophils, and induces eosinophil secretion and cytotoxicity. It also enhances

eosinophil responses to chemokines and αdβ2 integrins, and thereby promotes eosinophil adherence to vascular cell adhesion molecule (VCAM)-1 on endothelial cells (Borish and Steinke 2003).

IL-6 is produced mainly by mononuclear phagocytic cells, but also B and T cells, fibroblasts, endothelial cells, keratinocytes, and hepatocytes. IL-6 induces B lymphocytes to differentiate into plasma cells, and induces pyrexia and hepatocyte production of C- reactive protein (CRP). It activates T cells, promotes their growth, and differentiation. Furthermore it mediates anti-inflammatory effects, by inhibiting production of IL-1, and TNF-α. It also promotes IL-1ra synthesis (Borish and Steinke 2003).

IL-10 is produced by several different cells: Th1, Th2, Tr1 cells, cytotoxic T cells, B lymphocytes, mast cells, and mononuclear cells. It inhibits IFN-γ and IL-2 production of Th1 cells; IL-4 and IL-5 from Th2 cells; IL-1β, IL-6, IL-8, IL-12, and TNF-α from phagocytes; and IFN-γ and TNF-α by NK cells. IL-10 also inhibits the MHC II molecule, CD23, ICAM-1, and B7 expression of monocytes. Inhibition of B7 expression leads to the inhibition of the ability of APC to provide accessory signals to activate Th cells. IL-10 shortens the lifetime of eosinophils, and down-regulates IgE synthesis. On B cells, IL-10 plays an activating role; it stimulates cell proliferation and Ig secretion, enhances isotype switching to IgG4, and is a cofactor for cytotoxic T cells (Borish and Steinke 2003).

IL-12 is synthesized by monocytes, macrophages, B cells, DCs, Langerhans cells, and mast cells. It induces NK cell proliferation, cytotoxicity, and cytokine production. It also has effects on Th cell and cytotoxic lymphocyte proliferation. IL-12 up-regulates IL-18 receptor production, and together with IL-18 induces IFN-γ (Borish and Steinke 2003).

IFN-γ, a central Th1-associated cytokine, is synthesized by Th1 cells, cytotoxic T cells, and NK cells. It promotes cell-mediated immunity, up-regulates MHC class I and II expression, and activates antigen presentation and cytokine synthesis by monocytes. It promotes monocyte adherence, phagocytosis, secretion, and NO synthesis, which all results in macrophage

accumulation at sites of cellular immune responses. It also down-regulates allergic responses by inhibiting IL-4 effects on expression of low-affinity IgE receptors, and by switching off IgE production (Borish and Steinke 2003).

TGF-β has both inhibitory and stimulatory effects on cells. It is produced by chondrocytes, osteocytes, fibroblasts, platelets, monocytes, and Th3 cells. TGF-β inhibits functions of B cells, Th cells, and cytotoxic lymphocytes. It is inhibitory for Ig secretion of B cells and the cytotoxicity of mononuclear phagocytes and NK cells. In general it down-regulates proliferation of many different cell types. It enhances macrophage accumulation and promotes IgA isotype switching (Borish and Steinke 2003).

TNF-α, a central pro-inlammatory cytokine, is produced by neutrophils, activated lymphocytes, natural killer cells, endothelial cells, and mast cells. LPS is a potent inductor of TNF-α by monocytes. TNF induces cytotoxic effects and up-regulates adhesion molecules: intercellular adhesion molecule (ICAM)-1, VCAM-1, and E-selectin. It activates neutrophils, and promotes adherence and chemotaxis. Its adverse effects are chacexia, vascular leakage, a negative inotropic effect, and it is a mediator of toxic shock (Borish and Steinke 2003).

4.1.7 Adhesion molecules

Leukocytes entering from the circulation into the target tissue are an important part of the inflammation process. The first steps of this process are margination and rolling, which are mediated by selectins. At the time leukocytes are activated or adherent to activated endothelium, ICAM-1, together with its ligands, becomes responsible for a firmer adhesion of leukocytes and allows them to migrate into the tissue (reviewed by Patel et al. 2002;Montefort et al. 1993). Of the wide spectrum of currently recognised adhesion molecules, I describe here the ones studied in the present trial.

E-selectin is not continuously expressed on vascular endothelial cells, but its formation is stimulated by IL-1, LPS, and TNF-α, peak levels occurring in 4 hours after stimulus. E-selectin participates in migration and adhesion of neutrophils, and in recruitment of memory T cells and monocytes. Possibly it also plays a role in eosinophil adhesion and migration. Selectins function also as signalling molecules. E-selectin binding activates β2-integrins of neutrophils. In monocytes, E-selctin in soluble (s) form induces chemotaxis and phosphorylation of additional signalling proteins. In endothelial cells, selectins may enhance the changes in intracellular junctions that promote leukocyte extravasation, or enhance the down-regulation of cell adhesion molecules that would lead to dampening of leukocyte recruitment (Montefort et al. 1993; Patel et al. 2002).

ICAM-1 belongs to the immunoglobulin supergene family of adhesion molecules. Its ligands are lymphocyte function-associated antigen (LFA)-1 and Mac-1. It is constitutively expressed on different sites, although induced at sites of inflammation. It has been detected in normal tissues of the skin, kidney, liver, thymus, tonsil, and of lymph nodes, airways, and the intestine. The most prominent expression at these sites is on vascular endothelium, but it exists also on macrophages in the germinal centres, and on dendritic cells. It plays a role in T cell migration, and eosinophil-endothelium interaction. The latter is promoted because IL-5 enhances expression of Mac-1 and LFA-1 on eosinophils, thereby promoting their contact with ICAM-1. IFN-γ, IL-1, TNF-α, and LPS can up-regulate the basal expression of ICAM-1. Upregulation starts 2 to 4 hours after activation, depending on the cytokine involved (Montefort et al. 1993).

The consentration of soluble adhesion molecules is directly correlated with their expression on cell surfaces. Maximal release of E-selectin has occurred 6 to12 hours after activation, and for ICAM-1, the plateau was reached 24 hours after activation, lasting for 3 days. (Leeuwenberg et al. 1992). After shedding from endothelial cells, these soluble adhesion molecules may retain their capacity to bind to receptors, thereby potentially limiting the inflammatory process (Leeuwenberg et al. 1992; Montefort et al. 1993).

4.1.8 CRP as a marker of inflammation

In recent studies, low levels of CRP reflect subclinical inflammation such as in arteriosclerosis (Cesari et al. 2003). IL-6 induces gene activation of CRP in hepatocytes and stimulates CRP secretion (Li and Goldman 1996). Recent studies show diverse biological functions for CRP: It activates complement, which mediates phagocytosis (Volanakis 2001). In an earlier report, CRP did not promote formation of C5 convertase; therefore, complement activation initiated by CRP did not mediate proinflammatory reactions and membrane damage (Volanakis 2001). In vitro, human CRP inhibited production of inflammatory cytokines such as TNF- α and IFN- γ, and of chemokines, as well as enhanced IL-10 production on cultured encephalitogenic cells (Szalai et al. 2002). In vitro, peripheral blood mononuclear cells (PBMCs) incubated with CRP show increased anti-inflammatory IL-1ra synthesis (Tilg et al. 1993). Studies of its role in allergies are few. In acute allergic reactions, CRP and IL-6 levels correlated with each other (Fujii et al. 2001; Lin et al. 2001), and IL-6 correlated with extent of erythema (Lin et al. 2001).

4.2 Atopic Dermatitis (AD)

4.2.1 Clinical features and diagnosis of AD in infancy

Proposed diagnostic criteria for AD in infancy consist of three major features and four minor features (Seymour et al. 1987). The major features are family history of atopic disease, evidence of pruritic dermatitis, and typical facial or extensor or lichenified dermatitis. The minor features are: xerosis (cracked and roughened skin), ihcyotosis or hyperlinear palms, perifollicular accentuation, chronic scalp scaling, or peri-auricular fissures. A prospective study demonstrated that the minor features from among Hanifin's and Rajkas's original criteria for atopic dermatitis, as exhibited by 2-year old children, were xerosis, environmental factors influenced the course of eczema, and facial erythema (Böhme et al. 2000; Hanifin and Rajka 1980).

4.2.2 Prevalence of AD

The prevalence rate of AD in childhood is 10 to 15% in Europe (Wüthrich 1999). In a German cohort, the cumulative prevalence of AD by the age of 2 years was 21.5% (Illi et al. 2004). According to the ISAAC study, the prevalence rate of AD among children aged 6 to 7, was around 18% in Sweden, 8% in North America, and 11% in Australia (Williams et al. 1999). In Finland, the prevalence of AD was between 15 and 19% depending on the area studied (Remes et al. 1998). Incidence of AD is, according to one study, 2.4% per month during the first 3 months of infancy (Wahn et al. 1997). According to this same study, from infancy on, AD is more likely to occur in children from families with a positive family history of atopy.

4.2.3 Severity Scoring of Atopic Dermatitis (The SCORAD index)

The SCORAD index was established by the European Task Force on Atopic Dermatitis (1993). Intensity items are graded 0 to 3 with the aid of reference photographs. (0= absent, 1= mild, 2=

moderate, and 3= severe) Items graded are erythema, edema/papulation, oozing/ crusts, excoriations, lichenification, and dryness. Extent is graded by the rule of nine on front and back drawings, taking into account the inflammatory lesions, not dryness (scores 0-100). Subjective symptoms include pruritus and sleep loss, for both the average value for the last 3 days/nights, scored 0 to 10 each. Concerning young children and infants, parents answer on behalf of the patient. SCORAD was calculated by: extent/5 + 3.5 x intensity + subjective symptoms (max 103). Extent and subjective symptoms each account for 20% of the total score, and intensity accounts for the remaining 60%.

4.2.4 Pathogenesis

4.2.4.1 Histological features of AD lesions

Acute AD lesions are characterised by intercellular oedema and some infiltrative T-cells in the epidermis (Leung 1999). In the dermis, perivenular inflammatory infiltrate is characterised by CD4+ activated memory T cells and occasional monocyte-macrophages (Leung et al. 2004). In chronic lesions, the epidermis is hyperplastic with IgE-bearing Langerhans cells and with inflammatory dendritic epidermal cells, whereas in the dermis macrophages predominate (Leung 1999; Leung et al. 2004). In such lesions the number of mast cells and eosinophils is also increased .

4.2.4.2 Immune activation in patients with AD

Th2 deviation potentially plays an important role in AD. PBMCs from AD patients have been reported to express higher levels of IL-4receptor mRNA and to produce higher amounts of IL-4 than do normal controls (Renz et al. 1992). In another study, in vitro stimulation of PBMC from AD patients demonstrated an increase in IL-4 production (Campbell et al. 1998), whereas secretion of

IFN- γ was impaired (Campbell et al. 1998; Reinhold et al. 1988). In addition, after mitogen stimulation of PBMCs from AD patients, culture supernatants contained less IFN- γ, but increased IL-4 levels (Jujo et al. 1992). High IL-4 levels induced IgE production in PBMCs from nonatopics in a subsequent analysis (Jujo et al. 1992). In IgE-associated AD, patients showed IgE-mediated sensitisation and synthesis of Th2 cytokines (Novak and Bieber 2003).

In **acute and chronic AD lesions,** cytokine production differs. In situ hybridisation shows that both acute and chronic AD lesions had a greater number of IL-4 mRNA-, IL-5 mRNA-, and IL-13 mRNA- positive cells than did normal skin (Hamid et al. 1994; Hamid et al. 1996). Chronic AD lesions, on the other hand, had fewer IL-4 but a greater number of IL-5 mRNA-expressing cells than did acute lesions (Hamid et al. 1994). In another study, in eczematous skin, the majority of AD patients showed increased T cell expression of IFN- γ mRNA and protein (Grewe et al. 1994). In the same study, successful treatment of AD down-regulated IFN-γ mRNA expression. Moreover, during a house dust mite patch-test reaction in AD patients (in vivo model of AD), a biphasic T cell expression of cytokines appeared (Grewe et al. 1995). After 24 hours, increased T cell expression of IL-4 mRNA and protein was observable. After 48 hours IL-4 expression was down-regulated, and concomitantly IFN-γ mRNA expression was increased. In this study, IFN- γ production was preceded by IL-12 mRNA expression. Chronic AD lesions have also expressed an increased number of IL-12 mRNA cells compared to those in acute lesions or uninvolved skin (Hamid et al. 1996). IL-12 is suggested to play a role in modulation of chronic inflammation by inducing differentiation of T-cells into Th1-cells (Grewe et al. 1998).

Spontaneous **TGF-β** mRNA expression from unstimulated PBMCs was attenuated (Lee et al. 2000), and children with AD showed the low producer TGF-β1 genotype (Arkwright et al. 2001). Defects in the regulatory T system may play a role in AD.

On the other hand, over-expression of **IL-10** mRNA has been detectable in human atopic dermatitis lesions, and monocytes from AD patients have spontaneously produced more IL-10 than

controls (Ohmen et al. 1995). Spontaneous IL-10 mRNA expression in unstimulated PBMCs has also been increased in AD (Lee et al. 2000). A mouse model of allergic dermatitis, induced in IL-$10^{-/-}$ mice by epicutaneous sensitisation with OVA, showed that IL-10 promotes Th2 responses, suppresses Th1 responses, and enhances eosinophil accumulation in the skin (Laouini et al. 2003).

CRP and IL-6 levels correlated with each other in acute allergic reactions, (Fujii et al. 2001; Lin et al. 2001), and IL-6 correlated with extent of erythema (Lin et al. 2001).

Soluble ICAM-1 and sE-selectin can both be detected in plasma of both healthy and atopic children (Laan et al. 1998); and sE-selectin has a significant correlation with severity of AD (Laan et al. 1998; Wolkerstorfer et al. 1998). After intradermal allergen injection, E-selectin was expressed concomitantly with ICAM-1 and concurrently with the development of inflammatory cell infiltrates in all atopic patients, but not in controls (Leung et al. 1991).

4.2.5 Treatment and prognosis of AD

Treatment of AD is focused on relief of symptoms (Leung 1999). Hydration with emollients, and use of topical steroids to reduce skin inflammation, has been the basic treatment. With resistance to these therapies, oral and high-potency topical steroids are also used. A new treatment choice for children is tacrolimus and pimecrolimus ointments, potent topical immunosuppressive agents with no evidence of systemic side effects (Ashcroft et al. 2005; Reitamo et al. 2002). Occasionally, antibiotics against common *S. aureus* or other bacteria infections are also necessary. AD patients are at increased risk for herpes simplex and molluscum contagiosum infections (Hanifin 1991). Among all children with atopic dermatitis, certain foods can excacerbate symptoms with worsening of eczema in addition to immediate symptoms in more than half the cases (Werfel and Breuer 2004). Among children under 2 years, in one study of severe AD, 93% were sensitive to food allergens (Guillet and Guillet 1992). Investigations on benefits of elimination of offending food on AD symptoms gave controversial results. In one study, elimination of egg and milk showed no

benefit (Neild et al. 1986), while in another egg exclusion reduced AD symptoms moderately (Lever et al. 1998). The persistence rate of AD has ranged between 40% and 60% (Wüthrich 1999). In a German cohort of AD children, 43% were without symptoms at the age of 3, 38% had intermittent disease, and 19% had AD symptoms at least once a year (Illi et al. 2004).

4.3 Cow's Milk Allergy (CMA)

4.3.1 Symptoms of CMA

CMA infants react to proteins in cow's milk (CM). Casein constitutes 80% of milk proteins, and the remaining 20% are whey proteins; of the latter, the major proteins are β-lactoglobulin, α-lactalbumin, and bovine serum albumin (Heine et al. 2002). Symptoms occur via different organ systems. They range from cutaneous symptoms including AD, urticaria, and angioedema via respiratory symptoms of rhinitis, wheezing, and cough, to gastrointestinal symptoms like vomiting, diarrhoea, and gastroesophagal reflux. Cutaneous and gastrointestinal symptoms are most frequent. Among children with CMA the prevalence of AD seems to lie between 21% and 57% (reviewed by Bock and Sampson 1994; Novembre and Vierucci 2001). Among infants with CMA, mothers noticed infants' first adverse reactions to CM at a mean age of 2.9 months (Saarinen and Savilahti 2000). Symptoms usually appear shortly after introducing CM into the diet, but they can also appear during exclusive breast feeding (Saarinen and Savilahti 2000; Sicherer and Sampson 1999).

4.3.2 Prevalence of CMA

The approximate prevalence of CMA in industrialized countries lies between 2% and 3% in children under 2 years (reviewed by Novembre and Vierucci 2001; Heine et al. 2002). In a Finnish study with a cohort of 6209 infants, prevalence of CMA was 1.9% (Saarinen and Savilahti 2000).

4.3.3 Immune activation in CMA

Traditionally, immune reactions in CMA have been divided into IgE-mediated and non-IgE-mediated, the latter being poorly characterised. As many CMA reactions are IgE mediated, T cell responses in CMA patients show Th2 deviation. PBMCs of IgE-mediated CM- and egg-allergic

patients show Th2 biased reaction type, with increased IL-4 cytokine secretion (Campbell et al. 1998). Secretion of IFN-γ by PBMCs of infants with CMA was defective (Suomalainen et al. 1993). In addition, PBMCs of AD infants with CMA produced higher levels of IL-4, IL-5, and IL-13 than did AD infants without CMA, who had a Th1 skewed response with high levels of IFN-γ and low levels of IL-4, IL-5, and IL-13 (Schade et al. 2000). Children with the delayed type of food allergy, on the other hand, showed a Th1 deviation of immune responses in lamina propria cells (Veres et al. 2003). Schade et al found an association with spontaneous clinical tolerance to CM in CMA patients and a down-regulation of Th2 responses concomitantly with increased IFN-γ production (reviewed by Schade et al. 2003). This explain why CMA infants with Th2 responses need a strong Th1-biased reaction type to induce tolerance to CM. However, as a strong Th1-biased reaction type is potentially harmful, it was proposed that this reaction type would soon be down-regulated to a balanced response (Schade et al. 2003).

The activation state of food antigen-specific T cells is suggested to play role in the pathogenesis of CMA. One research group found that CM protein specific T cells from CMA infants expressed higher levels of CD25 and CD30 (markers of T cell activation) than did cells from non-CMA AD infants and healthy controls. After development of tolerance to CM, expression of CD 25 and CD 30 decreased (Schade et al. 2002).

Food allergy may require breakdown of oral tolerance. TGF-β and IL-10, produced by antigen specific-regulatory lymphocytes, are proposed to suppress reactivity of other lymphocytes in the intestinal mucosa (reviewed by Murch 2000). The gut mucosa of food-allergic children has been found to lack Th2 deviation, but to have a reduced number of TGF-β1-producing Th3 cells (Pérez-Machado et al. 2003). Tr1 cells represented the dominant subset specific for certain allergens in healthy individuals, while in allergic individuals allergen-specific IL-4 secreting T cells dominated (Akdis et al. 2004). Another study showed that during an elimination diet, production of TGF-β2 by stimulated PBMCs from AD infants with CMA was lower than in those AD infants

without CMA. After elimination, during oral challenge, cells from AD infants with CMA showed higher spontaneous production of IL-4 than did those without CMA (Rautava and Isolauri 2004). In another study, CM protein-specific T cells from CMA infants produced Th2 cytokines IL-4 and IL-13, and the level of production was correlated with CD25 surface marker. Milk-tolerant allergic controls had a high production of IL-10, which was correlated with IL-4, IFN-γ production, and CD25 expression. The authors proposed that activated T cells might contribute to tolerogenic immune responses through the production of IL-10 (Tiemessen et al. 2004).

Increased PBMC TNF-α release occurs in children with skin-symptomatic CMA (Benlounes et al. 1999), and in children with AD, plasma levels of TNF-α are increased (Sumimoto et al. 1992). Increased TNF-α secretion by the PBMC of CMA infants reduces the barrier capacity of intestinal cell lines in vitro (Heyman et al. 1994).

4.3.4 Markers of intestinal inflammation in CMA

TNF-α is a pro-inflammatory cytokine of both Th1- and Th2- type cells (Mosmann and Sad 1996) and a sign of increased intestinal permeability (Heyman et al. 1994). Increased faecal TNF-α has been detected after challenge in CMA infants with a delayed-type reaction (Majamaa et al. 1996) and with GI symptoms (Kapel et al. 1999).

Alpha-1 antitrypsin (AT) indicates protein loss in the intestine (Majamaa et al. 1996). CMA infants showed higher post-challenge AT levels than did non-CMA infants (Majamaa et al. 1996; Saarinen et al. 2002). Inflammation of mucosal membranes is associated with increased influx of plasma proteins into the secretions, as indicated by increased AT levels in induced sputum in asthmatic patients (Vignola et al. 1998), and increased faecal AT levels in patients with inflammatory bowel disorders (Bischoff et al. 1997; Thomas et al. 1981).

Eosinophil cationic protein (ECP) accumulates at sites of allergic inflammation and demonstrates local eosinophil degranulation in the gut (Bischoff 1996). Faecal ECP has remained

low in CMA infants after challenge (Kapel et al. 1999; Saarinen et al. 2002), except in infants with a delayed-type reaction who showed higher levels than did infants reacting immediately (Saarinen et al. 2002). In contrast, an increase in post-challenge ECP levels has occurred in infants with the immediate-type reaction (Majamaa et al. 1996).

Total IgA serves as a marker of maturity and of degree of local intestinal stimulation (Rognum et al. 1992). One study proposed that challenge with CM in both reacting and non-reacting infants results in increased levels of faecal IgA due to the more antigenic diet (Saarinen et al. 2002). In healthy infants, faecal IgA appears within the first week of life (Kuitunen and Savilahti 1995). Non-allergic infants tend to have more sIgA in saliva than do allergic ones, while those sensitised infants who develop allergies have lower sIgA in saliva than do those who do not (Böttcher et al. 2002). In an animal model, mice tolerant to food antigen have had increased levels of antigen-specific faecal IgA, and low levels of antigen-specific serum IgA, while in anaphylactic mice increased serum antigen-specific IgA has existed concomitantly with low faecal levels of IgA (Frossard et al. 2004).

4.3.5 Diagnosis of CMA

When CMA is suspected on clinical grounds, a CM protein elimination diet is initiated. For formula- fed infants soy protein based, extensively hydrolysed formulas (EHF), or amino acid-based formulas are substitutes. If the infant is breastfed, maternal elimination of CM may be needed. Under an elimination diet, good topical skin care is often required. After a succesful elimination of a minimum of 2 weeks, with disappearence of symptoms, an oral food challenge is initiated. (reviewed by Heine et al. 2002; Sicherer and Sampson 1999).

A double-blind placebo-controlled food (here CM) challenge (DBPCFC) is considered the gold standard for diagnosis of food allergy. The basic DBPCFC is performed with increasing doses, in one day (Heine et al. 2002; Sicherer and Sampson 1999). If late reactions are expected, the

challenge with the active and placebo milks can be prolonged until reactions can be expected (Bindslev-Jensen et al. 2004). DBPCFCs are recommended for studying late reactions and chronic symptoms such as AD (Bindslev-Jensen et al. 2004). As double-blind challenges are expensive and resource-consuming, open challenges with trained personnel are sufficient in infants and often preferred in clinical practice (Bindslev-Jensen et al. 2004).

Skin prick tests (SPTs) against CM and CM-specific IgE are used in clinical practise to distinguish IgE-mediated CMA from non-IgE-mediated disease type. In two different Finnish studies with infants under 12 months, a SPT wheal diameter ≥ 3 showed a respective sensitivity of 61% and 69%, specificity of 76% and 91%, positive predictive values of 71% and 79%, and negative predictive values of 67% and 85% (Saarinen et al. 2001; Vanto et al. 1999). For CM-specific IgE ≥ 0.7 kU/l, sensitivity was 45% and 58%, specificity was 87% and 88%, positive predictive values were 78% and 70%, and negative predictive values were 61% and 81% (Saarinen et al. 2001; Vanto et al. 1999).

4.3.6 Treatment and prognosis of CMA

At present, treatment of CMA is based on avoidance of CM protein. As during the diagnosis procedure, the elimination diet continues with soy protein-based, EHF, or amino acid-based formulas or with maternal elimination of CM (Heine et al. 2002). In Finland, soy protein-based formula is recommended for infants over 6 months of age, while extensively hydrolysed formulas are recommended for infants under 6 months, for those with widespread symptoms, and for those intolerant to soy protein-based formula (Vanto et al. 2004). EHFs are usually well tolerated in infants with CMA (Isolauri et al. 1995; Niggemann et al. 2001). In the absence of placebo-controlled studies, objective evidence for efficiency in alleviation of AD symptoms is weak. A very small proportion of CMA infants are intolerant to extensively hydrolysed formulas, and then a amino acid-based formula is recommended (Vanto et al. 2004). A new approach to treatment of

CMA might be induction of tolerance with increasing doses of CM proteins, over a time period of 6 months (Meglio et al. 2004). Overall prognosis for CMA is good: At the age of 1 year about 50% have recovered, 70% at 2 years, 85% at 3 years, and 90 to 95% at 5 and 10 years (reviewed by Heine et al. 2002; Novembre and Vierucci 2001).

4.4 Hygiene hypothesis

Strachan suggested the hygiene hypothesis for the first time in 1989 as an explanation for the increase in atopic diseases (Strachan 1989). This idea originated from epidemiological findings with an inverse correlation between family size and allergic rhinitis. The hypothesis proposed that in young children, infections contracted from older siblings may protect against allergic diseases.

4.4.1 Immunologic background of atopic diseases

During a normal pregnancy, immunity is skewed towards the Th2 cytokine pattern, contributing to succesful pregnancy (Wegmann et al. 1993). This is suggested by the finding that cells from foetoplacental tissues produced Th2-type cytokines during the whole pregnancy (Lin et al. 1993). Thus newborns, both atopic and non-atopics, show a weak Th2 type allergen-specific response (Prescott et al. 1999). Atopic individuals have a persistent Th2-skewed immune reaction type with insufficient Th1-type reactions, while non-atopic individuals gradually suppress the Th2 reaction type with a concomitant increase in Th1-type IFN- γ (Prescott et al. 1999; van der Velden et al. 2001) This change in cytokine pattern occurs in non-atopic children on average within the first year of life (Prescott et al. 1999), while among atopics, an adult-like cytokine pattern is achieved by age 6 (Macaubas et al. 2000). Based on this, the first years of life are claimed to form the critical period for development of Th cell memory and of the allergen-responder phenotype (Macaubas et al. 1999).

Atopic individuals also demonstrate defects in regulatory T cell responses (Lee et al. 2000; Pérez-Machado et al. 2003). CD4+CD25+ T cells from non-atopic donors suppress proliferation and Th2 responses in T cells from atopic donors, in vitro (Ling et al. 2004). The authors proposed that allergic diseases would result from an imbalance between allergen activation of regulatory T cells and of effector Th2 cells (Ling et al. 2004). This imbalance could result from a

decrease in suppression by regulatory T cells, or regulation could be conquered by strong activation signals (Ling et al. 2004). Recently it was proposed that when sufficient immunoregulation is lacking, depending on the individuals' own Th1/Th2 balance and genetic background, the individual will develop Th1 or Th2 type disease (Rook and Brunet 2005).

4.4.2 Environmental factors

Later, the change in immune pattern in non-atopic infants was explained by a modified version of the hygiene hypothesis, in which commensal gut microbiota and other microbial pressure would drive the immune system towards a Th1-type response (Holt et al. 1997). In addition, the microbial burden was proposed to act by increasing the activity of regulatory T cells (Bach 2002). These two possible mechanisms for microbial effects on immune cells are represented by lactobacilli in Figure 2. The latter theory would explain the increase in frequencies of both Th1 and Th2 disease types in the western world during thelast decades, while, from this perspective, the pure Th1/Th2 deviation theory seems not to fit (Bach 2002). At the same time the increase in allergic and autoimmune diseases occured, frequencies of infectious diseases like hepatitis A, mumps, tuberculosis, and measles decreased, which was suggested to occur due to improved hygienic conditions, vaccinations, and antibiotics (Bach 2002). Based on this, infections and other microbial burden may protect against allergic and autoimmune diseases.

Children living on farms, especially those exposed to livestock, are at decreased risk for hay fever and asthma (Von Ehrenstein et al. 2000). Furthermore, children living on farms are at decreased risk for hay fever or atopic sensitisation compared to children living in the same rural areas but whose parents are not farmers (Braun-Fahrländer et al. 1999). Increased endotoxin levels indoors in farmers' houses has been suggested as an explanation (von Mutius et al. 2000). High endotoxin levels from mattresses, reflecting environmental exposure to microbial products, were found to reduce risk for hay fever, atopic sensitisation, and atopic asthma in childhood both in farm

and non-farm families. This finding was associated with an inverse relation between endotoxin levels and leukocyte production of cytokines (TNF-α, IFN-γ, IL-10, IL-12), suggesting down-regulation of immune responses by activation of the innate immune system (Braun-Fahrländer et al. 2002). Pet ownership also protected against allergic rhinitis and asthma later in childhood (Hesselmar et al. 1999), and had an inverse association with prevalence of IgE-mediated allergies (Rönmark et al. 2003).

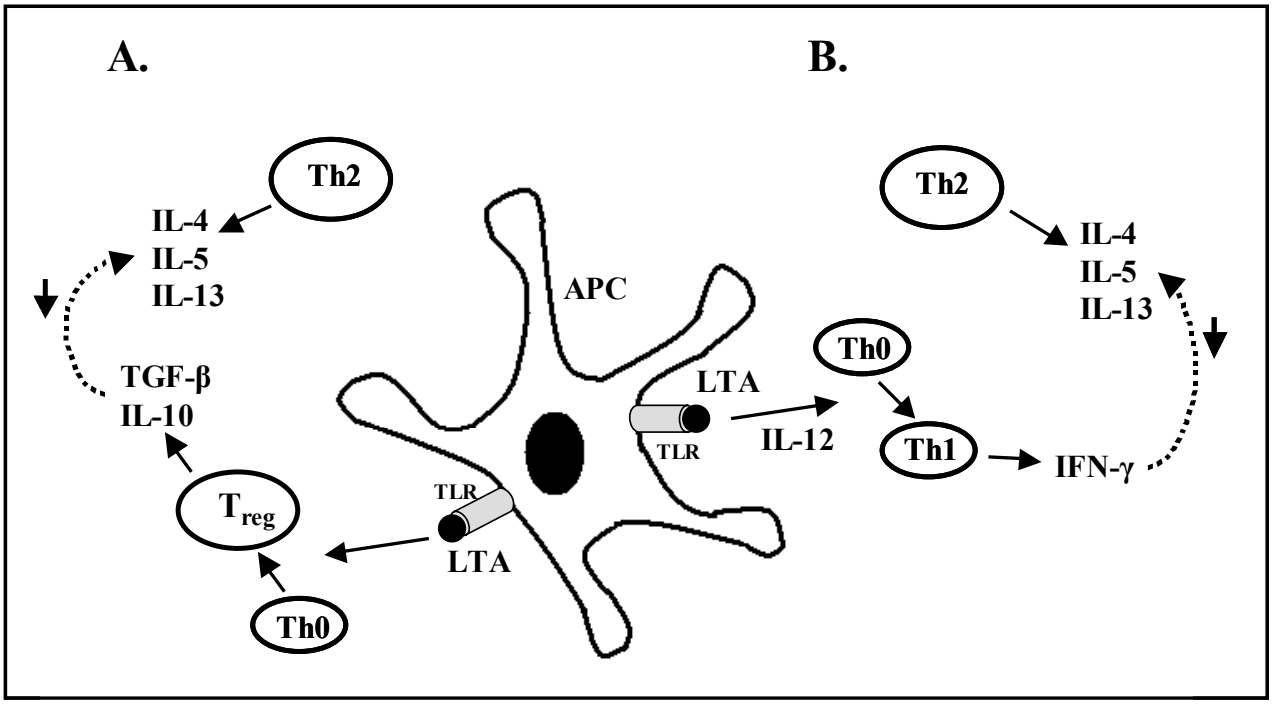


Figure 2. Two possible mechanisms by which lactobacilli may affect immune cells in allergies. A. Lipoteichoic acid (LTA) activates production of suppressive cytokines from T regulatory cells, which then down-regulate Th2 responses. B. LTA induces production of IL-12, which directs maturation of Th1 cells and subsequent production of IFN-γ, which inhibits production of Th2 cytokines. Abbreviations: APC, antigen-presenting cell; TLR, toll-like receptor

Sharp geographic variations in prevalence of allergies/atopy exist: In Finnish Karelia, birch- and timothy- specific IgE levels are higher than on the Russian side of Karelia (Moverare et al. 2004). Allergic symptoms also have been less common in Russia than in Finland (Vartiainen et al. 2002).

These variations in adjacent geographical areas, but on different sides of the border, are proposed to be due to environmental factors like lifestyle and standard of living (Vartiainen et al. 2002)

4.4.3 Orofaecal and other candidate infections

Exposure to orofaecal infections and food-borne microbes may protect against atopy. Atopy, defined by skin sensitisation, high specific IgE concentration, and prevalence of allergic rhinitis or asthma, was less common among military students with seropositivity to hepatitis A than among those with seronegativity (Matricardi et al. 1997). Moreover, male cadets exposed to *Helicobacter pylori*, *Toxoplasma gonadii*, or hepatitis A had less atopy or allergic asthma or rhinitis than did cadets without exposure to these agents (Matricardi et al. 2000). In a Finnish study, IgE antibodies in serum were more rare in individuals with *H. pylori* antibodies (Kosunen et al. 2002).

Measles is also mentioned when infections protecting against allergies is discussed. However, contradictory findings exist: In African children, measles infection protected against atopy, while in Finland among children vaccinated against measles, natural infection with measles was associated with increased risk for atopy (Shaheen et al. 1996; Paunio et al. 2000).

As mycobacterial infections are potential stimulators of Th1 responses, it has been proposed that exposure to them or BCG vaccination would protect against allergies. Indeed, positive tuberculin responses are inversely associated with the emergence of atopic sensitisation and asthma (Shirakawa et al. 1997). A later study failed to demonstrate that BCG vaccination can reduce atopic sensitisation in all children, but in children with an allergic predisposition, BCG demonstrated to reduce prevalence of asthma (Marks et al. 2003). This role for mycobacterial infections has been questioned, and these findings were explained by the fact that atopic individuals might exhibit a reduction in cutaneous delayed-type reactions (Kemp and Björkstén 2003).

Paradoxically, parasite infections, which are associated with the high levels of IgE, eosinophilia, and mastocytosis that are stimulated by Th2 type cytokines, are proposed to protect

against allergies. Concomitantly, reduced antigen-specific T cell proliferation and reduced cytokine production occur, which may be due to induction of suppressive cytokines by parasites. This immune suppression may be the mechanism behind the proposed protective effect against allergies (reviewed by Yazdanbakhsh et al. 2002). Opinions conflict conserningt these findings, as helminth infections were rare in industrialized countries before allergic diseases became common, and among countries like Estonia, where the prevalence of allergies is low, parasite infection is apparently rare (Romagnani 2004).

4.4.4 Gut microbiota

Initially, unborn infants are sterile. Type of delivery affects the formation of the gut microbiota: In infants delivered by caesarean section, intestinal microbial colonisation was delayed, and they later reached the same levels of bifidobacteria and lactobacilli as in vaginally delivered infants, but were less often colonised by *Bacteroides fragilis* type bacteria (Grönlund et al. 1999). Comparison of breast-feeding and bottle-feeding revealed that independent of feeding type, the gut microbiota was composed mainly of *E. coli* and bifidobacteria. Some differences, however, existed as in breast-fed infants bifidobacteria dominated, while the bottle-fed had both bifidobacteria and *Bacteroides* in similar amounts (Harmsen et al. 2000). Introduction of solid foods and withdrawal of breast milk were associated with major microbiota changes (Favier et al. 2002). This change and diversity led to formation of adult-like microbiota at an average age of 2 years (reviewed by Isolauri et al. 2004).

Hygienic conditions and an attempt at a sterile diet may provoke atopy, by influencing unfavourable gut microbiota that stimulates GALT (Matricardi et al. 2000). Indirectly, then, gut microbiota has been associated with development of allergies. In one study, allergic children were less often colonised with lactobacilli and bifidobacteria, and harboured higher counts of coliforms and *Staph. aureus* than did non-allergic children (Björksten et al. 1999). In another study faeces of atopic children contained more clostridia, and tended to have fewer bifidobacteria than did

nonatopics (Kalliomäki et al. 2001a). Contrasting findings also exist, as one study demonstrated that allergic children more often had lactobacilli in their faeces than did non atopic children (Björkstén et al. 2001). Additionally, allergic infants were less often colonised with enterococci and bifidobacteria and had higher counts of clostridia than did non allergic infants (Björkstén et al. 2001). In young AD patients, bifidobacteria counts were lower and occurrence of *Staphylococcus* higher than in age-matched controls (Watanabe et al. 2003). Frequency of Gram+ species among most predominant aerobes in faeces was also lower in atopic infants than in healthy infants (Kirjavainen et al. 2001). Differences have also been found in compositions of bifidobacteria: allergic infants were found to harbour *Bifidobacterium adolesscentis* in their faeces more often than did healthy infants, while *B. bifidum* made up the main microbiota in healthy infants (Ouwehand et al. 2001). Furthermore, allergic infants were colonised to a lesser extent with *B. infantis*, *B. breve*, and *B. longum* (Ouwehand et al. 2001).

In addition, delivery by caesarean section was associated with wheezing and allergic sensitisation, suggesting a role for gut microbiota (Negele et al. 2004). In one study, use of antibiotics during the first years of life was associated with an increased risk for asthma and allergic disorders in children with a genetic background (Droste et al. 2000). Another study found no association between childhood antibiotic use and atopy in adults (Cullinan et al. 2004). Children in families with an anthrophosophic lifestyle, who use fewer antibiotics, receive fewer vaccinations, and consume more fermented vegetables, which contain lactobacilli, were at decreased risk for atopy (Alm et al. 1999).

In animal models, intestinal colonisation plays a role in the regulation of oral tolerance (Maeda et al. 2001; Sudo et al. 1997). The effect of intestinal microbiota is reflected in the number of T cells in PPs, as germ-free mice show a reduced number of T cells. The number of T cells increased when mice were colonised with bifidobacteria and E.coli, representing Gram-positive and Gram-negative bacteria. Furthermore, germ-free mice failed to develop oral tolerance when T cells

were absent or their number was reduced in PPs. These findings suggest that gut the microbiota is important for development of the gut immune system and induction of oral tolerance.

4.5 Probiotics

4.5.1 Definition

According to Fuller's classical definition, probiotics are 'a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance' (Fuller 1991). A later definition of probiotics by the FAO/WHO is 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (2002). The idea of health-promoting effects of lactobacilli is by no means new, as Metchnikoff as early as 1910 proposed that lactobacilli may fight against intestinal putrefaction and contribute to long life (Metchnikoff 1910).

4.5.2 Colonisation and safety

Lactobacillus GG (LGG) is the most studied probiotic bacteria. Its ability to survive passage through the gastrointestinal tract in newborns has been shown in faecal samples (Millar et al. 1993; Sepp 1993), and by colonic biopsy specimens showing LGG attaching to colonic epithelial cells (Alander et al. 1999). LGG has colonised 67% of full-term infants receiving the probiotic strain, the colonisation lasted at least 2 weeks in most of the cases, and the supplementation did not interfere with establishment of normal gut microbiota (Sepp 1993). In pre-term infants, colonisation in the LGG group was seen in all infants, except in the one who received potent antibiotics immediately after birth. Colonisation declined after the end of LGG administration, but was detectable in four of seven infants 3 weeks after the end of supplementation (Millar et al. 1993). In adults, temporary LGG attachment lasted more than one week after discontinuation of LGG (Alander et al. 1999).

Several studies have shown that probiotic preparations containing lactobacilli and bifidobacteria are well tolerated in infants and children (Millar et al. 1993; Sepp 1993; Isolauri et al. 2000; Majamaa and Isolauri 1997; Rosenfeldt et al. 2003). As an exception, in one study with infants, heat-inactivated LGG was associated with adverse gastrointestinal symptoms and diarrhoea

(Kirjavainen et al. 2003). During the rapid increase in consumption of LGG products in the 1990s in Finland, incidence of LGG bacteraemia showed no increase, and proved very rare (Salminen et al. 2002). Most cases of bacteremia with lactobacilli occur in patients with severe underlying conditions (Borriello et al. 2003; Salminen et al. 2004).

4.5.3 Effects in treatment of AD and food allergies

The first study on probiotic effects on AD symptoms in CMA infants showed that treatment with LGG together with EHF alleviated AD symptoms, as placebo treatment together with EHF had no effect (Majamaa and Isolauri 1997). After 1-month treatment, SCORAD decreased from 26 to 15 in the LGG group, while in the placebo group it decreased from 21 to 19. One month after the end of the treatment, SCORAD was 14 in the placebo group, and 16 in the LGG group (Majamaa and Isolauri 1997). In a crossover study, 1- to 13- year-old children with AD were treated with a combination of two lactobacillus strains (*Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 122460) and with placebo for 6 weeks (Rosenfeldt et al. 2003) After active treatment, 56% of these children experienced alleviation of AD symptoms, but after placebo treatment, only 15% experienced any improvement. The total SCORAD showed no significant differences. In IgE-sensitised children, however, the SCORAD index decreased more in the probiotic group. In a recent study, 8-week supplementation of *L. fermentum* to 6- to 18-month-old children with moderate to severe AD demonstrated a significant reduction in SCORAD, compared to that of the placebo group (Weston et al. 2005). Among infants with AD, during exclusive breast-feeding the median SCORAD was 16, and weaning and use of EHF with *Bifidobactrium lactis* Bb-12 for 2 months improved AD symptoms significantly in all of 9 infants (median SCORAD 0), and EHF with LGG in all nine of those infants (median SCORAD 1) (Isolauri et al. 2000). At the same time, in the unsupplemented EHF group a significant change in SCORAD occurred in only four of nine patients, and the median SCORAD was 13.4. In a study with 35 infants, treatment effects of viable

LGG, inactivated LGG, and placebo, concomitantly with elimination diet, were comparable, and symptoms of AD were alleviated in infants with CMA (Kirjavainen et al. 2003). Viable LGG, however, tended to alleviate symptoms of AD more than did placebo (Kirjavainen et al. 2003). In adult food-allergic patients, treatment with *Bacillus subtilis* reduced symptoms of angioedema-urticaria more than did the placebo (Ciprandi et al. 1986).

4.5.4 Effects in prevention of atopic diseases

In a primary prevention study, by the age of 2, 23% of children who received probiotics perinatally for 6 months compared with 46% receiving placebo, suffered from AD. Probiotics had, however, no effect on severity of AD, as measured by SCORAD, nor on atopic sensitisation, as measured by increased IgE-levels or positive skin prick tests (Kalliomäki et al. 2001b). At the age of 4, cumulative prevalence of AD was 26% in the probiotic group compared with 46% in the placebo group (Kalliomäki et al. 2003). Additionally, in that study, probiotic treatment of breast-feeding mothers for 3 months after delivery raised levels of TGF-β2 in breast milk, and the risk for developing AD during first 2 years of life was reduced, compared to the placebo group figures (Rautava et al. 2002). In addition, oral colonisation of infants with probiotic *E. coli* strain reduced the risk for allergies after 10 and 20 years in pre-term and full-term infants, respectively (Lodinová-Zádníková et al. 2003).

4.5.5 Effects on intestinal inflammation, IgA, and intestinal permeability

Concentration of faecal AT decreased after 1-month LGG treatment in CMA infants, whereas placebo had no effect. Concentrations of TNF-α in faeces decreased also in the LGG group, and in the placebo group remained unchanged. ECP concentration however remained unaltered in both groups (Majamaa and Isolauri 1997).

During convalescence after rotavirus diarrhoea, viable LGG raised the numbers of IgA-secreting cells in serum (Kaila et al. 1992). In healthy children, a bifidobacteria-supplemented formula raised faecal IgA levels above basal values (Fukushima et al. 1998). In addition, intestinal IgA production in mice mesenteric lymph nodes and PP cells was reinforced by *Bifidobacterium bifidum* (Park et al. 2002).

In a double-blinded study, *L. rhamnosus* and *L. reuteri* supplementation for 6 weeks in AD children alleviated gastrointestinal symptoms in 39% of children compared to the 10% in placebo treatment. Concomitantly, gut permeability measurement by lactulose mannitol ratio was lower after probiotic treatment than after placebo treatment, and this ratio was positively associated with AD severity (Rosenfeldt et al. 2004). In a rat model, prolonged feeding with milk increased gut permeability to intact proteins, but LGG reversed this change (Isolauri et al. 1993).

4.5.6 Immunological effects in vivo

In an earlier study on infants with AD and CMA, secretion of IL-4 and IFN-γ by stimulated PBMCs remained unaltered during 4 week treatment with both LGG and placebo. In the same study, treatment with LGG showed no effects on TNF-α secretion of stimulated PBMCs (Majamaa and Isolauri 1997). In children with AD, combination of two lactobacillus strains for 6 weeks did not affect secretion of IL-4, IFN-γ, and IL-10 by stimulated PBMC (Rosenfeldt et al. 2003). In a study on AD infants, serum TGF-β1 concentration decreased after 2- month *Bifidobacterium lactis* Bb-12 treatment, but tended to increase after LGG treatment. In that study, concentration of TNF-α or sICAM-1 did not change with probiotics (Isolauri et al. 2000). In adult volunteers, milk increased the expression of phagocytic receptors in hypersensitive subjects, while administration of LGG prevented this increase (Pelto et al. 1998). In a control group, LGG had an immunostimulatory effect, because receptor expression rose during consumption of milk with LGG (Pelto et al. 1998).

A 1-month consumption of yoghurt containing *L. acidophilus* by adults with asthma tended to promote IFN-γ production by stimulated lymphocytes (Wheeler et al. 1997). A group of six healthy volunteers showed decreased IL-2 and IL-4 secretion capacity by stimulated naïve and memory T cells, after treatment with *L. rhamnosus* for 2 weeks. In the same study, PBMCs of six patients with Crohn's disease showed, after treatment with *L. rhamnosus* for 2 weeks, a decrease in IL-2 and IFN-γ production, the latter having been elevated initially (Braat et al. 2004). Furthermore, IL-10 secretion and expression of CD25 by CD4+ cells in both study groups after *L. rhamnosus* treatment was decreased (Braat et al. 2004). In healthy adult volunteers, fermented milk products supplemented with either *Lactobacillus acidophilus* La1 or with *Bifidobacterium bifidum*, both enhanced granulocyte and monocyte phagocytic activities compared to basal level, reflecting activation of non-specific immunity (Schiffrin et al. 1995).

4.5.7 Immunological effects in vitro

In vitro incubation of various lactobacillus strains with PBMCs from allergic patients stimulated with related allergen, inhibited production of Th2 cytokines (Pochard et al. 2002) (Figure 2). Human DCs derived from blood and lamina propria and cultured with components of the cell wall of VSL#3 probiotic preparation showed induction of IL-10 production (Hart et al. 2004). After the same culturing, T cells showed attenuated IFN-γ production, reflecting possible regulatory activities (Hart et al. 2004). In addition, human DCs, matured with *L. rhamnosus*, attenuated T cell proliferation and cytokine production by stimulated T cells, especially production of IL-2, IL-4, and IL-10 (Braat et al. 2004).

Lactobacillus rhamnosus species may also have stimulatory effects on PBMCs. Human PBMC, stimulated with *Lactobacillus rhamnosus* 509, *Lactobacillus rhamnosus* GG, and *Lactobacillus bulgaricus*, showed induction of IL-1β, IL-6, and TNF-α mRNA and protein production. IL-4 production was not induced by any of the lactobacillus strains, while all induced

weak IL-10 production. Except for *Lactobacillus bulgaricus*, all other lactobacillus strains induced IL-12 and IFN-γ production (Miettinen et al. 1998). In another in vitro study by Miettinen et al *Lactobacillus rhamnosus* 509 and *Lactobacillus rhamnosus* GG, among several lactobacilli, showed themselves to be the best stimulators of human PBMCs in producing TNF-α, IL-6, and IL-10 (Miettinen et al. 1996). In human macrophage cultures, *Lactobacillus rhamnosus* induced the highest levels of IL-10 among the lactobacilli studied, while *L. paracasei* was the strongest inducer of IL-12 (Hessle et al. 1999). The caco-2 cell line, resembling human epithelial cells, when cultured with different lactic acid bacteria, failed to induce production of proinflammatory IL-6 and IL-8 (Morita et al. 2002).

4.5.8 Immunological effects in animal models

In two separate studies, intra-gastric administration of heat-killed *L. casei Shirota* and *L. plantarum* L-137 both induced IL-12 production in an antigen-fed murine model, and concomitantly inhibited antigen-specific IgE production (Murosaki et al. 1998; Shida et al. 2002), and also diminished systemic anaphylaxis (Shida et al. 2002). In another study, OVA-primed murine splenocytes were restimulated in vitro with OVA and with *L. casei*, which resulted in increased production of IFN-γ and IL-12, inhibition of IL-4 and IL-5, and suppression of antigen-specific IgE secretion by OVA-stimulated splenocytes (Shida et al. 1998) (Figure 2).

Probiotics may also affect the T regulatory system (Figure 2). *L. paracasei* and LGG, when cultured with mice splenocytes, induced the highest IL-10 levels, while *L. paracasei* was the strongest IL-12 inducer (von der Weid et al. 2001). In the same study, in cultures of CD4+ T cells from naïve BALB7c mice with splenocytes, *L. paracasei* inhibited subsequent production of both Th1 and Th2 cytokines, maintained IL-10 production, and induced TGF-β from T cells, suggesting the development of T regulatory cells. In a murine model, treatment with VSL#3 probiotic preparation attenuated recurrent colitis compared to the effect of placebo, and this effect was

suggested to be attributed to lamina propria mononuclear cells, as their transfer to naïve mice reproduced protection (Di Giacinto et al. 2005). Additionally this probiotic enhanced IL-10 production, and numbers of CD4+ T cells bearing surface TGF-β in the form of latency-associated protein (Di Giacinto et al. 2005). In another study, a murine model of colitis, both *Lactobacillus salivarius* and *Bifidobacterium infantis* alleviated intestinal inflammation unlike the placebo, bifidobacterium reduced IFN-γ, TNF-α, and IL-12 production from stimulated splenocytes in vitro. With lactobacillus, however, a reduction was evident only in IFN-γ, and production of TGF-β was maintained in all groups. From resting PP cells, IFN-γ and TNF-α secretion was attenuated by the bifidobacterium strain (McCarthy et al. 2003). In a murine model resembling contact dermatitis, oral administration of *L. casei* reduced skin inflammation by inhibiting expansion of IFN-γ-producing CD8+ effector T cells (Chapat et al. 2004). This down-regulation required the presence of CD4+ cells, probably regulatory T cells. In another study, LGG down-regulated in vitro secretion of TNF-α by activated murine macrophages (Peña and Versalovic 2003).

Probiotic strain-specific differences exist in cytokine production. Murine DCs, cultured with different lactobacillus species, showed that *L. casei* is the strongest inducer of IL-12 and TNF-α, whereas *L. reuteri* showed no induction. A similar but less pronounced pattern was apparent for IL-6 and IL-10. Interestingly, *L. reuteri* inhibited *L. casei*-induced IL-12, IL-6, and TNF-α secretion, reflecting the possibility of one species being able to inhibit another species' effect (Christensen et al. 2002). Bifidobacteria isolated from faeces of allergic infants induced more IL-1, IL-6, IL-12, and TNF-α, and less IL-10 from murine macrophages than did bifidobacteria isolated from healthy adults, healthy infants, or dairy products (He et al. 2002).

5. AIMS OF THE STUDY

The purpose of this study was to evaluate the effects of two probiotic preparations on symptoms of AD and on immune responses in infants with AD and CMA.

Based on clinical studies, such infants demonstrate prominent Th2 type inflammatory responses and defects in production of Th1 type and suppressive cytokines. Induction of intestinal inflammation is also possible. Epidemiological data suggest that changes in gut microbiota, reduction in oro-faecal infections, and microbes in food are associated with the emergence of allergies. These changes are proposed to be counter-balanced by probiotics. Probiotic bacteria alleviated symptoms of AD and CMA in clinical trials. However, the mechanisms of probiotic effects have been mainly explored in *in vitro* studies and in animal models. Most of the clinical trials have failed to demonstrate the mechanisms of probiotic effects in allergies; while one earlier study suggested that suppression of intestinal inflammation may play a role.

Therefore, the specific aims of this study were to evaluate:

1) The effects of *Lactobacillus* GG and a mixture of probiotics on clinical symptoms of AD in infants with CMA, AD, and in a subgroup of IgE-associated AD.
2) The effects of probiotic bacteria on the immune response of infants with CMA, AD, and IgE-associated AD, a) by measuring cytokine production of stimulated PBMC and b) by characterising effects of probiotic bacteria on plasma levels of cytokines, on soluble adhesion molecules, and on CRP as an inflammation marker.
3) Probiotic effects on intestinal inflammation, characterised by faecal inflammation markers, and on faecal IgA, in infants with CMA, AD, and IgE-associated AD.
4) And search for associations between clinical effects and immunologic findings in infants with IgE-associated AD.

6. MATERIALS AND METHODS

6.1 Participants and study protocol

6.1.1 Participants

The clinical part of this study was carried out between November 1999 and March 2002 at the Skin and Allergy Hospital of Helsinki University Central Hospital. Infants fulfilling the following criteria were accepted: 1. Age under 12 months at the entrance of the study, 2. Symptoms suggesting CMA, the mandatory symptom being AD, 3. No regular use of probiotic preparations (longer than 1 week, and within 6 weeks before entering the study). Of the 431 infants referred from local health centres, 284 (66%) had parents who, after talking with a trained research nurse, wished to participate. Of the 252 infants meeting our inclusion criteria, 230 completed the study.

Of the 22 infants not completing the study; 2 moved away from Helsinki area, 11 did not start the elimination diet with EHF and topical skin treatment alone alleviated their symptoms, 4 were not tolerant to the EHF used, 3 had parents who found the study protocol too complicated, and 2 infants were hospitalised for severe AD and their study treatment was discontinued.

The ethics committee of the Hospital for Children and Adolescents, University of Helsinki, approved the study protocol. One parent of each infant gave written informed consent.

At the first visit, a comprehensive interview with questionnaires completed was performed with all the families to record data on symptoms prior to the study, feeding patterns, and other background information.

6.1.2 Assessment and treatment of AD

On every visit (Fig 3), I assessed the severity of AD by the Severity Scoring of Atopic Dermatitis (SCORAD) scale. SCORAD is calculated by: extent/5 + 3.5 x intensity + subjective symptoms (max 103). The extent is evaluated from 0 to 100% of the body surface. Intensity was estimated

with the aid of reference photographs, and consists of the sum of individual scores for erythema, oedema/papules, oozing/crusts, excoriation, lichenification, and skin dryness (each 0-3, max 18). One parent scores the subjective symptoms, which include pruritus and sleep-loss (scored 0-10 points each, max 20). Parents were instructed to treat the infant's skin with emollients every day and eczematous lesions with topical 1% hydrocortisone as needed but for a maximum period of 2 consecutive weeks (amount used recorded at every visit).

6.1.3 Elimination diet

At the first visit, a strict CM-elimination diet was initiated for infants and breast-feeding mothers (Fig 3). All infants received EHF (Peptidi-Tutteli®, Valio Ltd., Helsinki, Finland). If any other foods were suspected to cause symptoms, elimination diet was initiated based on clinical indications.

6.1.4 Treatment with probiotics

Infants were randomised in a double-blinded manner to receive one of three products for 4 weeks. This randomisation was performed with the computer generated block randomisation of six infants. The LGG group (n=80) received capsules containing *Lactobacillus rhamnosus* GG (ATCC 53103) (LGG) 5 x 10^9 colony-forming units (cfu); the MIX group (n=76), a mixture of 4 probiotics: LGG 5 x 10^9 cfu, *Lactobacillus rhamnosus* LC705 (LC705) 5 x 10^9 cfu, *Bifidobacterium breve* Bbi99 2 x 10^8 cfu, and *Propionibacterium freudenreichii ssp. Shermanii* JS (*Propionibacterium* JS) 2 x 10^9 cfu; and the placebo group (n=74), only the inert matrix material, microcrystalline cellulose. The capsule content was mixed with food twice daily. These products, supplied by Valio Ltd., were indistinguishable, as they looked, smelt, and tasted identical. Parents were asked to give no probiotic preparations to their infants during the study. The code was opened after the clinical data were analysed, and laboratory personell were unaware of the code also thereafter.

6.1.5 Skin prick test and serum IgE concentrations

At the first visit, SPTs were performed on the volar aspect of the forearm with commercial allergen extracts of eggwhite (Alyostal prick test®, Stallergenes SA, Antony, France), cat, dog, and birch (Soluprick®, ALK-Abelló, Hørsholm, Denmark) according to the standard technique (Dreborg et al. 1989). Duplicate tests were performed with fat-free CM; a panel of 10 widely used commercially available adapted CM, extensively hydrolysed, amino acid-, and soy protein-based infant formulas; cereal grains, and purified gliadin. A negative control solution (ALK-Abelló, Denmark) and histamine dihydrochloride at 10mg/ml (ALK-Abelló, Denmark) as a positive control were used. Reactions were read after 15 min, with any mean weal diameter ≥ 3 mm greater than the negative control considered positive. Concentrations of serum CM, and wheat-specific IgE were measured by the Pharmacia CAP system RAST FEIA (Pharmacia Ltd., Uppsala, Sweden).

6.1.6 Symptom diary

During the study, parents recorded daily in a symptom diary any skin, gastrointestinal, or respiratory symptom; the start of new solid foods; and use of antibiotics.

6.1.7 Diagnosis of cow's milk allergy

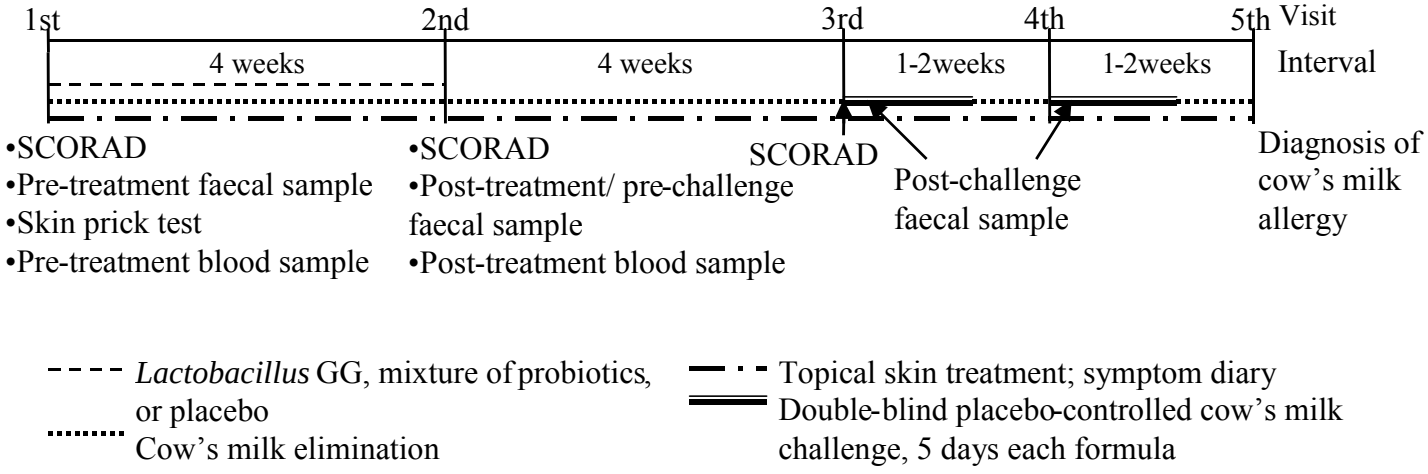
If the symptoms had considerably improved by the third visit (Fig 3), we started a DBPC cow's milk challenge, in which CM formula (Tutteli®, Valio Ltd.) was mixed with the EHF (1:2) to make it indistinguishable from the placebo formula (EHF alone). The challenge was started with drops of formula on the skin. Thereafter, the same formula was given orally in increasing doses at half-hour intervals: 2, 10, 50, and 100 ml on day one. If no adverse symptoms appeared during the challenge or 2-hour follow-up in the hospital, the challenge was continued at home. The infant received of the same formula 4 to 6 dl daily for the next 4 days. If any adverse reaction appeared at home, the

parents were advised to contact the research doctor by phone to consult whether or not to discontinue the challenge. At the fourth visit, after a wash-out period of 2 to 9 days, the challenge formula was changed and this procedure repeated. At the last visit, symptom diaries of the two challenge periods were compared, and a decision of symptomatic challenge period was made before the milk code was opened. CMA was diagnosed in infants who demonstrated urticaria, clear worsening of AD, vomiting, diarrhoea, physician-diagnosed wheezing, allergic rhinitis, or conjunctivitis during the challenge with CM-containing formula. In infants in whom the challenge was negative, CMA was excluded. Other food sensitivities were not studied by challenge test.

6.1.8 Definition of IgE-associated AD

Any infant with positive SPT or an antigen-specific IgE concentration ≥ 0.7 kU/l to any antigen tested were considered to have IgE-associated AD.

Figure 3 Study protocol (modified from the original publications)



6.2 Faecal samples

Faecal samples were collected from all infants at every visit (Fig2). All the samples were frozen at -20˚C within 15 minutes, and if taken at home, brought frozen to the hospital, and stored at -40˚C until inflammation marker analysis and at -70˚C until bacterial analysis.

6.2.1 Concentrations of bacteria in faecal samples (I)

From 52 infants, additional faecal samples were collected for bacterial analysis both at the first and second visits. The Starter Cultures Unit from Valio Ltd performed the bacterial analysis. Concentrations of anaerobic bacteria were determined with Brain Heart Infusion agar (Lab M, Lancashire, UK), of the lactic acid bacteria with MRS-agar (Oxoid Ltd., Basingstoke, UK), and that of the bifidobacteria with Raffinose-Bifidobacterium-agar (RB) (Hartemink et al. 1996). Concentrations of the two strains of lactobacilli were determined with MRS-vancomycin agar (Oxoid Ltd.) and that of the *Propionibacterium* JS with modified YEL agar (Suomalainen and Mäyrä-Mäkinen 1999). Limits of detection were 10^5 cfu/g for bifidobacteria and 10^3 cfu/g for the other bacteria.

6.2.2 Determination of faecal IgA, TNF- α, AT, and ECP (IV)

Pre-treatment faecal samples were collected on the day of the first visit, and post-treatment samples after the 4-week treatment, on the day of the second visit (Fig. 2). Of the 102 infants' samples randomly chosen for faecal analysis, paired samples (pre-treatment and post-treatment) were available for IgA analysis in 90, for TNF-α analysis in 87, for AT analysis in 91, and for ECP analysis in 81 infants. The post-challenge sample was the one after the active milk challenge (mean 8.2 h; range 0 to 94.3 h after). The wash-out period was short, and to avoid a carry-over effect from the active challenge, we chose the sample collected at visit 2 as the pre-challenge sample. Paired

samples (pre-challenge and post-challenge samples) from infants with CMA were available for IgA analysis from 66, from TNF-α analysis in 67, from AT analysis in 67, and from ECP analysis in 60 infants.

Faecal samples were homogenized with phosphate-buffered saline (PBS), weight/volume ratio 1:4, on a shaker, for 30 minutes at +4°C. The homogenates were centrifuged for 15 minutes at 10 000g at +4°C. The supernatants were collected and stored frozen at -70°C for later analysis.

To measure concentrations of total IgA in faecal extracts, the enzyme-linked immunosorbent assay (ELISA) was used. Microtitre plates (Nunc-Immuno Plates) were coated with anti-IgA antibodies (Dako, Glostrup, Denmark), which were diluted 1:1000 in 50 mM $NaHCO_3$, at pH 9.5, 100 µL/well, at +4°C overnight. The coated plates were washed with PBS (0.02 M Tris-0.5 M NaCl, pH 7.5) once, thereafter 2% bovine serum albumin-PBS was used to saturate them for 1 h at room temperature, and PBS again to wash them. Diluted (typically 1:10 000) in diluting buffer (1% BSA-0.05% Tween 20-PBS), faecal extracts and standards (Serum Protein Calibrator, Orion Diagnostica, Espoo, Finland) were distributed to microtitre plates (100 µL/well) and incubated overnight at room temperature. The plates were washed with 0.05% Tween-PBS three times. Alkaline phosphatase conjugated anti-human IgA (Dako), which was diluted (1:1000) in diluting buffer, was added, and the plates were developed as described previously for the CM-antibody assay (Savilahti et al. 1993). The detection limit of the total IgA assay was 5 µg IgA/L of the diluted faecal extract.

Faecal AT was measured by a single radial immunodiffusion method, with a detection limit of 0.08 mg/g. The antiserum (Orion Diagnostica) was diluted to 1:37.5 in the agar, and the Serum Protein Calibrator (Orion Diagnostica) was the standard. Diffusion was allowed to occur for 1 week at +4°C. ECP was measured by the Pharmacia CAP system (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden), with a detection limit of 16 µg/L, and TNF-α was determined by

the Quantikine HS TNF-α Immunoassay kit (R&D Systems, Inc., Minneapolis, USA), with a detection limit of2.0 pg/g.

6.3 Blood samples

Pre-treatment blood samples were collected at the first visit, and post-treatment samples after the 4-week treatment, on the second visit (Fig. 2).

6.3.1 Separation and stimulation of PBMC (II)

Cytokine production of the culture supernatants was measured in 119 infants; 63 of the blood samples were paired, 40 samples were from the first visit, and 16 samples from the second visit. PBMCs were isolated from blood-heparin samples by Ficoll-Hypaque (Pharmacia) centrifugation. PBMCs (2 x 10^6 / 2 ml) were resuspended in RPMI 1640 medium (GibcoBRL, Gaithersburg, MD, USA) containing 1% L-glutamine (GibcoBRL) and 5% AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Thereafter they were cultured and stimulated with monoclonal OKT3 (anti-CD3) antibody (1 μg/well) (R&D Systems Inc.) -coated plates (Nunc 24 wells, Roskilde, Denmark) in the presence of soluble anti-CD28 antibody (2 μg/well) (Becton Dickinson immunocytometry systems (BD), San Jose, CA, USA). Incubation was performed similarly but without stimulation to obtain control cultures (negative controls). After 24-h incubation, a supernatant sample was collected for further ELISA analysis, and the samples were frozen at -20˚C prior to analysis.

6.3.2 Determination of cytokines in culture supernatants (II)

ELISAs were used to measure quantities of IFN-γ, IL-5, IL-4, and IL-12 in the culture supernatants: monoclonal anti-human IFN-γ-antibody (Lot CD48432, Endogen, Woburn, MA, USA) at a concentration of 2 μg/ml (50 μg/well) was used to coat 96-well microplates (Nunc). A standard

curve was created with dilutions of recombinant human IFN-γ (Lot M070524, BD Pharmingen, San Diego, CA, USA). Supernatant samples (100 µl/well) and standards (100 µl/well) were incubated at 37°C for 2 hours. Biotinylated anti-human IFN-γ monoclonal antibody (Lot CD47529, Endogen) was added at a concentration of 0.25 µg/ml (50 µl/well) to measure bound IFN-γ. AP-Streptavidin Conjugate (Zymed Laboratories, San Francisco, CA, USA) served as the substrate. The detection limit of the assay was 8 pg/ml. IL-5 concentrations in the culture supernatants were measured by the same protocol using a purified rat anti-mouse/human IL-5 monoclonal antibody at 1 µg/ml (Lot M049743, Pharmingen) for coating, and biotinylated rat anti-human IL-5 monoclonal antibody to measure bound IL-5 at 0.5 µg/ml (Lot M057221, Pharmingen). Dilution of recombinant human IL-5 (Lot M021519, Pharmingen) was used to create a standard curve. The detection limit of the assay was 15 pg/ml. IL-4 concentrations in the culture supernatants were determined by a commercial human IL-4 ELISA Kit (Pelikine Compact™, Amsterdam, Netherlands) with a detection limit of 0.6 pg/ml. Total IL-12 concentrations were determined by commercial high-sensitivity sandwich ELISA (Quantikine® HS, R&D Systems, Abingdon, United Kingdom) according to manufacturer's instructions with a detection limit of 0.6 pg/ml.

6.3.3 Determination of adhesion molecules, TGF-β, and CRP in plasma (III)

Study III plasma samples of peripheral blood were obtained by centrifugation of the heparinized blood and stored frozen at -20˚C prior to analysis. We succeeded in receiveing paired plasma samples of 132 infants for ELISA analysis. Quantities of soluble (s)ICAM-1, sE-Selectin, TGF-β1, and TGF-β2 in the plasma samples were measured with ELISA according to manufacturer's instructions. Soluble ICAM-1 and sE-Selectin protein levels were measured with commercial sandwich ELISA kits (Parameter®, R&D Systems). Detection limits for the assays were 37.2 ng/ml, and 10.6 ng/ml, respectively. All plasma sE-Selectin and sICAM-1 values were above the detection limits. TGF-β1 and TGF-β2 concentrations in the plasma samples were determined by human TGF-

β1 and TGF-β2 ELISA kits (Quantikine®, R&D Systems) with detection limits of 84 pg/ml and 54.6 pg/ml, respectively. None of the TGF-β1 samples, 36% of pre-treatment TGF-β2 samples, and 37% of post-treatment TGF-β2 samples were below the detection limit. CRP concentrations were determined by immunoturbidimetric ultrasensitive CRP assay (Orion Diagnostica) with detection limit of 0.29 μg/ml. In CRP, 19% of samples were below the detection limit.

All ELISA assays were performed in duplicate, and the intensity of the colour was measured by multiscan MS version 8.0 (Labsystems Oy, Helsinki, Finland).

6.3.4 Determination of cytokines in plasma (III)

For the cytometric bead array, paired plasma samples of 121 infants were available. Capture beads of IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ were incubated with plasma samples or standards and mixed with the PE-conjugated detection antibodies according to manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA, USA). Standard concentrations of 625 pg/ml, 312.5 pg/ml, 156 pg/ml, 80 pg/ml, 40 pg/ml, 20 pg/ml, 10 pg/ml, 5 pg/ml, 2.5 pg/ml and assay diluent as a negative control were used to create a standard curve. Protein concentrations were measured on an FACScan flow cytometer (CellQuest software, BD) with a detection limit of 2.6 pg/ml for IL-2, 2.6 pg/ml for IL-4, 3.0 pg/ml for IL-6, 2.8 pg/ml for IL-10, 2.8 pg/ml for TNF-α, and 20 pg/ml for IFN-γ. In IL-6, 79% of pre-treatment samples and 73% of post-treatment samples were below the detection limit. For IL-10 and IL-4, 65% of pre-treatment samples and 58% of post-treatment samples were below the detection limit; 74% of IFN-γ samples were below the detection limit. All plasma IL-2 and TNF-α values were below detection limits. Results were analysed on BD CBA analysis software MAC OS version 9.

6.4 Statistical analysis

Study I: Sample size calculation was based on SCORAD. We assumed that the active treatments (*Lactobacillus* GG or the mixture of probiotics) would result in a difference of 8 points in SCORAD, compared to the placebo. Assuming a common standard deviation of 9.5 (Majamaa and Isolauri 1997), we estimated that for all paired comparisons a sample size of 40 CMA infants per group has 90% power to detect a difference statistically significant at the 0.05 level. Since CMA diagnosis was made after the treatment, and only half the infants that participated eventually had CMA, almost 80 infants per group had to be followed up.

The primary outcome variable was change in SCORAD. The interval after probiotic bacteria administration at which possible effects appear in humans is unknown. Therefore decided, before analysis, to calculate the changes from baseline (visit 1) at visit 2, after a 4-week treatment, and at visit 3, 4 weeks after the end of the treatment. Analysis of variance (ANOVA) was performed to compare the treatment groups with respect to the changes in SCORAD. Fisher's LSD test was used for comparisons between probiotic groups versus placebo. In Study I, treatment differences are given as means with standard deviation (SD). As we found significant interactions between treatment groups and IgE association, indicating that the treatment effect may be dependent on IgE association, we performed subgroup analyses to control for this interaction.

Studies III, and IV: Because distributions of faecal IgA, TNF- α, AT, and ECP (IV), and plasma CRP, E-Selectin, and ICAM-1 (III) were skewed, all comparisons were made after logarithmic transformation. Analysis of covariance (ANCOVA) was used for multiple comparisons. In ANCOVA, treatment groups were compared with each other with respect to post-treatment values/post-challenge values. Since differences existed in baseline values (pre-treatment values (at visit 1)/ pre-challenge values (at visit 2)), these were taken as covariates in the analysis. An antilogarithm was taken from baseline-adjusted geometric means to receive the initial unit of measurement. Fisher's LSD test served for comparisons between probiotic groups and placebo. For

comparisons between the paired measurements, we used a paired t-test. The data are presented with geometric means and 95% confidence intervals (CI).

Studies I, II, and III: The non-parametric tests were used for comparisons of changes in counts of faecal bacteria in Study I, of all variables in Study II, and of plasma cytokines IL-6, IL-10, IL-4, IFN-γ in Study III, since no variable was normally distributed and because of the great number of values were under the detection limit in Study III. The Kruskall-Wallis and Mann-Whitney U-test were used for comparisons of pre- and post-treatment values. The Wilcoxon signed-rank test served to compare the follow-up samples. Nonparametric tests were 2-tailed. Data in Study III are presented with mean, range, and upper quartile. In Study II, data are presented with median and range. As all plasma values of IL-2, and TNF-alpha were under the detection limits, they were not further analysed.

SCORAD change from first to third visit (SCORAD 3-1) was correlated with changes (after-before) and post-treatment levels of those markers that showed significant changes after probiotic therapy. In infants with IgE-associated AD, correlations were sought for IFN-γ, IL-6, CRP, and faecal IgA. The following cut-point values were used to categorise the above variables as dichotomous: SCORAD change from first to third visit (visit 3 minus visit1) < -30 belonged to the upper quartile of SCORAD changes, or <-19.2 belonged to the median group of SCORAD changes. IFN-γ, IL-6, CRP, and faecal IgA were categorized to show an increase or to be above the median (IFN-γ, CRP, and faecal IgA) / above the upper quartile (IL-6), post-treatment. Comparison of categorised variable was made with Pearson's chi-square test. Correlations between laboratory findings were made with the Spearman correlation.

Data were analysed with SPSS for Windows (versions 10.0 and 11.0, SPSS Inc, Chicago, USA) and with Statview for Windows (version 5.0, SAS Institute Inc). Statistical significance was defined as a p-value of ≤ 0.05.

7. RESULTS

7.1 Clinical characteristics (I and MV et al. unpublished results)

At entrance into the study, the infants were a mean 6.4 (range 1.4-11.9) months old, and 62% of them were males. Of all infants with AD, 120 had CMA, and in 110 infants, CMA was excluded. IgE-associated AD was detected in 136 infants. Age at start of the AD symptoms was 2.5 (0-9) months. The amount of topical 1% hydrocortisone used between the first and the third visit was a median 40 (range 0-500) grams. Solid foods were introduced at a mean age of 3.8 (1.5-6) months. Of all infants, 132 (57%) had at least one sibling, and 42 (18%) had a cat or a dog in the household. In 77 (33%) of the families, someone smoked. Parental atopy, diagnosed by a doctor, was detected in 166 (72%) of cases. Clinical features in treatment groups of all infants with AD are presented in Table 2.

Table 2.
Clinical characteristics in treatment groups of the whole study population. Figures are means (ranges) and numbers (percentage) of infants.

Variable	LGG	MIX	placebo
Number of patients	80	76	74
Age (months)	6.1 (1.8-11.1)	6.3 (1.4-11.4)	6.8 (2.4-11.9)
Exclusive breast feeding (months)	1.8 (0-5)	1.7 (0-5)	1.8 (0-6)
Age at introduction of CM formula	2.0 (0-10)	1.9 (0-8)	1.8 (0-10)
SCORAD at 1st visit	34.3 (4.6-73.5)	33.3 (7.6-82.5)	29.9 (6.1-52.2)
SCORAD at 2nd visit	17.7 (0-64.5)	19.3 (0-50.2)	15.7 (0-44.4)
Antibiotic treatments, 1st to 3rd visit, n (%)	20 (25)	28 (37)	20(27)

Among infants with IgE-associated AD, 75 (55%) had CMA, 69 (51%) had SPT positive to CM or an increased CM-specific IgE, 41(30%) had SPT positive to cereals or an increased whey-specific IgE, and 93 (68%) were SPT-positive to egg.

Among infants with CMA, 45 reacted at the active challenge immediately, within half an hour of the last dose, and a delayed-type reaction was detected in 75. At the start of reaction, mean time from last dose was 20.7 (0-74) hours, and mean cumulative dose of challenge formula was 517 (0-2162) ml perorally. Seven patients developed urticaria immediately after the

introduction of a drop of challenge milk on their skin or lips. For these infants, no oral challenge was performed. During the active challenge period, of the 120 infants with CMA: 49 demonstrated urticaria, 70 worsening of AD, 26 diarrhoea, 35 vomiting, 1 wheezing, 2 cough, 2 haziness, 10 allergic rhinitis, and 10 allergic conjunctival symptoms. All infants with allergic rhinoconjunctival or respiratory symptoms also demonstrated cutaneous symptoms. Of the 61 infants with GI symptoms, 3 demonstrated immediate type reaction and 5 had no symptoms other than GI ones at the challenge.

CMA infants were younger than non-CMA infants when they entered the study, whereas age at the start of AD symptoms did not differ between CMA and non-CMA infants (Table 3). Introduction of CM formula occurred at a younger age in non-CMA infants, and among these infants the use of CM formula before entering the study was more often regular, compared to that for CMA infants. CMA infants were exclusively breast fed longer and received irregularly (small amounts, a few times before the study) CM formula, compared to non-CMA infants. Severity of AD at first visit and occurrence of diarrhoea, respiratory symptoms, and sleep disturbances were comparable before the start of the study, while CMA infants more often demonstrated CM-SPT positivity and urticaria symptoms.

Table 3. Clinical characteristics in infants with CMA and in non-CMA infants.

	CMA infants n=120	**Non-CMA infants n=110**	**P-value**
Age at 1st visit (months)	5.9 (1.4-11.5)	6.9 (1.8-11.9)	0.003
Age at start of AD (months)	2.4 (0-7)	2.7 (0-9)	0.2
Exclusive breast feeding (months)	2.1 (0-6)	1.4 (0-5)	0.0008
Age at introduction of CM formula (months)	2.4 (0-10)	1.4 (0-6)	0.009
Regular use of CM formula	52 (43)	86 (78)	<0.0001
Irregular use of CM formula	55 (46)	16 (15)	<0.0001
SPT positive to CM	54 (45)	12 (11)	<0.0001
SCORAD at 1st visit	33.4 (4.6-82.5)	31.5 (7.6-73.5)	0.3
Diarrhoea prior to study	30 (25)	21 (19)	0.3
Urticaria prior to study	23 (19)	4 (4)	0.0003
Respiratory symptoms prior to study	22 (18)	15 (14)	0.3
Sleep disturbances prior to study	71 (59)	71 (65)	0.4
1% Hydrocortisone* (g), from 1st to 3rd visit	50(0-300)	31(0-500)	0.2

Figures are mean (range) and number (%) of patients, * median (range)

7.2 Cow's milk allergy (I-IV and MV et al. unpublished results)

Among infants with CMA, SCORAD change from the firs to the third visit (Table 4), or change from the first to the second visit (data not shown), did not differ between probiotic and placebo groups. Since antibiotics potentially interfere with the gut microbe balance, and the number of antibiotic courses was high and unevenly distributed among treatment groups, we made a secondary analysis after excluding infants who had received antibiotics between their first and third visits. Compared to the primary analysis, changes still remained non-significant (Table 4).

IFN-γ secretion in stimulated PBMCs of CMA infants differed significantly among treatment groups. The LGG group demonstrated an increase in IFN-γ secretion ($p=0.023$), but MIX and placebo treatments had no effect on IFN-γ response. Thus, the change in IFN-γ concentration was greater in the LGG group than in the placebo group ($p=0.006$, Figure 4A). In infants with CMA, IL-4 secretion of stimulated PBMCs was enhanced after treatment with MIX, and the change in IL-4 production was greater than in the placebo group, ($p=0.028$, Table 4, Figure 4B). In the LGG and placebo groups, no changes occurred in IL-4 secretion (Table 4). IL-5 secretion by PBMC was similar in all groups after the treatment period (data not shown).

The plasma level of IL-10 increased after the MIX treatment ($p=0.004$), while the post-treatment level, compared to the placebo group, showed no differences (Table 4). Levels of IL-6 in plasma showed no significant changes after probiotic or placebo treatment (Table 4). Adjusted post-treatment serum CRP levels showed no significant differences between treatment groups (Table 4).

Post-challenge faecal IgA, TNF-α, AT, and ECP, adjusted by pre-challenge values, demonstrated no significant differences between treatment groups in infants with CMA (Table 4, data not shown for TNF-α and ECP).

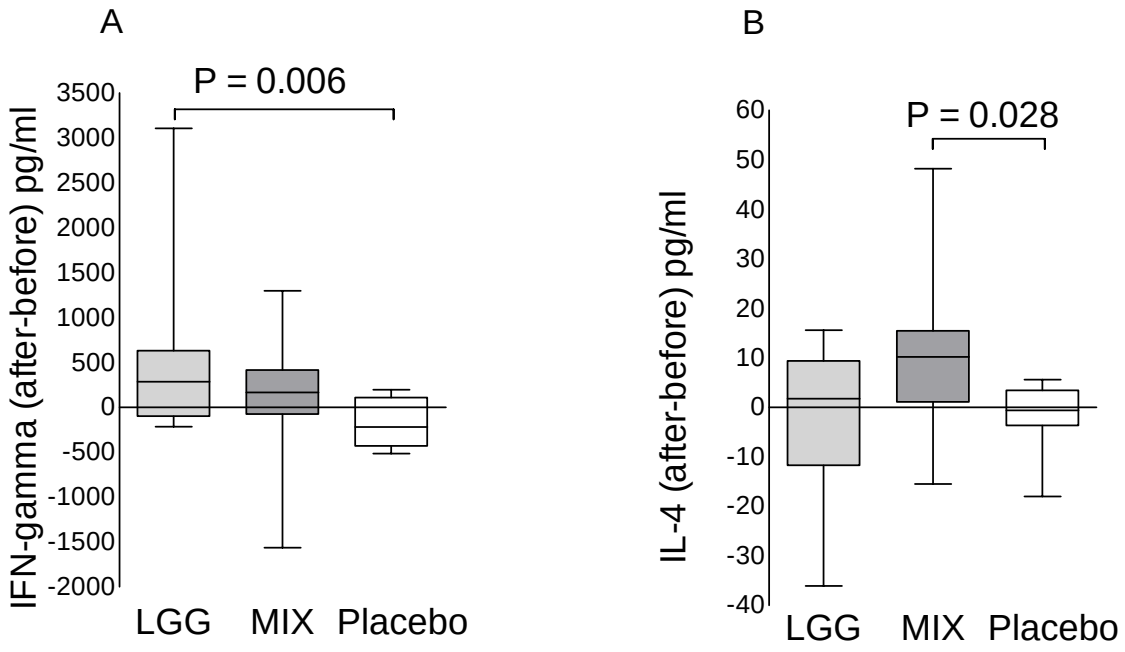


Figure 4. Infants with CMA; three treatment groups. Middle lines represent median, box borders upper and lower quartiles, and error bars represent range; P-values by Mann-Whitney U-test. A. Change in secretion of IFN-γ by stimulated PBMCs (post treatment- pre treatment), B. Change in IL-4 levels (post treatment- pre treatment).

Table 4. Clinical and immunologic findings in CMA: Changes in SCORAD from first to third visit in all infants and those receiving no antibiotics (AB-). Secretion of interferon-γ and IL-4 by PBMC before and after treatment; concentrations of IL-10, IL-6, and CRP in the sera; faecal concentrations of IgA and α1-antitrypsin (AT).

Parameters		**LGG**		**MIX**		**placebo**		**P-value**	**LGG vs. placebo**	**MIX vs. placebo**
			N		N		N	ANOVA/ ANCOVA	Fisher's LSD test	
SCORAD change Mean (SD)	From visit 1 to visit 3	-22.7 (17.5)	44	-19.4 (15.9)	44	-21.4 (13.6)	32	0.616	0.730	0.582
SCORAD change AB- Mean (SD)	From visit 1to visit 3	-25.9 (18.5)	32	-20.3 (12.5)	25	-22.2 (14.5)	25	0.397	0.390	0.661
CRP (µg/ml) Geom.mean (95%CI)	Post-treatment, adjusted by pre-treatment value	0.75 (0.51-0.93)	29	0.80 (0.52-0.82)	24	0.53 (0.32-0.89)	17	0.461	0.293	0.243
F IgA (g/L) Geom.mean	Post-challenge, adjusted by pre-challenge value	0.60	23	0.63	27	0.54	16	0.661	0.556	0.365
F AT (mg/g) Geom.mean	Post-challenge, adjusted by pre-challenge value	0.16	24	0.13	27	0.10	16	0.280	0.112	0.361
								K-W	Mann-Whitney	
IFN-γ (pg/mL) Median (range)	Before After	403 (90-1780) 829 (298-3501)[§]	12	437 (23-2431) 681 (331-1681)	12	836 (286-1595) 579 (316-1136)[§§]	9	**0.024**[a]	**0.006**[a]	0.058[a]
IL-4 (pg/mL) Median (range)	Before After	19 (9-74) 19 (8-46)	12	15 (4-49) 22 (13-65)[#]	12	21 (7-32) 18 (4-30)	9	0.060[a]	0.522[a]	**0.028**[a]
IL-10 (pg/mL) Mean (upper quartile, range)	Before After	1.7 (3.1, 0-7.1) 2.3 (4.7, 0-8.1)	25	1.5 (3.5, 0-4.9) 3.5(5.2, 0-16.8)[##]	21	1.7 (3.5, 0-6.7) 2.9 (4.6, 0-19.0)	16	0.974 0.574	0.864 0.885	0.868 0.404
IL-6 (pg/mL) Mean (upper quartile, range)	Before After	0.8 (0, 0-8.7) 2.7 (5.1, 0-20.3)	25	3.4 (4.9, 0-22.6) 1.9 (3.8, 0-17)	21	1.0 (0, 0-7.4) 1.3 (2.3, 0-9.5)	16	0.143 0.884	0.843 0.741	0.308 0.797

***** Kruskall-Wallis test, [a] tested change in concentration (after-before)

Follow-up samples of IFN-γ, IL-4, IL-6, and IL-10 -γ tested with Wilcoxon signed-rank test and presented with footnotes if $p<0.10$:

§ $p=0.023$, §§ $p=0.086$, # $p=0.034$, ## $p=0.004$

7.3 Atopic dermatitis (I-IV)

In the whole study group of infants with AD we found no differences after probiotic or placebo treatment in alleviation of AD symptoms, as measured by SCORAD change from the first to third visit (Table 5), nor from the first to second visit (data not shown). After excluding infants who had received antibiotics between the first and third visits, differences between probiotic and placebo groups were greater than those in the primary analyses, but these differences remained non-significant (Table 5).

Probiotic treatment demonstrated no significant effect on IFN-γ, IL-4, IL-5, or IL-12 secretion of stimulated PBMCs in all infants with AD (Table 5, for IL-5 and IL-12 data not shown).

The plasma level of IL-10 after MIX treatment increased significantly (p=0.016), while LGG or placebo treatments showed no significant changes (Figure 5A). However, post-treatment IL-10 was higher in the LGG (p=0.046) and MIX groups (p=0.039) than in the placebo group (Table 5). Plasma IL-6 showed no significant changes after probiotic or placebo treatments among all AD infants (Table 5). Plasma IL-4, TGF-β1, and TGF-β2 levels showed no differences between treatment groups in follow-up samples in the whole study population or in subgroup analyses (data not shown) Plasma IFN-γ levels were low, with no differences during follow-up, both in the whole study population and in subgroup analyses (data not shown).

In the whole study population, pre-treatment-adjusted serum CRP levels after probiotic or placebo treatment did not differ significantly (Table 5). After treatment, adjusted serum sICAM-1 ans sE-selctin levels were similar in all treatment groups in the whole study population, and in all subgroup analyses (data not shown).

In the whole study population, decrease in faecal IgA levels was significant during treatment in the placebo group (paired t-test p=0.010), but decreases in probiotic groups were non-significant (LGG p=0.114, and MIX p=0.114) (Fig. 5B). Thus, after treatment, faecal IgA, adjusted by pre-treatment value, tended to be higher in the LGG and MIX groups than in the placebo group

(Table 5) After treatment, adjusted faecal TNF-α showed no differences between treatment groups (data not shown). Levels of AT decreased significantly during the treatment period in the LGG (paired t-test, p=0.033) but not in the MIX (p=0.445) or in the placebo (p=0.111) groups (Fig. 5C). Post-treatment faecal AT, adjusted by pre-treatment values, showed, however, no differences between treatment groups (Table 5). Faecal ECP values decreased in all treatment groups during the treatment period (paired *t*-test, LGG p=0.0001; MIX p=0.0001, placebo p=0.0008). After treatment, adjusted faecal ECP levels in all treatment groups were similar (data not shown).

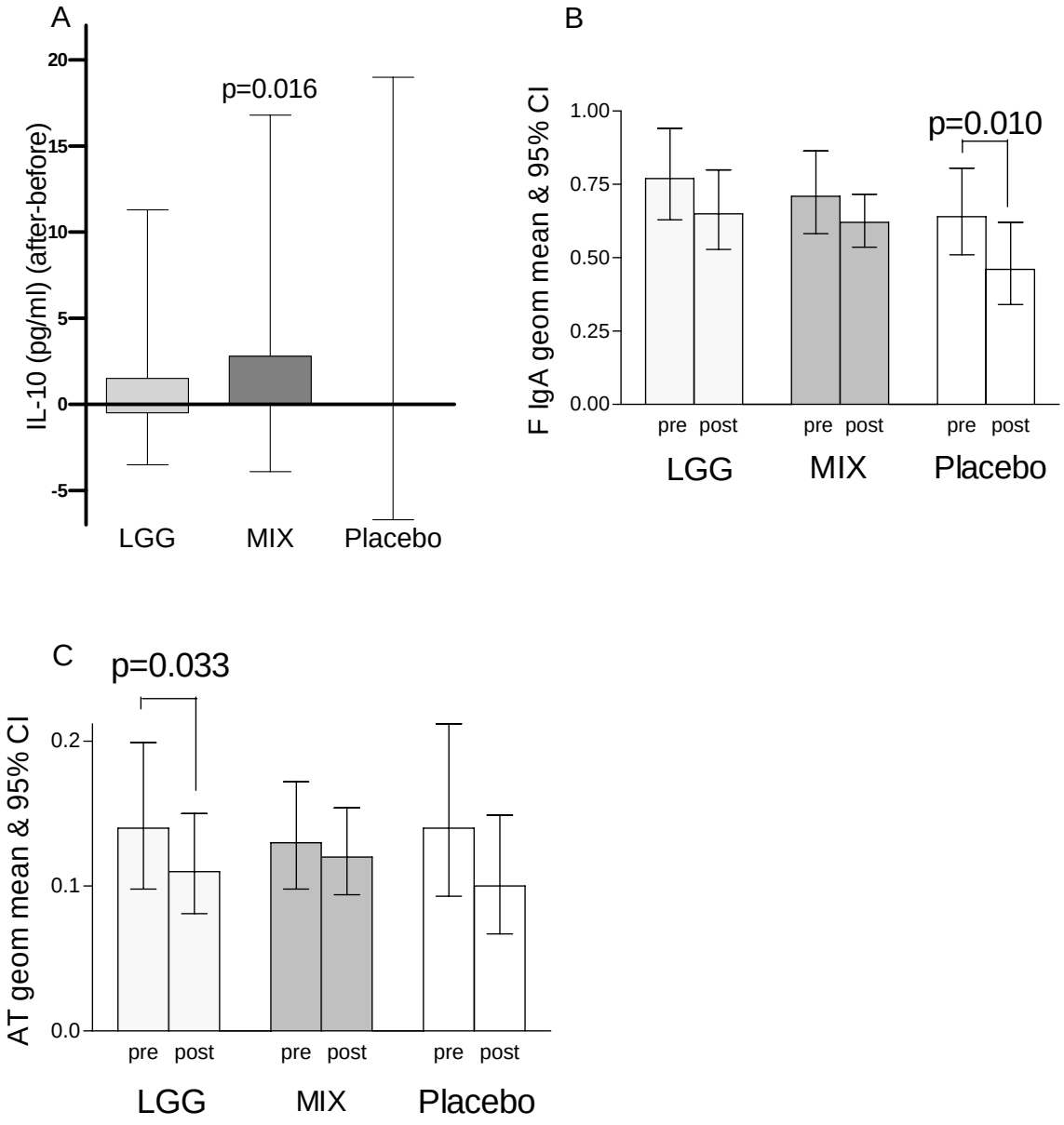


Figure 5. All infants with AD, three treatment groups. A.Change in serum IL-10 levels (post treatment- pre treatment), middle line representing median, box borders upper and lower quartiles, and error bars represent range, P-value by Wilcoxon Rank sign test. B. Unadjusted pre- and post-treatment faecal IgA geometric means and 95% CI. Decrease in placebo group, P-value by paired T-test. Post-treatment faecal IgA adjusted by pre-treatment value: *Fisher's LSD test LGG vs. placebo p=0.064, MIX vs. placebo p=0.064. C. Unadjusted pre- and post-treatment faecal α1-antitrypsin (AT) geometric means and 95% CI. Decrease in LGG group, P-value by paired T-test

Table 5. Clinical and immunologic findings in AD: Changes in SCORAD from first to third visit in all infants and those receiveing no antibiotics (AB-). Secretion of interferon-γ and IL-4 by PBMC before and after treatment; concentrations of IL-10, IL-6, and CRP in the sera; faecal concentrations of IgA and α1-antitrypsin (AT).

Parameters		**LGG**		**MIX**		**placebo**		**P-value**	**LGG vs. placebo**	**MIX vs. placebo**
			N		N		N	ANOVA/ ANCOVA	Fisher's LSD test	
SCORAD change Mean (SD)	From visit 1 to visit 3	-22.9 (16.1)	80	-20.4 (15.8)	76	-20.3 (12.6)	74	0.461	0.273	0.953
SCORAD change AB- Mean (SD)	From visit 1to visit 3	-25.4 (16.8)	60	-22.8 (14.5)	47	-20.9 (13.2)	54	0.268	0.107	0.519
CRP (µg/ml) Geom.mean (95%CI)	Post-treatment, adjusted by pre-treatment value	0.63 (0.48-0.84)	52	0.67 (0.49-0.91)	42	0.43 (0.31-0.60)	38	0.130	0.090	0.063
F IgA (g/L) Geom.mean	Post-treatment, adjusted by pre-treatment value	0.62	30	0.62	36	0.49	24	0.114	0.064	0.064
F AT (mg/g) Geom.mean	Post-treatment, adjusted by pre-treatment value	0.11	32	0.12	36	0.10	23	0.747	0.895	0.495
								K-W*	Mann-Whitney	
IFN-γ (pg/mL) Median (range)	Before After	780(90-1780) 859(298-3501)	24	492(23-2431) 663(59-1681)	21	955(231-3059) 765(316-2044)	17	0.114[a]	0.057[a]	0.101[a]
IL-4 (pg/mL) Median (range)	Before After	19(3-74) 19(7-46)	24	18(4-53) 23(8-65)	21	23(7-51) 14(4-46)	17	0.175[a]	0.375[a]	0.052[a]
IL-10 (pg/mL) Mean (upper quartile, range)	Before After	1.6 (3.0, 0-7.9) 2.4 (4.4, 0-11.3)	47	1.3 (3.4, 0-4.9) 2.5(3.8,0-16.8)[§]	38	1.2 (3.0, 0-6.7) 1.5 (2.5, 0-19)	36	0.726 0.079	0.423 **0.046**	0.658 **0.039**
IL-6 (pg/mL) Mean (upper quartile, range)	Before After	1.1 (0, 0-15.4)[#] 2.4(3.8,0-20.3)	47	2.3 (3.9. 0-22.6) 2.6(3.6, 0-35.8)	38	0.7 (0, 0-7.4) 2.5 (0, 0-61.2)	36	0.186 0.383	0.549 0.181	0.087 0.298

***** Kruskall-Wallis test, [a] tested change in concentration (after-before)

Follow-up samples of IFN-γ, IL-4, IL-6, and IL-10 tested with Wilcoxon signed-rank test and presented with footnotes if $p<0.10$:

§Change during MIX treatment, p=0.016, #Change during LGG treatment, p=0.070.

7.4 IgE-associated AD (I-IV and MV et al. unpublished results)

In infants with IgE-associated AD, symptoms of AD were alleviated more in the LGG than in the placebo group (P=0.036) between the first and third visit, while treatment with MIX showed no differences compared to placebo (Figure 6A, Table 6). We found no differences in alleviation of AD symptoms between the treatment groups from the first to second visit (data not shown). After the secondary analysis without infants who had received antibiotics, SCORAD decreased more for the LGG and MIX groups than for the placebo group (Table 6).

Among IgE-associated AD infants, secretion of IFN-γ by stimulated PBMCs increased in follow-up samples after the LGG treatment (P=0.048), while MIX and placebo treatments showed no effect on IFN-γ responses (Table 6). Change in concentration of IFN-γ (post treatment- pre treatment) was greater in the LGG than in the placebo group (p=0.017, Figure 6B). Treatment with probiotics showed no effects on IL-4 secretion (Table 6), or on IL-5 and IL-12 secretion in culture supernatants (data not shown). Level of plasma IL-6 increased during the treatment period in the LGG (p=0.023), but not in the MIX or placebo group (Fig. 6D, Table 6). Thus, analysed with the Mann-Whitney U-test, the post-treatment IL-6 level was higher in the LGG than in the placebo group (p=0.036, Table 6). In IgE-associated AEDS infants, post-treatment serum CRP, adjusted by pre-treatment value, was higher in the LGG group than in the placebo group (Table 6, Fig. 6D).

In infants with IgE-associated AD, treatment with probiotics showed no differences in post-treatment faecal IgA, TNF-α, AT, or ECP levels, adjusted by pre-treatment values (Table 6, data not shown for TNF-α, ECP). Post-challenge faecal IgA adjusted by pre challenge value was higher in the LGG group, than in the placebo group (P=0.012, Table 6)

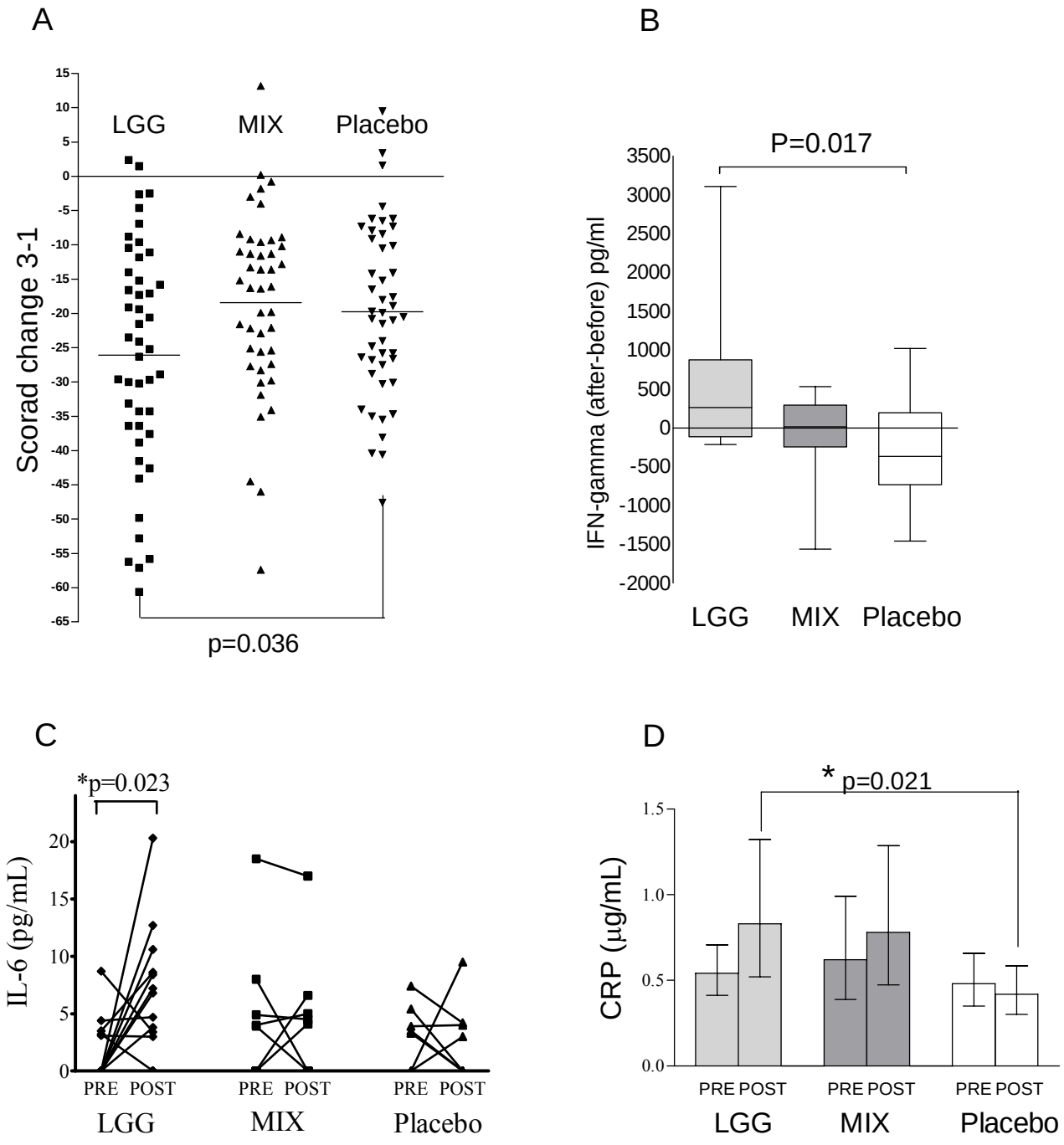


Figure 6. Infants with IgE-associated AD, three treatment groups: A. Change in SCORAD from first to third visit, means indicated with horizontal lines, P-value by Fisher's LSD test. B. Change in secretion of IFN-γ by stimulated PBMCs (post-treatment minus pre-treatment), middle line represents median, box borders upper and lower quartiles, and error bars represent range, P-value by Mann-Whitney U-test. C. Pre- and post-treatment serum IL-6, *P-value by Wilcoxon Rank sign test. D. Unadjusted pre- and post-treatment serum CRP geometric means and 95% CI. *Post-treatment serum CRP adjusted by pre-treatment value: Fisher's LSD test LGG vs. placebo.

Table 6. Clinical and immunologic findings in IgE-associated AD: Changes in SCORAD from the first to third visit in all infants and those not received antibiotics (AB-). Secretion of interferon-γ and IL-4 by PBMC before and after treatment; concentrations of IL-10, IL-6, and CRP in the sera; faecal concentrations of IgA and α1-antitrypsin (AT).

Parameters		**LGG**		**MIX**		**placebo**		**P-value**	**LGG vs. placebo**	**MIX vs. placebo**
			N		N		N	ANOVA/ ANCOVA	Fisher's LSD test	
SCORAD change Mean (SD)	From visit 1 to visit 3	**-26.1** (16.6)	46	**-18.4** (13.3)	44	**-19.8** (12.6)	46	**0.027**	**0.036**	0.655
SCORAD change AB- Mean (SD)	From visit 1 to visit 3	**-28.5** (17.5)	36	**-22.1** (12.8)	28	**-19.7** (13.3)	33	**0.044**	**0.016**	0.583
CRP (µg/ml) Geom.mean (95%CI)	Post-treatment, adjusted by pre-treatment value	0.83 (0.56-0.81)	31	0.79 (0.49-1.27)	21	0.42 (0.27-0.65)	26	**0.047**	**0.021**	0.055
F IgA (g/L) Geom.mean	Post-treatment, adjusted by pre-treatment value	0.61	18	0.56	19	0.46	14	0.310	0.138	0.283
F IgA (g/L) Geom.mean	Post-challenge, adjusted by pre-challenge value	0.74	17	0.60	19	0.44	12	**0.040**	**0.012**	0.105
F AT (mg/g) Geom.mean	Post-treatment, adjusted by pre-treatment value	0.11	19	0.13	19	0.10	14	0.430	0.513	0.197
								K-W*	Mann-Whitney	
IFN-γ (pg/mL) Median (range)	Before After	506 (90-1006) 840 (298-3501)§	14	952 (31-2431) 672 (289-1658)	12	1042(286-3059) 860 (316-2044)	12	**0.043**[a]	**0.017**[a]	0.319[a]
IL-4 (pg/mL) Median (range)	Before After	22 (3-74) 24 (8-46)	14	19 (4-53) 25 (8-50)	12	24 (9-51) 24 (11-46)	12	0.876[a]	0.899[a]	0.551[a]
IL-10 (pg/mL) Mean (upper quartile, range)	Before After	1.5 (3.0, 0-5.0) 2.3 (4.4, 0-11.3)	26	1.2 (3.3, 0-4.9) 2.1 (3.9, 0-5.5)§§	19	1.2 (3.2, 0-6.7) 2.0 (3.4, 0-19.0)	24	0.747 0.645	0.463 0.490	0.821 0.354
IL-6 (pg/mL) Mean (upper quartile, range)	Before After	0.9 (0.0, 0-8.7) 3.4 (6.9, 0-20.3)#	26	2.1 (3.9, 0-18.5) 2.0 (4.1, 0-17.0)	19	1.0 (0.0, 0-7.4) 0.9 (0.0, 0-9.5)	24	0.718 0.095	0.824 **0.036**	0.541 0.353

* Kruskall-Wallis test, [a] tested change in concentration (after-before). Follow-up samples of IFN-γ, IL-4, IL-6, and IL-10 tested with Wilcoxon signed-rank test and presented with footnotes if $p<0.10$: § $p=0.048$, §§ $p=0.075$, # $p=0.023$.

7.5 Faecal bacterial counts (I)

Before randomisation to the treatment groups, the LGG strain was detected in the faeces of 6 of 18 of the LGG group, 2 of 17 of the MIX group, and 3 of 17 of the placebo group (Table 7). Before treatment, faecal counts of the probiotic bacteria in all treatment groups were low. Immediately after the LGG treatment, LGG counts were high, and LC705 and *Propionbacterium* JS counts were low; and after the MIX treatment, LGG, LC705, and *Propionbacterium* JS counts were high. No changes occurred in the placebo group.

Treatment resulted in increases in total counts of lactobacilli in the probiotic groups and decreases in the placebo group. Changes in probiotic groups and the placebo group were significantly different (Table 7). Total counts of bifidobacteria showed no major changes.

Table 7. Median (range) counts given in logarithmic values of cfu/g of faeces and prevalence of probiotic bacteria in faeces at first and second visits in treatment groups. Prevalence column shows numbers (percentages) of infants with detectable levels of the corresponding strain.

		LGG n=18			**MIX** n=17			**placebo** n=17		
Strains	**Visit**	Counts (log cfu/g)		Preval. N (%)	Counts (log cfu/g)		Preval. N (%)	Counts (log cfu/g)		Preval. N (%)
LGG	**1**	3	(3 - 8.1)	6 (33)	3	(3 - 7.8)	2 (12)	3	(3 - 8.4)	3 (18)
	2	6.4	(3 - 8.5)	13 (72)	6.6	(3 - 8.9)	15 (88)	3	(3 - 7.8)	2 (12)
LC705	**1**	3	(3 - 3)	0 (0)	3	(3 - 3)	0 (0)	3	(3 - 3)	0 (0)
	2	3	(3 - 6.3)	2 (11)	5.8	(3 - 7.5)	9 (53)	3	(3 - 3)	0 (0)
***Propion-bacterium* JS**	**1**	3	(3 - 3)	0 (0)	3	(3 - 6.6)	2 (12)	3	(3 - 3)	0 (0)
	2	3	(3 - 3)	0 (0)	5.9	(3 - 7.2)	11 (65)	3	(3 - 3)	0 (0)
Bifidobacteria total	**1**	8.7	(5 - 10.9)	15 (83)	9.3	(5 - 11.3)	15 (88)	8.9	(5.3 - 11.3)	16 (94)
	2	8.3	(5 - 10.7)	14 (78)	8.9	(6 - 11.3)	17 (100)	9.1	(5 - 10.4)	14 (82)
Lactobacilli total	**1**	6.4†	(3 - 8.4)	12 (67)	5.5†	(3 - 8.7)	9 (53)	6.3	(3 - 9.5)	11 (65)
	2	6.9	(3 - 8.5)	14 (78)	6.8	(4.3 - 9)	17 (100)	4.3	(3 - 9.4)	9 (53)

†Change in lactobacilli counts: Kruskall-Wallis test p=0.009, LGG vs. placebo p=0.029, MIX vs. placebo p=0.002

7.6 Correlations of clinical outcome with laboratory findings (MV et al. unpublished results)

Among infants with IgE-associated AD, the SCORAD change above the upper quartile was associated with increase in IFN-γ production of stimulated PBMCs during the treatment period (Chi-Square p=0.036, Table 8), and with elevated IL-6 levels in plasma post-treatment (Chi-Square p=0.011). The SCORAD change above the median was associated with elevated post-treatment faecal IgA levels (Chi-Square p=0.033) For CRP, no significant differences was demonstrated.

Table 8. Association of clinical and immunologic findings.

	SCORAD change above upper quartile N=7/*N=13	**SCORAD change below upper quartile N=31/*N=56**	**p-value Chi-Square**
Increase in IFN-γ	86%	42%	0.036
Elevated IL-6 post-treatment*	54%	20%	0.011
	SCORAD change above median N=30	**SCORAD change below median N=25**	
Elevated faecal IgA post-treatment	57%	28%	0.033

7.7 Correlations of laboratory findings (MV et al. unpublished results)

Post-treatment IL-6 correlated directly with post-treatment CRP (Spearman correlation 0.424, $p<0.0001$), and with post-treatment IL-10 (Spearman correlation 0.268, p=0.003). Post-treatment CRP further correlated with post-treatment IL-10 (Spearman correlation 0.294, p=0.001). Post-treatment IL-6 and post-treatment faecal IgA showed no correlation with each other (Spearman correlation 0.106, p=0.458).

8. DISCUSSION

8.1 Probiotic effects in infants with AD and CMA

8.1.1 Clinical effect

In the whole study population of AD infants and in those with CMA, we found no effect of probiotic bacteria on alleviation of AD symptoms from the first to the second visit, nor from the first to the third visit. In the present study, the difference in reduction in SCORAD was less than 3 points between probiotic groups and the placebo group in the whole study population. In infants with CMA, the difference was less than 2 points. Two previous studies showed a 9- to 13-point greater reduction in SCORAD during LGG and bifidobacteria supplementation than during the placebo in infants with AD and CMA (Isolauri et al. 2000; Majamaa and Isolauri 1997). In a recent study, 8-week supplementation of *L. fermentum* for 6- to 18-month-old children with moderate to severe AD demonstrated a significant reduction in SCORAD, compared to that in the placebo group (Weston et al. 2005). Older children experienced an alleviation of AD symptoms with a combination of two lactobacillus strains, but the SCORAD showed no reduction in symptoms for the whole study population (Rosenfeldt et al. 2003). Unlike previous studies in which the placebo treatment with concomitant elimination of CM elicited only a minor change of 2 to 2.6 points in SCORAD (Isolauri et al. 2000; Majamaa and Isolauri 1997), in the present study the placebo group of CMA infants demonstrated a drop of 21.4 points in SCORAD. In part, this change was probably due to elimination of CM proteins. In CMA, elimination of allergenic CM proteins by use of EHF improves AD symptoms similarly to the effect of amino acid-based formulas (Isolauri et al. 1995; Niggemann et al. 2001). In those placebo-treated infants with no CMA, SCORAD reduction was 19.4 points, indicating a very good effect of careful topical treatment with emollients and 1% hydrocortisone acetate.

8.1.2 The MIX

The MIX treatment showed no effects in alleviation of AD symptoms, and immunologic effects differed from those of the LGG treatment. MIX seemed to enhance IL-10 production, as plasma levels increased in infants with AD and in CMA. In AD, post-treatment plasma IL-10 levels were higher in probiotic groups than in the placebo group, while in CMA, post-treatment levels showed no differences. In a previous study, LGG treatment for 4 weeks increased plasma IL-10 levels and augmented IL-10 secretion of PBMCs for children with AD (Pessi et al. 2000). In children with AD, a combination of two different lactobacillus strains demonstrated no effects on IL-10 production of PBMCs (Rosenfeldt et al. 2003). Can stimulation of IL-10 secretion explain the negligible clinical effect of the MIX? In a previous study, spontaneous monocyte production of IL-10 was higher in AD patients than in controls, and AD lesions demonstrated a relative over-expression of IL-10 mRNA (Ohmen et al. 1995). In a murine model of allergic dermatitis with epicutaneous OVA sensitisation, IL-10 enhanced IL-4 and IL-5 mRNA expression and promoted eosinophil accumulation in the skin (Laouini et al. 2003). On the other hand, in animal models, elevated IL-10 levels are associated with induction of oral tolerance (Faria et al. 2003; Zemann et al. 2003). Tolerance formation after CMA in children was associated with increased frequency of T-regulatory cells, potential producers of IL-10 (Karlsson et al. 2004). The immunomodulating function of IL-10 is diverse and may be characteristic of various diseases. In my opinion, induction of IL-10 may contribute to the MIX treatment's poor clinical outcome.

CMA and AD both are associated with Th2 type cytokine responses. (Campbell et al. 1998) (Renz et al. 1992) In the present study, pre-treatment IFN-γ secretion of PBMCs from CMA infants was lower than in infants with no CMA. In CMA, secretion of IFN-γ increased significantly after LGG treatment, compared to placebo. The MIX treatment tended to enhance IFN-γ secretion, but not significantly. MIX treatment enhanced secretion of IL-4 in PBMCs, whereas LGG and

placebo showed no effect. This change toward Th2 deviation after the MIX treatment may elicit an adverse effect on CMA and AD symptoms.

In the MIX preparation, the same amount of LGG was given as in the LGG group, which makes these differences in immunologic responses difficult to explain. A reason for these differences may be competition between various probiotic strains in the gut microbiota. Previously, lactobacilli demonstrated the replacement of bacterial pathogens in epithelial cell lines (Lee et al. 2003). Similarly, different bacteria strains in the MIX preparation may compete for the same receptors. One strain may also suppress another's inductive capacity, as seen with *L. reuteri*, which inhibited *L. casei*-induced cytokine secretion of murine dendritic cells (Christensen et al. 2002).

8.1.3 Faecal IgA, AT, and ECP

In the whole study population, faecal IgA decreased significantly during treatment period in the placebo group, while no decreases in probiotic groups were significant. Post-treatment faecal IgA tended to be higher in probiotic groups than with the placebo. The elimination diet, which was concomitant with the treatment period, may have caused a decrease in faecal IgA in the placebo group. Antigenic probiotic supplementation, on the other hand, may have stimulated IgA secretion and thus concealed the effect of dietary elimination.

In the whole study population, faecal AT decreased in the LGG group, while in MIX and placebo groups, no changes were significant. Post-treatment AT, adjusted by pre-treatment value showed, however, no differences between treatment groups. In a previous study, concentration of faecal AT decreased after 1-month LGG treatment in infants with CMA, and placebo had no effect (Majamaa and Isolauri 1997). Detection of AT in faeces indicates protein loss through inflamed mucosal membranes (Majamaa et al. 1996). Increased faecal AT levels were demonstrable after challenge in infants with CMA, in comparison to those in non-CMA infants (Saarinen et al. 2002; Majamaa et al. 1996).

Faecal ECP decreased in all treatment groups during treatment, with no differences in post-treatment values. In a previous study, faecal ECP levels showed no changes in LGG or in placebo groups (Majamaa and Isolauri 1997). ECP is induced at sites of allergic inflammation and demonstrates local eosinophil degranulation in the intestine (Bischoff 1996). Delayed-type reactions induce elevated faecal ECP levels in comparison to immediate-type reactions in challenge (Saarinen et al. 2002). In contrast, in another study, the immediate-type reaction in challenge elicited increased faecal ECP levels (Majamaa et al. 1996).

8.2 Probiotic effects in infants with IgE-associated AD

This DBPC study demonstrated an alleviation of AD symptoms after LGG treatment in infants with IgE-associated AD. Among these infants, LGG treatment augmented IFN-γ secretion of stimulated PBMCs, and increased plasma levels of IL-6, in comparison to placebo. In addition, serum CRP and post-challenge faecal IgA levels were higher in the LGG group. Interestingly I found that those infants with the greatest alleviation of AD symptoms showed a greater increase in IFN-γ secretion, higher IL-6 levels in plasma, and higher levels of faecal IgA after treatment compared to those who showed smaller alleviation of AD symptoms. I therefore infer that these cytokines and intestinal IgA may play a role in alleviation of AD symptoms. The MIX preparation showed no alleviation of AD symptoms and no significant changes in immunologic parameters in this subgroup of infants.

8.2.1 Clinical effect

The clinical effect of LGG could be detected only in infants with IgE-associated AD. Consistent with present findings, a recent study demonstrated a decrease in SCORAD after a combination of two lactobacillus strains in 1- to 13-year-old children with IgE-associated AD (Rosenfeldt et al. 2003). In two other studies on AD symptoms in infants with CMA, LGG and bifidobacteria supplementation alleviated AD more than did the placebo (Isolauri et al. 2000; Majamaa and

Isolauri 1997). Among these infants, IgE-sensitisation occurred in 4 to 37% depending on the antigen tested (Isolauri et al. 2000; Majamaa and Isolauri 1997). Altogether, a greater proportion of these infants may have been IgE-sensitised to at least one of the antigens, which may explain this difference in results. The clinical effect in the present study was first evident at the third visit, 4 weeks after the end of probiotic therapy, not immediately after treatment. Another study of AEDS children showed LGG to increase IL-10 concentration in sera 4 weeks after treatment, not immediately (Pessi et al. 2000).

8.2.2 Th1/Th2 balance

IgE-associated AD patients demonstrated a deviation in Th1/Th2 balance towards the Th2 type cytokine responses (Novak and Bieber 2003). In addition, after stimulation of PBMCs AD patients showed increased IL-4 secretion and impaired IFN-γ secretion (Jujo et al. 1992). Conversely, the majority of AD patients have demonstrated increased T cell expression of IFN- γ mRNA and protein in eczematous skin, and successful treatment of AD has down-regulated IFN-γ mRNA expression (Grewe et al. 1994). Among infants with IgE-associated AD, we demonstrated after LGG treatment an increase in IFN-γ secretion of stimulated PBMCs, and this change was greater in those who demonstrated a great alleviation of AD symptoms. Previous clinical studies have failed to demonstrate induction of Th1 type cytokines (Isolauri et al. 2000; Majamaa and Isolauri 1997) (Rosenfeldt et al. 2003). The present findings suggest that LGG treatment may enhance the Th1 cytokine profile and counter-balance Th2 type responses of AD infants, thereby contributing to alleviation of AD symptoms.

8.2.3 Innate immunity

Studies investigating the role of IL-6 in allergies are sparse. One earlier study demonstrated that in acute allergic reactions, IL-6 correlated with extent of erythema (Lin et al. 2001). In contrast, we

found that a greater reduction in SCORAD was associated with higher IL-6 levels in plasma. The induction of IL-6 suggests that cells of innate immunity are targets of probiotic action; in particular, dendritic cells in PPs are potent synthesizers of IL-6 (Sato et al. 2003). Epithelial cells also secrete IL-6, but an in vitro study showed epithelial cell line stimulated by lactobacilli not to be able to produce IL-6 (Morita et al. 2002). In this study, post-treatment IL-6 correlated directly with post-treatment CRP. This is in accordance with earlier findings that IL-6 induces gene activation of CRP in hepatocytes and stimulates CRP secretion (Li and Goldman 1996). CRP also correlated directly with post-treatment IL-10. In a previous study CRP enhanced IL-10 production on cultured encephalitogenic cells (Szalai et al. 2002).

8.2.4 Faecal IgA

After challenge, when the diet changed to one more antigenic, we could detect higher faecal IgA levels in the LGG group, than in the placebo group. Directly after treatment this difference was not significant in IgE-associated AD infants. Previously, LGG showed an increased number of IgA-secreting cells in blood of children recovering from rotavirus diarrhoea (Kaila et al. 1995). Supplementation of formula with bifidobacteria raised faecal IgA levels in healthy children (Fukushima et al. 1998). When human appendix B cells are cultured with IL-6, the number of IgA-producing cells increases. (Fujihashi et al. 1991; Pritts et al. 2002) IL-6 also regulates IgA production by PP B cells in the gut mucosa (Fujihashi et al. 1991; Pritts et al. 2002). In the present study, IL-6 levels were not correlated with faecal IgA levels; high faecal IgA levels were associated with a greater reduction in SCORAD. A possible mechanism for faecal IgA in alleviation of AD might be that it prevents the permeation of antigens by forming complexes with them, which then are trapped in the mucus and are excreted (reviewed by Mayer 2003).

8.3 Colonisation with probiotics

The use of probiotics before the study was restricted; a brief use was permitted up to 7 days, 6 weeks before entering the study. Use of probiotics was not restricted within families, and we did not record it. These two factors may have contributed to the detection of LGG in some infants before the study. We analysed only a limited number of faecal samples, but I believe that this proportion was representative.

I believe that treatment compliance was high, as in the probiotic groups' faecal prevalence of the probiotic strains after treatment was high. As reported by the parents, only seven of all the infants in the study had received fewer than 70% of the capsules. Total lactobacilli counts increased significantly in both probiotic groups. These changes alone do not explain differences in alleviation of AD or in immunologic markers, as increases in faecal lactobacilli were similar in LGG and MIX groups.

Antibiotics probably disturb the colonisation of probiotics (Alm et al. 2002), which may have caused reduction in treatment effect. In the MIX group, where 37% of infants had used antibiotics between the first and third visits, after exclusion of those who had received antibiotics, the reduction in SCORAD for AD patients increased by 2.4 points, and among infants with IgE-associated AD, the increase in reduction was 3.7 points. In the LGG group, the respective figures were 2.5 and 2.4 points. Antibiotics as such demonstrated no effect on AD, as evidenced by similar changes in SCORAD in the placebo group before and after exclusion of antibiotic-treated infants.

8.4 Methodological considerations

Randomisation was not fully successful, as baseline SCORAD differed among treatment groups, but not significantly (ANOVA p=0.175). We cannot, however, exclude the fact that some of the effect may be due to that. The randomisation was done in blocks of six infants with a computer, and it was double blind. Small blocks were used to exclude the variation by season in AD symptoms.

The treatment period may have been too short, as the clinical effect was first evident 8 weeks after the start of probiotic supplementation (4 weeks after the end). However, it is possible that detectable levels of probiotics may have been in the intestine also after the end of treatment for some weeks. Unfortunately, we did not analyse faecal bacterial concentrations after the second visit. Of previous studies, one demonstrated a clinical effect after a 4-week treatment with LGG (Majamaa and Isolauri 1997), and two others after 8-week supplementation with *L. fermentum* (Weston et al. 2005), LGG, and bifidobacteria (Isolauri et al. 2000).

The AD population was in many ways heterogeneous: It comprised infants with and without CMA, with and without IgE-association; the IgE-associated AD population of infants comprised infants with and without CMA; while the CMA population of infants was with and without IgE association.

The presence of skin-fixed and circulating antigen-specific IgE antibodies was assessed at studyentrance, at a mean age of 6.4 months. Categorising of infants to IgE-associated and non IgE-associated was performed according to these results. This classification may not have been completely accurate, as the sensitivity of these tests is rather low in infants, and some infants testing negative at 6 months may develop a positive reaction later. (Hill et al. 2004).

CMA was diagnosed after the treatment. We considered that it was ethically correct to eliminate the suspected CM allergen and to start skin treatment. Considerable improvement in symptoms is always required before the challenge. In the challenge, the short exposure with small amounts of CM provokes fewer symptoms, and the clinical effect would have been impossible to detect. Probably also the immunologic response after the challenge would have been weaker than before treatment. I therefore feel that randomisation before the challenge test was a correct decision.

As some false positive reactions occurred during placebo challenge, it is possible that some of the positive reactions during active challenge may have been false. In the challenge, 38% of CMA infants responded immediately, while in an unselected group of CMA infants in the

challenge, 49% showed an immediate type reaction within 2 hours (Saarinen et al. 2001). This difference is probably due to exclusion of infants solely with urticaria symptoms from the present study, as alleviation of AD symptoms was the primary outcome.

During treatment we demonstrated no significant changes in the plasma levels of IL-4, IFN-γ, TGF-β1, and TGF-β2. These cytokines are considered fundamental in regulation of allergic responses. Even though we showed no changes, this does not exclude the possibility that the cytokines are affected by probiotics. Cytokines and chemokines are local mediators, and only major changes may be sufficient to reflect changes in plasma.

We found increased IFN-γ secretion of anti-CD3- and anti-CD28-stimulated PBMCs. IL-12, secreted by monocytes, induces IFN-γ production. Here we found, however, no differences in IL-12 secretion of PBMCs, and 63% of samples were below the detection limit. Because T cells were stimulated with anti-CD3, which does not stimulate monocytes, the finding of low IL-12 production is reasonable.

8.5 Ethical considerations

At the start of our study, one earlier study showed that in CMA infants LGG alleviated symptoms of AD and intestinal inflammation (Majamaa and Isolauri 1997). Based on these findings, we assumed that CMA infants with AD would benefit from probiotic treatment. Moreover, those infants receiving placebo were treated according to common clinical practise with an elimination diet and topical skin treatment.

It is possible that this study protocol may have been stressful, at least to some families, with blood and faecal sampling, and prolonged elimination of CM prior to the challenge. As only 9% of families discontinued the study, however, I believe that the study was acceptable to most. At consecutive visits, all families received careful instruction on how to treat the skin and guidance as to the elimination diet. They also received the EHF free of charge during the CM

elimination period. Some families were very grateful for the opportunity provided to all, to be able to discuss allergy throughly with a doctor.

8.6 Future aspects

The result of the present study show that LGG treatment may alleviate AD symptoms in IgE-associated AD infants. Similar effects could not be demonstrated in all infants with AD, or in infants with CMA. Thus, the overall recommendation of LGG therapy for all infants with AD cannot be made. As the beneficial effect of LGG could be detected in a subgroup analysis, further investigation of the LGG effect in this patient population is necessary. Thus far, at least five studies have demonstrated the beneficial effects of various probiotics on AD symptoms. These findings are promising, and future studies need to uncover the specific patient populations and the specific probiotic strains which could elicit benefit.

Here we also demonstrated the immunologic effects of LGG and the MIX preparation. The clinical effect of LGG in IgE-associated AD infants was associated with an increase in secretion of IFN-γ, and elevated plasma IL-6 and faecal IgA levels after treatment. In the MIX group, IL-10 levels in plasma increased during treatment in AD and CMA, and IL-4 secretion of PBMCs increased in CMA. In the future, the role of these cytokines and faecal IgA should be evaluated in the pathogenesis and healing of AD. In addition, immunologic and clinical effects of the MIX preparation differed from those seen in the LGG group. As the MIX preparation contained a similar amount of LGG in addition to other probiotics, the modifying effects that different probiotic strains elicit on each other should be determined in detail.

9. CONCLUSIONS

This randomised DBPC study was undertaken to evaluate the effects of *Lactobacillus* GG and a mixture of probiotics on alleviation of AD and immunologic markers in infants with AD and CMA. The study population comprised 230 infants with AD and suspected CMA, who were given one of two probiotic treatments or placebo for 4 weeks, concomitantly with an elimination diet and topical skin treatment. Alleviation of AD was determined by reduction in SCORAD. The main conclusions of the study were:

1. Among infants with IgE-associated AD, LGG treatment showed to alleviate AD symptoms, compared to the placebo. The MIX treatment showed no such effects. In the whole study population or in CMA, probiotics demonstrated no alleviation of AD symptoms.

2. In infants with IgE-associated AD and in infants with CMA, LGG treatment enhanced the secretion of IFN-γ by stimulated PBMCs, indicating promotion of Th1-type responses. Among infants with IgE-associated AD, a great reduction in SCORAD was associated with an increase in IFN-γ secretion. In CMA, pre-treatment IFN-γ secretion was lower than in non-CMA infants.

3. In infants with IgE-associated AD, IL-6 and CRP levels in plasma were increased after LGG treatment, suggesting activation of innate immunity. A marked reduction in SCORAD was associated with elevated plasma IL-6, suggesting a role for innate immunity cells in the healing of IgE-associated AD.

4. Among infants with IgE-associated AD, post-challenge faecal IgA was more elevated in the LGG group, than in the placebo group. In the whole study population, faecal IgA decreased during the treatment period in the placebo group, while probiotic groups showed no significant decrease. Among those infants with the greatest alleviation of AD symptoms, higher faecal IgA levels appeared after treatment, compared to patients who showed smaller alleviation of AD symptom.

5. In AD and in CMA, plasma levels of IL-10 increased during the MIX treatment. In CMA, IL-4 secretion of stimulated PBMCs was elevated after the MIX treatment. These changes may explain the negligible clinical effect of the MIX.

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