Lactobacilli as antigen delivery system for mucosal tolerance induction in autoimmune disease

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Lactobacilli as antigen delivery system for mucosal tolerance induction in autoimmune disease

Lactobacillen als systeem voor produktie, transport en afgifte van antigeen voor de inductie van mucosale tolerantie in autoimmuunziekten

PROEFSCHRIFT

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Catharina Beatrix Maria Maassen

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Promotor:	Prof.dr. E.H.J.H.M. Claassen
Co-promotoren:	Dr. J.D. Laman Dr. W.J.A. Boersma
Overige leden:	Prof.dr. H.A. Drexhage Prof.dr. F.G.A. v.d. Meché Prof.dr. A.D.M.E. Osterhaus

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CONTENTS

Synopsis

Oral administration of autoantigens is a safe and convenient way to induce peripheral T-cell tolerance in animal models of autoimmune diseases such as multiple sclerosis (MS). However, oral administration of myelin has not been successful as treatment of MS patients yet. In order to increase the efficacy of oral tolerance induction, we use genetically modified lactobacilli which express myelin antigens. The lactobacilli serve as producers of the antigen and as vehicles to transport the antigen to the gut mucosa. The fact that those Gram-positive lactobacilli are generally regarded as safe (GRAS), makes them attractive candidates for this application.

It is known that lactobacilli may possess properties that influence the immune system, but such properties differ between *Lactobacillus* strains. Therefore *Lactobacillus* strain selection is very important. In order to select an appropriate *Lactobacillus* strain for the expression of myelin antigens, different wild type *Lactobacillus* strains were studied for several effects they have on the immune system after oral administration.

Of eight analyzed *Lactobacillus* strains, harvested in log phase, only three strains, *L. reuteri*, *L. brevis* and *L. fermentum* appeared to be able to enhance the specific antibody response against a parenterally administered T-cell dependent antigen. When the same strains were tested for their ability to induce cytokines in the gut villi upon oral administration, *L. reuteri* and *L. brevis* induced appearance of TNF-α, IL-1ß and IL-2 producing cells. In contrast, *L. casei* and *L. murines* tended to increase the number of TGF-ß and IL-10 producing cells. These results suggest that *L.casei* and *L. murines* might be suitable strains for the induction of oral tolerance.

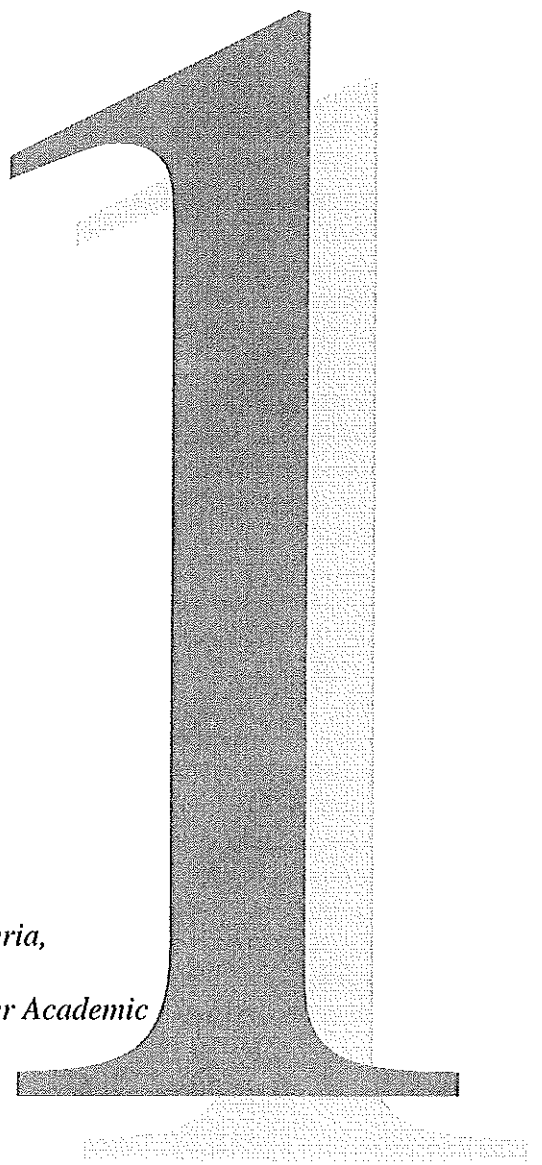
Determination of the specific IgG1/IgG2a ratio against a parenterally administered antigen, revealed a growth phase dependent skewing of T-cell pathways upon oral administration of some of the *Lactobacillus* strains. Feeding of stationary phase cultures of *L. casei* and *L. murines* induced higher IgG1/IgG2a ratios than after feeding of log phase cultures. Since IgG1 production is a reflection of Th2 responses and IgG2a reflects Th1 responses, these results imply that stationary phase cultures of those *Lactobacillus* strains skew more towards Th2, whereas log phase cultures direct the response towards Th1.

A series of vectors has been constructed which, upon transformation to lactobacilli, direct the expression of antigens either intracellularly or secreted into the environment. Among others, guinea pig myelin basic protein (gpMBP), human MBP, $MBP_{72\text{-}85}$ and $PLP_{139\text{-}151}$ were expressed by *L. casei*.

Nasal administration of $MBP_{72\text{-}85}$ prior to disease induction with the same peptide in Lewis rats, significantly reduced EAE severity. In contrast, two doses of gpMBP enhanced disease. Nasal administration of soluble extracts of *Lactobacillus* recombinants expressing gpMBP or $MBP_{72\text{-}85}$ did reduce EAE severity, although the content of myelin antigens in the extracts was low. Also oral administration of live *L.casei* recombinants expressing gpMBP or MBP72-85 prior to disease induction, significantly inhibited EAE in Lewis rats.

These studies provide 'proof of principle' that it is possible to reduce EAE by mucosal administration of recombinant lactobacilli expressing myelin antigens. Further studies are required to develop this approach for treatment of MS in humans.

General introduction



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1.1 Multiple sclerosis

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that affects CNS myelin which isolates nerve axons and allows saltatory pulse conduction. It is the most important chronic disabling neurological disease in young adults, with a mean age of onset around 30 years. It has a prevalence of approximately 1 per 1000 in Western European countries and it affects women versus men at a ratio of 1.5 to 1 (Sadovnick and Ebers, 1993). Although the cause and pathogenesis of MS are unknown, it is generally believed that MS is an autoimmune disease in which environmental factors as well as genetic factors are involved. Studies with monozygotic twins reveal an MS concordance rate of 20-30%, whereas this is 3-5% for dizygotic twins (reviewed by Ebers and Sadovnick, 1994). This high concordance rate is a strong argument in favor of a genetic basis of MS, but can also be used as an argument for an environmental involvement in MS. The role of environmental factors in MS is supported by the presence of geographic gradients in the distribution of the disease. The general pattern is that MS prevalence rises with increasing distance from the equator. The nature of these environmental factors is unknown. Dietary factors, climate variation and infectious agents have all been postulated to be involved in MS, but there is no definitive proof for involvement in MS of any of these factors. Especially herpes viruses have gained interest the last few years (Dalgleish, 1997).

The notion that MS is a multifactorial disease is strengthened by the fact that the clinical course of MS is highly variable for each individual patient, and can be divided into four categories (Lublin and Reingold, 1996). The first category is relapsing-remitting disease, in which discrete attacks are alternated with periods in which a patient returns to the pre-attack base line. In the relapsing-progressive form of MS, the patients do not return to base-line level in between attacks and disability increases in these patients. In chronic-progressive disease, the disease progresses without periods of stability. The uncommon fourth form is characterized by a progressive relapsing course of MS.

The most important pathological characteristics of MS are the presence of areas of demyelination (plaques) in the CNS white matter. In general, active demyelination is accompanied by inflammation. The inflammatory infiltrates are mainly composed of T lymphocytes, activated macrophages, microglia and some B cells. Proliferation of astrocytes accompanies the inflammatory changes (gliosis), eventually leading to scar formation. In inactive plaques, remyelination is often present at the lesion margin. The heterogeneity of lesions within and among different patients is large, as is the response to immunomodulatory treatments. These are additional arguments that the disease MS may be more heterogeneous than previously suspected (Lassmann *et al.*, 1998).

1.2 Therapies for MS

Non antigen-specific therapies

Rational development of immunotherapy for MS is hampered by limited insight into the effector cells and effector mechanisms involved in demyelination. Since an effective curative therapy for MS is not yet available, symptomatic treatment of the disease to improve the quality of life is still very important. Acute attacks in MS are usually treated with adrenocorticotropic hormone (ACTH) or the corticosteroids prednisone or methylprednisone, which have both immunosuppressive and anti-inflammatory properties. Based on the assumed inflammatory and immunologically mediated pathogenesis of MS, a large number of possible new therapies directed at immune intervention has been put forward (reviewed by Waubant *et al.,* 1997). IFNß-1a and IFNß-1b have been used in the clinic for treatment of relapsing-remitting patients for some time now. Although its mechanism of action *in vivo* are unknown, *in vitro* IFN-ß antagonizes IFN-γ, inhibits T-cell proliferation, it can restore T suppressor cell function and it can reduce T-cell migration by inhibiting the activity of T cell matrix metalloproteases (MMP)(reviewed by Yong *et al.,* 1998). These properties could be responsible for the positive effects of IFN-ß on relapse rate, disease activity (as measured by serial gadolinium-enhanced magnetic resonance imaging (MRI)) and disease progression (as measured by annual T2-weigthed MRI)(reviewed by Arnason, 1999).

Treatment with Copolymer-1 has also been approved for use in human. Copolymer-1 is a mixture of synthetic peptides, composed of four amino acids (Johnson *et al.,* 1995). There is evidence that Copolymer-1 blocks or competes with binding of encephalitogenic peptides to MHC class II molecules, this way reducing the autoimmune response (Aharoni *et al.,* 1999). Many more immunomodulatory compounds are used in MS treatment or are analysed in ongoing trials, but most of these therapies are not antigen-specific. Several non antigen-specific approaches are currently under investigation, in order to be used for application in human later, but also to reveal immunological mechanisms involved in autoimmune disease. Examples of such approaches are administration of cytokines or cytokine antagonists, blockade of migration of auto-reactive T-cells into the CNS and blocking lymphocyte co-stimulation by administration of anti-CD40 or anti-CD40 ligand monoclonal antibodies (Laman *et al.,* 1998a; Gerritse *et al.,* 1996). Such therapies may affect the entire immune system, both by suppression or by directing it into non-inflammatory immune responses. This could lead to unwanted side effects and a higher susceptibility for infectious diseases. Practically, this could mean that such treatments can not be given repeatedly or for a longer period.

Antigen-specific therapies

A major goal in the treatment of T-cell mediated autoimmune diseases is the establishment of a therapy that selectively targets only pathogenic T cells, leaving the remainder of the T-cell repertoire intact. One of the reasons that such antigen-specific

therapies are not easy to develop is the fact that the autoantigens in many autoimmune diseases and in MS in particular are as yet unknown. Nevertheless, extensive research in experimental autoimmune diseases in which the autoantigen is known, has provided information related to antigen-specific therapies. Some of the approaches for development of antigen-specific therapy are discussed below.

Intrathymic injection of auto-antigen

The thymus plays a major role in development of self-tolerance. Intrathymic injection of neonatal BB rats with pancreatic islets prevented insulitis (Koevary and Blomberg, 1992). It was possible to prevent experimental autoimmune encephalomyelitis (EAE), an animal model for MS, by intrathymic injection of myelin basic protein (MBP) or the immunodominant peptide of MBP_{71-90}, without otherwise compromising the peripheral lymphocyte pool in adult Lewis rats (Khoury *et al.*, 1993). This was also demonstrated by intrathymic injection of S-antigen in autoimmune uveoretinitis (Koevary and Caspi, 1997). However, intrathymic injection of acetylcholine receptor protein could not prevent experimental autoimmune myasthenia gravis, indicating that suppression of Th1 function alone may not be sufficient for the prevention of antibody-mediated autoimmune disease (Ohtsuru *et al.*, 1995). The mechanism of intrathymic injection of antigen for tolerance induction is unclear, but a role for CTLA4 has been postulated (Issazadeh *et al.*, 1999). This approach is not likely to be used in human autoimmune diseases, but it may help to elucidate the role of the thymus in acquired systemic tolerance of autoreactive T-cells.

Systemic antigen immunotherapy

It is well known that systemic injection of soluble deaggregated protein antigen specifically inhibits the induction of a subsequent T cell response to that antigen. Whether such suppression of the T cell response indeed is established and to what extent, is dependent on several parameters. The route of administration, the dose, the adjuvant and the nature of the antigen appear to be crucial parameters. Intravenous and intraperitoneal administrations are more effective in tolerance induction than the subcutaneous route. In most experimental set-ups, high doses of antigen are required. The efficacy of tolerance induction is enhanced by the use of adjuvant such as alum or incomplete Freund's adjuvant (IFA), which are known to induce CD4+ T helper 2 (Th2) function. Peptides or monomeric antigens are more potent tolerogens than aggregated proteins, which tend to be immunogenic. In addition, the immunodominance of an antigen or epitope, defined as the capacity to generate an immune-response, seems to correlate with its potential to induce hyporesponsiveness (Liblau *et al.*, 1997). Intravenous or intraperitoneal injection of myelin protein or peptides can prevent EAE (reviewed by Liblau et al. 1997).

Other approaches to systemically target antigen-specific T cells are the use of peptide analogues (also known as altered peptide ligands (APL)) and soluble peptide-MHC-complexes. Peptide analogues are capable of binding to the MHC molecule with

the same affinity as the wild-type peptide but have decreased affinity for the T-cell receptor (TCR) through mutations in primary TCR contact residues. Administration of peptide-MHC complexes is thought to act via occupation of the TCR of the targeted T cells. The T cells receive only signal one, without co-stimulation, leading to anergy and/or deletion of the autoreactive T cells.

APL as well as peptide-MHC complexes of myelin antigens can prevent EAE (reviewed by Liblau *et al.*, 1997)). Evidence for the treatment of established disease by systemic administration of antigen was obtained by Brocke *et al.* (1996), who used an APL of MBP_{87-99} in a T cell line specific for MBP_{87-99} induced EAE in $(PL/JxSJL/J)/F_1$ mice. A major drawback of the use of APLs and peptide-MHC complexes in humans is that they require knowledge of the peptide epitopes recognized by the pathogenic T cells in individual patients.

Oral and nasal tolerance

A promising approach to antigen-specific therapy in autoimmune diseases is the induction of peripheral T cell tolerance by oral or nasal administration of autoantigen, termed mucosal tolerance induction (definition see 1.4). Extensive research has been performed in animal autoimmune models, but also in humans, to induce tolerance by the oral and nasal route and to elucidate the mechanisms involved. Since the mucosal immune system has some unique features compared to the peripheral immune system, which play a role in the induction of mucosal tolerance, first some properties of the mucosal immune system will be discussed.

1.3 The mucosal immune system

Immune exclusion versus tolerance

The mucosal surface area of the respiratory tract and the gastrointestinal tract are over a hundred times the surface area of the skin. Such a large area allows efficient uptake of oxygen and absorption of nutrients and excretion of waste products. As a consequence, a large area of the body is continuously exposed to many pathogens of the external milieu. To cope with both pathogens and innocuous substances, the mucosal immune system has generated two major arms of immune reactivity (reviewed by Brandtzaeg, 1995; 1998; Brandtzaeg *et al.*, 1999). 1) Immune exclusion and elimination. Immune exclusion is a noninflammatory way of preventing colonization and penetration of harmful foreign material performed by the physical presence of indigenous bacteria leaving no niche for ingested pathogens, as well as by secretory IgA (and to some extent sIgM and IgG), which has a higher general cross-reactivity with antigens than has IgG (Waldman *et al.*, 1970, Shvartsman *et al.*, 1977). Immune elimination involves neutralization and removal of foreign material that has penetrated the epithelium by mainly proinflammatory, non-specific innate defense mechanisms like complement-activating IgG and IgM, and cytokines released from activated T cells and macrophages. 2) Oral tolerance. Since the mucosal epithelium has evolved for efficient transport of molecules, a continuous inflammatory response

evoked against indigenous microflora, ingested food antigens or inhaled antigens is undesirable. Therefore, under physiological circumstances, penetrating soluble antigens and the indigenous microflora mainly induce hyporesponsiveness. Since the mucosae are natural sites of tolerance induction, they can be used to reinduce tolerance against antigens to which tolerance has been lost and has resulted in autoimmune disease.

Respiratory tract versus gastrointestinal tract
The mucosae comprise a specific epithelial layer and underlying connective tissue that form the border between the internal and external milieu in the airway system, the gastrointestinal tract and other mucosal surfaces. It is a unique property of the intestinal immune system and some parts of the respiratory immune system that it is separated from the antigens by only one cell layer, and is not drained by lymphatic vessels (Neutra *et al.*, 1996). When comparing the immune system of the upper respiratory tract and the gastro-intestinal tract, some differences are obvious. One difference is the presence of large numbers of intraepithelial lymphocytes (IEL) in the gut villi, which are mainly of the CD8+ phenotype, in contrast to the low numbers of IEL in the airways, which are mostly CD4+ T cells (Abreu-Martin and Targan, 1996). Another difference is the distribution of subepithelial and intraepithelial professional antigen presenting cells (APC). In the gut, most APC are located under the epithelial cell layer, whereas many APCs of the upper respiratory tract are located between the epithelial cells (Holt *et al.*, 1990). How these differences may relate to mucosal tolerance induction is unclear. However, there is mounting evidence suggesting that the epithelium of the gastrointestinal tract, enterocytes, may act as antigen presenting cells (APC), preferentially stimulating CD8+ cells, probably IELs (Bland and Whiting, 1989; Yamamoto *et al.*, 1998). It has been postulated that (human) enterocytes present antigen to CD8+ cells via CD1d (Blumberg *et al.*, 1991). However, for proper activation co-stimulatory molecules are a prerequisite, but CD80 and CD86 costimulatory molecules have not been detected yet on enterocytes (Bloom *et al.*, 1995). This lack of costimulation may promote the induction of tolerance to luminal antigens In contrast, due to scarcity of such CD8+ IEL and the limited amount of antigen that enters via the airways, the induction of peripheral tolerance probably will be mainly regulated by CD4+ Th2 cells stimulated in draining lymph nodes by migrated dendritic cells (Brandtzaeg, 1996).

1.4 Mucosal tolerance induction

Definition
The definition of mucosal tolerance induction in this thesis is: Any mechanism by which a potentially injurious immune response is prevented, suppressed or shifted to a noninjurious class of immune response by mucosal (e.g. oral and nasal) administration of antigen (Weiner HL, 10th Int. Congress Mucosal Immunol., Amsterdam). In this thesis the injurious immune response is a Th1 mediated inflammation of the central

nervous system in EAE. Although the term oral administration strictly taken refers to administration of the antigen in the oral cavity, we use the term for intragastric intubation of the antigen in rodents, as is generally applied throughout the literature. On application of aerosolized antigen taken up via the nose, a small amount is thought to enter the gastro-intestinal tract, in contrast to administration of antigen in the nose by use of a pipet.

Mucosal tolerance induction in animal models

Oral tolerance was first described in 1911 when Wells fed hen egg proteins to guinea pigs and found them resistant to anaphylaxis when challenged with the same antigens (Wells, 1911). It was shown that this is an antigen specific phenomenon by numerous investigators who demonstrated that animals fed proteins such as ovalbumin (OVA) do not respond as well to these antigens when subsequently immunized, but do respond normally to other antigens (reviewed by Strobel and Mowat, 1998). An example of peripheral T cell tolerance induction without concomitant induction of B cell tolerance was observed after oral administration keyhole limpet hemocyanin (KLH) in humans (Husby *et al.,* 1994). Although the T cell responses were down-regulated, the oral administration of KLH primed the humoral response systemically as well as mucosally.

The T-cell hyporesponsiveness induced by autoantigen administered mucosally, could be beneficial in the treatment of autoimmune diseases. During the last decade, oral and nasal tolerance has been used successfully to treat autoimmune phenomena in animals (*Table 1*). Mucosal tolerance has been extensively studied, especially in Th1 mediated experimental autoimmune disease such as EAE. In addition to T cell mediated autoimmune diseases, the antibody-mediated experimental autoimmune diseases myasthenia gravis and thyroiditis could be suppressed by previous nasal and/or oral administration of acetylcholine receptor (AChR) or thyroglobulin, respectively (*Table 1*). A wide range of experimental diseases (*Table 1*) could be prevented by oral administration of the autoantigen. A complicating factor is that treatment of humans occurs when the disease is already fully established. Therefore, treatment of ongoing disease in chronic autoimmune models is clinically more relevant. Miller *et al.* (1993b) showed that orally MBP tolerized mice were not protected against adoptive transfer of EAE, indicating that suppression of active autoreactive T cells is much more difficult than prevention of induction. However, treatment of ongoing disease by oral administration of (auto)antigen has been demonstrated in several chronic autoimmune models such as EAE (*Table 2*)(al-Sabbagh *et al.,* 1996; Benson *et al.,* 1999; Brod *et al.,* 1991; Meyer *et al.,* 1996), collagen induced arthritis (CIA)(Khare *et al.,* 1995) and experimental autoimmune uveoretinitis (EAU)(Thurau *et al.,* 1997; Torseth and Gregerson, 1998).

Experimental autoimmune diseases have also been treated by nasal administration of (auto)antigen (*Table 1*). Nasal administration of high doses of AChR could ameliorate the manifestations of experimental autoimmune myasthenia gravis

(EAMG)(Shi *et al.*, 1999). Aerosol MBP decreased the severity of subsequent relapses in animals with EAE recovered from the first attack (al-Sabbagh *et al.*, 1996). Nasal administration of residues 139-151 from proteolipid protein ($PLP_{139-151}$) after onset of myelin induced EAE, reduced the number of relapses (Anderton and Wraith, 1998). Also nasal co-administration of MBP_{68-86} and MBP_{87-99} could reverse the disease in Lewis rats (Liu *et al.*, 1998). Aerosol insulin could treat IDDM in NOD mice (Harrison *et al.*, 1996).

Table 1. ***Oral and nasal tolerance induction in experimental autoimmune models***

	Human disease	Experimental autoimmune disease	Tolerogen	References
ORAL	multiple sclerosis	EAE	MBP (peptides), PLP (peptides)	see *Table 2*
	rheumatoid arthritis	CIA, AIA, AA	CII, CII peptides, inducing-antigen	1-13
	type I diabetes	IDDM	insulin (peptide), GAD65	14-18
	uveitis	EAU	S-Ag (peptides), IRBP (peptide)	19-27
	myasthenia gravis	EAMG	AChR	28, 29
	Guillain-Barré syndrome	EAN	bovine peripheral nerve myelin	30
	autoimmune thyroid disease	EAT	thyroglobulin	31, 32
	inflammatory bowel disease	EGC	haptenized colonic proteins, TNBS, colitis extracted proteins	33-35
NASAL	multiple sclerosis	EAE	gpMBP, MBP-peptides, $PLP_{139-151}$	1, 36-40, this thesis
	rheumatoid arthritis	CIA, AIA	CII, CII peptides or fragments, inducing-antigen peptide	1, 41-47
	type I diabetes	IDDM	insulin, GAD65-peptides	48, 49
	uveitis	EAU	retinal extract, IRBP, S-Ag	50, 51
	myasthenia gravis	EAMG	AChR (peptides)	52-55
	Guillain-Barré syndrome	EUN	P2 peptide 57-81	56

EAE: experimental autoimmune encephalomyelitis, CIA: collagen induced arthritis, AIA: antigen-induced arthritis, AA: adjuvant arthritis, IDDM: insulin-dependent diabetes mellitus, EAU: experimental autoimmune uveoritinitis, EAMG: experimental autoimmune myasthenia gravis, EAN: experimental autoimmune neuritis, EAT: experimental autoimmune thyroiditis, EGC: experimental granulomatous colitis, (gp)MBP: (guinea pig) myelin basic protein, PLP: proteolipid protein, CII: type II collagen, GAD65: glutamate decarboxylase peptide, S-Ag: S-antigen, AChR: acetylcholine receptor, TNBS: trinitrobenzene sulfonic acid, IRBP: interphotoreceptor retinol binding protein. [1]al-Sabbagh *et al.*, 1996; [2]Haque *et al.*, 1996; [3]Inada *et al.*, 1997; [4]Jorgensen *et al.*, 1998; [5]Khare *et al.*, 1995; [6]Myers *et al.*, 1998; [7]Thompson *et al.*, 1988; [8]Thompson *et al.*, 1993a; [9]Thompson *et al.*,1993b; [10]Thorbecke *et al.*, 1999; [11]Yoshino, 1996b; [12]Yoshino, 1996a; [13]Zhang *et al.*, 1990a; [14]Bergerot *et al.*, 1994; [15]Polanski *et al.*, 1997; [16]Ramiya *et al.*, 1997; [17]von Herrath MG *et al.*, 1996; [18]Zhang *et al.*, 1991; [19]Rizzo *et al.*, 1994; [20]Thurau *et al.*, 1991; [21]Thurau *et al.*, 1997; [22]Torseth and Gregerson, 1998; [23]Gregerson *et al.*, 1993; [24]Nussenblatt *et al.*, 1990; [25]Silver *et al.*, 1998; [26]Singh *et al.*, 1996; [27]Vrabec *et al.*, 1992; [28]Okumura *et al.*, 1994; [29]Wang *et al.*, 1993 , [30]Gaupp *et al.*, 1997 , [31]Guimaraes *et al.*, 1995; [32]Peterson and Braley-Mullen, 1995 ,[33]Elson *et al.*, 1996; [34]Neurath *et al.*, 1996; [35]Trop *et al.*, 1999; [36]Anderton and Wraith, 1998; [37]Liu *et al.*, 1998; [38]Bai *et al.*, 1997; [39]Li *et al.*, 1998; [40]Metzler and Wraith, 1993; [41]Bayrak and Mitchison, 1998; [42]Chu and Londei, 1999; [43]Matsumoto *et al.*, 1998; [44]Myers *et al.*, 1997; [45]Prakken *et al.*, 1997; [46]Staines *et al.*, 1996; [47]Verheijden *et al.*, 1997; [48]Harrison *et al.*, 1996; [49]Tian *et al.*, 1996; [50]Dick *et al.*, 1994; [51]Laliotou *et al.*, 1997; [52]Shi *et al.*, 1998a; [53]1999; [54]Karachunski *et al.*, 1997; [55]Ma *et al.*, 1995; [56]Zhu *et al.*, 1998.

Conversely, oral or nasal administration of antigen can lead to more severe clinical signs. Meyer *et al.* (1996) have shown that oral administration of a low dose of MBP enhanced the clinical signs of chronic EAE in B10.PL mice. As demonstrated in this thesis, nasal administration of two doses of gpMBP in Lewis rats enhanced the clinical signs of EAE in Lewis rats (Chapter 4.2). Nasal administration of MBP led to exacerbation of ongoing EAE in DA rats (Bai *et al.*, 1998). Exacerbation was also found when rats with mild EAU were treated with S-antigen peptides, although mild disease could be treated with high doses of antigen. Oral administration of autoantigen can also lead to induction of disease as oral administration of OVA induced cytotoxic T cells in B6 mice and increased incidence of diabetes in chimeric RIP-OVA mice with high levels of CD8+ T cells specific for OVA infiltrates in ß-islets (Blanas *et al.*, 1996). Terato *et al.* (1996) showed that oral administration of chicken collagen type II induced arthritis in DBA/1 mice.

Table 2a. ***Oral tolerance in rat EAE models***

Oral tolerogen	Animal	EAE model	Inducing antigen	Prevention/ treatment	References
gpMBP	Lewis rat	acute	gpMBP	prevention	Bitar and Whitacre, 1988; Higgins and Weiner, 1988; Miller *et al.*, 1992a
hMBP					Miller *et al.*, 1992a
rMBP					Miller *et al.*, 1992a
bMBP					Higgins and Weiner, 1988; Miller *et al.*, 1992a
$gpMBP_{1-37}$ $gpMBP_{44-89}$ $gpMBP_{90-170}$ bMBP enc. decapeptide bMBP non-enceph. S79	Lewis	acute	gpMBP	prevention	Higgins and Weiner, 1988
hMBP	Lewis	acute	hMBP	prevention	Bitar and Whitacre, 1988
gpMBP rMBP hMBP bMBP	Lewis	acute	rMBP	prevention	Miller *et al.*, 1992a
$gpMBP_{68-88}$	Lewis	acute	$gpMBP_{68-88}$	prevention	Javed *et al.*, 1995
$gpMBP_{68-88}$	Lewis	acute	$rMBP_{68-88}$	prevention	Javed *et al.*,1995
$gpMBP_{21-40}$ $gpMBP_{71-90}$	Lewis	acute	$gpMBP_{71-90}$	prevention	Miller *et al.*, 1993a
gpMBP gpmyelin	Lewis	chronic	gpSCH	treatment	Brod *et al.*, 1991

EAE can be induced in the susceptible Lewis rat with distinct myelin antigens. Oral administration of the disease inducing antigen or another myelin antigen can prevent or treat EAE. Studies describing effective oral tolerance protocols in the Lewis rat are listed.
gp: guinea pig, h: human, r: rat, b: bovine, MBP: myelin basic protein, SCH: spinal cord homogenate.

Apparently, there is a delicate balance between tolerance and immunity. The dosage, timing, frequency and route of application are important factors in the success of mucosal tolerance strategies for treatment of chronic autoimmune diseases. Many different EAE models have been used to demonstrate oral and nasal tolerance with many different antigens and protocols (*Table 1* and *2*) and these studies have been the basis for elucidation of mechanisms of mucosal tolerance. However, oral administration of (potential) autoantigens in humans has not been successful yet, as discussed below.

Table 2b. *Oral tolerance in guinea pig and mouse EAE models*

Oral tolerogen	Animal	EAE model	Inducing antigen	Prevention/ treatment	References
gpMBP gpmyelin bmyelin	strain 13 guinea pig	chronic	gpSCH	treatment	Brod *et al.*, 1991
hMBP bMBP gpMBP	Hartley guinea pig	acute	gpMBP	prevention	Miller *et al.*, 1992a
gpMBP rMBP hMBP bMBP mMBP bPLP	SJL/J mouse	acute	mmyelin	prevention	Miller *et al.*, 1992a Miller *et al.*, 1992a Miller *et al.*, 1992a Miller *et al.*, 1992a Miller *et al.*, 1992a; al-Sabbagh *et al.*, 1994 al-Sabbagh *et al.*, 1994
$PLP_{139-151}$	SJL/J	acute	$PLP_{139-151}$	prevention	Karpus *et al.*, 1996
mMBP bPLP	SJL/J	acute	$PLP_{140-159}$	prevention	al-Sabbagh *et al.*, 1994
gpMBP $gpMBP_{1-11}$ $gpMBP_{89-101}$ $PLP_{140-159}$ $PLP_{139-151}$ bPLP	SJL/J	acute	PLP	prevention	Santos *et al.*, 1994 al-Sabbagh *et al.*, 1994 al-Sabbagh *et al.*, 1994 al-Sabbagh *et al.*, 1994 Karpus *et al.*, 1996 Karpus *et al.*, 1996
bmyelin	SJL/J	chronic	mSCH	treatment	al-Sabbagh *et al.*, 1996
gpMBP	B10.PL mouse	acute/ chronic	gpMBP	prevention/ treatment	Meyer *et al.*, 1996
gpMBP	B10.PL	chronic	gpMBP	prevention/ treatment	Benson *et al.*, 1999

EAE can be induced in several susceptible animal species with distinct myelin antigens. Oral administration of the disease inducing antigen or another myelin antigen can prevent or treat EAE. Studies describing effective oral tolerance protocols in the guinea pig strain 13, Hartley guinea pig, SJL/J and B10.PL mice are listed.

gp: guinea pig, h: human, r: rat, b: bovine, m: mouse, MBP: myelin basic protein, PLP: proteolipid protein, SCH: spinal cord homogenate.

Oral tolerance induction in human trials

From animal experiments it is known that dose, timing, frequency and route of administration are important factors influencing the efficacy of mucosal tolerance induction. Additional complications in the treatment of humans with chronic autoimmune disease by mucosal administration of (auto)antigen are the established disease and the fact that in most cases the autoantigen is unknown. Despite these difficulties, several clinical trials have been initiated or are currently planned (*Table 3*). These initial trials suggest that there is no systemic toxicity or exacerbation of disease associated with the oral administration of antigen. Although some positive effects have been observed with low doses of collagen in human arthritis (Barnett *et al.*, 1998), consistent clinical efficacy has not yet been demonstrated. Results in humans, however, have mirrored several aspects of what has been observed in animal models. For instance, MS patients treated with oral bovine myelin contain MBP and PLP specific TGF-ß secreting in peripheral blood, in contrast to untreated patients (Fukaura *et al.*, 1996). The possible involvement of TGF-ß in oral tolerance will be discussed in the next paragraph.

Table 3. ***Oral tolerance induction in human autoimmune disease***

Disease trial	Oral antigen	References
Multiple sclerosis	bovine myelin	Fukaura *et al.*, 1996; Hohol *et al.*, 1996; Weiner *et al.*, 1993
Rheumatoid arthritis (also juvenile rheumatoid arthritis)	type II collagen	Barnett *et al.*, 1996; Barnett *et al.*, 1998; Sieper *et al.*, 1996; Trentham *et al.*, 1993
Uveitis	S-Ag, retinal antigens	Nussenblatt *et al.*, 1997
Thyroid disease	thyroglobulin	Lee *et al.*, 1998
Myasthenia gravis (planned)	acetylcholine receptor	Drachman *et al.*, 1996
Type I diabetes (planned in new-onset diabetes)	insulin	Carel and Bougneres, 1996

Proteins were taken orally as treatment in several human autoimmune diseases in order to induce oral tolerance leading to reduced disease activity. Those human trials are listed, as are two planned trials.
S-Ag: S-antigen, AChR: acetylcholine receptor

Mechanisms of mucosal tolerance

Three main mechanisms that have been identified by which mucosal tolerance is mediated include active suppression, anergy and clonal deletion. These mechanisms appear to be determined primarily by the dose of administered antigen. Low doses of antigen favor the generation of regulatory cells that suppress the specific immune response in the target organ, whereas high doses of antigen induce an antigen-specific anergic state or deletion of peripheral T cells. Active suppression is the mechanism of peripheral T cell tolerance induction which is mediated by regulatory cells that suppress the specific immune response in the target organ. The tolerogenic state can be adoptively transferred by the regulatory T cells from mucosally tolerized animals to naïve recipients. Several phenotypes of such regulatory T cells have been described.

Initially, regulatory T cells of the CD8+ subset have been described to be involved in oral tolerance (Nussenblatt *et al.*, 1990; Lider *et al.*, 1989). Miller *et al.* (1992b) have demonstrated that the suppressive activity of such CD8+ cells may be due to induction of TGF-ß. TGF-ß is generally a negative regulator of immune responses. Injection of TGF-ß has been shown to suppress EAE and other experimental autoimmune diseases (Kuruvilla *et al.*, 1991; Racke *et al.*, 1991). The appearance of TGF-ß in inflammatory infiltrates in the brain of animals recovering from EAE, indicates a down-regulatory role for TGF-ß in inflammatory processes (Khoury *et al.*, 1992). Another group of regulatory T cells, are CD4+ Th2 cells (e.g. Tian *et al.*, 1996; Chen *et al.*, 1995), producing non-inflammatory cytokines such as IL-4, IL-10 and TGF-ß. When in a Th1 mediated autoimmune disease, the inflammatory Th1 response shifts to a specific, non-inflammatory Th2 response, the mechanism of active suppression is further specified as immune deviation. However, LaFaille *et al.* (1997) have demonstrated that Th2 cells do have the potential to induce EAE in immunodeficient mice, implying that in the absence of a normal immune system Th2 cells can be equally pathogenic. The existence of a third T cell subset (Th3), potentially involved in induction/maintenance of mucosal tolerance, was proposed by Chen *et al.* (1994). These Th3 cells express CD4+ and produce TGF-ß mainly, sometimes with low co-expression of IL-4 or IFN-γ.

T cell anergy is defined as a cellular state in which a T cell is alive but fails to display certain functional responses when optimally stimulated through both its antigen-receptor and other receptors that are normally required for T cell activation (Schwartz, 1996). Anergy is induced when no or no proper co-stimulation is present (Lafferty *et al.*, 1983). *In vitro*, stimulation of anergic cells with antigen does not lead to proliferation and IL-2 production. Peripheral T cell tolerance can not be adoptively transferred to naïve animals with anergic antigen specific T cells.

Clonal deletion, peripheral deletion of antigen specific T-cells, has not yet been described in normal animals after mucosal administration of antigen. Deletion of specific T cells has only been shown after oral administration of very high doses of antigen in T cell receptor (TCR) transgenic mice (Chen *et al.*, 1995a; Whitacre *et al.*, 1996b). Circumstantial evidence for a role of clonal deletion as mechanism of mucosal tolerance has been provided by the observation that orally OVA tolerized lymphocytes die by apoptosis when cultured *in vitro* after an *in vivo* challenge with OVA (Garside *et al.*, 1996).

All described mechanisms target T cells specific for the mucosally administered antigen. However, the regulatory effector T cells can also suppress T cell responses evoked by other antigens. By secretion of suppressive cytokines such as TGF-ß and IL-10 into the microenvironment, an ongoing response against a co-localized antigen may be down-regulated (Anderton and Wraith, 1998; Bayrak and Mitchison, 1998; reviewed by Weiner, 1997). This mechanism of active suppression is further specified as bystander suppression. Successful induction of this bystander suppression is of major importance for the therapeutic use of mucosal tolerance in the treatment of

autoimmune disease, as it may obviate the need to identify and use specific autoantigens, and may circumvent complications of determinant/epitope spreading (reviewed by Vanderlugt *et al.*,1998).

Modulation of mucosal tolerance
When mucosal tolerance is mediated via active suppression, inducing Th2 or Th3 regulatory cells, any stimulus that favors Th1 versus Th2/Th3 responses could potentially abrogate mucosal tolerance (reviewed by Weiner, 1997). For example, high doses of IFN-γ abrogated oral tolerance (Zhang *et al.*, 1990b). Conversely, administration of factors that favor Th2/Th3 over Th1 may augment mucosal tolerance. Indeed it has been shown that factors such as oral IL-4 can enhance oral tolerance (Inobe *et al.*, 1998). Surprisingly, also administration of IL-2 potentiated oral tolerance in the uveoretinitis model possibly by increasing TGF-ß, IL-4 and IL-10 production in Peyer's patches (Rizzo *et al.*, 1994). Peripheral T cell tolerance was also increased by oral administration of cholera toxin subunit B (CTB) and LPS, often used to enhance the immunogenicity of (mucosally) administered antigens for vaccination purposes (Arakawa *et al.*, 1998; Bergerot *et al.*, 1997; Gaupp *et al.*, 1997; Khoury *et al.*, 1990; Sun *et al.*, 1996). Similar effects may be obtained with bacteria that can influence the immune response in a non-inflammatory fashion, an effect that could be obtained by oral administration of a specified *Lactobacillus* strain.

1.5 Lactobacilli

Microorganisms as antigen delivery system for vaccination purposes
Oral vaccination against certain viruses or bacteria appears to be feasible when administered attenuated (Kaul and Ogra, 1998). However, there always will be a (small) risk of infection. Oral vaccination solely with pathogenic protein antigens is difficult to achieve, since oral administration of soluble antigen normally leads to hyporesponsiveness. Therefore a wide range of antigen delivery systems is being developed, which enable antigen delivery at the proper sites of antigen presentation and in addition may fulfill an adjuvant function. Transformed microorganisms expressing heterologous proteins of the target pathogen are used as such antigen delivery systems to evoke a protective immune response against the pathogen. The strains of microorganisms employed are usually genetically attenuated mutants of pathogenic bacteria (the carrier) transformed with a vector designed to obtain an adequate expression of antigens from another pathogen. Such carriers can then be used as local antigen producers at the gut mucosa, with the additional advantage of circumventing expensive and difficult antigen purification processes. For instance, *Salmonella* strains have been used as carrier for hepatitis B virus antigens (Fairweather *et al.*, 1990). Other bacteria which are potentially useful as carriers include strains of *E. coli, Mycobacteria, Vibrio cholera* and *Shigella* (Butterton *et al.*, 1997; Killeen *et al.*, 1999; Noriega *et al.*, 1996; Hale, 1990). The effectiveness of pathogenic bacteria as carrier probably is related to the pathogenicity of the bacterial

strains. Most pathogenic bacteria have specific mechanisms to invade the human or animal body, normally evoking a strong immune response. This strong immune response against the carrier probably stimulates the immune response against the heterologous expressed antigen. However, due to the pathogenic character of most transformed microorganisms used for oral vaccination, their use as antigen carrier is limited. For this reason the use of a GRAS (generally regarded as safe) organism, such as *Lactobacillus*, as a safe antigen carrier for the delivery of foreign antigens in the gastrointestinal tract is to be preferred.

Microorganisms as antigen delivery system for tolerance induction
Using microorganisms as antigen delivery system is an approved method of vaccination in experimental animal models. We reasoned that the same technique could be used to establish the induction of oral tolerance for the treatment of autoimmune diseases. An ideal therapy consists of a continuous production and presence of the autoantigen at the site of tolerance induction, i.e. the gut associated lymphoid tissue (GALT). Oral administration and possibly subsequent colonization of microorganisms expressing autoantigens might realize the continuous presence of autoantigen at the gastro-intestinal tract. This approach provides several advantages over feeding purified autoantigens. Degradation of the autoantigens in the stomach and the rest of the gastrointestinal tract will be decreased and no bulk purification of human proteins is necessary, which makes this method also much cheaper. In addition, the use of recombinant lactobacilli increases safety of the method, because there is no risk of co-administering viruses or prions. When the transformed lactobacilli are able to colonize the gut, the antigens are released continuously in the gut, restricting the therapy to a single or a few administrations. Specified *Lactobacillus* strains could be suitable for this approach due to their GRAS-status and low intrinsic immunogenicity (Gerritse *et al.*, 1990).

Lactobacillus
Lactobacilli are Gram-positive rod-shaped lactic acid bacteria. Although there are some exceptions, most of the *Lactobacillus* strains are not pathogenic and lactobacilli are major constituents of the human and animal gut. Because of their harmless character they have the GRAS-status, and are frequently used in bio-processing and preservation of food and feed. Nowadays, lactobacilli are mainly known for their health stimulating properties. The importance of lactobacilli for human health was first recognized by Metchnikoff at the beginning of the century (Metchnikoff, 1908). He suggested that harmful effects of undesired bacteria could be overcome by establishing a new balance between intestinal bacteria, through ingestion of lactobacilli or fermented products made by these organisms. Especially during the last few decades, extensive research on the positive effects of *Lactobacillus* strains has been carried out, suggesting additional health and/or nutritional benefits for humans and animals by oral administration of lactobacilli. Such intrinsic properties include: anti-carcinogenic

activity, control of intestinal infections, improvement of lactose metabolism, improvement of gut barrier function, control of serum cholesterol levels and positive effects on allergy as well as on (experimental) autoimmune diseases (e.g. Matsuzaki *et al.*, 1990; Murosaki *et al.*, 1998; Perdigon *et al.*, 1995; Salminen *et al.*, 1996b; Shornikova *et al.*, 1997). It should be noted that not all strains from the very large and diverse (e.g. in fermentation pathways) genus *Lactobacillus* exhibit the same health-stimulating properties and may vary in strength of the effect obtained (Klein *et al.*, 1998). Some of the positive effects of lactobacilli are mediated by non-immune components such as modulation of vitamin production, enzymes, and antibiotics, but especially modulation of the immune system seems to play an important role (reviewed by Holzapfel *et al.*, 1998; Fernandes *et al.*, 1987; Rao and Shahani, 1987; Shahani *et al.*, 1977).

Immunomodulation by lactobacilli

One of the possible mechanisms for lactobacilli to influence allergy, autoimmunity, infection and carcinogenesis is by affecting cytokine expression in a specific or non-specific manner. Modification of the local cytokine profile in the gut probably is most effective. Therefore oral administration of lactobacilli could enhance protection and improve treatment of intestinal infections, food allergy and colon cancer by inducing or reducing levels of distinct cytokines. But apparently, oral lactobacilli can also influence non-mucosal disease sites, like autoimmune diseases affecting the joints or cancer of the pancreas and bladder. *Figure 1* shows which cytokines positively influence certain disorders when their expression is altered by oral administration of lactobacilli.

Effect of lactobacilli on cytokine expression in infection

It has been extensively reported that lactobacilli have probiotic effects (i.e. beneficial effects by improving the properties of the indigenous flora) which enhance protection against infections (Perdigon *et al.*, 1995, partially reviewed by Salminen *et al.*, 1996). These effects could be due to activation of innate immune defence effector functions (e.g. macrophages) and/or to support of the specific response against infectious agents by upregulation of IgA production. These two pathways are discussed below.

Many studies have shown that different strains of lactobacilli are able to activate macrophages *in vitro* and *in vivo* (Lehman *et al.*,1988; Nanno *et al.*,1989; Perdigon *et al.*,1986; Pool-Zobel *et al.*,1996; Tomita *et al.*,1993). Lactobacilli induce cytokines produced by macrophages, TNF-α, IL-1, IL-6, IL-12 and/or IFN-α/ß, in macrophage cell lines, human blood monocytes, spleen macrophages, Peyer's patch (PP) adherent cells or peritoneal macrophages (*Table 4*)(Kitazawa *et al.*, 1992; 1994; Klebanoff *et al.*, 1999; Marin *et al.*, 1998; Murosaki *et al.*, 1998; Popova *et al.*, 1993; Shida *et al.*, 1998; Tomita *et al.*, 1993). Since IFN-γ is the major macrophage-activating cytokine, induction of this cytokine by lactobacilli provides indirect evidence of macrophage-activating properties of lactobacilli (Laffineur *et al.*, 1996; Murosaki *et al.*, 1998; Shida *et al.*, 1998; Solis-Pereyra *et al.*, 1997). Intraperitoneal administration

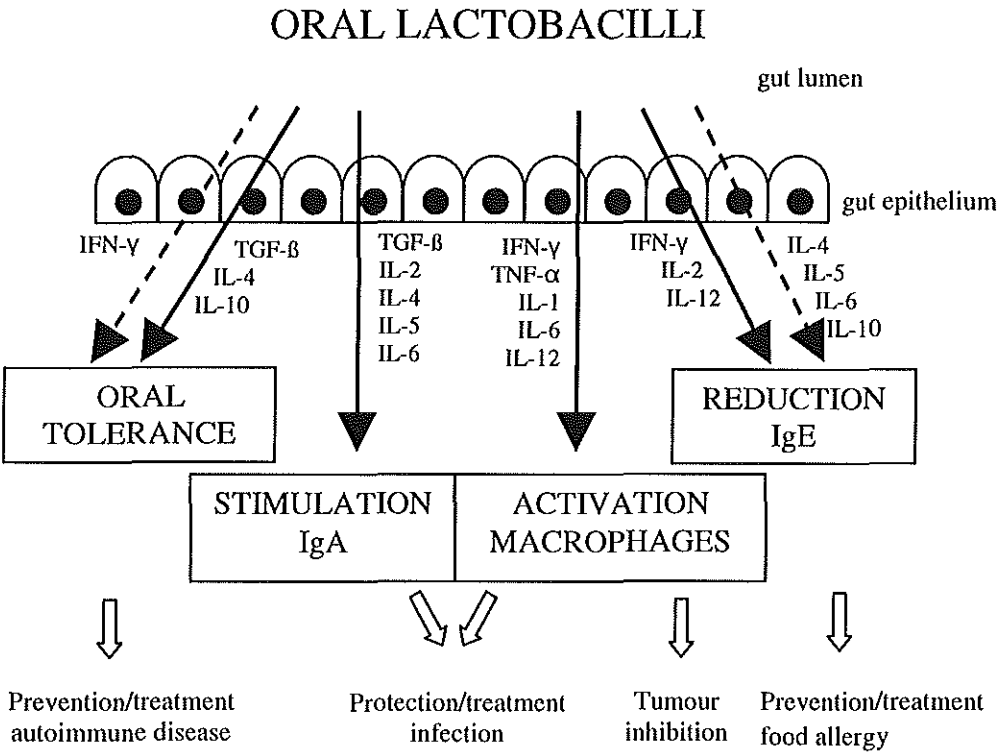


Figure 1. *Modulation of cytokines by oral administration of lactobacilli can affect disease.*
Oral administration of lactobacilli can modulate cytokine expression in the gastrointestinal tract and possibly elsewhere in the body. By inducing (lined arrows) and/or reducing (broken arrows) expression of one or more cytokines positive effects on infection and disease may be achieved. Enhancement of oral tolerance towards orally administered soluble (auto)antigens, even in the periphery, could limit autoimmune disease. Stimulation of IgA production and macrophage activation might improve protection against pathogens that enter *via* the gastrointestinal tract. The cytotoxic action of macrophages can inhibit tumour-growth. Reduction of IgE levels has positive effects on food allergy.

of *L. casei* cell wall, leading to macrophage activation, is now being used as a model for Kawasaki disease (cardiac arteritis)(Okitsu-Negishi *et al.*, 1996; Tomita *et al.*, 1993; Lehman *et al.*, 1988). When lactobacilli are added to *in vitro* cultures of mixed cell populations like splenocytes or human PBMC, upregulation or no effect on expression of IL-12, IL-1, IL-6, IFN-α/ß, IFNγ and TNF-α was found (Laffineur *et al.*, 1996; Matsushima *et al.*, 1998; Miettinen *et al.*, 1996, 1998; Murosaki *et al.*, 1998; Shida *et al.*, 1998; Solis-Pereyra *et al.*, 1997). This could reflect activation of the monocyte lineage, but may also be (partially) due to modulation of lymphocytes. The peptidoglycan layer of lactobacilli is able to activate macrophages to produce IL-1,

IL-6 and TNF-α by binding to the CD14 receptor (e.g. Schrijver *et al.,* 1999). However, there is some discrepancy between studies as to whether it is the peptidoglycan layer which is responsible for macrophage activation (de Ambrosini *et al.,* 1996). *In vivo* administration of lactobacilli can also result in activation of macrophages. In different studies one or more of the cytokines IL-12, IL-1, TNF-α and IL-6 were upregulated by peritoneal macrophages after oral, intramuscular or intraperitoneal administration of the lactobacilli (*Table 5*)(Murosaki *et al.,* 1998; Okitsu-Negishi *et al.,* 1996; Popova *et al.,* 1993; Saito *et al.,* 1987). Although almost all *Lactobacillus* strains could activate macrophages, the extent of cytokine induction differed between strains. TNF-α was also induced in the gut after oral administration of different *Lactobacillus* strains (Maassen *et al.,* 1999b). Significantly increased numbers of TNF-α producing cells in the gut villi and in the submucosa were demonstrated by immunohistochemistry after feeding BALB/c mice with *L. reuteri* (Chapter 2.2).

Lactobacilli can affect infection by macrophage activation (immune elimination), but also by stimulating IgA production (immune exclusion). It has been shown previously that lactobacilli are able to upregulate IgA production, locally as well as systemically (Perdigon *et al.,* 1991; Link-Amster *et al.,* 1994; Kaila *et al.,* 1995; Majamaa *et al.,* 1995). This is probably linked to upregulation of one or more cytokines. For instance, TGF-ß is necessary for the isotype switch to IgA (reviewed by Brandtzaeg, 1995). Terminal differentiation of B cells into plasma cells in the secretory tissues involves cytokines such as IL-5, IL-6 and IL-10 and possibly IFN-γ. These cytokines can all be produced by mucosal T-cells (Nilsen *et al.,* 1995). Little is known about the cytokine profile or other microenvironmental requirements for the enhancement of J chain production by mucosal B cells, a necessary component of secretory IgA and IgM. Probably IL-2, IL-5 and maybe IL-6 are involved in its upregulation, whereas IL-4 may have an opposing effect (Brandtzaeg, 1994). In a T-cell line the expression of IL-2 and IL-5 was enhanced by *L. bulgaricus* (Marin *et al.,* 1998), possibly correlating with the upregulation of J-chain expression by plasma cells (*Table 4*). In other cultures no effect or an increased expression of IFN-γ, IL-2, IL-4, IL-6 and IL-10 was measured (*Table 4*). It is noteworthy that IL-4 levels remained either unaffected or were down-regulated. This is consistent with the results found *in vivo* when, after intraperitoneal or oral application of lactobacilli, IL-4 levels were down-regulated (*Table 5*)(Kato *et al.,* 1998; Maassen *et al.,* 1999b; Matsuzaki *et al.,* 1998; Murosaki *et al.,* 1998b). In conclusion, lactobacilli may reduce infection by modulation cytokine expression.

In tumour growth

Lactobacilli show anti-tumour effects not only in rodents, but also in humans. In particular, intralesional injection of lactobacilli effectively inhibits tumour growth (lung carcinoma)(Masuno *et al.,* 1991), but also oral administration of lactobacilli can prevent tumour growth (colon/bladder cancer)(Aso *et al.,* 1995). IL-1 and TNF-α

Table 4a. Lactobacillus *strains affect cytokine expression* in vitro

(Possible) effect of lactobacilli	*Lactobacillus* strains	Technique used	Ref.
anti-tumour	*L. acidophilus DDS-1* *L. acidophilus NRRL 6934* *L. acidophilus NRRL B4527* *L. acidophilus NRRL 0734*	culture/ ELISA	1
immunomodulation	*L. bulgaricus Lr 78* *L. bulgaricus NCK 231*	culture/ ELISA	2
prevention IgE-mediated allergy	*L. casei Shirota*	culture/ ELISA	3
influence on vaginal physiology and host defense	*L. crispatus*	culture/ ELISA	4
induction of cardioangitis	*L. casei* (cell wall)	culture/ immunoradiometry (TNF-α) culture/ ELISA (IL-1ß, INF-γ)	5
resistance against bacterial infections	*L. bulgaricus* (lysozyme lysate)	thymocyte proliferation/ immuno-fluorescence	6
physiologically functional food	*L. gasseri*	culture/ 50% plaque reduction/ neutralization, rt-PCR	7
physiologically functional foods	*L. acidophilus* (4 strains)	culture/ neutralization	8
prevention/ treatment of food allergy	*L. plantarum L-137*	culture/ ELISA	9
prevention IgE-mediated allergy	*L. casei Shirota* *L. johnsonii JCM 0212*	culture/ ELISA	3
induction of pulpitis	*L. casei* (peptidoglycan)	rt-PCR	10
immunomodulation	*L. bulgaricus*	culture/ immunoradiometry	11
immunomodulation	*L. helveticus 5089* (medium)	culture/ ELISA	12
immunomodulation	*L. paracasei ssp paracasei E506* *L. paracasei ssp paracasei E510* *L. acidophilus E507* *L. plantarum E98* *L. rhamnosus E509* *L. rhamnosus GG E522* *L. bulgaricus E585*	culture/ ELISA, Northern blot	13, 14
immunomodulation	*L. bulgaricus Lr 78* *L. bulgaricus NCK 231*	culture/ ELISA	2*

Tables 4a and *4b* show an overview of studies on the *in vitro* effects of *Lactobacillus* strains on cytokine expression. From top to bottom the analyzed cytokine producing cells are divided into macrophages, mixed cell populations and T-cells. All experiments were performed with material from mice or humans. The experiments using human material are indicated in the column with cytokine producing cells. Northern blotting and rt-PCR were used to determine mRNA levels of cytokine genes. The cytokines analysed are indicated by ↑ (induction), ↓ (inhibition) or – (no effect). References including remarks: [1]Rangavajhyala et al., 1997 (*L. acidophilus DDS-1* far best inducer); [2]Marin et al., 1998; [3]Shida et al., 1998; [4]Klebanoff et al., 1999; [5]Tomita et al., 1993; [6]Popova et al., 1993; [7]Kitazawa et al., 1994; [8]Kitazawa et al., 1992; [9]Murosaki et al., 1998; [10]Matsushima et al., 1998; [11]Solis-Pereyra et al., 1997;

Table 4b. Lactobacillus *strains affect cytokine expression* in vitro

Lactobacillus strains	Cytokine producing cells analyzed	Cytokines analyzed												Ref.
		IL-12	IL-1	IL-1α	IL-1ß	TNF-α	INF-α/β	INF-γ	IL-2	IL-4	IL-5	IL-6	IL-10	
L. acidophilus DDS-1 *L. acidophilus NRRL 6934* *L. acidophilus NRRL B4527* *L. acidophilus NRRL 0734*	macrophage cell line (RAW 264.7)			↑ ↑ ↑ ↑		↑ ↑ ↑ ↑								1
L. bulgaricus Lr 78 *L. bulgaricus NCK 231*	macrophage cell line (RAW 264.7)					↑ ↑						↑ ↑		2
L. casei Shirota	macrophage cells J774.1	↑												3
L. crispatus	macrophage cell line (THP-1)				↑	↑								4
L. casei (cell wall)	human blood monocytes				↑	↑		-						5
L. bulgaricus (lysozyme lysate)	human blood monocytes		↑			↑								6
L. gasseri	spleen macrophages PP adherent cells						↑ ↑	- -						7
L. acidophilus (4 strains)	peritoneal macrophages						↑							8
L. plantarum L-137	peritoneal macrophages splenocytes	↑ ↑						↑						9
L. casei Shirota *L. johnsonii JCM 0212*	splenocytes	↑ -						↑ -		↓ -	↓ -			3
L. casei (peptidoglycan)	dental pulp cells											↑		10
L. bulgaricus	human PBMC				↑	↑		↑						11
L. helveticus 5089 (medium)	human PBMC							↑	↓					12
L. paracasei ssp parac.E506 *L. paracasei ssp parac.E510* *L. acidophilus E507* *L. plantarum E98* *L. rhamnosus E509* *L. rhamnosus GG E522* *L. bulgaricus E585*	human PBMC	 ↑ ↑ -			 ↑ ↑ ↑	↑ - ↑ ↑ ↑ ↑ ↑	 ↑ ↑ -			- - - -		- - - ↑ ↑ ↑ ↑	 ↑ ↑ ↑	13, 14
L. bulgaricus Lr 78 *L. bulgaricus NCK 231*	T-helper cell line (EL4.IL-2)								↑ ↑		↑ ↑			2*

[12]Laffineur et al., 1996 (in 2 out of 4 strains); [13]Miettinen et al., 1998; [14]Miettinen et al., 1996 (fixed bacteria: no cytokine induction, IL-18 induced by *L. rahmnosus* and *L. bulgaricus*, other strains not tested) * only with co-stimulation by phorbol 12-myristate-13-acetate.

Table 5a. Lactobacillus *strains affect cytokine expression* in vivo

(Possible) effect of lactobacilli	*Lactobacillus* strains	Route	Technique used	Ref.
resistance bacterial infections	*L. bulgaricus* (lysozyme lysate)	po	thymocyte proliferation/ immunofluorescence	1
anti-infection	*L. casei YIT9018*	im	^{3}H thymidine incorporation	2
induction of cardioangitis	*L. casei* (cell wall)	ip	culture/ ELISA (TNF-α), proliferation of B3B1 cells (IL-6), Northern blot (IL-1ß, TNF-α)	3
prevention/ treatment of food allergy	*L. plantarum L-137*	ip	culture/ ELISA	4
probiotic	*L. casei* *L. acidophilus* *L. helveticus* *L. gasseri* *L. reuteri*	po v	culture/ ELISA	5
reduction CIA	*L. casei Shirota*	po v	culture/ ELISA	6
prevention IDDM	*L. casei*	po nv	culture/ ELISA	7
treatment NIDDM	*L. casei*	po nv	culture/ ELISA	8
inhibition of IgE production	*L. casei Shirota*	po nv	culture/ ELISA	9
anti-tumour	*L. casei Shirota*	ipl	culture/ ELISA, rt-PCR	10, 11
immunomodulation	*L. reuteri* *L. brevis* *L. gasseri* *L. murines* *L. plantarum NCIB* *L. plantarum 14917* *L. casei* *L. fermentum*	po v	immunohistochemistry	12
immunomodulation	*L. brevis ssp. coagulans*	po v po nv	2'-5' A synthetase	13
treatment neoplastic disease	*L. bulgaricus* (lysozyme lysate)	po	ELISA	14
food processing	*L. bulgaricus*	ip	ELISA	15
anti-Trichinella spiralis infection	*L. casei*	ip v	ELISA	16
prevention/ treatment of food allergy	*L. plantarum L-137*	ip	ELISA	4
anti-tumour	*L. casei YIT9018*	il	^{3}H thymidine incorporation (IL-1, IL-2), ELISA (IFN-γ), cytostasis (TNF-α)	10, 17

Tables 5a and *5b* show an overview of studies on the *in vivo* effects of *Lactobacillus* strains on cytokine expression. From top to bottom the analyzed cytokine producing cells are divided into macrophages, mixed cell populations, T-cells and soluble components. All experiments were performed in mice or humans. The experiments in humans are indicated in the column with cytokine producing cells. The route of administration is indicated as follows; orally (po), intramuscular (im), intraperitoneally (ip), intrapleural (ipl), intralesional in the lung of tumour-bearing *L. casei* primed mice (il). Where known it was indicated whether viable (v) or non-viable (nv) lactobacilli were used. Experiments which demonstrated that lactobacilli had no effect on cytokine expression are not included in this table. Experiments in which mixed bacterial cultures

Table 5b. Lactobacillus *strains affect cytokine expression* in vivo

Lactobacillus strains	Cytokine producing cells analyzed	Cytokines analyzed												Ref.
		IL-12	IL-1	IL-1α	IL-1ß	TNF-α	INF-α/β	INF-γ	IL-2	IL-4	IL-5	IL-6	IL-10	
L. bulgaricus (lysozyme lysate)	peritoneal macrophages		↑											1
L. casei YIT9018	peritoneal macrophages		↑											2
L. casei (cell wall)	peritoneal macrophages				↑	↑						↑		3
L. plantarum L-137	peritoneal macrophages splenocytes	↑						-		↓				4
L. casei *L. acidophilus* *L. helveticus* *L. gasseri* *L. reuteri*	peritoneal leukocytes	↑ ↑ - - -				- - - - -		- ↓ ↓ ↓ ↓				↑ ↑ ↑ ↑ ↑		5
L. casei Shirota	splenocytes							↓		-				6
L. casei	splenocytes							↓	↑				↓	7
L. casei	splenocytes							↓	↓					8
L. casei Shirota	splenocytes	↑						↑	↑	↓	↓	↓	↓	9
L. casei Shirota	thoracic exudated cells	↑			↑	↑		↑				↑		10, 11
L. reuteri *L. brevis* *L. gasseri* *L. murines* *L. plantarum NCIB* *L. plantarum 14917* *L. casei* *L. fermentum*	gut villi			- - - - - - - -	↑ - - - - - - -	↑ ↑ ↑ ↑ ↑ ↑ ↑ -		- - - - - - - -	↑ ↑ - - - - - -	- - - - - - - -			- - - - - - - -	12
L. brevis ssp. coagulans (v) (nv)	human PBMC			↑ -										13
L. bulgaricus (lysozyme lysate)	serum					↑								14
L. bulgaricus	serum					↑	↑							15
L. casei	serum							↑						16
L. plantarum L-137	serum	↑												4
L. casei YIT9018	peritoneal exudate				↑	↑		↑	↑					10, 17

were used and where the effect of lactobacilli could not be positively identified were also excluded. ^{3}H-thymidine incorporation in cells dependent for their growth on the cytokine of interest, was used as a measure for specific cytokine production of the effector cells. Northern blotting and rt-PCR were used to determine mRNA levels of cytokine genes. The cytokines analysed are indicated by ↑ (induction), ↓ (inhibition) or – (no effect). References including remarks: [1]Popova et al., 1993; [2]Saito et al., 1987 (only with infection); [3]Okitsu-Negishi et al., 1996 (depicted results obtained from experiments done in BALB/c, in C3H/HeJ mice no cytokine induction); [4]Murosaki et al., 1998; [5]Tejada-Simon et al., 1999 (no effects detected in splenocytes and Peyer's patches); [6]Kato et al., 1998 (only after collagen immunization); [7]Matsuzaki et al., 1997a; [8]Matsuzaki et al., 1997b; [9]Matsuzaki et al., 1998; [10]Matsuzaki, 1998; [11]Matsuzaki et al., 1996; [12]Maassen et al., 1999b; [13]Kishi et al., 1996; [14]Davidkova et al., 1992; [15]Pereyra et al., 1991; [16]Bautista-Garfias et al., 1999; [17]Matsuzaki et al., 1990.

secreted by macrophages exhibit cytostatic and cytocidal effects on several tumour cell lines *in vitro* (Onozaki *et al.*, 1985; Urban *et al.*, 1986). It is thought that the tumour-suppressive activity of lactobacilli is dependent on the activation of macrophages producing IL-1 and TNF-α (*Table 4* and *5*)(Davidkova *et al.*, 1992; Matsuzaki *et al.*, 1996; Matsuzaki, 1998; Rangavajhyala *et al.*, 1997). IFN-γ not only activates macrophages but also natural killer cells, which can nonspecifically kill tumour cells. Therefore, induction of IFN-γ by lactobacilli could positively affect anti-tumour activity.

In autoimmunity

Many of the autoimmune diseases are chronic inflammatory disorders mediated by Th1 cytokines, such as IFN-γ and TNF-α (Liblau *et al.*, 1995; Powrie and Coffman, 1993). As discussed in section 1.4, oral administration of the autoantigen can prevent/treat experimental autoimmune diseases. Immune deviation towards Th2/Th3 can be the mechanism of oral tolerance. Because large amounts of autoantigen are necessary to skew the response towards the non-inflammatory side, a local environment more permissive for this type of response is desirable. Treatment with *Lactobacillus* strains that stimulate production of TGF-ß, IL-10 and IL-4 locally in the gut could possibly enhance tolerance induction against autoantigens. Unfortunately, a *Lactobacillus* strain with these inducing properties has not been identified yet. Only *L.casei* tended to enhance expression of both IL-10 and TGF-ß in the gut villi upon oral administration in mice, but these increases were not significant (Maassen *et al.*, 1999b). Down-regulation of the Th1 pathway can also be beneficial. Several reports show that *Lactobacillus* strains can positively affect experimental autoimmune diseases, such as arthritis and diabetes. In these studies at least IFN-γ was down regulated after oral administration of lactobacilli (Kato *et al.*, 1998; Matsuzaki *et al.*, 1997a; Matsuzaki *et al.*, 1997b).

In food allergy

Food allergy is thought to be caused by production of IgE against dietary antigens in atopic individuals, due to inappropriate generation and activation of Th2 cells. The Th2-type cytokine IL-4 not only induces switching of B cells to IgE-producing cells, but also inhibits the production of the Th1-type cytokine IFN-γ (Gascan *et al.*, 1991; Peleman *et al.*, 1989; Pene *et al.*, 1988). Conversely, IFN-γ inhibits the proliferation of Th2 cells and suppresses the switching of B cells. IL-12 is known to stimulate Th1 cells to produce IFN-γ, resulting in inhibition of Th2-type of immune responses, but IL-12 is also capable of preventing Th2 responses independently of IFN-γ (Kiniwa *et al.*, 1992). Lactobacilli that induce IL-12 and/or IFN-γ could therefore help to prevent or treat IgE-mediated food allergy. From *Table 4* and *5*, it is clear that some strains are able to induce IL-12 or IFN-γ, and sometimes both. When *L. casei Shirota* was administered orally, the Th2 response against OVA, which was injected i.p, was skewed towards a Th1 response, also reducing the OVA specific IgE response (Shida *et al.*, 1998). Even intraperitoneal administration of *L. plantarum L-137* reduced

casein specific IgG1 and IgE titers in casein fed mice (Murosaki *et al.,* 1998). This indicates that lactobacilli can have a role in treatment of food allergy (Shida *et al.,* 1998).

Concluding remarks

Clearly, selected *Lactobacillus* strains are able to modulate immune responses by affecting cytokine expression *in vitro* and *in vivo*, thus having the potential to suppress infection, autoimmunity, cancer and allergy. The most prominent feature of lactobacilli is that most strains can induce TNF-α, probably correlating with activation of macrophages. Although for most tested cytokines either no induction or enhanced expression was found, especially the data for cytokines IL-6 and IFN-γ were conflicting. This is probably due to the use of different mouse strains (Okitsu-Negishi *et al.,* 1996), routes of administration, doses, bacterial viability and effector cells in individual studies. For instance, *L. casei Shirota* showed opposite effects in different experimental set-ups (Kato *et al.,* 1998; Matsuzaki *et al.,* 1996; 1998; Matsuzaki, 1998). On the other hand, various *Lactobacillus* strains within the same experiment differentially affected cytokine expression, indicating that the obtained effect also depends on the *Lactobacillus* strain used (Maassen *et al.,* 1999b; Miettinen *et al.,* 1996; 1998; Shida *et al.,* 1998; Tejada-Simon *et al.,* 1999).

The levels of induction/reduction of cytokines vary greatly and are difficult to compare due to different techniques used. Furthermore, it is difficult to estimate the impact of up- or down-regulation of a particular cytokine *in vivo*. This hampers accurate investigations into application of lactobacilli for prevention or treatment of disease. Since many pathogens enter the body via the gastrointestinal tract, it is likely that the local cytokine profile at those sites is decisive for the kind of response. Some reports show that *Lactobacillus* strain induced cytokine expression is directly correlated with disease inhibition. Further study is needed to determine the effects of lactobacilli at the effector sites, including a complete as possible local cytokine profile. The cytokine profile induced by a particular *Lactobacillus* strain after oral administration may be crucial to decide which strains can be used as a probiotic, but also for other applications, such as oral vaccination with recombinant *Lactobacillus* strains and recombinant *Lactobacillus* strains as therapeutics in autoimmune disease.

Lactobacillus strain selection

Whether oral administration of recombinant lactobacilli expressing a pathogenic antigen or autoantigen results in the desired immune response does not only depend on the level of expression, location of expression (intracellularly, surface anchored or secreted)(discussed below), dose of lactobacilli and frequency of administration, but also the *Lactobacillus* strain used can be of major importance (Maassen *et al.,* 1999a; Pouwels *et al.,* 1996). The large genetic and physiologic diversity of this genus in many properties allows selection of the most appropriate strain for oral vaccination purposes as well as for oral tolerance induction. As discussed in the preceding sections *Lactobacillus* strains can influence the immune response by modulation of cytokine

expression. In addition, *Lactobacillus* strains may differ in many more properties such as colonization/persistence, immunogenicity and intrinsic adjuvanticity, here defined as the property to enhance the humoral response against a parenterally administered antigen. Although probably all *Lactobacillus* strains can maintain a stable population in germ-free animals or can colonize the human gut when applied at very young age before the gut flora is established, only some strains are able to persist for a longer time when administered to man or animal with a normal gut flora. Concerning intrinsic immunogenicity, it has been shown that lactobacilli can be found in the Peyer's patches (Claassen *et al.*, 1995), suggesting presentation to the mucosal immune system, but so far, none of the tested strains did evoke a high humoral immune response against itself after oral administration (Gerritse *et al.*, 1990). In order to select the best suitable strains and that way enhance the effectiveness of the *Lactobacillus* antigen delivery system for the use in vaccination and tolerization, several of the above mentioned properties have been investigated in this thesis.

Vector design

The expression vectors available for lactobacilli can direct the heterologous protein to the microenvironment (secretion), to the surface of the lactobacilli (surface-anchored expression) or retain the heterologous protein in the cytoplasm (intracellular expression). The location of the heterologous antigen may influence for example adherence, access to enterocytes or M-cells and preferential uptake by different APC. As suggested in the preceding sections, *Lactobacillus* strains can be used as delivery system in two distinct applications. They can be used as delivery system of pathogenic antigens in oral vaccination as well as delivery system of autoantigens in order to induce oral tolerance in autoimmune diseases. The success of these approaches depends on the appropriate presentation of the (auto)antigen at the mucosal immune system, leading to mucosal/systemic immune response or to systemic T cell tolerance, dependent on the application. Vector design may help to direct the immune response towards the desired immune response. However, it is unknown in what way an orally administered *Lactobacillus* strain has to present the antigens to the immune system. There are several methods of antigen presentation possible, dependent on the design of the vector. 1) The antigen is secreted by the lactobacilli in the gut. 2) The antigen is expressed intracellularly and is released from the cytoplasm of the lactobacilli upon death and cytolysis. Both methods lead to soluble antigen in the gut, which normally induces non-responsiveness towards the antigen. 3) The antigen is expressed intracellularly and presented to the GALT after processing of the lactobacilli by antigen presenting cells. 4) The antigen is expressed on the surface of the lactobacilli and is presented to the GALT as a particulate antigen. Theoretically, this seems the best option for the induction of a good immune response against a pathogenic antigen. Nevertheless, the most efficient route of antigen presentation for both applications in combination with an appropriate *Lactobacillus* strain has to be revealed by *in vivo* experiments.

1.6 Introduction to chapters

The aim of the study was to develop an antigen-specific therapy for EAE based on oral tolerance induction by recombinant lactobacilli. Before testing this approach in animal models, a suitable *Lactobacillus* strain had to be chosen and transformed to express myelin antigens.

Chapter 2. Properties of wild type Lactobacillus *strains: strain selection for mucosal tolerance induction and mucosal vaccination purposes*

Lactobacilli are attractive candidates for mucosal vaccination as well as for mucosal tolerance induction purposes. The large *Lactobacillus* genus and the diverse effects of its different species and strains on the immune system, necessitates careful selection of an appropriate strain for either of both purposes. In order to make a more directed *Lactobacillus* strain selection, several properties of wild type *Lactobacillus* strains which may have implications for induction of tolerance or immunity were investigated. *Chapter 2.1:* In this chapter the potential of recombinant lactobacilli in tolerance induction by oral administration of recombinant lactobacilli was shown. Just mixing autoantigen with a particular *Lactobacillus* strain did not affect the EAE disease course. However, oral administration of the wild type *Lactobacillus* strain alone had an adverse effect on EAE.

Chapter 2.2: The local cytokine environment in the gut plays a role in the effectiveness of inducing oral tolerance or immunity. Therefore the effect of orally administered wild type *Lactobacillus* strains on cytokine profiles in the gut villi was determined by immunohistochemistry. Some *Lactobacillus* strains were able to induce cytokines such as IL-2, IL-1ß and TNF-α. In the same chapter the enhancement of the specific humoral response against a parenterally administered T-cell dependent antigen by orally administered *Lactobacillus* strains (adjuvanticity), was investigated in Th2-biased BALB/c mice. The *Lactobacillus* strains that induced the abovementioned cytokines also showed adjuvant activity.

Chapter 2.3: Four of the *Lactobacillus* strains analyzed in chapter 2.2 were analyzed in a similar experimental set-up for their adjuvant activity in Th1-biased SJL/J mice. In this mouse strain no adjuvant activity could be detected, but further analysis of the antibody responses evoked in the SJL/J mice revealed a growth phase dependent effect on the IgG1/IgG2a ratio. This indicates a growth phase dependent skewing of T cell pathways by orally administered *Lactobacillus* strains.

Chapter 3. Genetic engineering of Lactobacillus *strains*

Based upon the results described in chapter 2, a choice was made which *Lactobacillus* strain to transform with myelin antigens for the use in mucosal tolerance induction experiments in EAE.

Chapter 3.1: This chapter describes the development of vectors allowing expression of heterologous antigens by lactobacilli. It was shown that dependent on the vector, the heterologous proteins are indeed directed to the surface of the

bacterium, secreted into the environment or retained intracellularly. Parenteral immunization of recombinant lactobacilli evoked specific antibody responses against the expressed antigens, confirming expression of the antigen by the lactobacilli.

Chapter 3.2: Because most of the *Lactobacillus* strains did not readily accept plasmid ligation mixtures for transformation, intact plasmids needed to be purified from *L. casei*, in order to transform other *Lactobacillus* strains. In this chapter an easy and rapid method is described to isolate plasmids from lysis-resistant lactobacilli.

Chapter 4. Mucosal tolerance induction with recombinant lactobacilli
In the last experimental part of this thesis we combined the results obtained in the previous chapters to induce mucosal tolerance with recombinant lactobacilli. We selected *Lactobacillus casei* based on its effects on the immune system (intrinsic adjuvanticity, intrinsic immunogenicity, induction of cytokine profile)(chapter 2). A panel of recombinant *L. casei* expressing myelin antigens was generated and analyzed (chapter 3.1). A selection of recombinants was tested for its ability to reduce EAE (chapter 4.2).

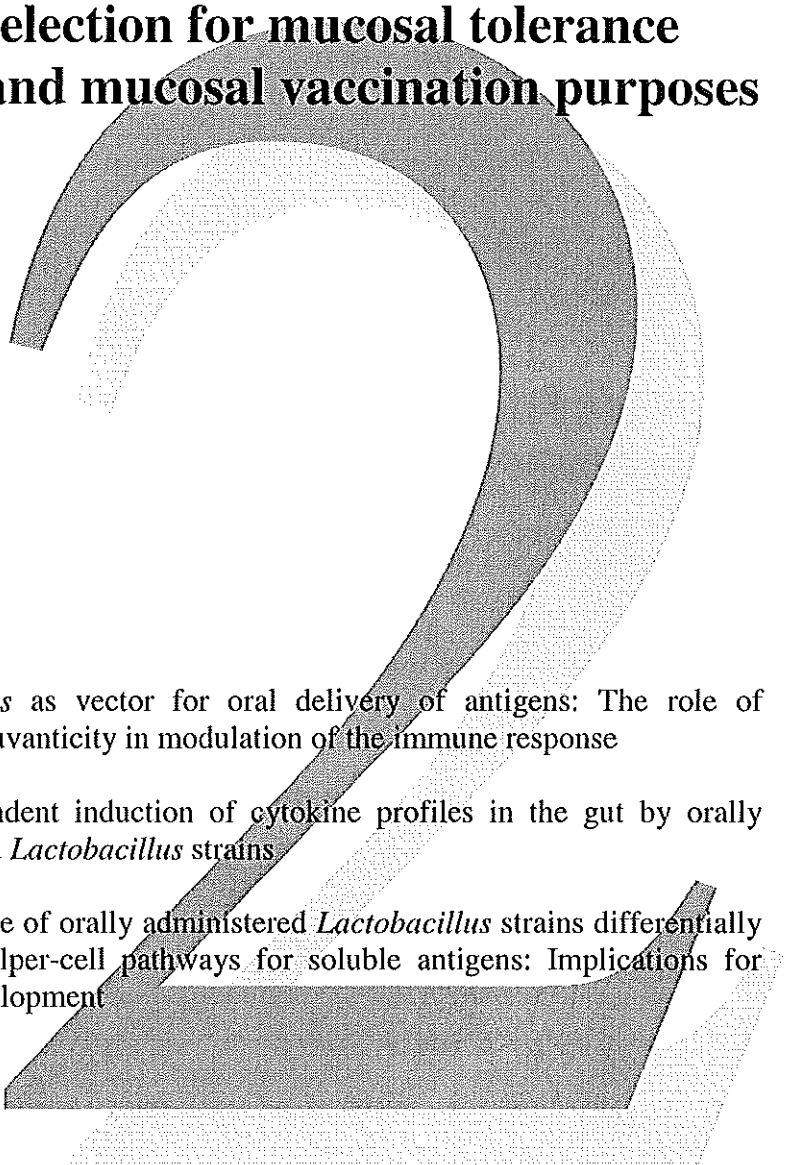
Chapter 4.1: Before the effectiveness of recombinant lactobacilli expressing myelin antigens in preventing EAE by mucosal administration could be investigated, a dose response analysis was performed with $PLP_{139\text{-}151}$ for the induction of EAE. This was done in order to select a dose of $PLP_{139\text{-}151}$ that induced medium disease severity, permitting modulation.

Chapter 4.2: In this chapter we provide 'proof of principle' that it is possible to reduce EAE by mucosal administration of recombinant lactobacilli expressing myelin antigens. It was demonstrated that nasal administration of *Lactobacillus* extracts containing myelin antigens could reduce the EAE disease burden when administered prior to disease induction. In addition, it was demonstrated that live recombinant lactobacilli expressing myelin antigens were able to reduce the EAE disease burden when administered orally prior to disease induction.

The nasal tolerance experiments in the Lewis rats were performed in an EAE model not previously used for nasal tolerance induction. In addition, we demonstrated that nasal administration of purified MBP could enhance the disease burden in this EAE model.

Chapter 5. Summarizing discussion
The discussion starts with the rationale behind the *Lactobacillus* strain selection for mucosal tolerance experiments in EAE. This is followed by a discussion about intranasal versus oral tolerance induction. In the subsequent part is discussed what might happen after oral administration of recombinant lactobacilli. Finally, approaches for future development of this approach for clinical use are discussed.

Properties of wild type *Lactobacillus* strains: strain selection for mucosal tolerance induction and mucosal vaccination purposes



2.1 *Lactobacillus* as vector for oral delivery of antigens: The role of intrinsic adjuvanticity in modulation of the immune response

2.2 Strain dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains

2.3 Growth phase of orally administered *Lactobacillus* strains differentially affects T helper-cell pathways for soluble antigens: Implications for vaccine development

Chapter 2.1

Lactobacillus as a vector for oral delivery of antigens: The role of intrinsic adjuvanticity in modulation of the immune response

C.B.M. Maassen, K. Gerritse, R.J. Leer, D. Heemskerk, W.J.A. Boersma and E. Claassen

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Summary

Oral delivery of antigens under specific conditions induces systemic immunotolerance. For this purpose non-pathogenic genetically engineered lactobacilli can be used as delivery vehicles. The intrinsic adjuvant activity of Lactobacillus *strains could play a role in tolerization versus induction of immune responsiveness. To assess whether a soluble antigen orally delivered by a particulate vehicle can suppress the systemic immune response, we orally administered ß-galactosidase expressed in* Lactobacillus plantarum 80, *a strain with relatively low adjuvanticity. This was followed by an i.p. immunization with ß-galactosidase. The pre-treatment led to a suppression of the anti-galactosidase antibody response, indicating that oral antigen delivery by a* Lactobacillus *strain with low adjuvant properties can have down modulatory effects.*

To assess whether in a mouse autoimmune model lactobacilli can promote tolerance induction, we orally co-administered Lactobacillus casei, *a strain with a relatively high adjuvant activity, with an encephalitogenic PLP peptide. After this pre-treatment of SJL mice, experimental autoimmune encephalomyelitis (EAE) was induced with the same peptide. This treatment did not result in tolerance, but instead enhanced this autoimmune disease.*

This study indicates that the intrinsic adjuvant activity of Lactobacillus *strains affects the immune response in a stimulating or inhibiting manner, in conjunction with the dose and character of the antigen. This has implications for vaccine development for infectious diseases and therapy of autoimmune disease.*

Introduction

Recent developments have brought clinical application of the modulation of the immune system within reach. Application is for example the enhancement of the immune response for vaccines for infectious diseases, while response suppression or tolerance induction against autoantigens may be the goal in therapy of autoimmune diseases. Systemic immune responses can be modulated via the gut associated lymphoid tissues (GALT) through oral administration of immunogenic or tolerogenic proteins. Immune stimulation by oral administration of antigen is safe and simple. Oral vaccines are advantageous in many aspects over vaccines parenterally administered. The gut mucosa forms a natural site for suppression or downregulation of the immune response against a.o. food antigens. This property may be taken advantage of for the treatment of autoimmune disease. It has been shown in rats and mice that it is possible to prevent experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis, by oral administration of myelin proteins identical to those used for disease induction (Hafler and Weiner, 1995; Whitacre *et al.*, 1996a). Clinical trials suggest that MS patients could benefit from oral administration of autoantigen (Weiner *et al.*, 1993).

The complexity of the antigen may be a major factor determining tolerance induction versus enhancement of the immune response. Soluble proteins are more likely to induce tolerance, whereas particulate antigens, like antigens expressed on the cell surface of bacteria tend to stimulate immune responses (Mondino *et al.*, 1996). But the modulatory effects of oral antigen administration also depend on other factors such as size, dose, immunization regimen of the antigen, the use of adjuvants and use of live or dead bacteria.

An important obstacle in mucosal immune modulation is the difficulty in establishing effective local dosages of the antigen or tolerogen in the gut as a result of the risk of proteolytic degradation of the antigen. To prevent this degradation of antigens in the stomach and to improve the efficiency of oral immunization, specific protective carrier systems can be used. Inert particles but also transformed bacteria expressing antigens or tolerogens can fulfill this purpose. Additional advantages of the latter are the local production of the antigens at the gut mucosa and the intrinsic adjuvanticity. However, many bacteria are potential pathogens and are therefore not suitable for use in humans. An attractive alternative is the use of *Lactobacillus* strains, which are gram positive commensals of the human gut and which are generally regarded as safe (GRAS status). In previous studies we have shown that orally administered lactobacilli can be very efficient antigen carriers as reflected by enhanced antibody responses against the hapten TNP conjugated to the lactobacilli (Gerritse *et al.*, 1990; 1991). Furthermore, gene transfer and expression systems for efficient production of foreign antigens by a variety of *Lactobacillus* species have been developed recently (Posno *et al.*, 1991b; Pouwels *et al.*, 1992). In addition, it has been shown that oral administration of lactobacilli can have immunostimulating

effects in humans (Isolauri *et al.*, 1995). Several dairy companies use *Lactobacillus* strains in a new generation of health foods, because of the putative health-stimulating properties resulting from their intrinsic adjuvanticity. It should be emphasized that the *Lactobacillus* genus consists of a wide variety of species that among others share the property of the production of lactic acid, but have a wide variety in fermentation pathways, growth characteristics and cell wall composition. Consequently, they differ in their intrinsic adjuvant properties, although the mechanism of adjuvanticity is not known.

In this study we investigated whether oral administration of two distinct *Lactobacillus* strains can be used to positively or negatively modulate the systemic immune response. In the first case we used a *Lactobacillus* strain with low intrinsic adjuvanticity as a vehicle for the antigen ß-galactosidase. ß-galactosidase was administered orally either as an expression product of *Lactobacillus plantarum 80* (intracellular) or dissolved in PBS. Antibody responses were measured after intraperitoneal immunization with ß-galactosidase.

In addition we investigated whether a *Lactobacillus* strain with high intrinsic adjuvanticity, *L. casei*, could promote systemic tolerance induction. The effect of oral administration of *L. casei* with and without co-administration of $PLP_{139-151}$ prior to EAE disease induction with the same peptide was studied. We discuss implications of our data for selection of appropriate *Lactobacillus* strains for vaccine versus tolerance induction approaches.

Materials and methods

Construction of Lactobacillus plantarum 80 *producing* E. coli *ß-galactosidase*

For the intracellular expression of ß-galactosidase by *Lactobacillus plantarum 80* (LP80), LP80 was transformed with pCBH72. To construct pCBH72, the promoter of the *xylR* gene in pLPCR2 (Carlsson and Bratthall, 1985; Forrest, 1988) was replaced by a fragment comprising the promoter of the conjugated bile acid hydrolase gene of *Lacto8bacillus plantarum 80* (LP80)(Christaens *et al.*, 1992) followed by insertion of the *E. coli lacZ* gene, encoding for ß-galactosidase into the multiple cloning site.

LP80 was cultured and transformed by electroporation as described previously (Christaens *et al.*, 1992). LP80 transformants were selected on solid MRS medium supplemented with 10 µg/ml erythromycin. ß-galactosidase production was confirmed in the lysate of LP80 transformants by standard gel electrophoresis, western blotting and an enzymatic assay. ß-galactosidase producing and non ß-galactosidase producing LP were designated $LP80^+$ and LP80-, respectively. The ß-galactosidase expression levels in $LP80^+$ were approximately 1-2% of total protein contents.

Oral administration of $LP80^+$ *and immunization with ß-galactosidase*

To assess whether oral administration of ß-galactosidase expressed intracellulary in *Lactobacillus plantarum 80* ($LP80^+$) induces suppression of a systemic antibody

response, six groups of three female BALB/c mice (10-12 weeks of age) received different doses of live LP80$^+$ (50, 100, 500 or 1000 µg dry weight) by intragastric intubation. As controls, wild type *Lactobacillus plantarum 80* (1000 µg dry weight LP80-) or PBS only were administered by intragastric intubation. For a continuous delivery of antigen during a period of ten days, lactobacilli were administered (500 µl) five times with a two day interval. At days 3 and 31 after the last oral administration all mice were immunized i.p. with 8 µg ß-galactosidase in 200 µl PBS. This amount is the lowest dose of antigen sufficient to mount an anti-ß-galactosidase serum Ig plateau level response after an i.p. booster injection in low dose ß-galactosidase primed mice (data not shown). Serum anti-ß-galactosidase responses were determined in a direct ELISA (see below).

Adoptive transfer of spleen cells
To assess whether, in an alternative experimental setting, the results which were obtained by oral administration of LP80$^+$ were an intrinsic property of the lymphoid cells of the systemic immune system, an adoptive transfer to naive animals was performed. Spleen cells from mice pre-treated as stated above were injected into naive irradiated mice. Mice were sacrificed by CO_2 euthanasia and the spleens were removed. Erythrocytes were removed by standard ammoniumchloride treatment. Cell suspensions of individual spleens (500µl) were injected i.v. into naive 5 Gy irradiated recipient BALB/c mice (10-12 weeks old). Two days after cell transfer the mice were immunized with 8 µg ß-galactosidase in 200µl PBS i.p. The sera were screened for anti-ß-galactosidase antibody reactivity in a direct ELISA.

ELISA
PVC microtiter plates were coated with ß-galactosidase in PBS (5 µg/ml, 50 µl/well) overnight at 4 C. Subsequently free sites were blocked for 30 min at 25 C with gelatin (0.5 mg gelatin/ml PBS, 50 µl/well) to prevent non-specific antibody binding. After washing with PBS-G (0.1 mg gelatin/ml PBS), the plates were incubated for 60 min at 25 C with dilutions of immune sera. The plates were washed with PBS-G and incubated with alkaline-phosphatase labelled goat anti-mouse total IgM + IgG antibodies (KPL, Inc., Gaithersburg) for 60 min at 25 C. The plates were washed with PBS-G and incubated with para-nitrophenyl phosphate (1 mg/ml 10 mM dietanolamine,1 mM $MgCl_2$, pH 9.8) as enzyme substrate. After incubation for 30 min at 25 C the absorption was determined at 405 nm.

Peptide synthesis, purification and characterization
The synthesis of the EAE-inducing PLP-peptide was carried out with 9-fluororenylmethoxycarbonyl (f-moc) protected amino acids, using routine solid phase synthesis method with an automated Milligen 9050 Continuous Flow Synthesizer (Millipore Co, Bedford, MA, USA). The PLP-peptide is a 13-mer amino

acid sequence analogous to residue numbers 139-151 of the rat PLP sequence: NH_2.His-Cys-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys-Phe-COOH. Side-chains were protected as follows: asparagine: 2,4,6-trimethoxybenzyl; histidine and cysteine: trityl; lysine: tertiar-butoxycarbonyl. Peptide cleavage and side-chain deprotection of the completed peptide were performed by trifluoracid treatment (Van Denderen *et al.*, 1987). Peptide purification was performed according to Shively (1986). Amino acid composition of the synthetic peptide was confirmed by HPLC analysis according to the method of Janssen *et al.* (1986).

Oral administration of $PLP_{139-151}$ and Lactobacillus casei ATCC393

To assess whether oral administration of wild type *Lactobacillus casei* together with $PLP_{139-151}$ has an effect on EAE, female SJL/J mice (12-15 weeks old, 8 mice per group) were pre-treated by oral administration of PBS only (group A), PLP peptide (group B and C), *Lactobacillus casei* (group D) or *Lactobacillus casei* and $PLP_{139-151}$ (group E and F). For a continuous delivery of antigens during a period of ten days, administration of suspensions or solutions (500 µl per administration) was repeated five times at two-day intervals. PLP-peptide was administered in two amounts: 100 µg (group B and E) and 500 µg (group C and F). *Lactobacillus casei* suspensions, administered to mice of group D, E and F, contained $5*10^8$ CFU per mouse per administration. EAE induction was performed three days after the last oral administration. The mice were followed until day 43.

EAE induction after oral pre-treatment

EAE induction was performed by s.c. injection of 150 µg $PLP_{139-151}$ emulsified in CFA (100 µl/injection site) at two sites in the abdominal flanks. The emulsion contained per 100 µl: 25 µg *Mycobacterium tuberculosis* (H37RA, Difco) in 50 µl CFA and 75 µg peptide in 50 µl PBS. In addition each mouse was injected i.v. on day 0 and 2, with 200 µl of a *Bordetella pertussis* suspension containing $2*10^{10}$ bacteria in PBS. The severity of EAE clinical signs was evaluated each day according to the following criteria: Disability scale (DAS) grade 0 = no clinical signs, grade 1 = tail paralysis, grade 2 = mild paraparesis and ataxia of the hind legs, grade 3 = severe paraparesis or ataxia of the hind legs, grade 4 = moribund, grade 5 = death due to EAE (Gerritse *et al.*, 1996).

Results

Oral administration of a Lactobacillus *strain with low adjuvant activity producing ß-galactosidase.*

To investigate the immunomodulating properties of lactobacilli overproducing an intracellularly expressed heterologous antigen, we fed BALB/c mice up to 1mg LP80 which either produce ß-galactosidase or not. After the first i.p. immunization of ß-galactosidase in pre-treated mice no specific anti-ß-galactosidase Ig-total serum

responses were observed. After the second immunization, anti-ß-galactosidase serum antibody responses were measured with a maximum at day 8 (*Figure 1*). A reduction in the anti-ß-galactosidase serum responses was observed after the second immunization with ß-galactosidase in all groups of mice orally pre-treated with ß-galactosidase containing lactobacilli (*Figure 1*). The anti-ß-galactosidase antibody responses of the animals which received 50, 100, 500 and 1000 µg transformed LP80$^+$ orally, were reduced with 64%, 51%, 27% and 39% respectively as compared to the anti-ß-galactosidase response measured in control animals which received identical doses of non-transformed LP80. In conclusion, lower doses of ß-galactosidase administered orally by a *Lactobacillus* strain appeared to have better downmodulating effects than higher doses.

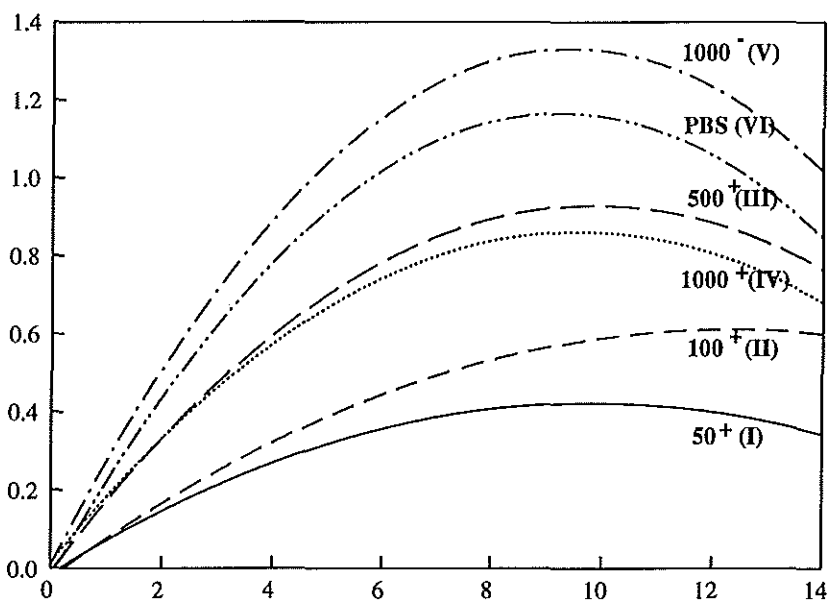

Figure 1. ***Anti-ß-galactosidase antibody responses after oral administration of LP80, a strain with low adjuvant activity.***
The anti ß-galactosidase serum antibody responses on day 0, 5, 8 and 14 after the second ß-galactosidase immunization were measured in direct ELISA. The results are expressed as the average absorbance at 405 nm of serum dilution 1/800 of three mice per group. The animals of the four test groups received 50, 100, 500 and 1000 µg *Lactobacillus plantarum* expressing ß-galactosidase (LP80$^+$) orally, prior to two i.p. ß-galactosidase immunizations spaced 30 days apart. The animals of the control groups received wild type *Lactobacillus plantarum* (LP80-) or PBS only, prior to two i.p. ß-galactosidase immunizations.

Post-transfer anti-ß-galactosidase serum antibody responses

To confirm whether the reduction of the anti-ß-galactosidase antibody response in the groups of mice pre-treated with LP80$^+$ was due to the intrinsic property of the lymphoid cells, spleen cells of these mice were transfered to naive mice. Mice which received spleen cells of donor mice pre-treated by oral administration of LP80$^+$ showed a decreased antibody response after a subsequent injection with ß-galactosidase in three out of four test groups, as compared to mice which received spleen cells from mice pre-treated with PBS. The lowest anti-ß-galactosidase responses were found in sera from mice, which received the spleen cells of donor mice orally pre-treated with a low dose of ß-galactosidase producing LP80$^+$ (100µg) (*Figure 2*). The highest anti-ß-galactosidase responses were observed in sera from the mice which received spleen cells of donor mice pre-treated with 500µg LP80$^+$. These donor mice showed the lowest reduction (27%) in anti-ß-galactosidase antibody responses after the second immunization with ß-galactosidase before transfer. In contrast to the other three groups, the anti-ß-galactosidase responses of this group of

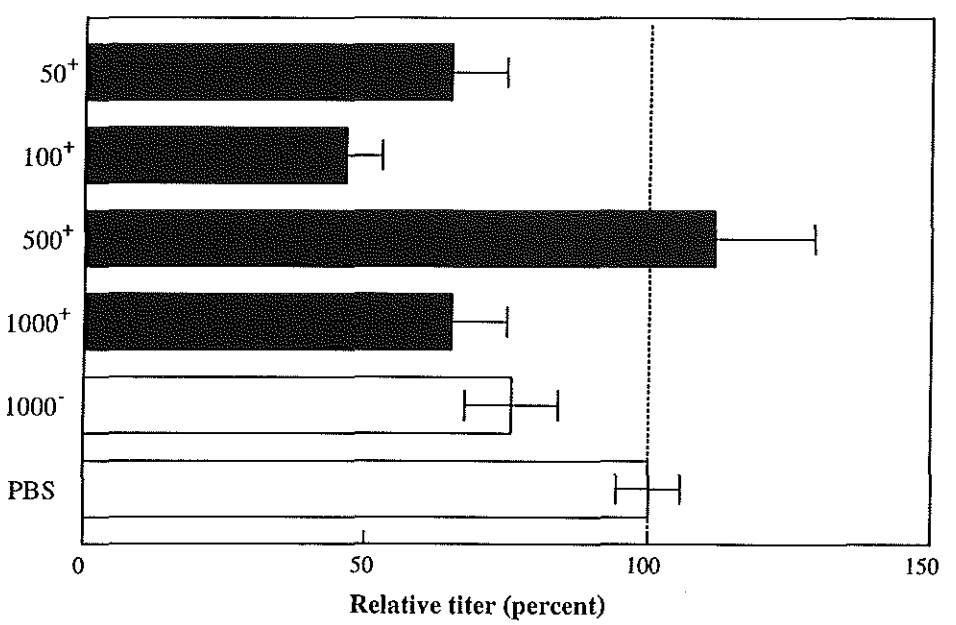

Figure 2. ***Relative anti-ß-galactosidase titers after adoptive transfer of spleen cells of mice orally pre-treated with LP80$^+$.***

Mice received spleen cells of mice pre-treated orally with LP80$^+$, LP80$^-$ or PBS followed by two i.p. immunizations of ß-galactosidase. The anti ß-galactosidase serum antibody responses on day 8 after post-transfer ß-galactosidase injection were measured in direct ELISA. The average titer of the control recipient animals which received the spleen cells of the PBS pre-treated donor animals was set at 100%. The results are expressed as the average of relative titers. Standard deviations are indicated.

recipient mice were not reduced as compared to the responses of both control groups of mice. These data partially confirm that the reduction of the immune response in mice pre-treated orally with lactobacilli expressing ß-galactosidase is due to an intrinsic property of the lymphoid spleen cells.

Role of high adjuvanticity of Lactobacillus casei *in EAE induction*
To investigate whether oral administration of a *Lactobacillus* strain with high intrinsic adjuvant activity can promote tolerance induction of orally administered antigen, we orally co-administered wild type *Lactobacillus casei* and $PLP_{139-151}$, prior to EAE induction with the same encephalitogenic PLP peptide. Oral administration of *Lactobacillus casei* only (group D) resulted in enhanced disease, as compared to the course of disease in animals which were not pre-treated with *Lactobacillus casei* (A, B and C) (*Figure 3*). Animals which were pre-treated with PBS only (group A) followed by EAE induction developed a moderate form of EAE, with the highest average DAS score (1.38) at day 31. The peak levels of the DAS scores of animals of groups B (1.63) and C (1.75), which were pre-treated by oral administration of different doses of 100 and 500 µg PLP-peptide respectively prior to EAE induction, were reached on day 34 and 32 respectively. During the entire course of the disease, the average DAS scores of the animals of groups B (100 µg $PLP_{139-151}$) and C (500 µg $PLP_{139-151}$) were slightly higher than the animals of group A (PBS) (*Figure 3*). The oral administration of $PLP_{139-151}$ simultaneously with *Lactobacillus casei* (groups E [100 µg]) and F [500 µg]) resulted in enhancement of EAE, comparable to administration of *Lactobacillus casei* only (E and F vs D) (*Figure 3*). Peak levels of average DAS scores of the animals of group E (3.14) and F (3.38) were slightly higher compared to group D (2.71) which received *Lactobacillus casei* only.

Discussion
We showed that oral administration of ß-galactosidase produced by a *Lactobacillus* strain, with low adjuvant properties, may induce a state of peripheral immune suppression, reflected by a reduced anti-ß-galactosidase antibody response after parenteral immunization with ß-galactosidase. This finding was confirmed in an adoptive transfer of the spleen cells to naive recipient mice. In contrast, the oral administration of a *Lactobacillus* strain with high adjuvanticity, together with an EAE inducing peptide, results in stimulation of the system immune system as is reflected by EAE disease enhancement. These findings demonstrate that dependent on their intrinsic adjuvant properties, oral administration of different *Lactobacillus* strains can have either immune stimulating or immune inhibiting effects.

The oral pre-treatment of BALB/c mice with the intact protein ß-galactosidase expressed within *Lactobacillus plantarum 80*, prior to i.p. immunization with ß-galactosidase, induced a reduction in anti-ß-galactosidase antibody responses. The observation that small amounts of orally administered antigen were more efficient in

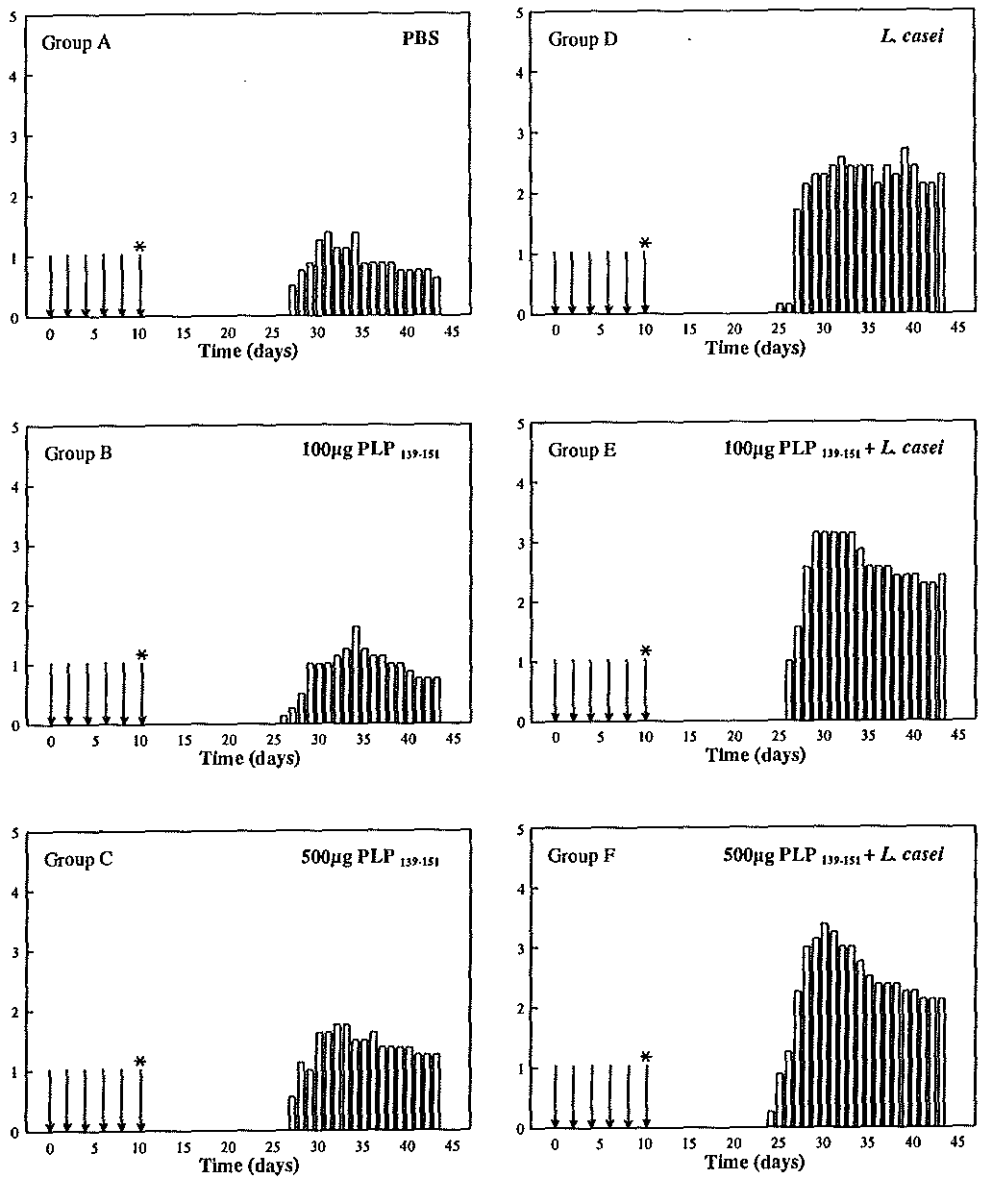

Figure 3. ***Role of high adjuvanticity of*** **Lactobacillus casei** ***in EAE induction***
Mice were orally pre-treated with *L. casei* (group D), *L. casei* together with $PLP_{139-151}$ (group E and F), $PLP_{139-151}$ only (group B and C) or PBS only (group A) prior to EAE induction with $PLP_{139-151}$. Oral administrations were performed on day 0, 2, 4, 6 and 8 (arrows). EAE was induced on day 10 (asterisk). The severity of clinical signs was evaluated every day according to the criteria as described in Materials and methods. The clinical signs are expressed as average DAS units per group per day.

inducing non-responsiveness as compared to large amounts of orally administered antigen suggests that low zone tolerance for ß-galactosidase exists (Hafler and Weiner, 1995). In the present experiments only a state of incomplete low zone tolerance was observed. This might be explained by differences in antigen processing of intact lactobacilli with cytoplasmic ß-galactosidase and free soluble ß-galactosidase derived from lactobacilli degraded in the gastro-intestinal tract. It is noteworthy that live lactobacilli were used, and therefore ß-galactosidase could also have been produced in the gut. The fact that the antigen probably was present in both particulate and soluble form, might have influenced immunological (un)-responsiveness.

Rigorously controlled conditions are required for *in vivo* modulation. To be able to evaluate up- and down modulatory effects of *Lactobacillus* strains on EAE disease course, a dose of the encephalitogenic peptide needs to be chosen, which induces a medium level of disease. To determine this dose, the intensity of disease was evaluated in a dose-response experiment with $PLP_{139-151}$ (data not shown). Oral pre-treatment of SJL mice with *Lactobacillus casei* (group D) prior to EAE induction resulted in a considerable enhancement of disease (*Figure 3*) as compared to the course of disease of animals which were orally pre-treated with PBS (group A). We expected that oral pre-treatment with soluble $PLP_{139-151}$ peptide alone would induce systemic immune tolerance in SJL mice. However, we did not find any reduction of the disease after oral pre-treatment with $PLP_{139-151}$ (group B and C). There even seems to be a slight enhancement of the disease. In the experiment with ß-galactosidase described here, it has been shown that, among other factors the dose is of importance for the outcome. In the EAE experiment the dose of antigen but also the treatment regimen could be responsible for the absence of down modulatory effects of $PLP_{139-151}$. When *Lactobacillus casei* was co-administered with $PLP_{139-151}$ (group E and F) prior to EAE induction, a marked enhancement of disease (*Figure 3*) was observed as compared to animals which received the PLP peptide only (group B and C). The severity of clinical signs was similarly enhanced when *Lactobacillus casei* were orally administered with or without peptide (group D vs E and F). Therefore the enhancement of disease is most likely due to the high adjuvant properties of this *Lactobacillus* strain.

The adjuvant properties of lactobacilli may influence whether the immune system is activated or tolerized, upon oral delivery of a particular *Lactobacillus* strain expressing antigen. This suggests that dependent on the aim of the specific application an appropriate *Lactobacillus* strain needs to be chosen with respect to its adjuvant properties. The results implicate that for tolerance induction a *Lactobacillus* strain with no or low intrinsic adjuvant activity is the best choice for antigen delivery. In contrast, for immune stimulating approaches, like vaccines for infectious diseases, *Lactobacillus* strains with high intrinsic adjuvanticity are better candidates. It is clear that some *Lactobacillus* strains can exert non-specific immunostimulatory effects (adjuvanticity) (Bloksma *et al.*, 1979). This seems mainly due to wall components of

the bacteria (Henderson *et al.*, 1996). However, lactobacilli do not produce LPS and the mechanism (component) of adjuvant activity of lactobacilli is still unknown.

Because immune-stimulating effects are easier to achieve with particulate antigens, we have constructed vectors for lactobacilli which are able to anchor (viral) antigens to the cell-surface of the lactobacilli. Probably, for the best immune stimulating results these vectors need to be transformed into *Lactobacillus* strains with high adjuvanticity. For vaccine applications vectors expressing virus epitopes on the cell-surface of the *Lactobacillus casei* have been made. In contrast, soluble antigens presented at the gut mucosa normally induce tolerance. We are currently developing *Lactobacillus* constructs which are designed to secrete the antigen, which could be applied to treat autoimmune disease. These vectors are being transformed into *Lactobacillus* strains with low adjuvanticity. The tolerizing properties of oral administration of low adjuving *Lactobacillus* strains excreting myelin proteins on EAE will be investigated. For further optimalisation of the immunization/tolerization protocols the expression levels of antigens expressed by the lactobacilli need to be controlled and standardized.

In conclusion, the results of this study indicate that lactobacilli are promising candidates for the delivery of immune stimulating signals for vaccine applications as well as for the induction of peripheral tolerance against putative autoantigens in human autoimmune diseases, depending on strain characteristics and expression levels of the (auto)antigens.

Chapter 2.2

Strain dependent induction of cytokine profiles in the gut by orally administered *Lactobacillus* strains

C.B.M. Maassen, J.C.P.A. van Holten, Fräncis Balk, M.J. Heijne den Bak-Glashouwer, R.J. Leer, J.D. Laman, W.J.A. Boersma and E. Claassen

Vaccine, in press

Summary

Different Lactobacillus *strains are frequently used in consumer food products. In addition, recombinant lactobacilli which contain novel expression vectors can now be used in immunotherapeutic applications such as oral vaccination strategies and in T-cell tolerance induction approaches for autoimmune disease. Both for food and clinical applications of lactobacilli, proper selection of wild type strains is crucial.*

For that purpose, eight different common Lactobacillus *strains were analysed with respect to mucosal induction of pro- and anti-inflammatory cytokines, IgA producing plasma cells in the gut, as well as systemic antibody responses against a parenterally administered antigen. Immunohistochemical analysis of cytokine producing cells in the gut villi showed no significant induction of the cytokines IL-1α, IFN-γ, IL-4 or IL-10 after oral administration of wild type Lactobacillus strains. In contrast, oral administration of* L. reuteri *and* L. brevis *induced expression of the pro-inflammatory/Th1 cytokines TNF-α, IL-2 and/or IL-1ß. Oral administration of these two strains and* L. fermentum *also significantly enhanced the IgG response against parenterally administered haptenated chicken gamma globulin (TNP-CGG). The five other strains did not show this adjuvanticity.* L. reuteri *induced relatively high levels of IgG2a compared to L. murines, a non-adjuving* Lactobacillus *strain.*

These findings imply that different Lactobacillus *strains induce distinct mucosal cytokine profiles and possess differential intrinsic adjuvanticity. This suggests that rational Lactobacillus strain selection provides a strategy to influence cytokine expression and thereby influence immune responses.*

Introduction

The capability of the mucosal immune system to act in both an immunogenic as well as in a tolerogenic manner protects our body against infection as well as inadvertent inflammation. The specific functions of mucosa-lined organs, e.g. the exchange of gasses (lung) and uptake of nutrients (gut), are essential and therefore the occurrence of destructive inflammatory immune responses at those sites is undesirable. Therefore, normally T-cell tolerance is induced against ingested food antigens. Nevertheless, the mucosal immune system is responsible for over 50% of the daily production of immunoglobulins (Mestecky and McGhee, 1987), reflecting continuous protective immune reactions. However, experimental induction of immune responses has proven difficult, as the precise nature of the parameters which determine the induction of such responses at the mucosa are poorly defined (Mestecky *et al.*, 1997). It is likely that local cytokine profiles may favour either Th1 type immunogenic responses or Th2 type tolerogenic responses (Gonnella *et al.*, 1998).

Vaccination against infectious diseases versus peripheral T-cell tolerance induction in autoimmune therapy upon oral antigen administration is thought to be at least partly dependent on particulate versus soluble nature of the antigen, respectively (Benson *et al.*, 1999; Fairweather *et al.*, 1990; Melamed *et al.*, 1996). It has previously been shown that lactobacilli with surface-linked haptens can induce an antibody response when administered orally (Gerritse *et al.*, 1990). For oral vaccine applications recombinant lactobacilli have now been constructed which express heterologous protein on the surface of the bacteria, which serve as a particulate antigen for induction of immune responses and B-cell memory (Maassen *et al.*, 1999a). Orally fed soluble autoantigens can induce peripheral T cell tolerance and thereby prevent the induction of EAE (experimental autoimmune encephalomyelitis), an animal model for multiple sclerosis (Higgins and Weiner, 1988; Whitacre *et al.*, 1991). In order to induce peripheral T-cell tolerance with the use of genetically engineered lactobacilli, recombinant lactobacilli have been designed to produce and locally secrete soluble autoantigens in medium or *in vivo* (Maassen *et al.*, 1999a).

Lactobacilli are frequently used in dairy products because of their health promoting effects such as the non-specific enhancement of the immune response (adjuvanticity), control of intestinal infections, control of serum cholesterol levels and anti-carcinogenic activity (Bloksma *et al.*, 1979). Oral administration of these diverse species of Gram-positive lactic acid bacteria with the generally regarded as safe (GRAS) status is economical and simple (Pouwels *et al.*, 1996). Since the bacterial properties required for the different applications are clearly distinct, strain selection is very important for wild type as well as recombinant lactobacilli. Therefore, in this study two properties of *Lactobacillus* strains were investigated in order to more rationally select appropriate *Lactobacillus* strains for use in oral vaccination and oral tolerance induction. First, the capability of *Lactobacillus* strains to induce specific cytokines at the mucosa by oral administration was determined in BALB/c mice,

which are Th2-cell biased (Sun *et al.*, 1997; Nishimura *et al.*, 1997). Mice were immunized i.p. with Chikungunya virus and fed eight different *Lactobacillus* strains for four days. Actual *in vivo* cytokine production was demonstrated immunohistochemically in gut tissue sections. That oral administration of an antigen can indeed lead to increased cytokine production in the gut has previously been shown with the model antigen ovalbumin (OVA) in an OVA T-cell receptor transgenic mouse (Gonnella *et al.*, 1998). Here we show that oral lactobacilli can influence local cytokine production after parenteral immunisation with a pathogen (Chikungunya virus) in BALB/c mice. Second, in order to assess whether orally administered *Lactobacillus* strains were able to non-specifically enhance the humoral response (adjuvanticity) and whether adjuvanticity correlated with the induced cytokine profiles, systemic antibody responses of mice immunised intraperitoneally with trinitrophenyl conjugated to chicken gamma globulin (TNP-CGG) were analysed.

Materials and methods

Bacteria

Eight *Lactobacillus* strains were used in this study. *Lactobacillus reuteri ML1*, *L. brevis ML12*, *L. gasseri ML21* and *L. murines CNRZ* were originally isolated from mouse. Strain *L. casei ATCC 393* was isolated from cheese and strain *L. plantarum ATCC 14917* was isolated from sauerkraut (Pouwels *et al.*, 1996). The strains *L. fermentum 104R* and *L. plantarum NCIB 8826* were isolated from pig and human saliva, respectively (Pouwels *et al.*, 1996).

Animals and antigens

Female BALB/c mice were purchased from Charles River Laboratories (Sulzfeld, Germany). These mice were kept under filtertop hoods. The mice were 6-10 weeks old at the start of the experiments. Experiments were performed according to regulations in the Dutch law on animal experimentation. BALB/c mice are Th2-biased, favoring antibody responses (Sun *et al.*, 1997; Nishimura *et al.*, 1997).

Trinitrophenyl (TNP) was conjugated to chicken gamma globulin (CGG)(Sigma Chemical, La Jolla, CA) according to the method described by Claassen and Van Rooijen (1984). TNP-CGG was used, as a soluble protein antigen, because the dose used without adjuvant will give a suboptimal immune response. Chikungunya virus was cultured and inactivated by UV according to the method described by Nakao and Hotta (1973). This pathogen was used since it is an ARBO (arthropod borne, insect vector) virus causing disease in man and animal, which is being widely used as a model for other ARBO viruses (Greiser-Wilke *et al.*, 1989).

For the detection of cytokines the following antibodies were used. Mouse antibodies against human IL-1α and IL-1β were obtained from the Instituto Ricerche Immunobiologiche Siena, Italy. The IL-2 specific rat MAb, S4B6, was a kind gift of

Dr. T. Mosmann, Department of Medical Microbiology and Immunology, University of Alberta, Canada (Mosmann *et al.*, 1986). The rat MAb 11B11, directed to IL-4, was a kind gift of Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD (Ohara and Paul, 1985). SXC-1-biotin, a rat MAb against IL-10, was obtained form Pharmingen (San Diego, CA). The murine Mab DB-1 directed to IFN-γ and rabbit serum against TNF-α were both obtained from Dr. P.H. van der Meide from the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands. All antibodies were crossreactive with their murine counterparts, but not with eachother. Goat anti Mouse-IgA-HRP (horseradish peroxidase) was obtained from KPL (Gaithersburg, MD). Streptavidin-HRP was obtained from Life Technologies (Gaithersburg, MD) and Swine anti Rabbit-Ig-HRP from Dako A/S (Glostrup, Denmark).

Oral administration of Lactobacillus *strains*
All strains were cultured in MRS broth (Difco, Detroit, MI) at 37°C until the OD_{690} was 1.1 (end log phase). The cells were harvested by centrifugation at 4000g for 20 min at 4°C, washed twice with PBS and once with 0.2M $NaHCO_3$. Before use, the cells were resuspended in 0.2M $NaHCO_3$ buffer (pH 8.4) to compensate for the acidic gastric environment. Each mouse received approximately 10^{10} CFU lactobacilli via the oral route. Administration was performed intragastrically.

Adjuvant activity of orally administered Lactobacillus *strains*
Eight different *Lactobacillus* strains were orally administered to groups of 3 mice for 4 consecutive days. On the first day the mice were also immunized intraperitoneally (i.p.) with 25μg TNP-CGG in PBS. Oral administration of lactobacilli was repeated on days 21, 22, 23 and 24. The booster immunisation with TNP-CGG was performed on day 21. Serum was collected on day 7 and 14 after prime and booster immunizations. Specific antibodies against CGG and TNP were detected by ELISA.

ELISA
Polyvinyl chloride (PVC) microtiter plates (Titertek, Flow Laboratories, Irvine, Scotland) were coated with CGG (5μg/ml, 50μl/well) or TNP-BSA (5μg/ml, 50μl/well) overnight at 4°C. To block non-specific antibody binding the plates were incubated for 1 hour with 0.2% gelatine in PBS (50μl/well) at room temperature. Subsequently the plates were incubated for 1 hour at 25°C with dilutions of TNP-CGG induced anti-sera and preimmune sera to correct for background reactivity. After washing, the plates were incubated with either alkaline phosphatase-labelled goat-anti mouse IgG or anti-IgM (H+L)(KPL). For the detection of CGG specific IgG1 or IgG2a antibodies, rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibodies (ICN Immunobiologicals, Costa Mesa, CA) were used respectively, followed by 1 hour incubation with alkaline phosphatase-labelled swine anti-rabbit Ig

antibodies (Dako A/S). After addition of the substrate paranitrophenyl phosphate, the absorbance was read at 405nm. An IgG1 monoclonal antibody directed against CGG was used as reference for detection of IgG1 CGG specific antibodies on each ELISA-plate. A polyclonal mouse serum containing high levels of IgG2a antibodies specific for CGG was used as reference in the ELISA's to detect IgG2a specific antibodies against CGG. Sera of mice immunized with TNP-CGG in specol (water-in-oil adjuvant) served as reference serum for relative concentrations of IgG specific antibodies against TNP and CGG.

Relative concentrations of IgG1, IgG2a and total IgG were calculated after subtraction of the absorbance of preimmune sera at the corresponding dilutions. The linear part of the reference curve was used to perform linear regression. Only those measurements of the test sera falling within the same absorbance range as reference samples used for regression (with comparable slope), were used for the calculation of the relative concentrations of IgG1, IgG2a and IgG in arbitrary units (a.u.).

Immunohistochemical detection of cytokine producing cells in gut villi induced by lactobacilli

Eight different *Lactobacillus* strains were orally administered to groups of 3 mice on days 0, 2, 4 and 6, in order to obtain a high continuous level of administered lactobacilli in the gastrointestinal tract during the experiment. On day 0 the mice were also immunized i.p. with 25µg UV-inactivated Chikungunya virus in PBS. One day after the last feeding the mice were euthanized. The first 10 cm of the small intestine containing the most Peyer's patches were taken out and rinsed in PBS. Swiss rolls were made by rolling up the small intestine around a match stick, whereby the terminal end is on the center of the role and the "stomach" end is on the outside. Subsequently, these swiss roles were frozen in liquid nitrogen.

Mouse cytokines were detected as described before (Herzenberg *et al.*, 1997). Briefly, frozen sections (-20°C) of 8 µm were thaw-mounted on silane coated glass slides and kept overnight under high humidity at RT. The next day, slides were air-dried for 1 hour and fixed at room temperature in acetone containing 0.02% H_2O_2. After air-drying for at least 10 minutes, sections were incubated with pre-determined optimal dilutions of primary reagents overnight at 4°C, in humidified atmosphere. Primary reagents were diluted in PBS containing 0.1% BSA. Incubations with secondary reagents diluted in PBS containing 1% BSA were done during 1 h at room temperature under high humidity. Between the incubation steps slides were washed with PBS. Biotinylated antibodies followed bij streptavidin-HRP were used for detection of IL-1α, IL-1β, IL-2, IL-4, IL-10 and IFN-γ. TNF-α was detected with swine anti rabbit-Ig-HRP conjugate. IgA containing plasmacells were directly detected with goat anti mouse-IgA-HRP. Incubation with AEC (3-amino-9-ethylcarbazole) substrate for 10 minutes was used to reveal HRP activity (bright red precipitate) (Jeurissen *et al.*, 1997). After washing, slides were counterstained with

hematoxylin and embedded with glycerol/gelatin. Primary antibody/reagent omission control stainings were performed for the different reagents used.

Quantitation was performed by counting cytokine positive cells by light microscopy. Comparable areas of gut villi were scored for positive cells by two independent observers blinded to treatment. The average number of positive cells per five villi per mouse was determined. Significance of differences between all groups were determined by a single factor anova. Only the significant differences of the *Lactobacillus* fed groups as compared to the $NaHCO_3$ fed group i.p. immunized with virus are indicated.

Statistical analysis

A single factor ANOVA was used to analyse the data. Since per group all mice were kept in one cage, it is allowed to use the t-test to calculate the least significant differences. When $p<0.05$ the difference was interpreted as significant. This approach was used to compare the total IgG antibody responses, the IgG1/IgG2a ratios and the number of cytokine positive cells.

Results

Adjuvant activity of orally administered Lactobacillus *strains*

In order to examine whether there is a difference between distinct *Lactobacillus* strains in their ability to influence the humoral immune response when administered orally to BALB/c mice, which are Th2-cell biased (Sun *et al.*, 1997; Nishimura *et al.*, 1997), eight different strains were tested. Seven days after booster immunisation TNP and CGG specific IgG titers were determined in sera of mice treated according to the immunisation protocol. No differences could be detected between the levels of IgM antibodies against CGG and TNP 7 days after prime and booster immunization. Differences in specific IgG titer were interpreted as adjuvant effects of *Lactobacillus* strains (*Figure 1*). Although most of the tested strains did not show any effect as compared to the control group immunized with TNP-CGG which received $NaHCO_3$ orally, *L. reuteri* significantly enhanced the specific CGG antibody response ($p<0.01$). *L. reuteri* appeared to be significantly different from all groups, except from *L. brevis* (significance not indicated in *Figure 1B*). When the same sera were tested for the presence of TNP specific antibodies, again *L. reuteri* ($p<0.01$), but also *L. brevis* ($p<0.01$) and *L. fermentum* ($p<0.05$) showed significant enhancement of the humoral response as compared to the TNP-CGG immunized group of mice which orally received $NaHCO_3$ only (*Figure 1A*). This indicates that some orally administered *Lactobacillus* strains, but not others, can non-specifically enhance the humoral response against a parenterally immunized antigen (adjuvanticity).

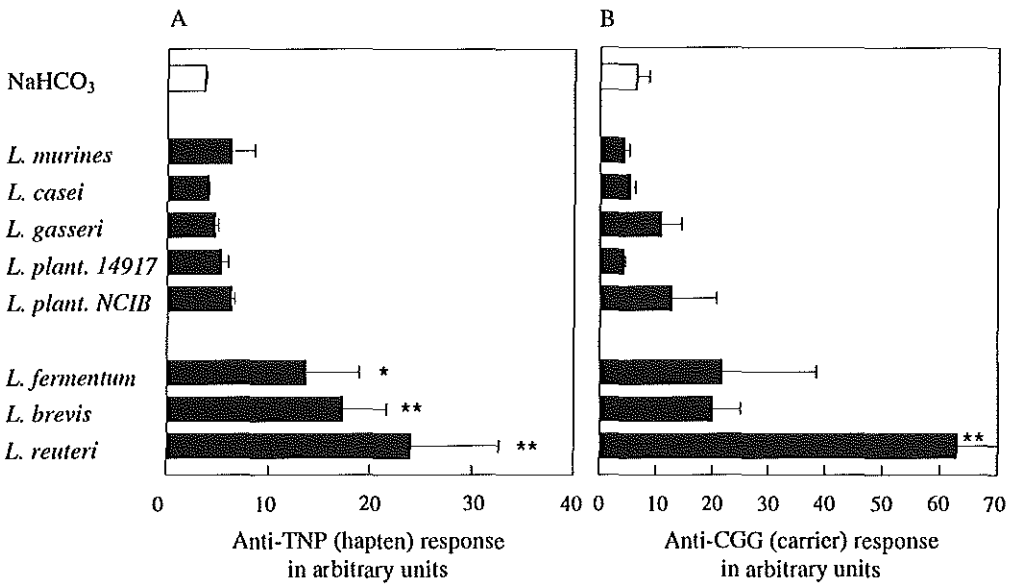

Figure 1. ***Adjuvant activity of orally administered*** **Lactobacillus** ***strains.***
The effect of orally administered *Lactobacillus* strains on the antibody response against the thymus dependent antigen TNP-CGG administered intraperitoneally was analyzed by ELISA. The mice received a *Lactobacillus* strain or $NaHCO_3$ only orally on 4 consecutive days. On the first day the mice were also immunized i.p. with TNP-CGG in PBS. From day 21 on this protocol was repeated. The left panel shows the mean IgG antibody response ± SEM against TNP per group in arbitrary units 7 days after the second immunization. The right panel shows the IgG antibody response ± SEM against the carrier protein CGG. Only significant differences compared to the $NaHCO_3$ group are indicated by asteriks. No antibody responses were detected in mice orally fed $NaHCO_3$ and immunized with PBS (data not shown).
*: $p<0.05$, ** : $p<0.01$, *plant.: plantarum*

IgG1/IgG2a ratio after oral administration of Lactobacillus *strains*

It was demonstrated that the quantity of the antibody response evoked against the parenterally immunized antigen CGG-TNP could be enhanced by some of the orally administered *Lactobacillus* strains. Since it is generally accepted that the IgG1 response reflects activity of Th2 CD4+ T-cells and IgG2a is a reflection of Th1-activity (see Martin and Lew, 1998; O'Garra, 1998 and references therein), the difference in the quality of the immune response was determined by analysing those antibody isotypes. The IgG1 and IgG2a responses against CGG were detected after oral administration of 4 *Lactobacillus* strains. The antibody responses were expressed as IgG1/IgG2a ratios *(Figure 2)*. Only the antibody response against CGG was detected since the IgG2a responses against TNP were in most cases too low to be calculated. Oral administration of *L. reuteri* and *L. plantarum NCIB* induced relatively high IgG2a levels against CGG when compared to the control group which was fed buffer only, although the differences did not reach significance. The reduced IgG1/IgG2a ratio was significant compared to oral administration of *L. murines*,

which slightly increased the IgG1/IgG2a ratio. The IgG1/IgG2a ratio induced by *L. murines* was also significantly higher than after parenteral immunization of CGG-TNP in specol, a water-in-oil adjuvant. The ratio detected after oral administration of *L. casei* was comparable to the ratio of the control group fed buffer and the group fed *L. murines*.

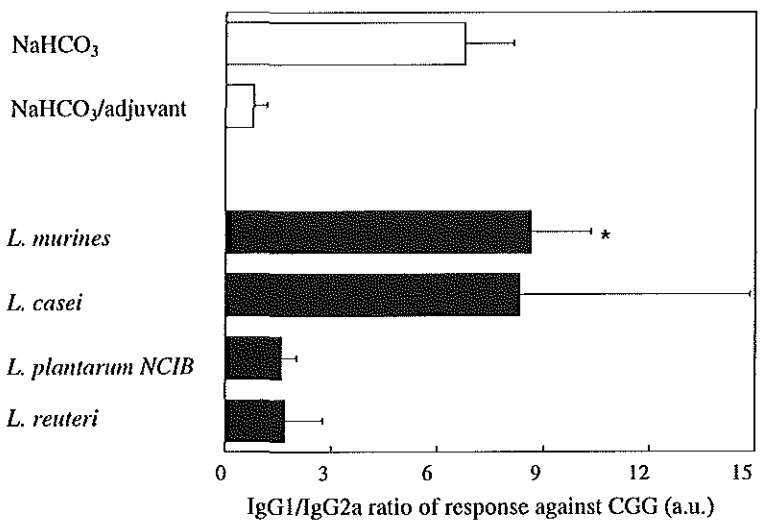

Figure 2. ***IgG1/IgG2a ratio of anti-CGG response after oral administration of*** **Lactobacillus** ***strains.*** The effect of 4 orally administered *Lactobacillus* strains on the IgG1/IgG2a ratio of the response against CGG was analyzed by ELISA. The mice received a *Lactobacillus* strain or $NaHCO_3$ only orally on 4 consecutive days. On the first day the mice were also immunized i.p. with TNP-CGG in PBS. A control group was fed $NaHCO_3$ and immunized i.p. with TNP-CGG in specol ($NaHCO_3$/adjuvant). From day 21 on this protocol was repeated. Seven days after the second immunization the IgG1 and IgG2a responses against CGG were calculated in arbitrary units with the use of reference curves. The mean IgG1/IgG2a ratio per group ±SEM is shown. *: $p<0.05$ compared to the groups fed *L. plantarum NCIB, L. reuteri* and the group immunized with TNP-CGG in adjuvant.

Immunohistochemical detection of IgA and cytokines in gut villi

To investigate whether orally administered *Lactobacillus* strains are able to induce cytokines and IgA at the mucosa, BALB/c mice were immunized i.p. with Chikungunya virus and fed wild type lactobacilli for four days. Seven days after administration of the inactivated pathogen the animals were sacrificed and frozen gut tissue sections were analysed for IgA and cytokine production. In *Figure 3A* the production of IgA in this primary immune response is shown in a complete cross-section of the duodenum including a Peyer's patch (lower right corner). In all animals we could find massive amounts of IgA in the B (plasma) cells located centrally in the villi (*Figure 3B*). Hardly any antibody producing B-cells were seen in the Peyer's patches. As *Figure 3B* shows, the lamina propria lymphocytes were the ones exclusively involved in antibody production. No IgA containing cells were seen in the

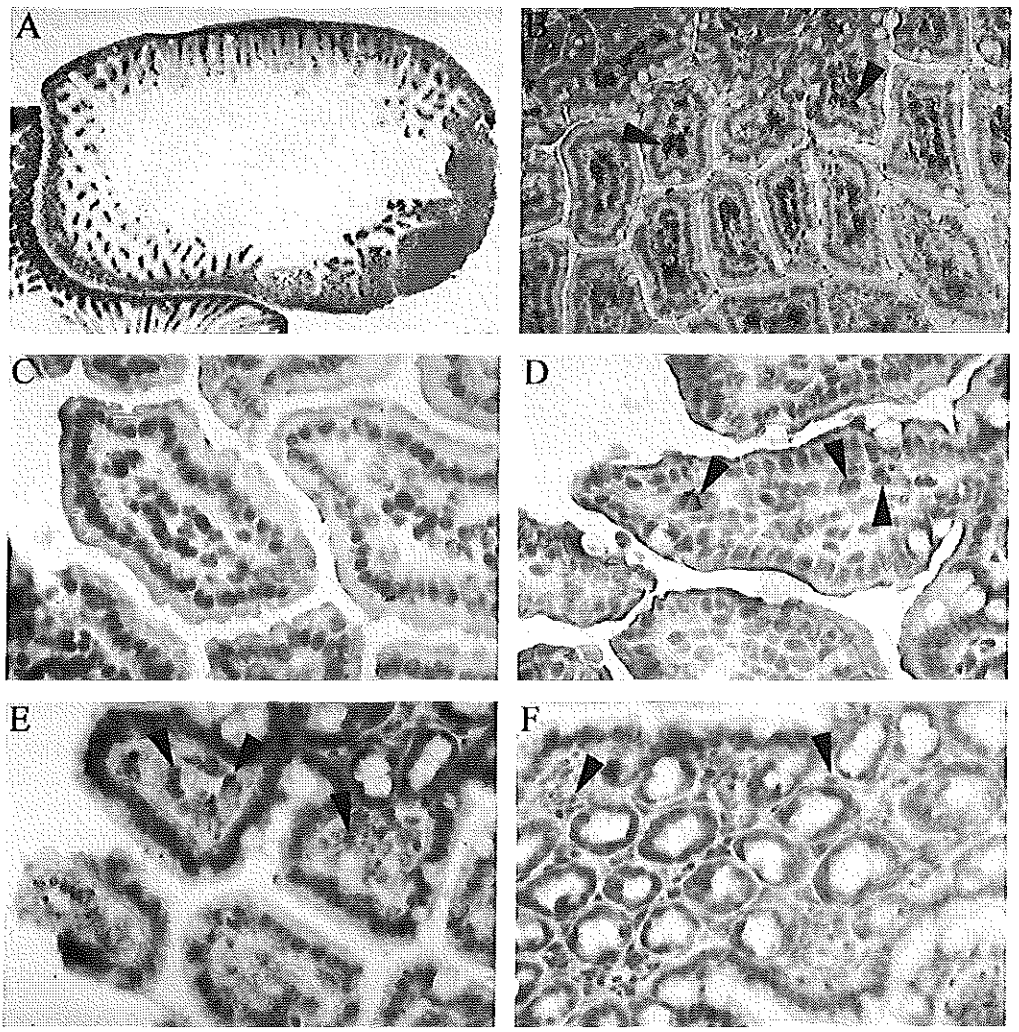

Figure 3. ***Immunohistochemical detection of IgA and cytokine-producing cells in gut villi***

A) IgA producing cells in the duodenum of a mouse immunised with Chikungunya virus.

B) Lamina propria plasma cells stained for IgA of a mouse fed with *L. reuteri* and immunised with Chikungunya virus.

C) Absence of IL-2 producing cells in gut villi stained for IL-2 of a mouse immunised with PBS and fed $NaHCO_3$.

D) IL-2 producing cells in gut villi of a mouse immunised with Chikungunya virus and fed $NaHCO_3$.

E) TNF-α producing cells in the lamina propria of a mouse fed *L. reuteri* and immunised with Chikungunya virus.

F) Localisation of TNF-α positive cells in submucosa of mouse described in E.

Some of the positively stained cells are indicated by arrows. For color figures see the original paper.

outer rim of the villi. *Figure 3B* also gives a clear visual indication of the maximum number of cells per villus (8-10) that can be observed for both IgA production as well as for cytokine production by T-cells (9-12, see also *Figure 3E*).

The background number of positive cells was different for all cytokines, as shown in a quantitative way in *Figure 4*. *Figure 3C* demonstrates that for IL-2 no cytokine producing cells are found when the animals are given $NaHCO_3$ orally and PBS intraperitoneally (no virus). It should be noted that sections on the same glass slide included positive tissue controls (van den Eertwegh *et al.*, 1991) for IL-2. Brown (not red) rims circumfering the villi were found in all samples stained for IL-2 with AEC (for revelation of horseradish peroxidase activity), even in the absence of IL-2 positive cells, representing non-specific staining.

After intraperitoneal immunisation with Chikungunya virus, but without oral pretreatment with lactobacilli, IL-2 positive cells could be demonstrated in the lamina propria of the villi (identical to the localisation pattern of IgA) as indicated by arrows (*Figure 3D*). As is clear from *Figure 3D* the cells were not always homogeneously distributed over the tissue when relatively small numbers (0.2-2 on average per villus) are found.

When antigen was given in conjuction with oral lactobacilli, the number of cytokine producing cells increased in a strain-dependent manner (*Figure 4 and 5*). Most cytokine producing cells (±10 per villus) were found for TNF-α in *L. reuteri* treated animals as shown in *Figure 3E*.

In the submucosa, where Brunner's glands are located, TNF-α was also found in the endothelial cells after oral administration of *L. reuteri* (*Figure 3F*). This localisation pattern was not observed for any of the other cytokines studied.

Quantitative analysis of cytokine producing cells in gut villi induced by lactobacilli

In order to investigate whether *Lactobacillus* strains are able to differentially induce cytokines at the gut mucosa after oral administration in Chikungunya-immunized mice, the frequency of cytokine producing cells was determined. The levels of IgG and IgM antibodies against Chikungunya virus in sera obtained at sacrifice, were too low to be correlated to the found cytokine profiles. The cytokines IL-1α, IL-1ß, IL-2, IL-4, IL-10, IFN-γ and TNF-α were immunohistochemically analysed by counting the number of cytokine producing cells in gut villi. The background level of positive cells differed for each cytokine. Of the cytokines tested, only IL-4 was hardly detectable in all treatment groups, despite the fact that positive control tissue, (TNP-ficoll reactive spleen) contained numerous IL-4 producing cells (van den Eertwegh *et al.*, 1991; 1993). The rest of the tested cytokines can be roughly divided into two groups. Some cytokines are not induced by oral administration ofany of the tested *Lactobacillus* strains when compared to the control group which wasimmunized i.p. with virus and orally received $NaHCO_3$ only (group $NaHCO_3$) (*Figure 4*). Other cytokines are induced by one or more *Lactobacillus* strains (*Figure 5*). Cytokines that were not induced are IL-1α, IL-4, IFN-γ and IL-10. A low level of

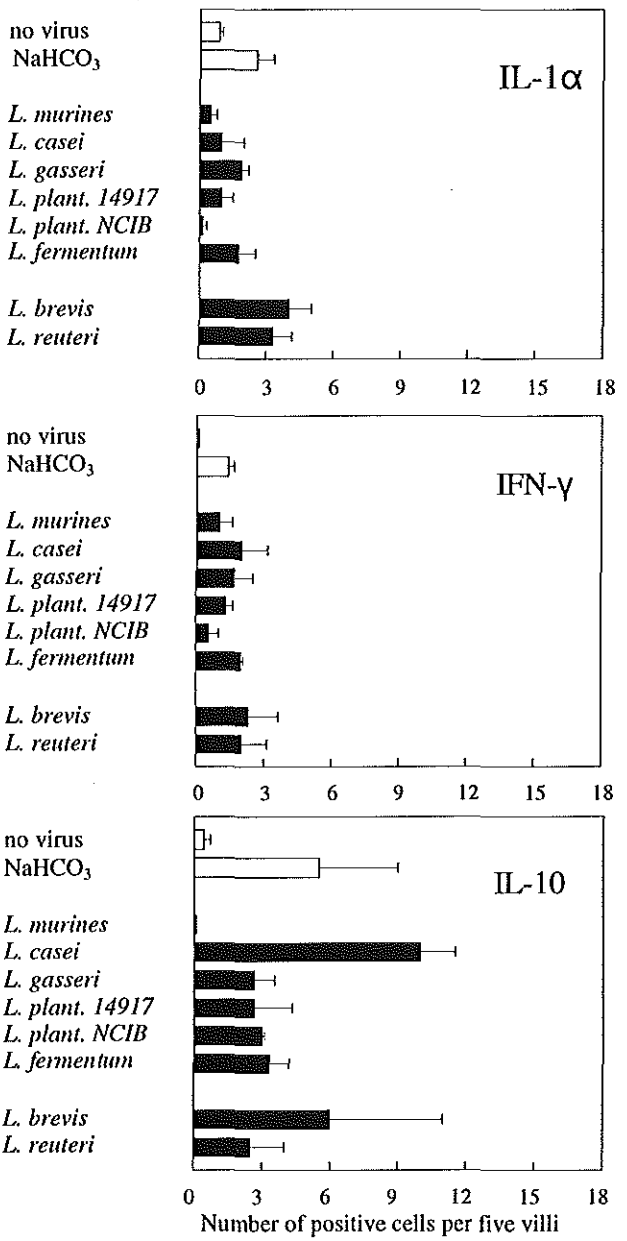

Figure 4. ***Quantitative analysis of background cytokine producing cells in gut villi.***
A quantitation on the immunohistochemically stained sections from mice immunized with Chikungunya virus and fed different *Lactobacillus* species was performed. Animals in the control groups were fed $NaHCO_3$ only and were immunized with TNP-CGG ($NaHCO_3$ group) or PBS (no antigen). Sections of the duodenum were stained for the cytokines IL-1α, IFN-γ and IL-10. The mean number of positive cells per five villi per group ± SEM is shown.

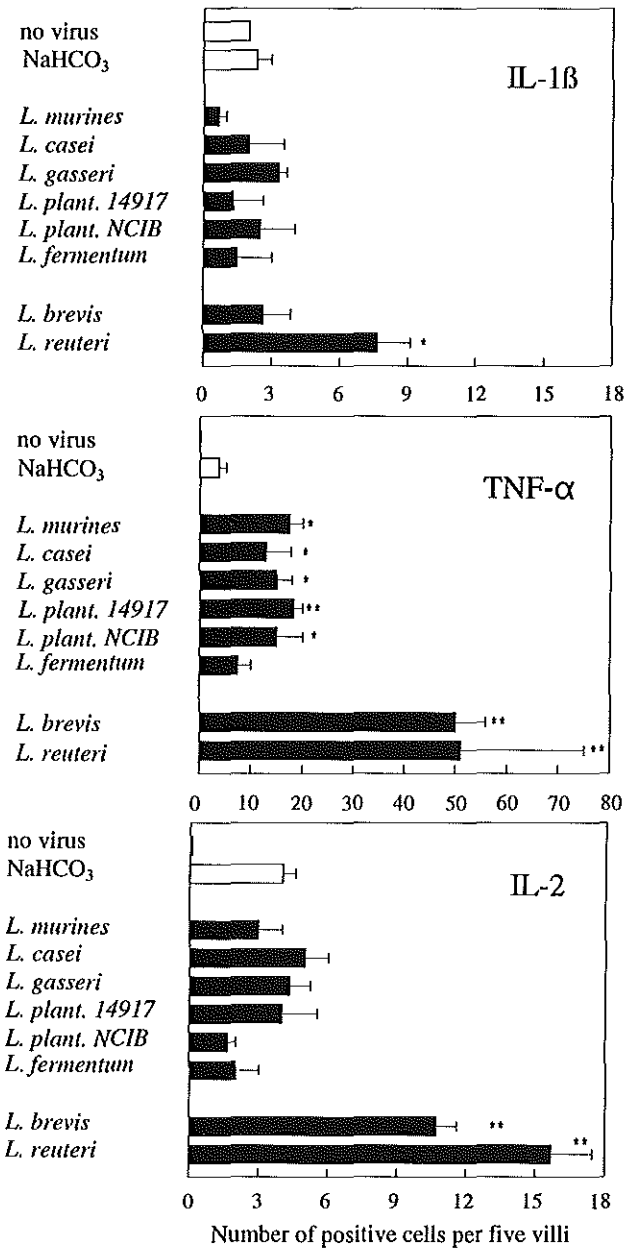

Figure 5. ***Quantitative analysis of cytokine producing cells in gut villi induced by lactobacilli.***
A quantitation on the immunohistochemically stained sections from mice immunized with Chikungunya virus and fed different *Lactobacillus* species was performed. Animals in the control groups were fed $NaHCO_3$ only and were immunized with TNP-CGG ($NaHCO_3$ group) or PBS (no antigen). Sections of the duodenum were stained for the cytokines IL-1ß, TNF-α and IL-2. The mean number of positive cells per five villi per group ± SEM is shown. Only significant differences compared to the $NaHCO_3$ fed group are indicated by asterisks. *: $p<0.05$, **: $p<0.01$

IFN-γ and IL-1α positive cells could be detected in all mice, but there was no significant difference with the $NaHCO_3$ fed group (*Figure 4*). For IL-10 we observed that i.p. immunisation with virus only (group $NaHCO_3$) increased the frequency of IL-10 producing cells above background (no virus), although this difference did not reach significance (*Figure 4*). No further enhancement by oral administration of lactobacilli was observed. Only *L. casei* tended to further increase IL-10 producing cells as compared to the virus immunized group which received $NaHCO_3$ orally (group $NaHCO_3$), but this increase was not significantly different either. The group of cytokines that can be induced by oral administration of wild type *Lactobacillus* strains consists of IL-1ß, IL-2 and TNF-α (*Figure 5*). An enhancement of TNF-α levels in the gut villi could be detected after oral administration of all of the tested *Lactobacillus* strains, except for *L. fermentum,* when compared to the group fed $NaHCO_3$ only (group $NaHCO_3$)(*Figure 5*). *L. reuteri* and *L. brevis* both showed an approximately 15-fold increase in the number of TNF-α positive cells. Like TNF-α, IL-2 is already induced in the gut by parenteral immunisation with Chikungunya virus alone (compare group 'no virus' with group '$NaHCO_3$')(see *Figure 3D*), but is significantly further increased by oral administration of *L. reuteri* and *L. brevis (Figure 5). L. reuteri* is also able to induce IL-1ß (*Figure 5*). Clearly, there are *Lactobacillus* strains which are able to induce an inflammatory cytokine profile (*L.reuteri, L. brevis*).

Discussion

This study shows that orally administered wild type *Lactobacillus* strains can induce differential cytokine profiles in the gut of Th2-biased BALB/c mice. The *Lactobacillus* strains *L. reuteri* and *L. brevis* induce several of the proinflammatory and/or Th1 cytokines IL-1ß, IL-2 and TNF-α but not anti-inflammatory Th2 cytokines such as IL-10 and IL-4 (*Figure 4*). These same *Lactobacillus* strains were able to significantly enhance the systemic antibody response against a parenterally immunized antigen. In the current dogma, Th1 cells stimulate IgG2a antibodies production regulated by IFN-γ, whereas IgG1 antibodies are induced under control of the Th2 cytokine IL-4. Analysis of the antibody isotypes induced after oral administration of *L. reuteri* revealed that high IgG1 as well as high IgG2a levels could be detected. However, after oral administration of *L. reuteri* the IgG1/IgG2a ratio of antibodies directed against CGG were (significantly) lower than the IgG1/IgG2a ratios after oral administration of buffer or non-adjuving *Lactobacillus* strains. These results indicate that *L. reuteri* and probably also *L. brevis* are inducers of the cellular as well as humoral responses. Most of the tested *Lactobacillus* strains did not affect the systemic humoral immune response and showed antibody levels similar to the control group that orally received $NaHCO_3$ only. A possible explanation is that these strains act as natural gut commensals of the BALB/c mice and do not disturb the existing immune status. This is in accordance with the observation that lactobacilli are not or hardly immunogenic (Maassen *et al.*, 1999c). Possibly, the *Lactobacillus*

strains that induce IL-2 and high levels of IL-1ß and TNF-α and enhance the humoral immune response do evoke an immune response against themselves, leading to local inflammation. It should also be kept in mind that mucosal cytokine induction may not be directly linked to systemic antibody secretion. That there can be a relation between mucosal cytokine production and systemic antibody responses has been demonstrated by e.g. Marinaro *et al.* (1999) and Shi *et al.* (1998b).

Most cytokines are differentially regulated, which may result for instance in the presence of the Th1 cytokine IL-2 without the presence of IFN-γ, which is also a Th1 cytokine (van den Eertwegh *et al.*, 1992). In addition, due to different kinetics of expression of the cytokines, analysis at another timepoint may show induction of other cytokines, such as IFN-γ. Also IL-1α and IL-1ß appear to be under separate transcriptional control, which may lead to expression of one form of IL-1 without the other form (Dinarello, 1998).

Most *Lactobacillus* strains induce TNF-α, although there is a strain dependent difference in the number of TNF-α containing cells which is induced. This has also been seen after parenteral injection of cell wall components of *L. casei*, where *L. casei* components led to coronary arteritis due to activation of macrophages, which produced mainly IL-1 and TNF-α (Lehman *et al.*, 1988; Okitsu-Negishi *et al.*, 1996; Tomita *et al.*, 1993). *In vitro,* an increased production of IL-1 and TNF-α or IFN-γ by macrophages exposed to various strains of *Lactobacillus* could be demonstrated, as well as enhanced phagocytosis (Perdigon *et al.*, 1986; Rangavajhyala *et al.*, 1997; Kitazawa *et al.*, 1994). From these studies, it seems that components in the cell wall of lactobacilli are responsible for this activation. That this is not necessary due to the peptidoglycan, a major cell wall component of Gram-positive bacteria, was demonstrated by de Ambrosini *et al.* (1996) who showed that after oral administration of peptidoglycan of four different Gram-positive bacteria only the *L. casei* cell wall preparation could activate macrophages. Although no double staining was performed to determine which cells produce the detected cytokine, due to results obtained in other studies it is likely to assume that the induced TNF-α and IL-1ß are produced by macrophages. Whether also IL-10 was produced by macrophages can not be excluded. TNF-α containing cells were located in the villi like all other cytokine producing cells. In addition to the TNF-α positive cells in the villi, TNF-α was also found to be produced by cells in the submucosa after oral administration of *L. reuteri*, but not after administration of other *Lactobacillus* strains. Also in humans with appendicitis, TNF-α can be found in the submucosa (Wang *et al.*, 1996).

Although inflammatory/Th1 cytokine inducing *Lactobacillus* strains and non-cytokine inducing strains were identified, our panel did not contain a Th2 cytokine inducing strain despite the fact that a Th2-biased mouse strain was used. Only *L. casei* tended to induce IL-10 and TGF-ß. Although TGF-ß was clearly found to be high for *L. casei* and *L. murines*, the TGF-ß data in general were not always clear to interpret and are therefore not shown. IL-10 and TGFß have

immunosuppressive effects on Th1 cells and are thought to be involved in oral tolerance (Miller *et al.*, 1992b). An IL-10/TGF-ß cytokine profile in the lamina propria can be found in ovalbumin (OVA) T cell receptor transgenic mice after oral administration of OVA, leading to T-cell tolerance towards OVA (Gonnella *et al.*, 1998). From our panel of *Lactobacillus* strains, no *Lactobacillus* strain emerged, inducing a local cytokine environment permissive for oral tolerance induction.

As mentioned above, orally administered lactobacilli can activate macrophages. The known tumor-suppressive activity of lactobacilli is thought to be due to the cytotoxic action of TNF-α and IL-1 produced by macrophages (Kato *et al.*, 1994; Nanno *et al.*, 1989; Pool-Zobel *et al.*, 1996; Matsuzaki *et al.*, 1990; 1996; Perdigon *et al.*, 1995). It seems that most of the tested *Lactobacillus* strains do have this anti-tumor property, but not all. Also many orally administered *Lactobacillus* strains, like *L. casei, L. reuteri, L. bulgaricus* and *L. acidophilus* seem able to protect against infections (Alak *et al.*, 1997; De Simone *et al.*, 1988; Kabir *et al.*, 1997; Perdigon *et al.*, 1990; 1991; Wagner *et al.*, 1997). Intrinsic properties of different *Lactobacillus* strains can play a role in the effectiveness of the treatment. Therefore, the choice of the *Lactobacillus* strain is of major importance. Properties that can play a role are the capability to induce cytokines in the gut and stimulate antibody responses (adjuvanticity), but also adhesion and colonization properties of the strains. Even the growth phase of the strain can be of importance, since we have shown that log phase versus stationary phase cultures differentially influence the IgG1/IgG2a ratio of systemic antibody responses against protein antigen dependent on the growth phase (Maassen *et al.*, 1999c).

Lactobacilli can also be used for oral vaccination and immunotherapeutic purposes in autoimmune diseases. Therefore, recombinant lactobacilli were developed which express bacterial or viral antigens on the cell surface (Maassen *et al.*, 1999a). These immunogenic particles can be used as oral vaccines. In contrast, the soluble autoantigen secreted by other recombinant lactobacilli probably will induce peripheral T-cell tolerance and protect against experimental autoimmune diseases like experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (Maassen *et al.*, 1999a). For oral tolerance induction with a recombinant *Lactobacillus* strain secreting autoantigens, a wild type strain without adjuvanticity but able to induce Th2 cytokines might be optimal. Such a strain did not emerge from the current analyzed panel. On the other hand, the *Lactobacillus* strains *L. reuteri* and *L. brevis* are good candidates for the expression of immunogenic antigens for oral vaccination purposes since they may stimulate innate, cellular and humoral immune responses.

Acknowledgements

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Chapter 2.3

Growth phase of orally administered *Lactobacillus* strains differentially affects T helper-cell pathways for soluble antigens: Implications for vaccine development

C.B.M. Maassen, W.J.A. Boersma, J.C.P.A. van Holten, E. Claassen and J.D. Laman

Submitted for publication

Summary

Lactobacillus *strains with probiotic activity are major constituents of numerous common food products. Due to their 'generally regarded as safe'-status,* Lactobacillus *strains can also be genetically engineered for use in oral immunotherapeutic applications, such as vaccination and T lymphocyte tolerance induction in autoimmune disease.*

In the current study we demonstrate that the growth phase of orally administered individual Lactobacillus *strains can differentially affect systemic activity of T helper cell type 1 (Th1) and T helper 2 (Th2) pathways supporting antigen-specific antibody formation (IgG2a versus IgG1 subclass, respectively). Mice were orally fed different wild type Lactobacillus strains in log phase or stationary phase and immunized intraperitoneally with a T-cell dependent protein antigen. Sera were evaluated for the ratio of antigen-specific IgG1 and IgG2a antibodies. Stationary* Lactobacillus murines *and* Lactobacillus casei *cultures, but not two other* Lactobacillus *strains, evoked significantly higher IgG1/IgG2a ratios than log phase cultures, implying increased activity of the Th2-pathway. Despite normal variation in antibody responses against TNP-CGG among individual mice, a high correlation was found between the IgG1 and IgG2a responses of mice within experimental groups. This differential activation of Th-pathways is likely due to growth phase-dependent differences in bacterial cell composition.*

As growth phase-dependent skewing of T-cell pathways may inadvertently affect allergic and (auto)-immune responses, the current findings strongly caution against unidimensional views on the oral administration of individual Lactobacillus *strains for probiotic or immunotherapeutic purposes, but also suggest additional possibilities for immune modulation.*

Introduction

The genus *Lactobacillus* belongs to the family of Lactobacteriaceae, also known as lactic acid bacteria, a group of microorganisms that have been used for centuries in bio-processing and preservation of food and feed. Some fermented dairy products containing *Lactobacillus* species are believed to have certain health promoting properties for humans. This assumption was originally based upon Metchnikoff's theory (Metchnikoff, 1908) that harmful effects of undesired bacteria can be overcome by establishing a new balance between intestinal bacteria, through ingestion of lactobacilli or fermented products made by these organisms. Research carried out during the last few decades has resulted in additional claims of health and/or nutritional benefits for humans and animals associated with the consumption of fermented milk products. Such claims include; control of intestinal infections, control of serum cholesterol levels, enhancement of immune responses (adjuvanticity), and anti-carcinogenic activity (Perdigon *et al.*, 1995; Pouwels *et al.*, 1996).

Because lactobacilli are commensals of the gut and are generally regarded as safe (GRAS-status)(Maassen *et al.*, 1999a), lactobacilli are good candidates for use as production and delivery hosts of heterologous proteins in food but also health care products. Genetically engineered lactobacilli expressing bacterial or viral proteins on the cell surface can potentially be used as oral vaccines (Maassen *et al.*, 1999a). On the other hand, *Lactobacillus* recombinants secreting tolerogenic autoantigens can be used to induce systemic peripheral T-cell tolerance by oral administration for treatment of autoimmune disease (Maassen *et al.*, 1999a).

Due to the diversity of species within the *Lactobacillus* genus not each *Lactobacillus* strain possesses (all) of the above mentioned health stimulating properties. For instance, it has been shown that *Lactobacillus* strains differ in their ability to enhance the humoral immune response (adjuvant activity) and that they induce distinct cytokine profiles in the gut after oral administration (Maassen *et al.*, 1999b). This implies that strain selection is a crucial issue for the use of wild type *Lactobacillus* strains in food products, but also when recombinant lactobacilli are to be used to promote different types of immune responses such as vaccination, T-cell tolerance induction and treatment of allergy.

Most immune responses are centrally regulated by the activity of CD4-positive T helper (Th) cells. In mice and humans, two functionally polarized responses of Th cells can be distinguished mediated by Th1 versus Th2 cells (reviewed by Romagnani, 1997). The distinction depends on the profile of cytokines which these Th cell subsets secrete. Th1 cells produce proinflammatory cytokines like interleukin 2 (IL-2), IL-12, tumor necrosis factor alfa (TNF-α) and interferon gamma (IFN-γ). Th1 cells promote cellular immune responses including macrophage activation, delayed type hypersensitivity (DTH) and cytotoxicity. Th1 cells positively contribute to the humoral (antibody) response to a limited extent by supporting the production of the IgG2a antibody subclass, but they inhibit production of several other subclasses like IgG1. Although the Th1 pathway is crucial to protection against many pathogens, it

may also be the predominating type of response in chronic inflammatory autoimmune diseases like multiple sclerosis and insulin-dependent diabetes mellitus (Romagnani, 1997; Laman *et al.*, 1998b; O'Garra, 1998).

Th2 cells produce IL-4, IL-5 and IL-10, which are generally regarded to be anti-inflammatory. These cytokines are involved in stimulating strong antibody production by B cells (e.g. IgG1, IgM and IgE), stimulation of eosinophils and mast cells, as well as deactivation of macrophages. Dominant Th2 responses may contribute to diseases such as atopic allergy. In animal studies, the IgG1/IgG2a ratio is an accepted and convenient marker for differential Th2-Th1 reactivity (see O'Garra, 1998; Martin and Lew, 1998 and references therein).

A multitude of approaches to skew T helper cell pathways to treat human diseases is under intense investigation (Weiner, 1997). When oral biotherapy with wild type *Lactobacillus* strains or genetically engineered recombinants (Maassen *et al.*, 1999a) is considered, the way these strains impinge on T helper pathways is an important factor determining successful application. Therefore, the aim of the present study was to establish whether orally administered individual *Lactobacillus* strains can differentially affect T-helper cell pathways as reflected in IgG1 (Th2) versus IgG2a (Th1) antibody responses against systemically administered exogenous protein antigen, and whether such T-helper cell skewing is dependent on the growth phase of the lactobacilli.

Materials and Methods

Animals and Lactobacillus strains

Female SJL mice (Erasmus University Rotterdam, The Netherlands) were kept under filtertop hoods in a DII facility with free access to mouse chow and acidified water (pH 2.8). Experiments were performed according to regulations in the Dutch law on animal experimentation.

The *Lactobacillus* strains *L. reuteri ML1* and *L. murines CNRZ* were originally isolated from mouse. The strains *L. casei ATCC 393* and *L. plantarum NCIB 8826* were originally isolated respectively from cheese and human saliva.

Culturing of Lactobacillus strains

All *Lactobacillus* strains were cultured overnight, in 5 ml MRS broth followed by 15 ml MRS broth (Difco, Detroit, MI), before inoculation in 500ml MRS broth (1:50). The cultures were grown to late log phase (6 hours) or stationary phase (16 hours). The cells were harvested, washed twice with PBS and once with 0.2M $NaHCO_3$. This extensive washing was performed to ensure no secreted *Lactobacillus* products were present in the suspension. Before use the cells were resuspended in 0.2M $NaHCO_3$. From this suspension a sample was taken for plating on MRS broth plates for the calculation of the number of colony forming units (CFU) as well as to rule out contamination.

Oral administration of Lactobacillus strains and immunisation schedule
To investigate the effect of orally administered lactobacilli on the humoral immune response, mice orally received wild type lactobacilli, prepared as described above, on four consecutive days. Each animal received approximately 10^{10} CFU per intragastric administration. On the first day the mice were also immunized intraperitoneally with 25 µg of the hapten trinitrophenyl (TNP) conjugated to the thymus dependent antigen chicken gamma globulin (CGG) (Sigma Chemical, La Jolla, CA). One control group was immunized with TNP-CGG in a water-in-oil adjuvant, specol (Bokhout *et al.*, 1981), which resembles Freund's incomplete adjuvant. From day 49 onwards oral administration of lactobacilli as well as intraperitoneal immunisation with TNP-CGG was repeated as above. This day was chosen as titers had declined to below 50% of maximal values. Blood samples were taken before the start of the experiment and every 7 days.

Semi-quantitative ELISA
Serum titers of CGG and TNP specific antibodies after immunisation with TNP-CGG were determined by ELISA. Polyvinyl chloride (PVC) microtiter plates (Titertek, Flow Laboratories, Irvine, Scotland) were coated with CGG (5µg/ml, 50µl/well) or TNP-BSA (5µg/ml, 50µl/well) overnight at 4°C. Non-specific antibody binding was blocked by incubation with 0.2% gelatin in PBS (50µl/well) for 1 hour at 25°C. Subsequently the plates were incubated for 1 hour at 25°C with dilutions of TNP-CGG induced antisera and preimmune sera to correct for background reactivity. For the detection of IgG antibodies specific for CGG or TNP, alkaline phosphatase-labelled goat anti-mouse IgG (KPL, Gaithersburg, MD) was used. For the detection of CGG or TNP specific IgG1 or IgG2a antibodies, rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibodies (ICN Immunobiologicals, Costa Mesa, CA) were used respectively, followed by 1 hour incubation with alkaline phosphatase-labelled swine anti-rabbit Ig antibodies (Dako A/S, Glostrup, Denmark). After addition of the substrate paranitrophenyl phosphate, the absorbance was read at 405nm. An IgG1 monoclonal antibody directed against CGG was used as reference for detection of IgG and IgG1 CGG specific antibodies on each ELISA-plate. A polyclonal mouse serum containing high levels of IgG2a antibodies specific for CGG was used as reference in the ELISA's to detect IgG2a specific antibodies against CGG. The reference used in all TNP-specific ELISA's was a polyclonal mouse serum with high levels of IgG1 and IgG2a TNP-specific antibodies.

Relative concentrations of IgG1, IgG2a and total IgG were calculated after subtraction of the absorbance of preimmune sera at the corresponding dilutions. The linear part of the reference curve was used to perform linear regression. Only those measurements of the test sera falling within the same absorbance range as reference samples used for regression (with comparable slope), were used for the calculation of the relative concentrations of IgG1, IgG2a and IgG in arbitrary units (a.u.).

To detect antibody responses against *Lactobacillus murines* after oral

administration, PVC plates were coated with fragmented *L. murines* (50ng/ml, 50µl/well). The fragments were obtained by sonification of cells harvested after overnight growth in MRS broth. The following incubation steps and development were performed as described for total IgG. Serum obtained after intraperitoneal immunisation of mice with *L. casei* in adjuvant (Difco's complete adjuvant, Difco) was used as reference.

Statistical analysis

A single factor ANOVA was used to analyse the data. Because the groups of mice were too large to be held in one cage, it was calculated whether there were differences between cages holding mice of the same group. Since this was not the case, the t-alfa for student's t-test could be used to calculate the least significant differences instead of the F-alfa of the F-test. When $p<0.05$ the difference was interpreted as significant. This approach was used to compare the total IgG antibody responses and the IgG1/IgG2a responses of the groups on one day (*Figure 1* and *Figure 2*). When the IgG1/IgG2a ratio's were compared over time (7, 14 and 21 days after the second immunisation), linear regression was performed on the data of each individual animal. The same procedure as used for the single data points was applied to the regression lines (*Figure 5*). Differences between the intercepts indicate different IgG1/IgG2a ratio's. Distinct slopes indicate difference in change of the ratio's over time. Linear regression was performed to calculate the correlation lines in *Figure 4*.

Results and Discussion

Four wild type Lactobacillus *strains do not enhance systemic antibody responses*

In order to investigate whether different *Lactobacillus* strains have different intrinsic adjuvanticity (defined as the capability to non-specifically enhance the antibody response), four wild type *Lactobacillus* strains were orally administered to SJL/J mice (n=5). SJL/J mice are biased towards the Th1 pathway and are susceptible to induction of experimental autoimmune diseases such as EAE (experimental autoimmune encephalomyelitis), a model for multiple sclerosis. The bacteria were grown to log phase (6h) or stationary phase (16h) to investigate whether the intrinsic adjuvanticity is growth phase dependent. The mice received one of the wild type *Lactobacillus* strains, *L. plantarum NCIB, L. reuteri, L. murines* or *L. casei* in $NaHCO_3$ orally on four consecutive days. The control groups received $NaHCO_3$ only. On the first day, the mice were also immunized i.p. with TNP-CGG in PBS. One control group was immunized with TNP-CGG in adjuvant. The immunisation with TNP-CGG as well as the oral administration of lactobacilli was repeated from day 49 onwards. Total IgG antibody responses against CGG were determined in sera collected 14 days after the second immunisation (*Figure 1*). A reference curve was used to calculate the arbitrary units (a.u.). None of the tested *Lactobacillus* strains exhibited intrinsic adjuvanticity in SJL mice, as no significant differences between groups fed *Lactobacillus* strains and the control group immunized with TNP-CGG in PBS and fed with $NaHCO_3$ only were

seen. These data are consistent with results obtained with log phase *Lactobacillus* strains in the Th2-biased BALB/c mouse strain, except for *L. reuteri* (Maassen *et al.*, 1999b). This strain did enhance the antibody response against CGG in BALB/c mice. In addition, no significant differences between log and stationary phase lactobacilli fed groups were present in SJL/J mice, indicating that in this Th1-biased mouse strain, the growth phase of these *Lactobacillus* species does not affect adjuvant activity. As expected, the control group immunized with TNP-CGG in adjuvant showed a significant and strong increase in CGG-specific antibody levels when compared to all other groups ($p<0.01$). Total IgG responses against TNP were lower but comparable to the anti-CGG responses.

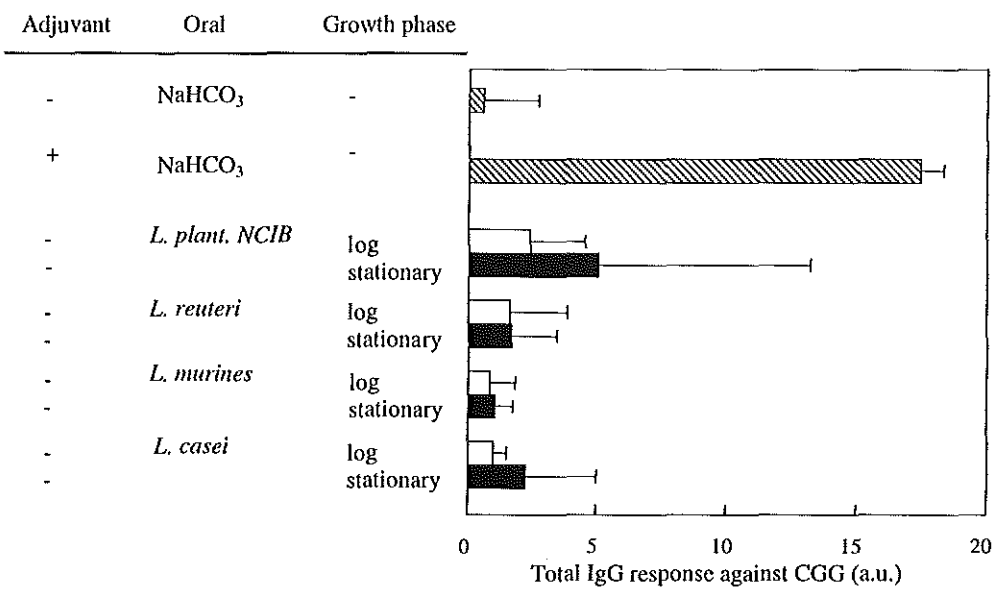


Figure 1. ***Four wild type*** **Lactobacillus** ***strains do not enhance systemic antibody responses***
Total IgG antibody responses against CGG were determined in sera of mice immunized i.p. with TNP-CGG in PBS (n=5). One control group was immunized with TNP-CGG in adjuvant. The mice received wild type lactobacilli in $NaHCO_3$ orally on four consecutive days. The lactobacilli were grown to log phase (white bars) or stationary phase (black bars). The control groups received $NaHCO_3$ buffer only (hatched bars). The immunisation with TNP-CGG as well as the oral administration procedure was repeated from day 49 onwards. Results (± SEM) obtained with sera collected 14 days after this second immunisation are shown. A reference curve was used to calculate the arbitrary units.

Two wild type Lactobacillus *strains skew the IgG1/IgG2a ratio dependent on growth phase*

Although no significant differences occurred in the total IgG response of mice fed log phase or stationary phase cultures of the same *Lactobacillus* strain, the antibody response might be qualitatively different. It is generally accepted that the IgG1 response reflects activity of Th2 CD4+ T-cells, where IgG2a results from Th1-activity

(see O'Garra, 1998; Martin and Lew, 1998 and references therein). Therefore the IgG1 and IgG2a responses against CGG were calculated for sera obtained 14 days after the second immunisation and were expressed as the IgG1/IgG2a ratio (*Figure 2*). Oral administration of a stationary phase culture of *L. casei* or *L. murines* lead to a significantly higher IgG1/IgG2a ratio than log phase bacteria of the same strain ($p<0.01$). Although *L. plantarum NCIB* induced a similar pattern of IgG1/IgG2a ratio's, the difference did not reach significance. *L. reuteri* did not show a difference in IgG1/IgG2a ratio between log and stationary phase cultures. Because of the differences found between *Lactobacillus* strains, it can be concluded that this isotype skewing is growth phase- as well as bacterial strain-dependent. Although the anti-TNP IgG2a responses in many cases were too low to calculate the IgG1/IgG2a ratio, the ratio's that were calculated showed a similar trend as the ratio's calculated for anti-CGG (data not shown).

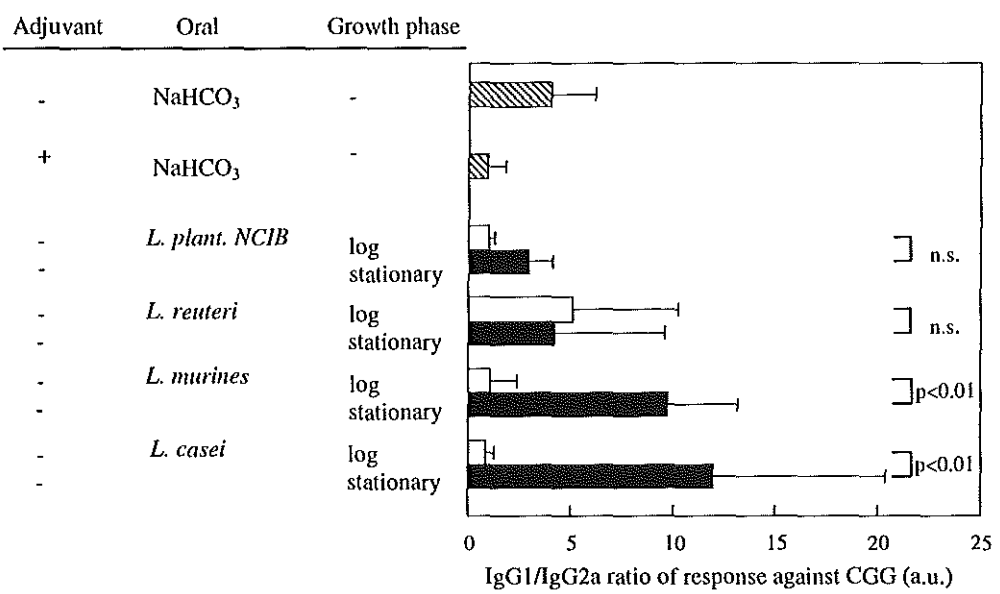


Figure 2. ***Two wild type*** **Lactobacillus** ***strains skew the IgG1/IgG2a ratio dependent on growth phase***
Mice (n=5) were immunised i.p. with TNP-CGG in PBS and fed with wild type *Lactobacillus* strains in log (white bars) or stationary phase (black bars). The control groups orally received $NaHCO_3$ only (hatched bars) and were immunised i.p. with TNP-CGG in PBS or adjuvant. The IgG1 and IgG2a responses were calculated in arbitrary units with the use of reference curves. The ratio between IgG1 and IgG2a antibodies specific for CGG was calculated for sera obtained 14 days after the second immunisation with TNP-CGG (±SEM). Significance of differences observed between groups of mice fed log or stationary phase cultures of the same *Lactobacillus* strain is indicated.

No systemic antibody response against L. murines *after oral administration*
From the previous experiments it was clear that some *Lactobacillus* strains are able to skew the Th1 and Th2 response dependent on their growth phase. In addition to *L. casei,* also *L. murines* showed a growth phase-dependent difference in IgG1/IgG2a ratio. *L. murines* was selected for further investigation. One of the rationales to use lactobacilli for oral vaccination or other therapeutical purposes is the fact that they are commensals of the human gut (Perdigon *et al.*, 1986; Pouwels *et al.*, 1993) and are generally regarded as safe (GRAS-status). In addition, they should be non-immunogenic reflected in an inability to evoke antibody responses against themselves. To evaluate this in the current experimental setting, a similar experimental design as in *Figures 1* and *2* was used. Total IgG and IgM responses of SJL/J mice (n=8) against *L. murines* were determined by ELISA in pooled sera obtained 21 days after the second intraperitoneal immunisation with TNP-CGG and oral administration of *L. murines*. *Figure 3* shows that, as expected, the treatment protocol did not induce IgG responses against *L. murines* itself, nor IgM (data not shown). In contrast, when separate mice were immunized intraperitoneally with *L. murines* in adjuvant as a positive control group, high IgG responses against *L. murines* itself occurred. These data confirm that oral administration of *L. murines* does not induce antibody responses against the bacteria, and imply that growth phase-dependent skewing of the TNP-CGG specific IgG1/IgG2a ratio is not related to an antibody response against the bacteria.

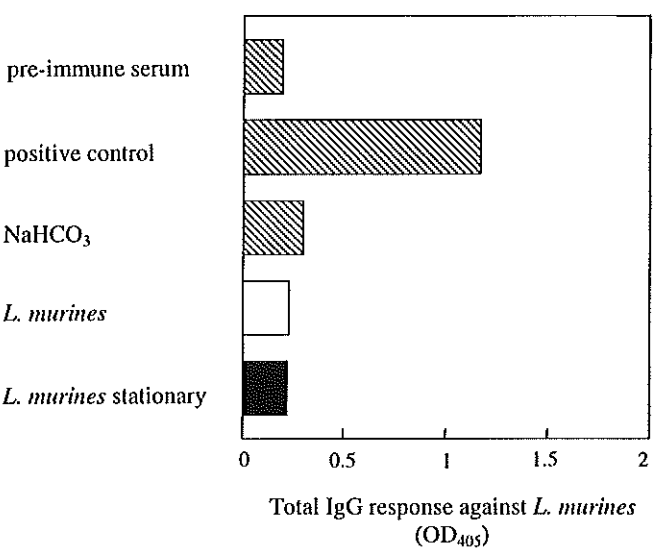


Figure 3. ***No systemic antibody response against*** **L. murines** ***after oral administration***
The total IgG antibody response against *L. murines* was determined after oral administration of the lactobacilli to mice (n=8) in sera obtained 21 days after the second immunization with TNP-CGG. The anti-*L. murines* antibody response is indicated in absorbance at 405 nm of a 1:100 dilution of the pooled sera. Pooled pre-immune serum was used as negative control serum and serum obtained from mice immunized i.p. with *L. casei* in adjuvant was used as reference serum.

L. murines *of different growth phases induce distinct but constant IgG1/IgG2a ratio's*
The skewing of IgG1/IgG2a ratio's as depicted in *Figures 1* and *2* was further confirmed in the experiment described above (*Figure 3*). The total IgG responses against CGG and TNP were similar to the data presented in *Figure 1*, although the total IgG response against CGG after oral administration of *L. murines* in stationary phase to mice (n=8) was more pronounced (data not shown). From the previous experiments (*Figures 1* and *2*) it was apparent that the IgG1/IgG2a ratio is independent of the level of the total IgG response. This raised the question whether the IgG1/IgG2a ratio is also independent of the level of the IgG1 and IgG2a response per animal, i.e. whether the ratio is constant. Therefore, in *Figure 4* the IgG1 response of individual animals is depicted against the IgG2a response of the same animal, with each circle representing an individual animal. Because comparable observations were made for time points 7, 14 and 21 days, only data are shown from groups which received *L. murines* in log or stationary phase 21 days after the second immunization.

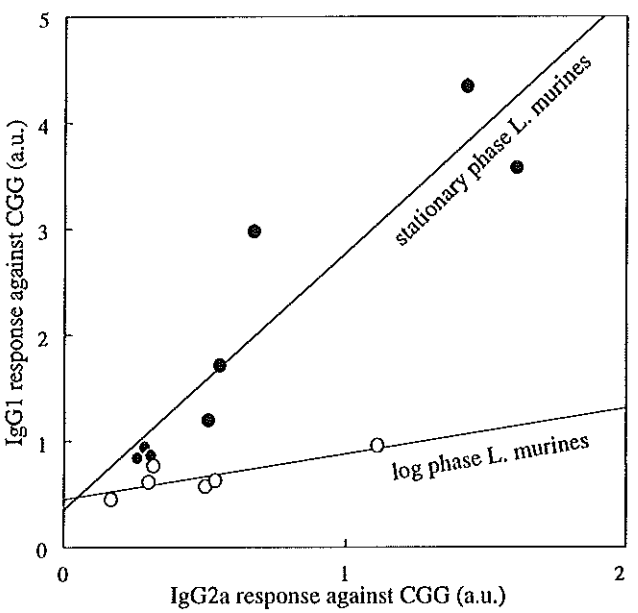


Figure 4. L. murines *of different growth phases induce distinct but constant IgG1/IgG2a ratio's*
Mice (n=8) were fed *L. murines* in stationary or log phase. Sera obtained 21 days after the second immunisation with TNP-CGG were tested for IgG1 and IgG2a antibody responses against CGG. IgG1 and IgG2a responses are indicated in arbitrary units which were calculated with the use of reference curves. The IgG2a response of two animals in the group fed log phase *L. murines* was too low to be calculated. These mice were excluded from the analysis to prevent undue further emphasis on the existing clear difference between the groups. Each circle represents an individual animal. Mice fed log phase *L. murines* are indicated by O and mice fed stationary phase *L. murines* are indicated by ●. The correlation lines were calculated by linear regression. The variance is 0.67 for the group fed log phase *L. murines* and 0.85 for the group fed stationary phase cultures. R= 0.43 for the group fed log phase cultures and R= 2.43 for the group fed stationary phase cultures.

Two conclusions can be drawn from this figure: First, there is a strong correlation between the IgG1 and IgG2a response of each individual animal within an experimental group (variance = 0.67 for the group fed log phase cultures and variance = 0.85 for the group fed stationary phase cultures), despite considerable differences in IgG1 and IgG2a responses between the animals. This strong correlation indicates that the IgG1/IgG2a ratio of individual animals within an experimental group is highly comparable. This was demonstrated not only for the groups fed *L. murines* but also for the control groups fed buffer only. Second, the different slopes of the correlation lines of the groups fed log phase (R=0.43) or stationary phase (R=2.43) *L. murines* indicate that the IgG1/IgG2a ratio's between those groups are different (see *Figure 2* and *Figure 5*), confirming that the growth phase of orally administered lactobacilli differentially affects T-cell pathways.

IgG subclass skewing by stationary phase L. murines *decreases but persists over time*
In order to investigate whether the antigen specific IgG1/IgG2a ratio's persist over time, the ratio between IgG1 and IgG2a antibodies specific for CGG was calculated for sera obtained at 7, 14 and 21 days after the second intraperitoneal immunisation of mice (n=8) with TNP-CGG. *Figure 5A* shows the mean IgG1/IgG2a ratio of responses against CGG in mice fed *L. murines* in log phase and *Figure 5B L. murines* in stationary phase. When the ratio's were compared over time, the group fed *L. murines* in log phase showed a significantly lower IgG1/IgG2a ratio than the stationary phase fed group (intercept difference with $p<0.05$). This is consistent with the data presented in *Figure 2*. Furthermore, the IgG1/IgG2a ratio decreased significantly with time when *L. murines* in stationary phase were administered (R= -1.04), in contrast to a comparable IgG1/IgG2a ratio over time when *L. murines* in log phase was given (R= -0.07) (slope difference with $p<0.05$). This change in IgG1/IgG2a ratio over time was not seen in the control groups (R=0.06 for the buffer fed group and R=0.02 for the group immunized with TNP-CGG in adjuvant).

Perspectives
This study shows that orally administered individual *Lactobacillus* strains are able to differentially affect CD4-positive T helper-cell pathways supporting systemic antigen-specific antibody production. Stationary phase cultures skewed T-cell responses to the Th2 pathway as reflected by increased production of the IgG1 subclass. This skewing was evident even while the Th1-biased SJL/J mouse strain was used. The most likely explanation for these findings is the occurrence of growth phase-dependent differences in bacterial cell wall composition, for which there is ample prior evidence, including proinflammatory compounds (Davies *et al.*, 1991; Derre *et al.*, 1999; Graumann *et al.*, 1999; Henderson *et al.*, 1996; Hu and Coates, 1999). Another not mutually exclusive explanation is that log phase versus stationary lactobacilli differentially affect dendritic cell populations (DC1 versus DC2), which govern directional activation and polarization of T-cell subsets during antigen presentation (Rissoan *et al.*, 1999).

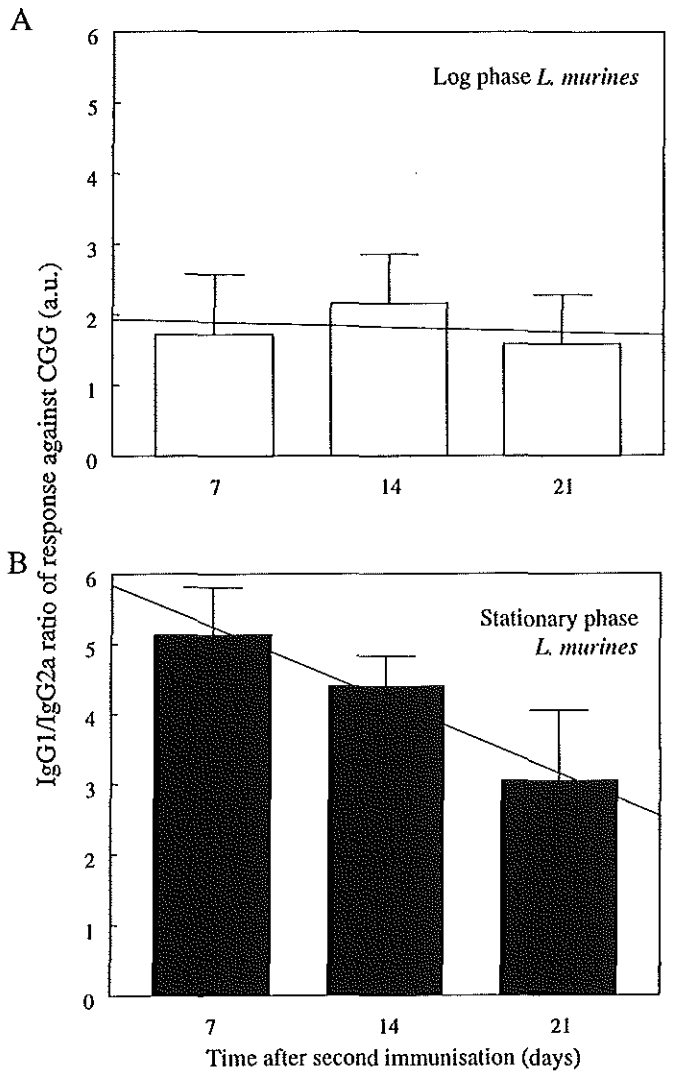


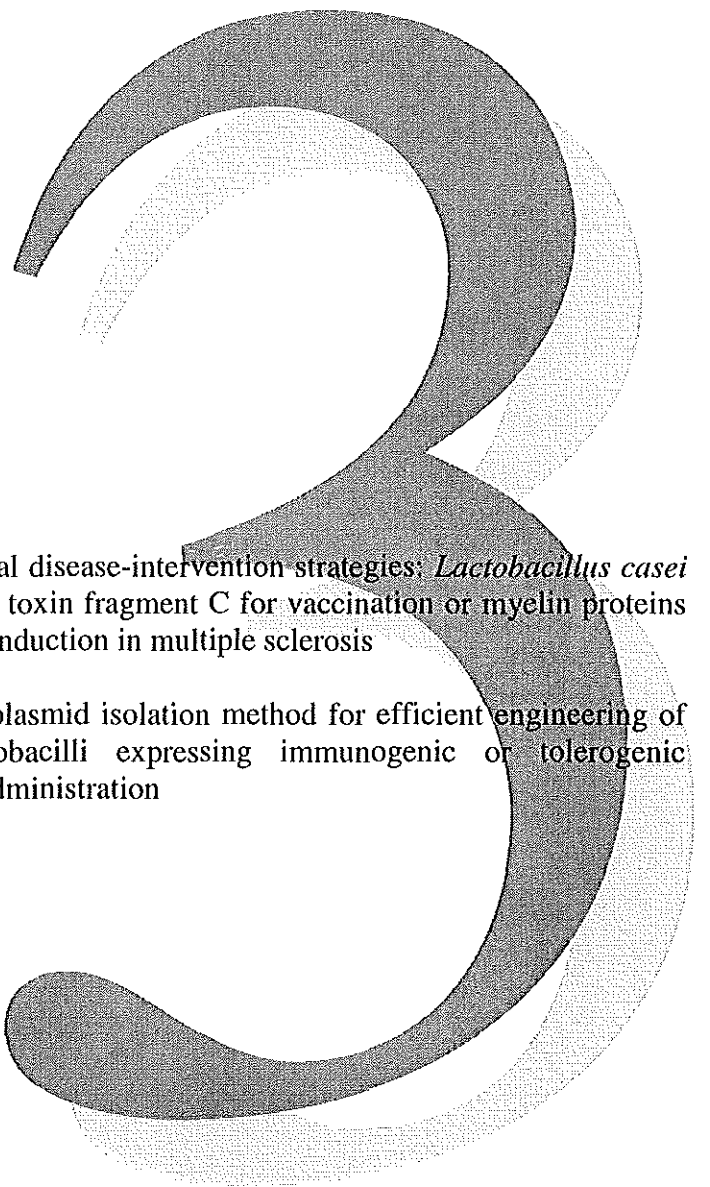
Figure 5. ***IgG subclass skewing by stationary phase L. murines decreases but persists over time***
The ratio of IgG1 and IgG2a antibodies specific for CGG was calculated for sera obtained 7, 14 and 21 days after the second immunisation.
(A) IgG1/IgG2a ratio of response against CGG in mice fed *L. murines* in log phase (±SEM).
(B) IgG1/IgG2a ratio of response against CGG in mice fed *L. murines* in stationary phase (±SEM).
The IgG1/IgG2a ratio decreased significantly ($p<0.05$) over time when *L. murines* in stationary phase was fed ($R= -1.04$), in contrast to a similar IgG1/IgG2a ratio over time when *L. murines* in log phase was fed ($R= -0.07$).

Growth phase dependent skewing of T helper cell pathways has important consequences for industrial application of *Lactobacillus* strains, as skewing may affect desired probiotic activities of food products. With respect to immunotherapeutic applications, including genetically engineered lactobacilli, our data caution that oral administration may inadvertently promote autoimmune or allergic reactions. However, this property of some *Lactobacillus* strains may also offer promising opportunities for immune modulation. Oral vaccination strategies (Clements, 1997; Robinson *et al.*, 1997) may exploit skewing properties to enhance T helper cell pathways dependent on the desired protective response, for example induction of cell mediated immunity versus antibody production, or preferential induction of specific functional IgG subclasses (e.g. differential complement fixation by IgG1 versus IgG2a). Oral *Lactobacillus* administration strategies for peripheral T cell tolerance induction as a treatment of autoimmune disease (Maassen *et al.*, 1999a; Weiner, 1997) may benefit from skewing towards Th2 responses, whereas reduction of Th2 activity is required in treatment of allergy.

The current findings strongly emphasize the need for rational *Lactobacillus* strain selection and detailed evaluation prior to application in food or health care products, and imply that the bacterial growth phase is a crucial parameter allowing additional manipulation of immune responses by oral administration of lactobacilli.

Acknowledgements
We thank dr. J. de Bree for help with statistical analysis.

Genetic engineering of *Lactobacillus* strains



3.1 Instruments for oral disease-intervention strategies: *Lactobacillus casei* expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis

3.2 A rapid and safe plasmid isolation method for efficient engineering of recombinant lactobacilli expressing immunogenic or tolerogenic epitopes for oral administration

Chapter 3.1

Instruments for oral disease-intervention strategies: recombinant *Lactobacillus* casei expressing tetanus toxin fragment C for vaccination or myelin proteins for oral tolerance induction in multiple sclerosis

C.B.M. Maassen, J.D. Laman, M.J. Heijne den Bak-Glashouwer, F.J. Tielen, J.C.P.A. van Holten, L. Hoogteijling, C. Antonissen, R.J. Leer, P.H. Pouwels, W.J.A. Boersma and D.M. Shaw

Vaccine 17: 2117-2128, 1999

Summary

Lactobacillus *strains possess properties that make them attractive candidates as vehicles for oral administration of therapeutics. In this report we describe the construction and analysis of recombinant* Lactobacillus casei *applicable in oral vaccination against an infectious disease (tetanus) and in oral tolerance induction for intervention in an autoimmune disease, multiple sclerosis.*

Recombinant Lactobacillus casei *which express surface-anchored tetanus toxin fragment C (TTFC) were generated. Quantitative analysis by flow cytometry demonstrated a high level of cell wall-bound expression of TTFC and immunogenicity was demonstrated by parenteral immunisation with whole cell extracts of the recombinants.*

A series of expression vectors was constructed to secrete human myelin basic protein (hMBP) or hMBP as a fusion protein with ß-glucuronidase from Escherichia coli. *These heterologous products produced by* L. casei *were detected in the growth medium and parenteral immunisation with this medium evoked antibodies against hMBP, confirming that secretion indeed had occurred.*

Based on the different localisation of the heterologous proteins, lactobacilli expressing surface-anchored TTFC are ideally suited for the induction of antibody responses, whereas lactobacilli that secrete myelin proteins can be used for the induction of peripheral T-cell tolerance. In conclusion, the specific technology described here allows the construction of a wide array of safe live recombinant lactobacilli which may prove to be useful in oral intervention strategies for the prevention of infectious diseases or treatment of autoimmune diseases.

Introduction

Among the acquired disorders of the central nervous system (CNS) are infectious and autoimmune diseases. For the prevention of infectious diseases, oral vaccines possess a variety of advantages over parenterally administered vaccines (Czerkinksy and Holmgren, 1995). Similarly, as an intervention in autoimmune diseases, autoantigens can be administered orally to restore peripheral T-cell tolerance. Active vaccination as opposed to the induction of T-cell tolerance following oral administration of antigens is considered to be crucially dependent on the particulate or soluble nature of the antigen delivered to the intestinal tract (Brandtzaeg, 1996; Gebert *et al.*, 1996). Therefore, in the current study, we have developed various recombinant *Lactobacillus* strains which differ in the form of expression of the antigen, facilitating either surface-anchored or secreted heterologous antigen expression.

It has long been recognized that feeding of soluble proteins can result in a state of immunological non-responsiveness termed oral tolerance (Strobel *et al.*, 1983; Mowat, 1985). This natural feature of the gut mucosa can be exploited to generate tolerance towards autoantigens. We have previously shown that oral tolerance to ß-galactosidase can be induced with *L. plantarum* transformed to express ß-galactosidase (Maassen *et al.*, 1998a). In this context, it has been reported that oral administration of MBP can result in inhibition of experimental autoimmune encephalomyelitis (EAE) in Lewis rats, a model for multiple sclerosis (MS)(Higgins and Weiner, 1988; Bitar and Whitacre, 1988).

The mucosal surfaces of the intestinal tract are designed for the uptake of nutrients. Immune responses towards dietary antigens are unwanted and therefore suppressed and as a result it is very difficult to evoke systemic immune responses following delivery of antigens to the mucosal surfaces. Enhanced immunogenicity following oral administration is achieved by presenting antigen to the immune system in association with inert particles, bacteria (Challacombe and Tomasi, 1980) or live viral vectors (reviewed for vaccinia by Moss (1996)). Due to the many advantages of oral vaccination, the 'Children's Vaccine Initiative' (CVI) of the World Health Organization (WHO) supports the development of a new generation of vaccines that can be administered orally.

Lactobacillus species are members of the diverse group of Gram-positive lactic acid bacteria that are generally recognized as safe (GRAS). These bacteria are potentially food grade, a status corroborating their particular suitability for oral therapeutical applications. Presentation of antigens on the surface of lactobacilli is attractive for vaccine design, especially because the peptidoglycan layer of some strains appears to exhibit natural immuno-adjuvanticity (Perdigon *et al.*, 1991; Boersma *et al.*, 1994; Link-Amster *et al.*, 1994; Pouwels *et al.*, 1996). The chemical coupling of the hapten trinitrophenyl (TNP) to the surface of lactobacilli has resulted in the induction of significant TNP-specific antibody responses following oral immunization (Gerritse *et al.*, 1990). In contrast to immunogenic applications of the strains expressing the antigen at the cell surface, recombinant lactobacilli that secrete

(auto)antigens locally in the gastrointestinal tract may also be used for the induction of peripheral T-cell tolerance following oral administration.

Here we describe the construction of recombinant *L. casei* expressing tetanus toxin fragment C (TTFC) on the cell surface. Tetanus is an infectious disease affecting the CNS of which over 400.000 children die worldwide each year. Infection with *Clostridium tetani* results in the production of tetanus toxin, which enters the CNS via the blood, leading to muscle cramping and eventually to death. TTFC is a non-toxic but immunogenic 47kDa fragment of the tetanus holotoxin heavy chain which is capable of inducing protective antibody responses, and hence TTFC is a rational choice for inclusion in a subunit (Fairweather *et al.*, 1990; Robinson *et al.*, 1997). In this study we examine the expression of TTFC by recombinant *L. casei* using both immunochemistry and flow cytometry analysis. The immunogenicity of the transformed cells was evaluated following parenteral immunisation.

For oral tolerance induction, *L. casei* recombinants expressing myelin antigens were constructed. Multiple sclerosis is a T cell mediated autoimmune disease of the CNS that affects the myelin sheath, which insulates the nerve axons. EAE (experimental autoimmune encephalomyelitis), a model for MS, can be induced in several animal species by imunization with myelin antigens such as MBP. Two vectors were created directing secretion of the encephalitogenic/tolerogenic compound hMBP or hMBP fused to ß-glucuronidase. In this study we report the analysis of the MBP recombinants and the immunogenicity of the hMBP secreted by transformed *L.casei* following immunisation of mice with concentrated growth medium.

Consequently, based on this generic technology a wide array of recombinant lactobacilli can be designed for application in oral intervention strategies for the prevention and treatment of infectious as well as autoimmune diseases.

Materials and methods

Bacterial strains and vector construction

The *E. coli* strain DH5α (Hanahan, 1983) was used as host strain for the construction of shuttle vectors. *L. casei* (ATCC 393) was cultured in MRS broth (Difco Laboratories, Detroit, MI). General molecular cloning, PCR techniques and transformation of *Lactobacillus casei* were carried out essentially as described elsewhere (Sambrook *et al.*, 1989; Posno *et al.*, 1991a).

A series of TTFC expression vectors was constructed, based upon the constitutive *ldh* (L-(+)-lactate dehydrogenase gene) promoter of *L. casei* and regulatable *amy* (α-amylase gene) promoter of *L. amylovorus,* as described previously (Pouwels *et al.*, 1996). The TTFC gene, kindly provided by Dr. A. Mercenier (Transgene, Strasbourg, France), containing a *Bam*HI site at its 5' end was extended by PCR at the 3' end using designed primers in order to obtain *Xho*I and *Nco*I sites. A *Bam*HI-*Nco*I DNA fragment was cloned into the multiple cloning site (MCS) of pTUAT (*Figure1*). The pTUT/pTUAT vector series were developed for convenient analysis of DNA sequences and to simplify construction of the desired DNA fragment

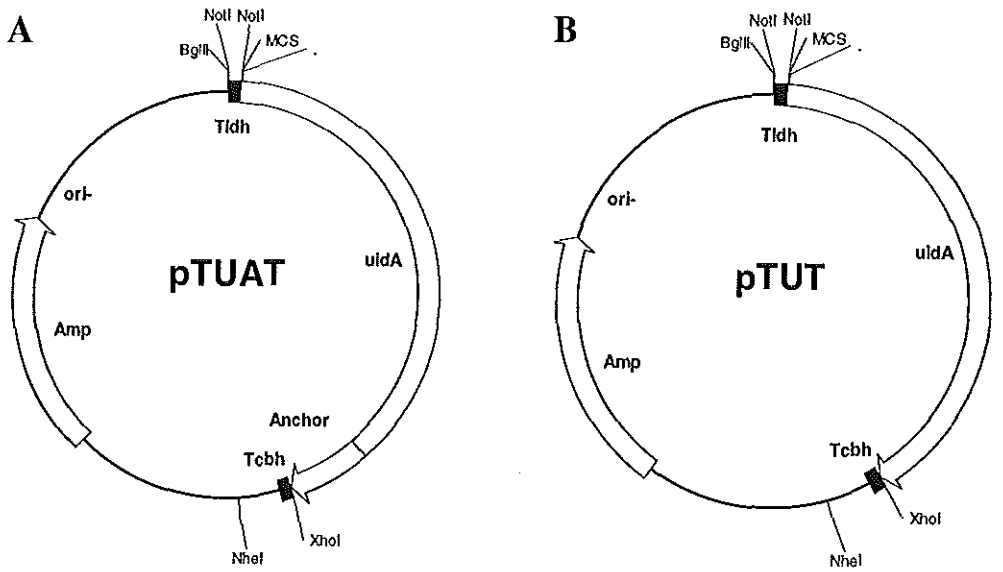


Figure 1. ***pTUT/pTUAT vectors to simplify cloning DNA fragments into*** **Lactobacillus** ***expression vectors***
The pTUAT (*Figure 1A*) and pTUT (*Figure 1B*) vector series are based on the pGEM-3 vector (Promega, Madison, WI). In brief, the multiple cloning site (MCS) of pGEM-3 was replaced by sequences encompassing a fragment flanked by *Not*I sites containing the *ldh* gene transcription terminator sequences, the *uidA* gene encoding ß-glucuronidase and the transcription terminator sequence of the *L. plantarum cbh* gene. In case of pTUAT the ß-glucuronidase open reading frame (orf) sequence was fused in frame with that of a sequence encoding the sequences responsible for the anchoring of *prtP* of *L. casei ATCC 393*. The MCS, which is direct in front of the uidA gene sequence, contains several restriction sites including *BamH*I and *Nco*I.

into the pLP400/500 series of *Lactobacillus/E.coli* shuttle vectors (Pouwels *et al.*, 1996). Subsequently, the *Bam*HI/*Nhe*I fragment containing TTFC-*uidA* was exchanged with the *Bam*HI/*Nhe*I fragment of the expression-secretion vectors of the pLP400/500 series. In the pLP401-TTFC/U expression vector TTFC is fused to the marker gene *uidA*, which encodes ß-glucuronidase. This expression vector is under the transcriptional control of the promoter and secretion signal sequence of the regulatable amylase gene of *L. amylovorus* (*amy*). The fusion TTFC-*uidA* open reading frame is in frame with a sequence coding for the cell wall anchor sequence of the *prtP* gene of *L.casei* in order to expose the synthesized product at the cell surface of the bacterium. The pLP501 expression vector resembles the pLP401 expression vector, however expression is under the transcriptional control of the constitutive *ldh* promoter and translocation is facilitated by fusion of the orf to the secretion signal sequences of the *prtP* gene of *L. casei*. By digestion with *Xho*I the *uidA* gene sequencewas removed resulting in expression vectors pLP401-TTFC and pLP501-TTFC (*Figure 2*).

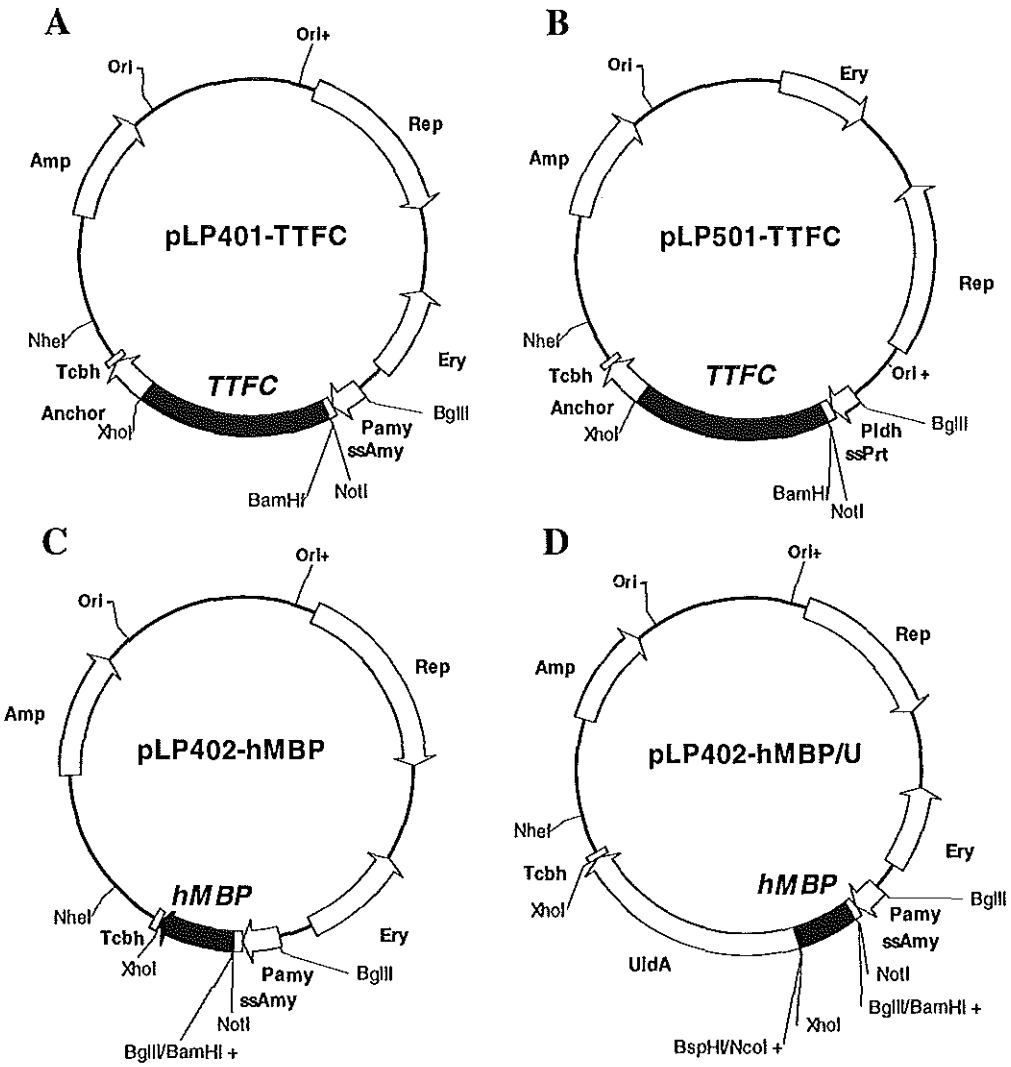


Figure 2. Lactobacillus *expression vectors for secretion or surface-anchored expression of heterologous proteins*

(A) pLP401-TTFC vector mediating surface-anchored expression of TTFC under control of the regulatable *amylase* promoter. (B) pLP501-TTFC vector mediating surface-anchored expression of TTFC under control of the constitutive lactate dehydrogenase promoter. (C) pLP402-hMBP vector mediating secretion of hMBP under control of the regulatable *amylase* promoter. (D) pLP402-hMBP/U vector mediating secretion of hMBP fused to ß-glucuronidase under control of the regulatable *amylase* promoter. P_{amy} = promoter sequence of the α-*amylase* gene of *L. amylovorus*. ssAmy = sequences encoding secretion signal (36aa) of the α-amylase gene of *L. casei* and the N-terminus (26aa) of α-amylase gene of *L. casei*. P_{ldh} = promoter sequences of the *ldh* gene of *L. casei*. ssPrt = sequences encoding secretion signal (33aa) of the *prt* P gene of *L. casei* and the N-terminus (36aa) of the *prt* P gene of *L. casei*. Anchor = anchor peptide (117aa) encoding sequence of *L. casei*. T_{cbh} = transcription terminator sequence of the *cbh* gene of *L. plantarum 80*. *UidA* = sequences encoding β-glucuronidase. *rep* = *repA* gene of plasmid p353-2 from *L. pentosis*. *ery* = erythromycine resistance gene. *amp* = ampicilline resistance gene. ori = origin of replication (ori+ =ori *E. coli*, ori- = ori *Lactobacillus*).

A series of expression vectors was constructed to secrete human MBP or hMBP fused to *uidA* under transcriptional control of the *amy* promoter, in a similar fashion as used for the TTFC vectors. The 513 bp DNA coding for human MBP (ATCC clone M13577) was extended at its 3' end with a *Bsp*HI site and at the 5' end with a *Bgl*II site using PCR. The *Bgl*II – *Bsp*HI MBP DNA fragment was cloned in pTUT (*Figure 1*), following digestion of pTUT with *Bam*HI and *Nco*I. Subsequently, the *Bgl*II/*Nhe*I fragment containing the insert was exchanged with the *Bam*HI/*Nhe*I fragment of pLP402 creating pLP402-hMBP/u (*Figure 2D*). Digestion with *Xho*I removed the *uidA* gene, resulting in the vector containing only hMBP (*Figure 2C*). The pLP400/500 vectors are stable in *E. coli* due to the presence of the *ldh* terminator (T_{ldh})(Pouwels *et al.*, 1996). After removal of the T_{ldh} terminator by *Not*I digestion, ligation of the vectors juxtaposed the heterologous protein encoding sequences in frame with the first few codons of the translation initiation region present downstream of the promoter sequences, allowing heterologous gene expression in *Lactobacillus* species (*Figure 2*).

Expression analysis of heterologous gene products

For expression analyses, recombinant *L. casei* containing the vectors pLP401 and pLP501 expressing TTFC (designated pLP401-TTFC and pLP501-TTFC) or containing the vector pLP402 expressing hMBP or hMBP fused to ß-glucuronidase (designated pLP402-hMBP and pLP402-hMBP/U) were routinely prepared from glycerol stocks by two consecutive overnight (o/n) cultures without aeration of a 1:50 dilution in MRS medium containing 5 µg/ml erythromycin.

Transformants with expression vectors containing the constitutive *ldh* promoter were subsequently grown in antibiotic selective MRS medium or *Lactobacillus* carrying medium (LCM)(Efthymiou and Hansen, 1962) containing 1% glucose (w/v) at 37°C, using a 1:50 dilution of the last o/n culture. In order to induce expression of the heterologous proteins in transformants containing plasmids under the transcriptional control of the regulatable α-amy promoter, cells were grown in mannitol (1% w/v) supplemented LCM or modified MRS medium (mMRS) (1% w/v proteose peptone, 0.5% w/v yeast extract, 0.2% w/v meat extract, 0.1% v/v Tween 80, 37mM sodium acetate, 0.8 mM magnesium sulfate, 0.24 mM manganese sulfate, 8.8 mM diammonium citrate in 0.1M potassium phosphate buffer [pH 7.0]). Cells were harvested at defined time points, pelleted by centrifugation and suspended in phosphate buffered saline (PBS). Cell extracts were obtained from the bacteria by sonicating the cells four times on a 30 sec on/30 sec off cycle using an MSE Soniprep 150 sonicator at 10 amplitude microns (Sanyo Gallenkamp PLC, Leicester, UK), to release both cytoplasmic and cell wall-bound proteins. After centrifugation the soluble fraction was used for immunoblot analysis.

To detect heterologous proteins secreted into the medium, the culture medium was collected after spinning down the cells and filtered through a 0.2 µm filter to remove the remainder of cells. After addition of 0.2mg/ml trypsin inhibitor (Sigma

Chemical, La Jolla, CA) the culture medium was freeze-dried and concentrated approximately 200-fold in PBS. Dilutions of these concentrated media are used for immunoblot analysis. Proteins in cell extracts or culture medium were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)(7.5, 10 or 14% acrylamide, 400 mM Tris pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 200V for 45 minutes. Protein was transferred electrophoretically onto nitrocellulose using a Bio-Rad Miniprotean II blotting unit (Bio-Rad Laboratories, Hercules, CA). Immunoblots were incubated with optimally diluted rabbit anti-TTFC (Calbiochem-Novabiochem International, San Diego, CA), rabbit anti-hMBP or rabbit anti-β-glucuronidase antiserum. Antibodies against hMBP and β-glucuronidase were induced by immunization of New Zealand White rabbits with human MBP and ß-glucuronidase in complete adjuvant containing *Mycobacterium tuberculosis H37Ra* (Difco Laboratories). The second incubation step was performed with swine anti-rabbit Ig-HRP (Dako A/S, Glostrup, Denmark). The immunoblots were incubated with ECL detection reagents (Amersham Life Science Ltd, Buckinghamshire, UK). A light sensitive film (Kodak X-omat) was exposed to the blots for a variety of time periods before development. In all immunoblots, recombinant TTFC antigen (List Biological Laboratories, Campbell, CA), bovine MBP (purified according to Deibler *et al.*, 1972) or ß-glucuronidase (Sigma Chemical) were used as references for the immuno-reagents. Culture medium of various recombinant *L. casei* strains expressing fusion proteins with ß-glucuronidase were used as controls for secretion or lack of secretion of heterologous proteins into the culture medium in immunoblot experiments. pLP402-HA/U, which secretes the 111-120 peptide of hemagglutinin of influenza (HA111-120) fused to β-glucuronidase, was used as a positive control for secretion (Hackett *et al.*, 1985). Surface-expressed TTFC fused to ß-glucuronidase (pLP401-TTFC/U) and intracellularly expressed TTFC fusion protein (pLP503-TTFC/U) are examples of heterologous proteins that are not directed to the medium and were utilized as negative controls. The amount of soluble heterologous hMBP-ß-glucuronidase was calculated by comparison of several dilutions of extracts of transformants with a reference of purified ß-glucuronidase in immunoblot analysis making use of Bio-1D V6.32 software (Vilber Lourmat, Marne la Vallée, France).

Flow cytometry analysis

At defined time points bacteria were prepared for analysis by FACScan. Cells were washed twice with FACS fluid (FF), Facsflow™ (Becton-Dickinson, San Jose, CA) with 1% BSA and 0.1% sodium azide. Cells were resuspended in 200µl FF containing optimal dilutions of antibodies specific for TTFC, MBP or β-glucuronidase and incubated for one hour at 4°C. Cells were again washed twice in FF. Bound antibody was detected by incubation with fluorescein isothiocyanate-conjugated (FITC) labeled goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, PA) at a dilution of 1:1500 for 1h at 4°C. Cells were then washed twice prior to analysis on a flow cytometer (FACScan™, Becton-Dickinson). CellQuest® software

was used for analysis of the data. A gate was set for events representing lactobacilli as determined by cytograms of forward and side scatter to enable exclusion of debris. Control strains were wild type *L. casei* or recombinant strains expressing an irrelevant protein harvested in the same growth phase. For control stainings normal rabbit serum was used or the antigen-specific primary antibody was omitted. For each sample, data was collected for 10,000 or 20,000 gated events. The fluorescence obtained from bacterial cell suspensions was represented by fluorescence histograms and mean channel intensities were calculated.

For quantification of surface expression, *L. casei* pLP401-TTFC cells were analysed by examining TTFC antibody binding at the point of antibody saturation. This precise determination of fluorescence levels at the point of saturation of binding sites was achieved empirically following the generation of a titration curve for the TTFC-specific polyclonal antibody.

The conversion of measured fluorescence intensities into values representing the number of TTFC molecules accessible for binding on the surface (ABC) was undertaken using the Quantum25® FITC Kit calibration beads (Flow Cytometry Standards, Leiden, The Netherlands). To enable quantitative estimates of the numbers of antibodies bound per cell, peak channel data generated from the calibration beads was regressed against the defined values of molecules of equivalent soluble fluorochrome (MESF) possessed by each bead population. Because of the broader distribution of channel intensities for stained cells, extrapolation of the MESF value for pLP401-TTFC vectors was then undertaken using the mean channel fluorescence intensity obtained at antibody saturation of the lactobacilli presenting TTFC on the cell surface. Following corrections for fluorochrome/protein (F/P) ratios in the anti-rabbit FITC conjugate (second) step an estimation of numbers of surface molecules available for binding on the surface of the recombinant lactobacilli was made.

Parenteral immunisation with L. casei *recombinants*

In order to demonstrate the immunogenicity of the expressed heterologous proteins, mice were immunized parenterally with cell extracts or concentrated growth medium of the *L. casei* recombinants. Cell extracts of the recombinants pLP401-TTFC and pLP501-TTFC were obtained as described above. Three BALB/c mice per group were immunized s.c. with an emulsion of 112.5µl total cell extract and 137.5µl Difco's complete adjuvant with *M. tuberculosis* H37Ra (Difco Laboratories). Concentrated culture medium from the recombinants pLP402-hMBP and pLPhMBP/u was used for immunisation. Three SJL/J mice per group were immunized s.c. with 250µl concentrated culture medium emulsified in Difco's complete adjuvant or i.p. in incomplete adjuvant (Difco Laboratories). The immunisation procedure was repeated 28 days after the first immunisation. Serum was collected for analysis in ELISA every seven days.

TTFC and MBP specific ELISA

To test serum for the presence of anti-TTFC or anti-MBP antibodies, Nunc immuno plates (Life Technologies, Gaithersburg,MD) were coated with tetanus toxoid (RIVM, Bilthoven, The Netherlands)(5µg/ml, 50µl/well) in 0.05M carbonate-bicarbonate buffer (pH 9.6) or guinea pig MBP (5µg/ml, 50µl/well) in PBS overnight at 4°C. Non-specific binding was blocked by incubation with 3% BSA in PBS (150µl/well) for 1 hour at 37°C. Subsequently the plates were incubated for 1 hour at 37°C with dilutions of sera of mice immunized with cell extracts or culture medium of *L. casei* recombinants and preimmunization sera to correct for background reactivity. For the detection of IgG antibodies specific for TTFC or MBP, alkaline phosphatase-labelled goat anti mouse IgG (γ) (KPL, Gaithersburg, MD) was used (1h, 37°C). After addition of the substrate 4-nitrophenylphosphate, the absorbance at 405nm was read at several time points. Endpoint titer was defined as the highest dilution which gives an absorbance above twice the absorbance with normal mouse serum.

Results

TTFC-expressing recombinant L. casei

Construction of TTFC-expressing *L. casei* transformants

To enable the construction of TTFC-expressing *L. casei* vectors, the 1329bp DNA coding for TTFC was cloned into the *Lactobacillus* expression vectors pLP401 and pLP501 (*Figure 2*). The pLP401 vectors drive expression via the regulatable *α-amy* promoter and include the secretion signal and N-terminus of the amylase gene of *L. amylovorus*. The pLP501 vectors contain the promoter sequence of the *ldh* gene *of L. casei* and include the secretion signal and N-terminus of the *prtP* gene of *L. casei*. Both the pLP401 and the pLP501 vectors contain the anchor sequence of the *prtP* gene of *L. casei*, resulting in expression of the TTFC-anchor protein at the cell surface. Candidate vectors were constructed in *E. coli DH5α* hosts and after removal of the *ldh* terminator introduced in *L. casei* by electroporation.

Immunoblot analysis of TTFC-expressing *L. casei* transformants

Expression of TTFC was demonstrated by immunoblot analysis using a rabbit polyclonal TTFC-specific antiserum (*Figure 3A*). Cell extracts were prepared of the *L. casei* transformant carrying pLP401-TTFC approximately 8 hours (log phase) after inoculation in LCM supplemented with mannitol at OD_{690}= 0.6. Expression of TTFC in the pLP501-TTFC strain, in which expression is driven by P*ldh*, is not particularly time dependent (data not shown). This confirmed the constitutive expression by pLP501-TTFC during growth, whereas in transformants containing pLP401-TTFC, TTFC synthesis was induced by derepression of the *α-amy* promoter following replacement of glucose in the growth medium by mannitol. Expression was demonstrated to be optimal at the end of the log phase (OD_{690}=0.6). *Figure 3A* demonstrates that the amount of TTFC product present in pLP501-TTFC (*lane 2*) is higher than in pLP401-TTFC (*lane 4*). The TTFC products of both vectors are larger

than the purified TTFC (*lane 5*) due to additional elements such as the anchor sequence. The apparent molecular weight of these TTFC products on immunoblot is approximately 75 kDa. In extracts of either of the transformants types a variety of breakdown products was observed. This in contrast with pLP503-TTFC transformants which retain the TTFC product intracellularly. The breakdown products of this recombinant are less abundant (*Figure 3A, lane 6*). This difference in breakdown products between anchored/secreted products and intracellular products holds true for all pLP400/500 vectors constructed so far. *L. casei* transformed with the pLP401/pLP501 vectors with irrelevant sequences demonstrated no corresponding heterologous product expression (*Figure 3A, lane 1 and 3*).

L. casei producing MBP and MBP fusion products

For oral tolerance induction *Lactobacillus* transformants containing expression vectors with which secretion of heterologous proteins can be achieved were constructed. DNA encoding human MBP was cloned into the expression vector pLP402 in frame with or without the sequence of β-glucuronidase (*Figure 2C/D*). These pLP402 plasmids drive the expression via the regulatable *α-amy* promoter and contain the secretion signal and N-terminus of the amylase gene of *L. amylovorus*.

Expression of both fusion products was demonstrated in extracts by immunoblot analysis using antiserum against hMBP (*Figure 3B, lane 3 and Figure 3C, lane 3*). HMBP fused to ß-glucuronidase was also detected after incubation of the immunoblots with anti-β-glucuronidase serum (*Figure 3D, lane 1*). Semi-quantitative analysis yielded a calculated amount of 1-1.5% MBP-β-glucuronidase fusion protein as a percentage of total protein.

Secretion of MBP and MBP fusion products into the medium

Culture medium used for the propagation of the recombinant lactobacilli was analysed in order to detect whether the cells secreted MBP or MBP fusion proteins. Using immunoblot analysis of cell extracts, the time point of optimal expression was determined to be 12 hours after initiation of growth in mannitol-supplemented mMRS medium (OD_{690}=1.1). The fusion protein hMBP fused to β-glucuronidase was detectable in 50-fold concentrated medium (=750µl medium) in low concentration using the β-glucuronidase specific anti-serum (*Figure 3C, lane 3*). The breakdown products, which are abundantly present, are specific for ß-glucuronidase. This indicates that the product is, at least partially, secreted. The fusion product of hMBP with ß-glucuronidase could not be detected in the media when anti-hMBP serum is used (data not shown). Other secreted fusion proteins could be detected in the medium, such as the influenza peptide fused to ß-glucuronidase of *L. casei* pLP402-HA/U (*Figure 3D, lane 4*). That this product is probably intact can be concluded from the fact that the MW of this product is higher than secreted ß-glucuronidase from the *L. casei* pLP402/u recombinant (*Figure 3D, lane 8*). During the secretion process the signal sequence is cleaved off.. Therefore, the secreted

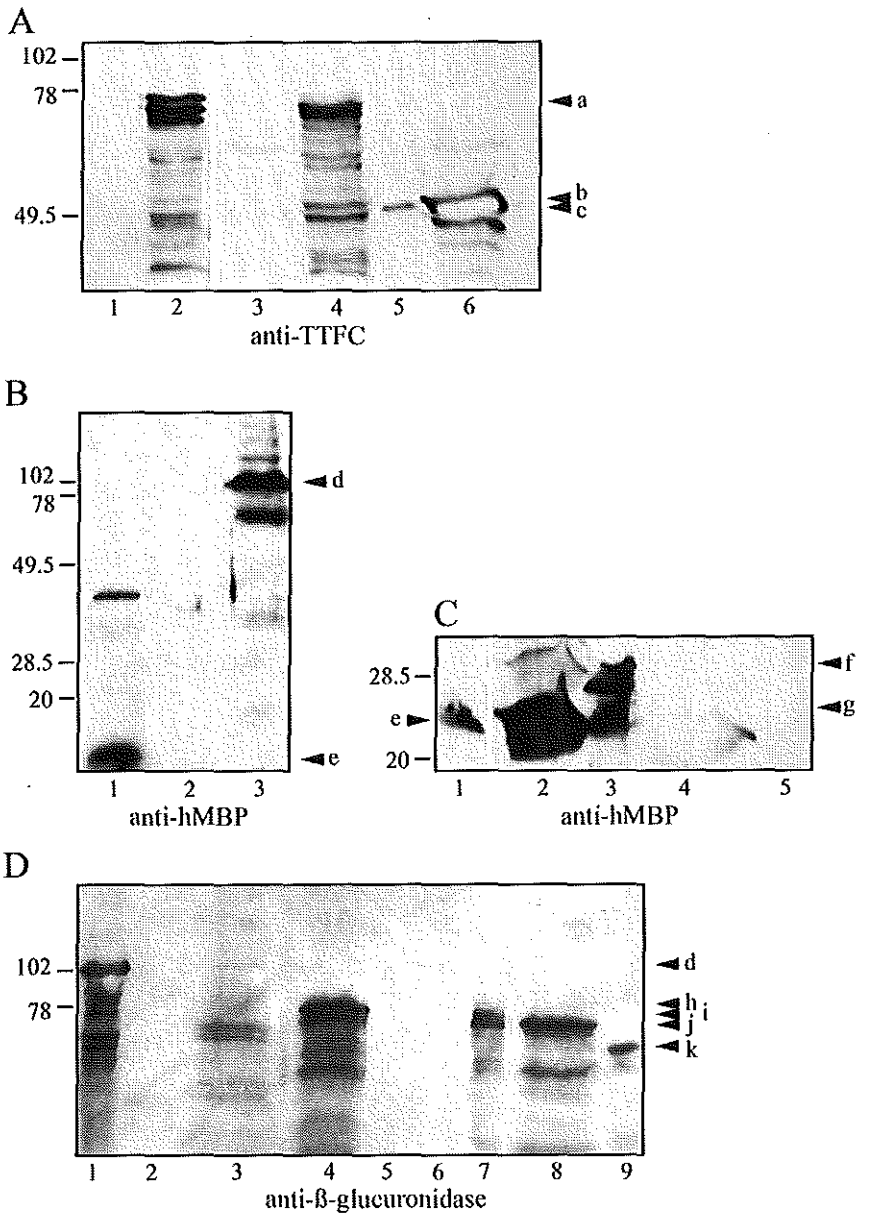


Figure 3. ***Immunoblot analysis of*** **Lactobacillus casei** ***recombinants***
(A) *Expression of TTFC by recombinants carrying pLP401-TTFC (lane 4) or pLP501-TTFC (lane 2).* When the culture reached an optical density of 0.6 at 690 nm after derepression of the α-amy promoter, cell extracts were prepared and analysed by immunoblot. Nitrocellulose membranes were stained with serum specific for TTFC. Purified TTFC was used as positive control (lane 5) and cell extracts of wild type *L. casei* carrying an pLP501 vector with an irrelevant insert (lane 1) or a pLP401 vector with an irrelevant insert (lane 3) were used as negative controls. pLP503-TTFC, which retains the TTFC product

products are smaller than the heterologous proteins detected in the cell extracts, as can be seen by comparison of the cell extract (Figure 3D, lane 7) with medium of L. casei pLP402/u (Figure 3D, lane 8). In concentrated culture medium from two control recombinant *L. casei* strains which expressed irrelevant antigens fused to ß-glucuronidase either intracellular (pLP503-TTFC/U) (*Figure 3D, lane 5*) or anchored to the bacterial surface (pLP401-TTFC/U) (*Figure 3D, lane 6*), no heterologous proteins were detected using antiserum specific for β-glucuronidase, as expected. This indicated that the positive signals obtained with the medium samples of the recombinants are unlikely to result from leakage or lysis of cells.

Although the large hMBP-ß-glucuronidase protein is not efficiently secreted, the much smaller hMBP product from the pLP402-hMBP transformant was secreted well. This product was clearly demonstrated in 25 fold-concentrated culture medium on a immunoblot incubated with anti-hMBP serum (*Figure 3C, lane 2*). All medium samples have the property that they run in a wide pattern (*Fig 3D, lane 3, 4 and 8*), but they show very wide and diffuse bands in the region of the smaller proteins (*Figure 3C, lane 2*). As a negative control pLP402/u, which secretes only ß-glucuronidase, did not show a specific reactivity, as expected (*Figure 3C, lane 4*).

Flow cytometric analysis and selection

Flow cytometry analysis of surface association of TTFC

Presentation of the TTFC antigen on the surface of *L. casei* transformed with plasmids pLP401-TTFC or pLP501-TTFC was demonstrated by FACScan analysis of recombinant lactobacilli. TTFC gene expression by *L. casei* was analysed using a polyclonal rabbit TTFC-specific antiserum in the period of 4 to 9 h following the onset of

intracellularly, is shown in lane 6. (B) *Expression of hMBP fused to ß-glucuronidase by recombinants carrying pLP402-hMBP/U.* At an optical density of 1.1 at 690 nm, cell extracts were prepared and analysed by immunoblot. Nitrocellulose membrane was stained with serum specific for hMBP. Purified bovine MBP was used as positive control antigen (lane 1) and a cell extract of *L. casei* expressing ß-glucuronidase (pLP402-U)(lane 2) was used as a negative control. (C) *Expression of hMBP in cell extracts and in culture medium.* Cell extracts (lane 3) and culture medium (lane 2) of *L. casei* recombinants pLP402-hMBP were analysed by immunoblot for the presence of heterologous hMBP. Nitrocellulose membrane was stained with serum specific for hMBP. Purified bovine MBP was used as positive control antigen (lane 1) and a cell extract (lane 5) and concentrated culture medium (lane 4) *of L. casei* with pLP402-U were used as negative controls. (D) *Detection of secreted ß-glucuronidase fusion products.* At an optical density of 1.1 at 690 nm, cell extracts and culture medium were prepared for analysis by immunoblot. Nitrocellulose membrane was stained with serum specific for ß-glucuronidase. Lane 1 showed hMBP-ß-glucuronidase in a cell extract from pLP402-hMBP/U. Lane 2 is kept empty, to be sure that the secreted product in medium from pLP402-hMBP/U in lane 3 can not be due to overflow. Lane 4 and lane 8 show the secreted products in medium from pLP402-HA/U and pLP402-U respectively. Lane 5 and 6 showed the concentrated media of pLP503-TTFC (intracellular TTFC) and pLP401-TTFC (anchored TTFC) respectively. In lane 7, pLP402-U extract was analysed. Purified ß-glucuronidase was used as positive control antigen in lane 9.
a= heterologous anchored TTFC, b= intracellular TTFC, c= purified TTFC, d= hMBP-ß-glucuronidase, e= purified MBP, f= non-secreted hMBP, g= secreted hMBP, h =secreted HA-ß-glucuronidase, i= non-secreted ß-glucuronidase, j= secreted ß-glucuronidase, k= purified ß-glucuronidase. Sizes of the low range marker are indicated in kDa.

growth. Data obtained 7 h after inoculation are presented (*Figure 4A*). Mean fluorescence levels observed with pLP501-TTFC after staining with non-immune rabbit serum were used as control (*Figure 4A*). A higher level of surface-anchored exposure of TTFC under control of the *ldh* promoter (pLP501-TTFC) in comparison to that of the surface-anchored exposure of TTFC under control of the α-*amy* promoter (pLP401-TTFC)(Pouwels and Leer, 1993) was indicated by the distinct mean channel fluoresence intensities of the two recombinants at an equivalent time point. Similar results were obtained at other time points (results not shown).

Quantitation of the number of TTFC molecules present on the cell surface

To estimate the mean number of TTFC molecules present on the surface of the cell, mean channel intensities obtained following staining of *L. casei* pLP401-TTFC and *L. casei* pLP501-TTFC with anti-TTFC were used for calculation. As shown in *Table 1*, after 7 hours of growth approximately $3.9x10^3$ and $1.4x10^3$ TTFC molecules were found on the surface of the pLP501-TTFC and pLP401-TTFC recombinant *L. casei*, respectively.

Table 1. *Quantitation of the number of TTFC molecules expressed on the cell surface of recombinant L. casei.*

Cells	Mean channel fluorescence	MESF	F/P ratio	Surface-molecules / per cell
pLP401-TTFC	21	4282	3.1	$1.4*10^3$
pLP501-TTFC	55	12170	3.1	$3.9*10^3$

Analysis of secreted hMBP and hMBP fusion products

In order to determine whether it is possible to detect epitope-specific sequences associated with the cell wall of heterologous proteins which are directed to be secreted, transformants secreting MBP and MBP fusion proteins were analysed by flow cytometry, at time points of optimal expression as determined by immunoblot analysis (data not shown). The cells were incubated with specific antiserum against β-glucuronidase or against MBP. Wild type *L. casei* as well as *L. casei* transformed the vector pLP402-U, which secreted β-glucuronidase, were used as controls. As shown in *Figure 4B and 4C*, it was possible to detect myelin proteins fused to ß-glucuronidase associated with the cell surface by flow cytometry. Significant levels of hMBP-β-glucuronidase (mean channel fluorescence = 13.5 channels) could be detected using anti-β-glucuronidase serum as compared to the wild type *L. casei* (mean channel fluorescence ≈ 2.5). Using anti-MBP serum, the hMBP fusion protein (*Figure 4C*) and hMBP (*Figure 4D*) only could also be detected, although the level of surface expression of hMBP only was very low (mean channel fluorescence = 8.1

versus 5.1 of pLP402-U). The increase in fluorescence intensity of pLP402-MBP/U compared to the control recombinant pLP402-U, which only secretes ß-glucuronidase (mean fluorescence = 15.5 respectively 7.7 channels) (*Figure 4C*) was slightly less than the increase in fluorescence intensity of the control strains and the recombinant pLP402-MBP/U detected with anti-ß-glucuronidase (*Figure 4B*).

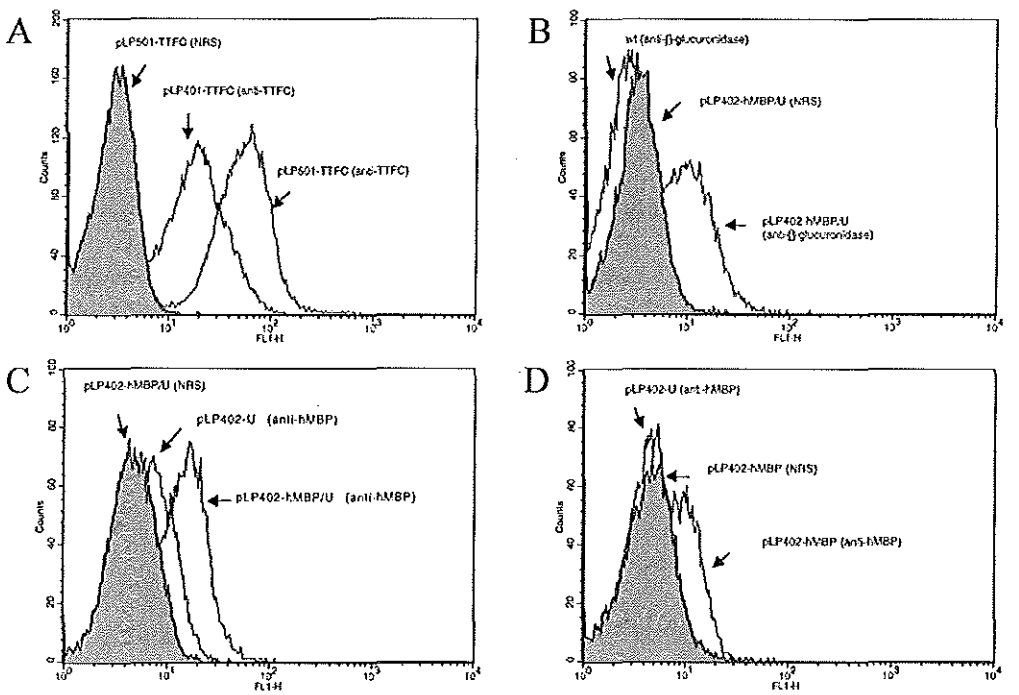


Figure 4. ***Flow cytometry analysis of*** **Lactobacillus casei** ***recombinants***
(A) *Surface-association of TTFC.* TTFC was detected on the cell surface of *Lactobacillus casei* recombinants with pLP401-TTFC (α-*amy* promoter) or pLP501-TTFC (*ldh* promoter) by incubation of cells with rabbit antibodies specific for TTFC and stained with FITC labelled goat anti-rabbit serum. Recombinant pLP501-TTFC incubated with normal rabbit serum (NRS) was used as negative control. (B) *Surface-association of MBP-β-glucuronidase using antiserum against β-glucuronidase. Lactobacillus casei* recombinants secreting MBP-β-glucuronidase (pLP402-MBP/U) showed surface-association when the cells were incubated with rabbit antibodies against β-glucuronidase and stained with FITC labelled goat anti-rabbit serum. Wild type *Lactobacillus casei* (wt) incubated with serum specific for ß-glucuronidase and pLP402-MBP/U incubated with normal rabbit serum (NRS) were used as negative controls. (C) *Surface-association of hMBP-β-glucuronidase using hMBP specific antiserum.* pLP402-MBP/U recombinants were incubated with rabbit antibodies directed against MBP and stained with FITC labelled goat anti-rabbit serum. Recombinant *L. casei* secreting β-glucuronidase only (pLP402-U) incubated with serum specific for hMBP and pLP402-MBP/U incubated with normal rabbit serum (NRS) were used as negative controls. (D) *Surface-association of hMBP using hMBP specific antiserum.* pLP402-hMBP recombinants were incubated with rabbit antibodies directed against hMBP and stained with FITC labelled goat anti-rabbit serum. Recombinant *L. casei* secreting ß-glucuronidase only pLP402-U) incubated with serum specific for hMBP and pLP402-hMBP incubated with normal rabbit serum (NRS) were used as negative controls.

Immunogenicity of heterologous proteins expressed by L. casei

To determine whether the heterologous TTFC proteins expressed by *L. casei* recombinants pLP401-TTFC and pLP501-TTFC were immunogenic, BALB/c mice were immunized with total cell extracts of these recombinants. Immunogenicity of TTFC construct pLP501 was demonstrated in a tetanus toxoid specific ELISA within 14 days after the second immunization with an IgG endpoint titer of $10^{3.2}$ (Log_{10}) for the pooled mice sera. The TTFC construct pLP401-TTFC showed an endpoint titer of $10^{2.9}$ (Log_{10}) 21 days after the second immunization.

SJL/J mice were immunized with cell-free culture medium of *L. casei* recombinants pLP402-hMBP and pLP402-hMBP/u to demonstrate immunogenicity as well as secretion of the heterologous proteins into the medium. Immunogenicity of secreted MBP was shown by IgG endpoint titers of $10^{2.3}$ (Log_{10}) for pLP402-hMBP and $10^{2.9}$ (Log^{10}) for pLP402-hMBP/u after 21 days with negligible values at 14 days. None of the selected control recombinants (i.e. *L. casei* pLP402/u) induced MBP or TTFC antibodies, as expected.

Discussion

In this study we describe the construction and characterisation of *Lactobacillus* expression/delivery vectors as potential vaccines or therapeutics for diseases of the central nervous system. Essentially, similar vectors are designed for use of two distinct applications; live oral vaccines to actively immunise versus live oral therapeutics to restore peripheral T cell tolerance in autoimmune disease. The distinct effects are facilitated through the precise location of the antigen; surface-anchored antigen-expression for the vaccine application versus antigen secretion into the local environment for tolerance induction (Brandtzaeg, 1996; Gebert *et al.*, 1996).

Two plasmids were constructed to enable surface-anchored expression of TTFC. The expression is under transcriptional control of the constitutive *ldh* promoter (pLP501-TTFC) or under the control of the *α-amy* promoter (pLP401-TTFC), which is repressed in the presence of glucose. For application in vaccine strategies, the number of molecules of the antigen present on the cell-surface is considered important for immunogenicity. Flow cytometry has previously been used to determine the composition of bacterial cultures, in which each bacterial species has its own characteristic 'signature' (Pinder *et al.*, 1990). In addition, heterologous proteins have also been demonstrated on the surface of other recombinant Gram-positive bacteria such as *Staphylococcus xylosus* (Samuelson *et al.*, 1995). Flow cytometry, to our best knowledge, has not been used before to detect heterologous proteins associated with the *Lactobacillus* surface. We have shown that this technology allows an estimation of the relative number of molecules expressed on the cell wall by individual cells. In the utilisation of a polyclonal antibody we assumed that each TTFC molecule is bound by only one antibody molecule, enabling the relative measurements between recombinant lactobacilli. With this strategy it was demonstrated that for *L. casei* pLP501-TTFC, under control of the constitutive *ldh* promoter, the number of TTFC molecules per

cell, present on the *Lactobacillus* cell wall is three times higher than observed for *L. casei* pLP401-TTFC which expresses TTFC under the control of the regulatable *α-amy* promoter. The difference in expression level can be explained by the fact that the promoter sequences used have different transcription activities (Pouwels *et al.*, 1996). The different secretion signals (proteinase versus amylase) inherent to the vectors may also lead to higher translocation efficiencies in the pLP501-TTFC recombinant and may result in elevated levels of TTFC on the cell wall accessible for binding by antibody (Pouwels and Leer, 1993; Hols *et al.*, 1997). The difference in level of expression/surface association may also account for the higher levels of TTFC specific antibodies found after parenteral immunisation with total cell extracts of *L. casei* pLP501-TTFC as compared to pLP401-TTFC. Although both recombinant strains appear to be immunogenic, the results argue for the use in vaccine applications of recombinant strains utilising the constitutive promoter. The breakdown products present in extracts obtained from either the pLP401 or pLP501 recombinants, which are not as abundantly present in extracts obtained from pLP503 recombinants maintaining the heterologous proteins intracellular, may well be due to activity of proteases present at the surface of the bacterium or in the medium.

Vectors for the use in oral tolerance studies preferably should secrete the tolerogen (Brandtzaeg, 1996; Gebert *et al.*, 1996). Therefore, two vectors that secrete hMBP (pLP402-hMBP) or hMBP fused to ß-glucuronidase were constructed and analysed. Although secretion of both products was demonstrated in immunoblot analysis using protein samples prepared from the culture medium, there was a clear difference in the levels of secretion. The quantity of hMBP-ß-glucuronidase fusion protein actually present in the culture medium was very low, and the product apparently was digested into two main products. However, this fusion product was detectable by flow cytometry using either anti-MBP or anti-ß-glucuronidase antibodies. This suggests that although the protein is translocated over the membrane, it did not undergo signal peptide cleavage or was degraded by extracellular proteases. When cleavage does not occur within the encephalitogenic epitopes, the secreted protein could still maintain its tolerogenicity. Association of the mature form of translocated heterologous proteins with the cell wall has been described for Gram-positive bacteria (Suanders *et al.*, 1987; Simonen and Palva, 1993; Wells *et al.*, 1993). The smaller hMBP protein without ß-glucuronidase is much more efficiently secreted than hMBP fused to ß-glucuronidase. Following analysis of this recombinant by flow cytometry, we only found a very low surface-association of hMBP. Apparently, this product is indeed quite efficiently secreted into its environment. We have shown that protein secretion into the medium from recombinants transformed with vectors using the same secretion signals (pLP402-hMBP, pLP402-hMBP/U, pLP402-HA/U, pLP402-U) does not occur at similar levels. These differences probably arise from biochemical properties of the protein itself (e.g. size, hydrophobicity, conformation). In addition, varying sensitivities of each protein for proteases may affect the amount of protein found in the medium. Introduction of pLP402-MBP/U in alternative

Lactobacillus strains may result in an increase of the level of secretion of this particular protein.

Immunisation of mice with concentrated culture medium of the hMBP recombinants evoked MBP-specific antibodies, from which it could be concluded that the heterologous proteins were immunogenic and secreted into the medium. However, the antibody levels induced by medium containing the hMBP-ß-glucuronidase fusion protein were higher than the levels induced by medium containing hMBP.

Host strain selection is a factor that may impact significantly on the capacity to successfully induce antibodies or restore peripheral T-cell tolerance by oral administration. Recently, *Lactobacillus* strains have been identified that persist for extended periods in the intestinal tract of mice (D.M. Shaw, unpublished) as well as in humans in comparison to the prototype *L. casei*, and this property may improve efficacy of oral vaccination and of tolerance induction considerably. We have previously shown that induction of oral tolerance can be induced with *L. plantarum* transformed to express β-galactosidase (Maassen *et al.*, 1998a) and mucosal antibody responses can be induced with orally administered trinitrophenyl-*Lactobacillus acidophilus* conjugates (Gerritse *et al.*, 1991).

In conclusion, the generic technology described here allows the construction of a multitude of safe recombinant lactobacilli which may prove advantageous in oral intervention strategies for the prevention and treatment of infectious and autoimmune diseases of the central nervous system. Currently, recombinant lactobacilli expressing TTFC or viral antigens are tested in animal models for their ability to induce specific antibodies after oral administration. Whether orally administered recombinant lactobacilli secreting myelin antigens are capable of inducing peripheral T-cell tolerance is currently investigated in mouse and rat EAE-models for multiple sclerosis.

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Chapter 3.2

A rapid and safe plasmid isolation method for efficient engineering of recombinant lactobacilli expressing immunogenic or tolerogenic epitopes for oral administration

C.B.M. Maassen

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Summary

Recombinant lactobacilli are being developed which can be used as expression and delivery vectors of heterologous antigens in oral vaccination and other therapeutic applications. Because most Lactobacillus *strains do not accept ligation mixtures, sufficiently pure plasmid DNA needs to be isolated from* L. casei *to transform other* Lactobacillus *strains. The isolation of plasmid DNA from Gram-positive lactobacilli is complicated by the resilience of the peptidoglycan layer. Here a rapid, safe and efficient method is described that combines enzymatic breakdown of the cell wall and purification of the plasmid by commercially available DNA-binding columns. For the lysis-resistant* Lactobacillus casei *strain, this method yields high levels of pure plasmid DNA that can be used for common molecular techniques, such as digestion and transformation, with high efficiency.*

Introduction

Lactobacilli are lactic acid bacteria, which are founs as commensals of the human gut. Lactobacilli are increasingly being used in food products. In addition, pharmaceutical applications are being developed, such as genetically engineered lactobacilli as oral vaccines and for oral T-cell tolerance induction for the treatment of autoimmune diseases such as multiple sclerosis (MS) (Pouwels *et al.*, 1996; Maassen *et al.*, 1999a). Currently, in our laboratory, recombinant lactobacilli which express viral and microbial heterologous antigens on the cell surface are orally administered for the induction of antibodies in mice. Lactobacilli which secrete autoantigens such as myelin basic protein (MBP), are administered orally for the induction of peripheral T-cell tolerance in a mouse model for MS (experimental autoimmune encephalomyelitis, EAE).

Because most *Lactobacillus* strains do not accept ligation mixtures, the DNA required to transform these strains needs to be isolated from *Lactobacillus casei* ATCC 393, which can be transformed with ligated DNA. For efficient transformation of *Lactobacillus* strains, highly pure DNA is required. Current isolation techniques used to obtain large quantities of plasmid DNA consist of a cell lysis method followed by alkaline lysis, phenol-extraction and cesium chloride gradient centrifugation. The major obstacle in these isolation procedures is the efficient lysis of the Gram-positive cell wall. This can be achieved by mechanical or enzymatic procedures (Klaenhammer and Sutherland, 1980; Posno *et al.*, 1991a; Frère, 1994; Reinkemeier *et al.*, 1996). *Lactobacillus* plasmid DNA obtained after alkaline lysis and phenol-extraction contains contaminating chromosomal DNA and cell wall debris. Therefore additional cesium chloride equilibrium density gradient centrifugation is necessary to obtain plasmid DNA of high purity suitable for electroporation (Sambrook *et al.*, 1989). The procedure is time-consuming (2-3 days) and requires the use of toxic chemicals.

As a consequence, there is clearly a need for a more rapid, high yield isolation method to obtain plasmid DNA from lactobacilli, which does not require the use of hazardous agents. The aim of this study was to develop a simple method that yields large amounts of pure plasmid DNA from several different *Lactobacillus* strains, including lysis-resistant strains such as *L. casei*. Furthermore, this method should be more rapid than methods such as cesium chloride gradient centrifugation and has the advantage that no hazardous agents such as phenol and ethidium bromide need to be used. This method would simplify the development of recombinant lactobacilli, which can be used in various therapeutic applications.

Materials and methods

Plasmid isolation

Recombinant *Lactobacillus* strains were cultured overnight in 500 ml MRS (Difco Laboratories, Detroit, MI). Lysis of the lactobacilli was performed essentially as described by Posno *et al.* (1991a). The cells were harvested by centrifugation at 4000g for 20 min. The cells were washed twice in 25 ml 20 mM Tris/HCl buffer pH 8. The

pellet was suspended in 20 ml Tris/HCl (20 mM/pH 8) with 5 mg/ml hen egg white lysozyme (Boehringer Mannheim GmbH, Mannheim, Germany) and 20 ml of 24% polyethylene glycol 20,000 (Fluka Chemie AG, Buchs, Switzerland). After 2 h of incubation at 37°C, the cells were centrifuged and washed again with Tris/HCl (20 mM/pH 8). After discarding the supernatant the cells were taken up in the solution buffer E1 of the commercial kit (Jetstar plasmid maxi kit, Genomed GmbH, Bad Oeynhausen, Germany). The manufacturer's protocol, comprising alkaline lysis and subsequent neutralization with potassium acetate, was followed. After precipitation of the proteins present in this solution, the resulting DNA solution was applied to the provided column. After washing, the DNA was eluted with the buffer provided. Finally, the DNA was concentrated by alcohol precipitation and dissolved in 200 µl TE buffer (10 mM Tris/HCl pH 8, 1 mM EDTA). Digestion of the isolated DNA and analysis by agarose gel electrophoresis were carried out essentially as described by Sambrook *et al.* (1989).

Electroporation of Lactobacillus strains

Electroporation of *L. casei* was conducted as described by Posno *et al.* (1988), slightly adjusted from the method described by Chassy and Flickinger (1987). Electroporation of *L. plantarum 256* was essentially performed as described for *L. plantarum 80* by Josson *et al.* (1989). Briefly, an overnight culture of *L. plantarum 256* was diluted (1/50) in MRS broth and incubated anaerobically at 37°C. The cells were harvested in the mid-log phase, washed twice with "MilliQ" water, and washed once in 30% (w/v) PEG-1000. The cells were resuspended in 30% PEG-1000 in 1/50 of the original culture volume. Plasmid DNA (1 µg) was mixed with 100µl of the cell suspension in a disposable electroporation cuvette with an interelectrode distance of 0.2 cm. A single pulse of 8500 V/cm^2 was delivered (100Ω parallel resistor and 25 µF capacitance settings, Gene Pulser and Pulsecontroller from Bio-Rad). Following the pulse, the cell suspension was diluted with 900 µl MRS broth and incubated for 2 h at 37°C. Transformants were selected by plating the cells on MRS agar plates containing erythromycin. Transformants were visible after approximately 48 h.

Immunoblot analysis of Lactobacillus *transformants*

Immunoblots were performed in order to investigate the expression of heterologous proteins by *L. casei* and *L. plantarum* transformants. To induce expression, cells were grown in modified MRS medium (mMRS) (1% w/v proteose peptone, 0.5% w/v yeast extract, 0.2% w/v meat extract, 0.1% v/v Tween 80, 3.7mM sodium acetate, 0.8mM magnesium sulfate, 0.24mM manganese sulfate, 8.8mM diammonium citrate in potassium phosphate buffer (pH 7.0) containing 1% w/v mannitol) at 37°C, using a 1/50 dilution of an overnight culture. Cells were harvested in exponential phase, pelleted by centrifugation and suspended in phosphate buffered saline (PBS). Cell extracts were obtained from the bacteria by sonicating the cells four times on a 30 sec on/30 sec off cycle using an MSE Soniprep 150 sonicator at 10 amplitude microns

(Sanyo Gallenkamp PLC, Leicester, UK), to release both cytoplasmic and cell wall-bound proteins. After centrifugation the soluble fraction was used for immunoblot analysis. Proteins in cell extracts (30µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), (7.5% acrylamide for the UreB transformants and 11% acrylamide for the MBP/U transformants, 400 mM Tris pH 8.8) and run in a 25 mM Tris, 192 mM glycine buffer (pH 8.3) at 200V for 45 min. Protein was transferred electrophoretically onto nitrocellulose using a Bio-Rad Miniprotean II blotting unit (Bio-Rad Laboratories, Hercules, CA). Immunoblots blots were incubated with polyclonal rabbit anti-*H. pylori* (Simoons-Smit *et al.*, 1996) or rabbit anti-MBP serum. The second incubation step was performed with swine anti-rabbit Ig-HRP (Dako A/S, Glostrup, Denmark). The immunoblots were incubated with ECL detection reagents (Amersham Life Science Ltd, Buckinghamshire, UK). A light sensitive film (Kodak X-omat) was exposed to the blots for 1 min before development.

Results and Discussion

Isolation of plasmid DNA from L. casei *recombinants*

Two recombinant *Lactobacillus casei* strains were used to demonstrate the advantages of this novel plasmid isolation method. The first *L. casei* recombinant contained a vector (pLP402-MBP/U)(Pouwels *et al.*, 1996) with an insert coding for myelin basic protein (MBP), which is a central nervous system protein thought to be involved in the pathogenesis of multiple sclerosis. The heterologous MBP protein is fused to the marker protein ß-glucuronidase. Because this fusion protein will be secreted, these transformants can be used for oral tolerance induction in an animal model for multiple sclerosis (EAE). The second *Lactobacillus casei* recombinant contained the vector pLP401-UreB (Pouwels *et al.*, 1996) with an insert encoding UreB, a subunit of the urease enzyme of *Helicobacter pylori.* The UreB-product is expressed on the cell surface, because the vector also contains an anchor sequence. UreB transformants have proposed for use in oral vaccination experiments and plasmid DNA from both recombinants were isolated with the present novel isolation procedure. The ratio of A_{260} over A_{280} of the purified DNA was approximately 1.8, indicating that the DNA was indeed highly purified. The yield obtained from a 500 ml culture was approximately 200 µg of plasmid DNA. This is similar to the amounts of DNA usually obtained with the classic method using alkaline lysis and cesium chloride DNA banding.

Restriction analysis of isolated plasmid DNA

Two vectors isolated from the highly lysis-resistant *Lactobacillus casei* were digested with two restriction enzymes at the same time. The vector pLP402-MBP/U was digested with the restriction enzymes *BamH*I and *Pvu*I for 2 h at 37°C. The vector pLP401-UreB was digested with two other enzymes, *Pst*I and *Nhe*I. Both constructs were fully digested, confirming the high purity of the plasmids (*Figure 1*).

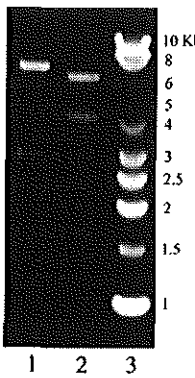

Figure 1. *Restriction analysis of plasmids isolated from L. casei*
The vector encoding the urease subunit B of *H. pylori* was digested with *Pst*I and *Nhe*I (lane 1). The vector encoding myelin basic protein fused to ß-glucuronidase was digested with *BamH*I and *Pvu*I (lane 2). For each restriction enzyme only one site was present. Lane 3, molecular weight DNA ladder.

Transformation of isolated plasmid DNA to other Lactobacillus *strains*
To assess whether the plasmids isolated by this method were sufficiently pure for use in transformation, the plasmids pLP402-MBP/U and pLP401-UreB were transformed to *L. casei* and *L. plantarum 256* by electroporation. Transformation efficiencies of 10^6-10^7 transformants per µg plasmid DNA were obtained for *L. casei* and 10^5-10^6 transformants per µg DNA for *L. plantarum*. These efficiencies are equal to those obtained with plasmid DNA purified by CsCl gradient centrifugation. This suggests that the plasmid DNA is highly pure and that limited breakdown of the DNA occurs.

Expression of heterologous proteins by L. casei *and* L. plantarum
In order to demonstrate that the *L. casei* and *L. plantarum* transformants are able to express heterologous proteins, extracts of these transformants were analysed by immunoblotting. After electroporation of the isolated plasmid DNA, the transformants were grown under conditions that permit expression of the heterologous proteins to occur. After 8 h of culture the cells were harvested and extracts were made. The soluble fraction of the extracts was analysed for expression by immunoblotting. *Figure 2A* shows the expression of UreB in *L. casei* containing pLP401-UreB (lane 3) as compared to wild type *L. casei* (lane 2) which, as expected, does not show any expression. UreB can also be demonstrated in *L. plantarum* pLP401-UreB extracts (lane 4). Wild type *L. plantarum* is negative for UreB (lane 5). As a positive control recombinant UreB (66kDa) isolated from *E. coli* was used (lane 1). UreB from *L. casei/L. plantarum* is larger (82kDa) due to the presence of signal peptide and anchor sequences. Expression of MBP fused to ß-glucuronidase is demonstrated in *Figure 2B*. Lane 3 shows the expression by *L. casei* pLP402-MBP/U and lane 4 shows the expression in *L. plantarum* pLP402-MBP/U. Again the wild type strains were used as negative controls (lane 2 = *L. casei*, lane 5 = *L. plantarum 256*). The size difference between the heterologous MBP, which runs at 85kDa (lane 3 and 4), and purified guinea pig MBP (21kDa)(lane 1) is mainly due to the fusion of the heterologous MBP with ß-glucuronidase. The additional bands present in the lanes with heterologous proteins result from cross-reaction of the polyclonal rabbit sera with *Lactobacillus*

antigens when those bands are also present in the corresponding wild type lane. All bands of smaller size, which are not due to such cross-reactivity, are breakdown products of the heterologous proteins. These results clearly demonstrated that *Lactobacillus* strains transformed with plasmids isolated using the present method are able to express the heterologous proteins that are encoded on the plasmids.

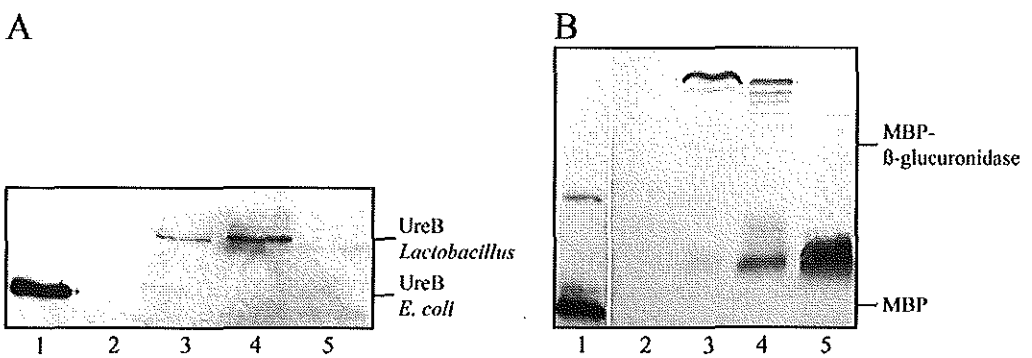

Figure 2.
(A) ***Immunoblot analysis of*** **Lactobacillus** ***transformants expressing UreB***
Lane 1, positive control of recombinant UreB isolated from *E. coli*. Lane 2, wild type *L. casei*. Lane 3, *L. casei* containing pLP401-UreB. Lane 4, *L. plantarum* containing pLP401-UreB. Lane 5, wild type *L. plantarum*. The immunoblot was incubated with anti-*H. pylori* serum.
(B) ***Immunoblot analysis of*** **Lactobacillus** ***transformants expressing MBP***
Lane 1, purified MBP. Lane 2, wild type *L. casei*. Lane 3, *L. casei* containing pLP402-MBP/U. Lane 4, *L. plantarum* containing pLP402-MBP/U. Lane 5, wild type *L. plantarum*. The immunoblot was incubated with anti-MBP serum.

Plasmid isolation from Lactobacillus plantarum 256

In order to investigate whether this method is more generally applicable to various *Lactobacillus* strains, plasmid DNA from *L. plantarum 256* transformed with the MBP containing plasmid was re-isolated as described above. Plasmid DNA was also isolated from *L. plantarum 256* transformed with a plasmid encoding tetanus toxin fragment C (pLP401-TTFC), which was isolated using a classic method followed by cesium chloride gradient centrifugation (Posno *et al.*, 1988). *Figure 3* shows that, from this *Lactobacillus* strain also, plasmid DNA could be isolated using this method. Breakdown of plasmid DNA, as reflected by the DNA smear in *Figure* 3, can probably be diminished when the incubation time with lysozyme is shortened, because the stringent lysis protocol for *L. casei* was also used for *L. plantarum*.

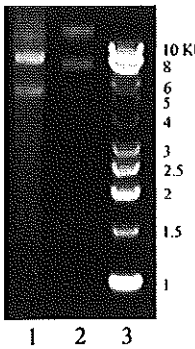

Figure 3. ***Plasmids isolated from L. plantarum 256***
The vectors pLP402-MBP/U (lane 1) and pLP401-TTFC (lane 2) were isolated from *L. plantarum 256* with the method described above and analysed on a 1% agarose gel. Lane 3, molecular weight DNA ladder.

Conclusion

In conclusion, this new method for the isolation of plasmid DNA from lysis-resistant lactobacilli has major advantages over the classic method that requires phenol-extraction and cesium chloride gradient centrifugation. This new method takes approximately five hours to perform instead of approximately two days, does not employ highly toxic reagents, is considerably cheaper and is easy to perform. This method provides high yields of pure DNA, which can be used for restriction analysis, transformation and other molecular techniques. This plasmid isolation technique is also applicable to other Gram-positive bacteria, and will improve the efficient engineering of biotechnologically and therapeutically useful lactobacilli recombinants for oral administration.

Acknowledgements

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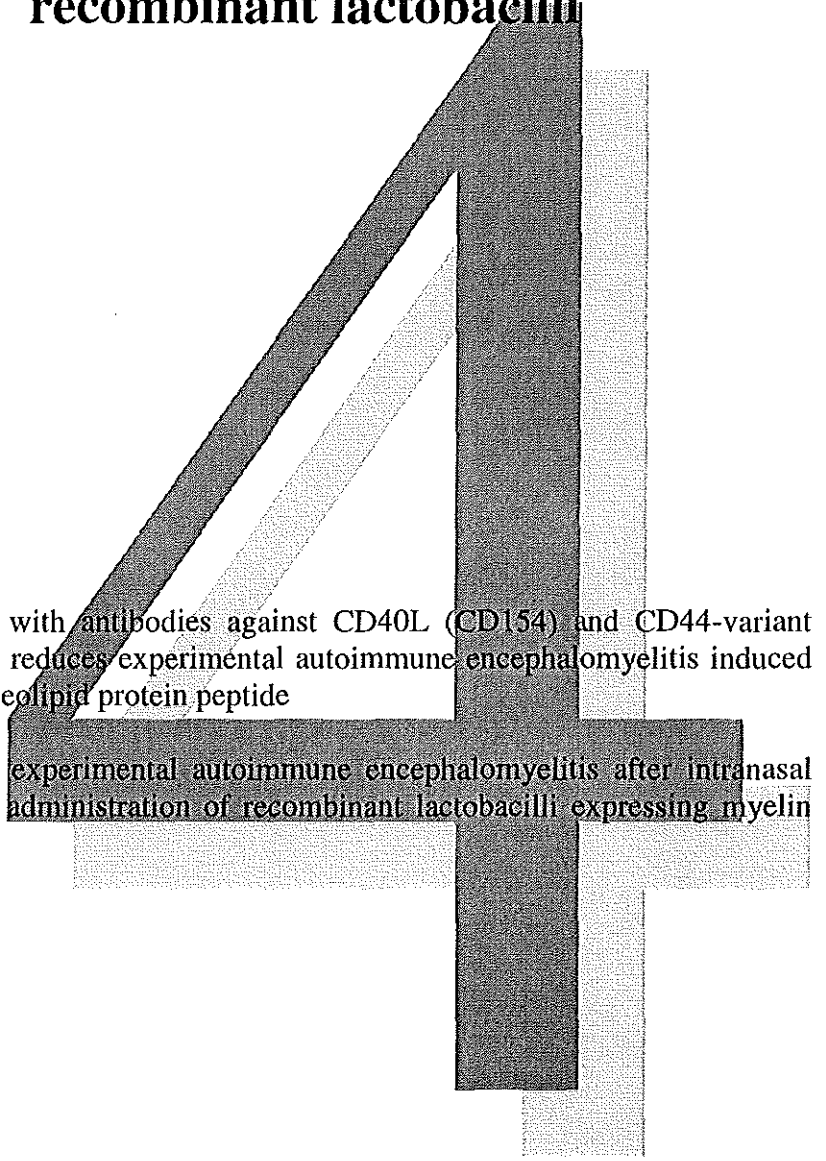
Mucosal tolerance induction with recombinant lactobacilli



4.1 Therapy with antibodies against CD40L (CD154) and CD44-variant isoforms reduces experimental autoimmune encephalomyelitis induced by a proteolipid protein peptide

4.2 Reduced experimental autoimmune encephalomyelitis after intranasal and oral administration of recombinant lactobacilli expressing myelin antigens

Chapter 4.1

Therapy with antibodies against CD40L (CD154) and CD44-variant isoforms reduces experimental autoimmune encephalomyelitis induced by a proteolipid protein peptide

J.D. Laman, C.B.M. Maassen, M.M. Schellekens, L. Visser, M. Kap, E. de Jong, M. van Puijenbroek, M.J.B. van Stipdonk, M. van Meurs, C. Schwärzler and U. Günthert

Multiple Sclerosis 4: 147-153, 1998

Summary

Interactions between mononuclear cells are required for the formation of inflammatory infiltrates in the CNS and the activation of cellular effector functions provoking demyelination in MS. Membrane-expressed costimulatory molecules are crucial to such interactions. We therefore investigated whether two costimulatory molecules, CD40L (CD154, expressed on activated CD4-positive T cells) and selected CD44-variant isoforms (expressed on activated CD4-positive T cells), are targets for immunotherapy in MS. The model of experimental autoimmune encephalomyelitis (EAE) induced in SJL-mice by immunization with a peptide derived from the proteolipid protein ($PLP_{139-151}$) was optimized to address these questions. A previous observation that anti-CD40L (CD154) monoclonal antibodies can effectively prevent EAE in this model was confirmed, and extended by demonstrating that CD40 is expressed by cells of the monocytic lineage infiltrating the spinal cord. In vivo treatment with antibody against the standard isoform of CD44 (CD44s or CD44H) did not affect disease burden. In contrast, combined treatment with antibodies against the isoforms CD44v6, v7 and v10, which are thought to be involved in inflammatory processes, reduced the disease burden considerably. In addition, CD44v10-expressing cells were detected in the spinal cord. These data support the idea that CD40-CD40L interactions form a target for immunotherapy of MS, and indicate that cells expressing CD44v6, v7 and/or v10-containing isoforms have such potential as well.

Introduction

Temporary and cumulative neurological dysfunction of multiple sclerosis (MS) patients results from chronic autoimmune inflammatory events within the central nervous system (CNS), leading to edema, demyelination of nerve axons, and axonal loss. Perivascular and periventricular inflammatory infiltrates of mononuclear cells containing mostly macrophages and CD4+ T-cells most probably affect these three processes, but the effector mechanisms involved have been elusive thus far. The formation of such infiltrates and activation of cellular effector mechanisms requires intercellular communication mediated by soluble mediators such as cytokines, as well as costimulatory (accessory) molecules on the cell surface.

Immunotherapy of MS with antibodies directed against costimulatory molecules such as CD40L, CD80/CD86 and CD44-isoforms, may affect one ore more of the following events: (i) Peripheral activation of autoreactive T-cells; (ii) Adhesion, blood-brain barrier transmigration, and migration of mononuclear cells within the CNS; (iii) Peripheral or local (CNS) cellular activation events, leading to further (co)-stimulation of T-cells, and/or induction of cytokines, chemokines and proteolytic enzymes. In the current study, the co-stimulatory CD40L and CD44-isoform molecules were evaluated as candidate targets for therapy, in a mouse EAE model.

We have previously shown that CD40-CD40L interactions are involved in EAE and MS, and that this interaction is indeed a target for therapy of MS (Gerritse *et al.*, 1996). CD40 is expressed on antigen presenting cells, such as dendritic cells, macrophages, microglia, endothelial cells and B cells. Interaction with CD40L expressed by activated, antigen-experienced CD4+ T-cells can trigger a wide array of effector functions of both the antigen presenting cell and the T cell itself (Laman *et al.*, 1996). In human MS autopsy brain, CD40 is expressed on the surface of monocytic cells within mononuclear infiltrates of the CNS. Functionally, we have demonstrated that development of EAE in SJL mice immunized with the proteolipid protein (PLP) peptide residues 139-151 can be prevented by treatment with anti-CD40L monoclonal antibody (Gerritse *et al.*, 1996). Finally, we have demonstrated that CD40 and CD40L are expressed in the CNS of marmoset monkeys with demyelinating EAE in relation to expression of pro-inflammatory and anti-inflammatory cytokines (Laman *et al.*, 1998c). This novel model is useful for the evaluation of human-specific immunotherapeutics.

In view of the proposed roles of differential CD44-isoform expression in tumor metastasis and chronic (autoimmune) inflammation, we hypothesized that CD44 variant isoforms are also involved in the development of MS and EAE (Rosenberg *et al.*, 1995; Günthert, 1996).

CD44 (formerly also called Pgp-1, gp90Hermes and H-CAM) has been implicated to act in processes such as pattern formation in embryogenesis, formation of the CNS, hematopoiesis, lymphocyte homing and activation, tumor dissemination and inflammatory reactions (Kaayk *et al.*, 1997). CD44 is an 80-250 kDa type I (extracellular N-terminus) transmembrane glycoprotein, encoded by only one gene.

Due to the complex RNA splicing mechanism of CD44 and post-translational modifications, about a thousand CD44-isoforms can potentially be generated. In practice, about one hundred different functional isoforms have been identified to date. The combination of structural features of CD44-isoforms allows high versatility in a wide array of cellular adhesion and activation events under physiological as well as pathological conditions. The CD44H (hematopoietic) or CD44s (standard) isoform is expressed widely, but no clear functional defects are apparent in CD44s knockout mice. CD44-variant isoforms, however, are only expressed on haematopoietic precursor cells, transiently during activation, and on memory cells. The pro-inflammatory cytokines TNF-alpha and IFN-gamma upregulate CD44 expression. Multiple ligands exist for CD44s, including hyaluronate, fibronectin, osteopontin, and collagen types I and IV, which are all components of the extracellular matrix (Lesley *et al.*, 1993). By virtue of glycosylation and heparan sulfate side chains, CD44v3 isoforms can also capture and concentrate cytokines as well as other soluble components, and present these to neighboring cells. The functions of CD44 and its variants still remain unknown, and putative ligands for these molecules await identification.

The aim of the current study was to provide further evidence for the involvement of CD40L in EAE, and to establish whether CD44-variant isoforms could also form a target for therapy in MS. The model of EAE induced in the SJL-mouse by immunization with $PLP_{139-151}$ peptide was further optimized to address these questions.

Materials and methods

Animals

Female SJL-mice were bred and maintained under SPF-conditions with free access to 0.9 MRAD-irradiated pelleted food (Hope Farms, Woerden, The Netherlands) and acidified water (pH 2.2) at the animal facility of the Department of Immunology, Erasmus University Rotterdam. This mouse strain is derived from the original SJL/J (Jackson) line, and has a low microbiological burden as assessed by routine screening. This strain is characterized by low IgE levels in naïve mice and animals display markedly more docile behavior than the original line. All experiments were performed with animals of 8-12 weeks old, at TNO-PG in Leiden, according to regulations in the Dutch law on animal experimentation.

EAE-induction

EAE was induced by immunization with $PLP_{139-151}$ peptide and additional treatment with *Bordetella pertussis* to affect blood-brain barrier integrity. This is a classic and widely used EAE-model in mice. Animals develop paralytic disease 10-11 days after disease induction and recover spontaneously around day 20 (Kono *et al.*, 1988; Sobel *et al.*, 1990; Gerritse *et al.*, 1996). Animals also show weight loss up to 25% concurrent with paralytic disease. On day 0, animals were injected subcutaneously with 50-100 micrograms (or other doses in the *in vivo* titration experiments) of peptide

(sequence HSLGKWLGHPDKF), divided over the four flanks, with 50 microliter per site. The peptide in PBS was mixed at 1:1 in Difco complete adjuvant (Difco 3113-60-5, with *Mycobacterium tuberculosis* H37RA). At day 1 and 3, animals received intravenous injections with 10^{10} heat-killed *Bordetella pertussis* bacteria from the vaccine preparation used for vaccination of humans (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Clinical disease was monitored daily from day 7 onward by weighing the mice, and by grading symptoms of paralysis using an internationally accepted clinical scoring scale, ranging from 0 to 5 (0 = no disease, 0.5 = tail paresis or partial paralysis, 1 = complete tail paralysis, 2 = paraparesis: limb weakness and tail paralysis, 2.5 = partial limb paralysis, 3 = complete hind- or front limb paralysis, 3.5 = paraplegia, 4 = quadriplegia, moribund, 5 = death due to EAE).

Treatment with antibodies against costimulatory molecules

To interfere with development of EAE, animals were treated intraperitoneally with protein A-purified monoclonal antibody preparations from the day of immunization onward. For CD40L, this treatment has previously been shown to effectively prevent EAE, even when treatment was started shortly before onset of clinical signs (Gerritse *et al.*, 1996). For CD44-variant isoforms such experiments have not been reported yet. In the current study, anti-CD40L treatment was performed to confirm previous findings, and to extend these by analysis of spinal cord mononuclear cell infiltrates for expression of CD40. In addition, anti-CD40L treatment served as an internal treatment control for the anti-CD44 isoform experiments. Animals were treated by i.p. injection of 250 microgram of antibody against CD40L and CD44s on day 0, 2 and 4, or on day 0, 2, 4 and 6 with a combination of antibodies against CD44 v6, v7 and v10. This protocol was based on previous experiments where anti-CD40L successfully prevented EAE (Gerritse *et al.*, 1996). The rat IM-7 antibody (IgG2b) directed against the mouse CD44s isoform was used, as well as mouse antibodies against isoforms v6 (LN 6.1), v7 (LN 7.1) and v10 (LN 10.1). These antibodies are further referred to as anti-v6, anti-v7 and anti-v10. CD40 was detected by means of FGK-45 (Rolink *et al.*, 1996). As the blocking antibody against CD40L is raised in hamster, hamster immunoglobulin (Hig) was used as a negative control antibody lacking blocking activity (Noelle *et al.*, 1992).

Immunohistochemistry

To evaluate expression of CD40, CD40L and CD44-variant isoforms in the CNS of animals used in the experiments above, immunohistochemistry was performed on frozen sections from brain and spinal cord of mice, using biotinylated preparations of the antibodies mentioned above, according to standard methods with previously described modifications (Gerritse *et al.*, 1996; Laman *et al.*, 1998c).

Results

In vivo *titration of* $PLP_{139-151}$

Animal models for EAE and other autoimmune diseases are notoriously sensitive to minute changes in a wide array of variables, including the origin, age, sex, hormonal and microbiological status of the animals, as well as the dose and composition of the disease-inducing antigen, and of the adjuvant used. In addition, for modulation experiments in such animal models, it is a prerequisite that the dose of antigen and the level of clinical disease are carefully balanced to be able to detect both disease-reducing and disease-aggravating effects of treatment. In other words, the lowest dose of antigen inducing a 100% incidence of disease should be used, and not a higher dose inducing plateau-levels of disease, obscuring modulatory effects of experimental treatment. For that reason, the EAE $PLP_{139-151}$ peptide was titrated *in vivo* in SJL-mice to accurately determine the optimal dose allowing modulation by treatment with antibodies or by oral feeding of *Lactobacillus* strains genetically engineered to express myelin antigens for mucosal T cell tolerance induction (Maassen *et al.*, 1999a). *Table 1* shows two independent *in vivo* titration experiments performed. Both the disease incidence and the level of disease of individual mice need to be considered. As is clear from comparing the upper and lower panel of the *Table*, peptide doses of over 25 microgram induce a 100% disease incidence. The low dose of 3.125 microgram of peptide is ineffective in inducing disease. The 6.25 microgram dose does induce disease, but only in a single animal out of three, with a delayed onset

Table 1. In vivo *titration of the encephalitogenic* $PLP_{139-151}$ *peptide*

Peptide dose (μg)	Incidence	Mean day of onset ±SD[a]	Mean max. score ±SD[b]
25	3/4	11.3±1.5	2.7±0.3
50	4/4	10.5±0.6	3.3±0.3
75	4/4	10.5±0.6	3.4±0.3
100	4/4	10.5±1.0	2.6±1.1
0	0/4	-	-
3.13	0/3	-	-
6.25	1/3	16[c]	1[c]
12.5	3/4	13.0±1.7	2.2±1.0
25	3/3	11.0±0.0	3.3±0.3
50	2/2	11.5±0.7	3.3±0.4
100	4/4	11.3±1.0	3.4±1.2[d]
200	3/4	11.0±0.0	3.0±0.0
400	4/4	10.8±0.5	3.3±0.5
800	3/3	11.3±0.6	3/3±0.3

Two independent experiments using partially overlapping dose ranges, are shown in the upper and lower part of the table. [a]mean day of disease onset of animals with clinical signs. [b]mean maximal disease score of animals with clinical signs. [c]data of a single individual animal with clinical signs. [d]one animal in this group died of EAE (clinical score 5).

and a lower disease score than that of animals receiveing higher doses. 12.5 microgram induces disease in three out of four animals, of which two have disease scores comparaple to animals treated with higher peptide doses. Doses of 25 microgram and higher lead to a 100% incidence. Interestingly, the higher doses of peptide (even up to 800 microgram per mouse) did not lead to death from EAE. Only one animal in the 100 microgram dose group died of EAE (clinical score of 5: lower panel). This is clearly a desirable aspect of a model for MS, in which death due to acute inflammation of the CNS is not typical at all. On the basis of these results, a dosage of 50-100 microgram peptide was considered to be optimal for use in subsequent experiments, resulting in a 100% incidence of disease.

Prevention of EAE by antibodies against CD40L and CD44-variant isoforms
To assess whether the standard form of CD44 (CD44s) is involved in development of EAE, animals were treated with a specific antibody (IM-7). Control animals were either treated with an irrelevant antibody (disease control group) or with anti-CD40L (blocking of disease control group). Although the irrelevant antibody did not interfere with disease as expected (*Figure 1*, top panel) and anti-CD40L antibody blocked disease effectively (middle panel), the monoclonal antibody against CD44s did not affect disease burden (lower panel)(Gerritse *et al.*, 1996).This indicates that either treatment was not effective in the regimen used, or that the standard isoform CD44s is not involved in development of EAE. As it has been claimed that specific isoforms of CD44 are involved in (autoimmune) inflammatory disorders such as colitis and arthritis, we next investigated whether CD44v6, v7 and v10 have a role in development of EAE in this model. Therefore, animals were treated with a combination of three different monoclonal antibodies against the CD44 isoforms v6, v7, and v10 on days 0, 2, 4 and 6 after EAE-induction. As can be seen in *Figure 2*, animals treated with the irrelevant antibody developed disease and recovered spontaneously, following normal disease kinetics (top panel). According to expectation, anti-CD40L antibody again effectively blocked development of EAE in this administration regimen (middle panel). Interestingly, administration of antibodies against v6, v7 and v10 considerably reduced EAE disease burden (lower panel). Importantly, one out of four animals was totally protected, and a second one only displayed minimal clinical symptoms for a period of three days versus eight days in the controls. The other two animals developed disease at a regular level, consistent with the all-or-none character of the model as was found in the *in vivo* titration experiments described above.

Immunohistochemistry
To assess whether CD40, CD40L and CD44-variant isoforms are expressed within mononuclear cell infiltrates within the CNS, brain and spinal cord tissue of the animals from the experiment was evaluated *in situ* by immunohistochemistry. As animals were sacrificed after resolution of the disease, it should be kept in mind that

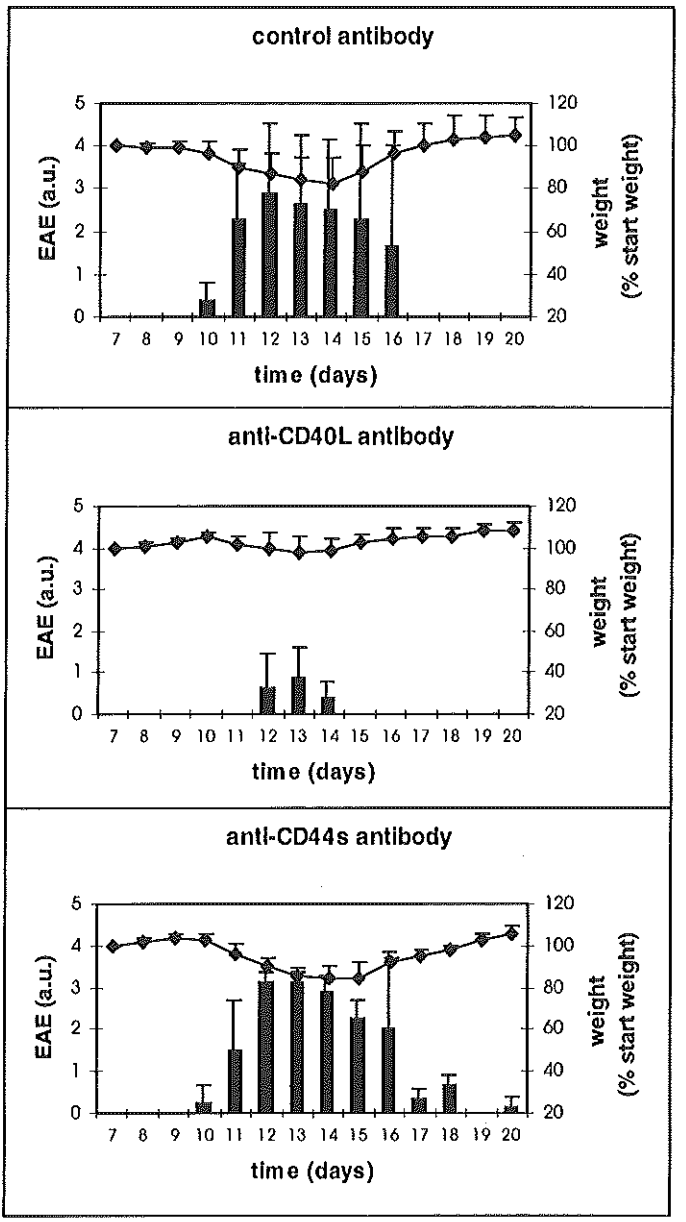


Figure 1. ***Treatment with anti-CD44s antibody does not prevent EAE***
Four SJL mice per group were treated with $PLP_{139-151}$ peptide to induce EAE. On days 0, 2 and 4 after immunization they received intraperitoneal injections with irrelevant control antibody (top panel: disease control group), anti-CD40L antibody (middle panel: disease blockade control group), or anti-CD44s antibody IM-7 (lower panel). Graphs show averages of four mice, with the line indicating the weight of the animals as a percentage of the start weight, and the bars represent clinical signs of disease, scored on a scale from 0 to 5 (see Materials and methods section).

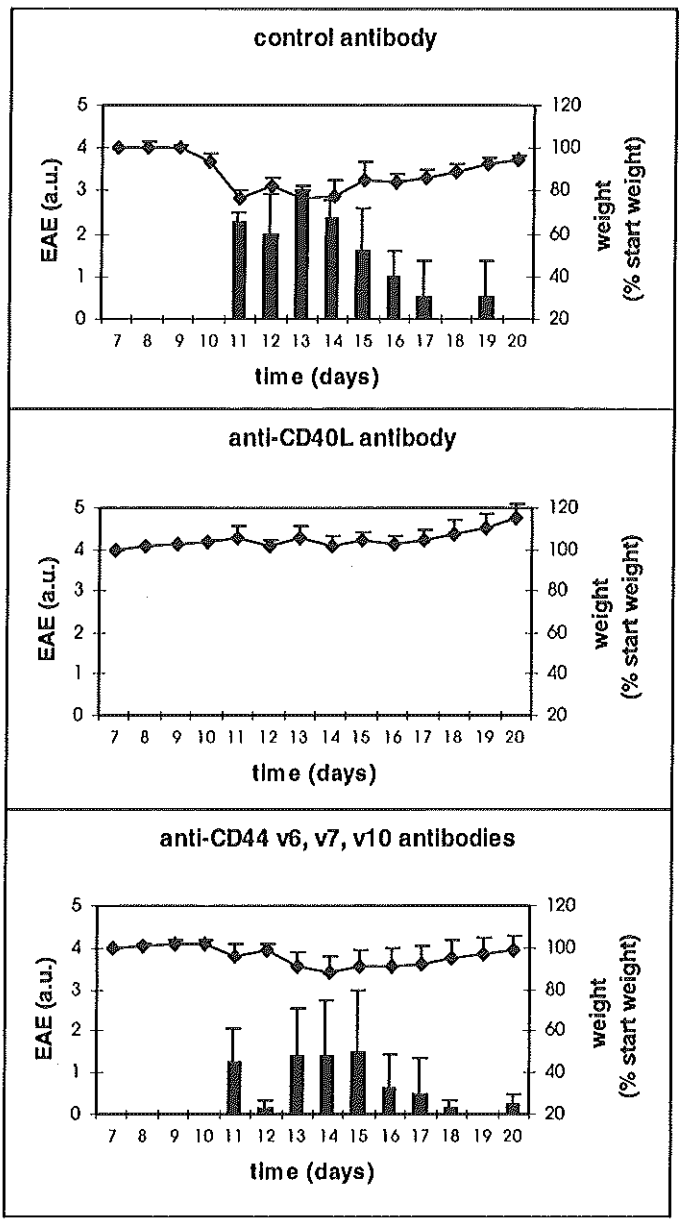


Figure 2. ***Treatment with anti-CD44 v6, v7 and v10 reduces EAE disease burden***
Four SJL mice per group were treated with $PLP_{139-151}$ peptide to induce EAE. On days 0, 2, 4 and 6 after immunization they received intraperitoneal injections with irrelevant control antibody (top panel: disease control group), anti-CD40L antibody (middle panel: disease blockade control group), or anti-CD44 v6, v7 and v10 antibody (lower panel). Graphs show averages of four mice, and are organized as described in the legend to *Figure 1*.

only late-stage infiltrates were analysed. In general, the infiltrates within the CNS of animals treated with anti-CD44-variant antibodies or anti-CD40L antibodies were markedly reduced in number and size compared to animals treated with irrelevant antibody (data not shown), indicating that antibody treatment directly or indirectly interfered with the local inflammatory process. CD40L-positive cells were only occasionally found within the infiltrates (not shown), consistent with findings in human MS brain (Gerritse *et al.*, 1996). However, we demonstrated for the first time that CD40 is clearly and abundantly expressed in mononuclear cell infiltrates of the mouse spinal cord (*Figure 3a*). Similar to the situation in human MS autopsy brain and marmoset EAE brain, CD40 was abundantly expressed by cells with intracellular

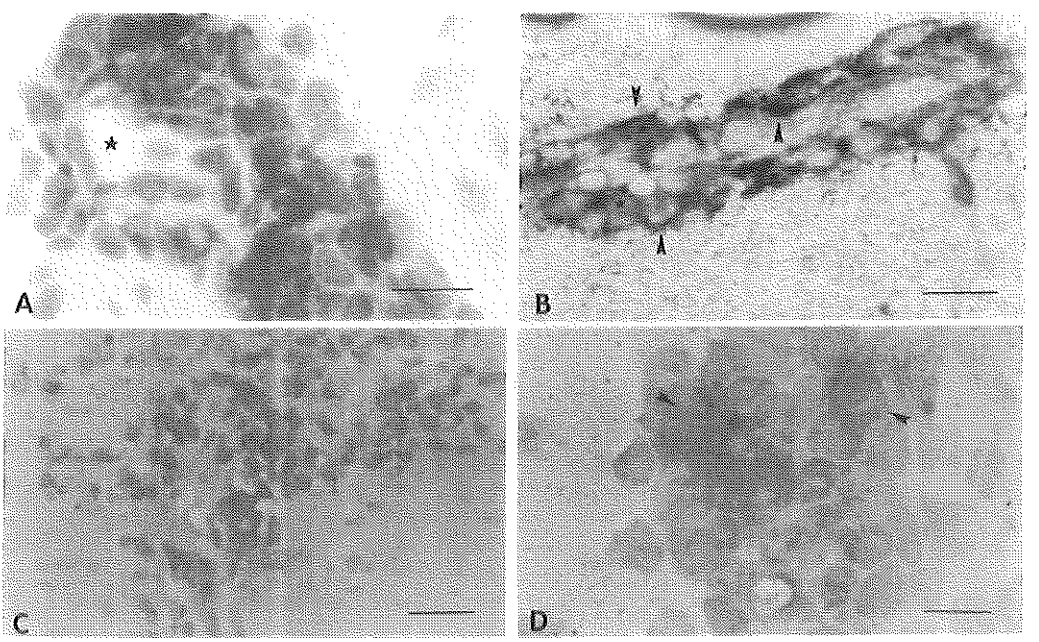


Figure 3. ***Expression of CD40 and CD44 isoforms in the spinal cord***
Control antibody-treated animals from the experiment shown in *Figure 3* were sacrificed after resolution of disease and frozen spinal cord tissue analyzed by immunohistochemistry for expression of CD40, CD40L and CD44 isoforms using the respective specific monoclonal antibodies (see Materials and methods). (A) Abundant expression of CD40 by cells present within perivascular mononuclear cell infiltrates in the spinal cord. Star indicates vessel. Bar indicates 15 micrometer. (B) CD40 is expressed on the surface of cells (examples indicated by arrowheads) by cells in the perivascular mononuclear cell infiltrates. Some of the CD40-positive cells belong to the monocytic lineage as indicated by the activity of intracellular acid phosphatase, a lysosomal enzyme (examples indicated by stars). Bar indicates 20 micrometer. (C) Abundant expression of CD44s by mononuclear cells within the spinal cord of an animal treated with control antibody, as detected by antibody IM-7. Bar indicates 50 micrometer. (D) Expression of the CD44v10 isoform (red) within the spinal cord of an SJL-mouse treated with control antibody (examples indicated by arrowheads). Bar indicates 10 micrometer.

acid phosphatase activity, characteristic for cells of the monocytic lineage (*Figure 3b*)(Laman *et al.*, 1998c). Single CD40-positive cells were also present (again similar to human MS and marmoset EAE), which might represent B cells or other antigen presenting cells lacking detectable acid phosphatase activity. Furthermore, cells with clear acid phosphatase activity but lacking CD40 expression were found too, indicating that local conditions do not necessarily induce CD40 expression of cells of the monocytic lineage. CD44s was clearly present within mononuclear cell infiltrates as can be seen in *Figure 3c*. In addition, expression of CD44v10, but not v6 or v6, by cells within the infiltrates could be demonstrated in the CNS of animals treated with irrelevant antibody after resolution of the disease (see *Figure 3d*), indicating that this isoform is indeed expressed in the target organ.

Discussion

This study confirms previous findings that anti-CD40L treatment can prevent EAE induced by $PLP_{139-151}$ in SJL-mice, and extends this concept by showing that CD40 is expressed abundantly in the spinal cord by infiltrating mononuclear cells. In addition, it is shown that combined treatment with antibodies against the CD44 variant isoforms v6, v7 and v10 reduces EAE disease burden, and that CD44s and CD44v10 are expressed by inflammatory cells in the spinal cord.

Although CD40-CD40L interactions have initially been described with respect to physiological T-B cell communication, it has now become clear that these interactions are also involved in B cell-driven autoimmune disease, and in autoimmune diseases driven by T cells and macrophages such as EAE in SJL-mice (Durie *et al.*, 1994; Gerritse *et al.*, 1996; Laman *et al.*, 1996; Mohan *et al.*, 1995). In addition, CD40-CD40L interactions are relevant for the chronic inflammatory process underlying atherosclerosis (Laman *et al.*, 1997). We have previously proposed that the CD40-CD40L interaction is a target for immunotherapy of MS (Gerritse *et al.*, 1996). Further support for this idea is provided by data in the current study showing that CD40 is expressed in the spinal cord of SJL-mice, mostly on the membrane of infiltrating cells of the monocytic lineage characterized by intracellular acid phosphatase activity. This is fully concordant with what was described previously for human MS autopsy brain and marmoset monkeys (*Callithrix jacchus*) with demyelinating EAE, a recently developed model for MS allowing evaluation of human-specific reagents (Gerritse *et al.*, 1996; Laman *et al.*, 1998c). The local expression of CD40 strongly suggests that HLA class II-restricted antigen presentation is ongoing, and that effector functions of macrophages expressing CD40 can also be triggered by CD40L-expressing activated CD4+ T cells. *In vitro*, such T cell/macrophage interactions can lead to production of nitric oxide, pro-inflammatory cytokines (including interleukins 1, 8, 12 and TNF-alpha), and matrix metalloproteinases (MMP-9: gelatinase B), which are all compounds thought to be involved in the immunopathogenesis of MS as well as other chronic inflammatory diseases (Laman *et al.*, 1997).

In view of the role of CD40-CD40L interactions in MS/EAE, and data on the involvement of CD44-variant isoforms in inflammation, we hypothesized that specific variant isoforms of CD44 might be involved in development of MS and EAE as well. Interestingly, it has been shown previously that CD40-CD40L interactions can activate CD44s expression *in vitro*, thereby functionally linking these receptor-coreceptor systems (Guo *et al.*, 1996). CD44-isoforms could be involved in the process of leukocyte extravasation (DeGrendele *et al.*, 1997) and trans-blood-brain barrier migration, as well as cellular interactions within perivascular and periventricular infiltrates, triggering effector functions that evoke MS and EAE-pathology (Wittig *et al.*, 1997). The concept that CD44-directed therapy may have promise for treatment of MS is supported by studies describing successful CD44-directed immunotherapy of experimental tumor formation, colitis and the inflammatory autoimmune disease arthritis (Mikecz *et al.*, 1995; Verdrengh *et al.*, 1995; Brennan *et al.*, 1997; Croft *et al.*, 1997).

Our *in vivo* treatment experiments with an antibody against CD44s (IM-7: see *Figure 1*) might argue against an involvement of the CD44s molecule, despite its expression on cells within infiltrates of the CNS. However, it cannot be excluded at this stage that factors inherent to the current experimental setting have influenced the findings, such as high and ubiquitous expression of CD44s on diverse cell types, limiting the amount of antibody available for blocking migration or effector functions of mononuclear cells effecting clinical disease. In addition, antibody characteristics such as affinity and functional capabilities of the Fc-portion of this IgG2b molecule may limit its blocking efficacy. In contrast, combined treatment with antibodies against v6, v7 and v10 effectively prevented development of EAE in half of the animals (*Figure 2*). CD44 isoforms (by staining with IM-7 antibody) and CD44v10 separately (by staining with a specific antibody) were found to be expressed in the spinal cord of animals treated with the control antibody (*Figure 3*). Clearly, it needs to be addressed whether treatment with antibodies against v6, v7 and v10 can prevent disease individually. Also, further evaluation of *in situ* expression of the isoforms in relation to disease stage is required.

What activities of CD44 might specific antibodies be blocking to prevent EAE? In comparison to other chronic inflammatory diseases, the insight into the roles of CD44-variant isoforms in EAE and MS is limited as yet. It has been described that CD44 is expressed within the CNS of SJL-mice with EAE by CD4+ T-cells of the activated memory/effector cell phenotype known to effect disease (Zeine and Owens, 1992). Possibly, CD44-expression by activated T-cells supports passage over the blood-brain barrier, but it is also conceivable that CD44 mediates activation of cellular effector functions within the mononuclear cell infiltrates in the CNS (Merrill *et al.*, 1992). CD44 also participates in binding of T cells to astrocytes (Haegel *et al.*, 1993).

Current experimentation is focusing on expression of CD44-isoforms in human MS and marmoset EAE brain, and on the role of selected isoforms in EAE in SJL-mice. To this end, both treatment with single isoform-specific antibodies and

isoform knockout mice are being used. In due course, such experiments should provide further insight into the mechanisms of CD44-involvement in inflammation of the CNS, as well as into the feasibility of CD44-isoform directed immunotherapy of MS.

Acknowledgements
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Chapter 4.2

Reduced experimental autoimmune encephalomyelitis after intranasal and oral administration of recombinant lactobacilli expressing myelin antigens

C.B.M. Maassen, J.D. Laman, J.C.P.A. van Holten, L. Hoogteijling, L. Groenewegen, L. Visser, M.M. Schellekens, W.J.A. Boersma and E. Claassen

Submitted for publication

Summary

Oral administration of autoantigens is potentially a safe and convenient way to induce peripheral T cell tolerance in autoimmune diseases like multiple sclerosis. To increase the efficacy of oral tolerance induction and obviate the need for purification of human myelin proteins, we use genetically modified lactobacilli that locally secrete the antigen. A panel of recombinant Lactobacillus *strains was constructed which secrete myelin proteins and peptides, including human and guinea pig MBP and* $PLP_{139\text{-}151}$*. In this study we examined whether these* Lactobacillus *recombinants are able to induce oral and nasal tolerance in an animal model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Lewis rats and SJL/J mice received soluble cell extracts of* Lactobacillus *transformants intranasally three times prior to induction of EAE. For the induction of oral tolerance, rats and mice were fed live transformed lactobacilli for 20 days. Ten days after the first oral administration EAE was induced. Intranasal administration of extracts containing gpMBP or* $MBP_{72\text{-}85}$ *significantly inhibited EAE in Lewis rats. Extracts of control transformants did not reduce EAE. Live lactobacilli expressing guinea pig* $MBP_{72\text{-}85}$ *fused to β-gluc were also able to significantly reduce disease burden when administered orally, in contrast to total guinea pig MBP. Also in SJL/J mice* Lactobacillus *constructs tended to reduce disease after oral or nasal administration. In conclusion, these experiments provide proof of principle that lactobacilli expressing myelin antigens reduce EAE disease burden after mucosal administration. Optimization of myelin expression levels and administration regimens will further increase efficacy of peripheral T-cell tolerance induction in rodents.*

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that leads to destruction of CNS myelin. It is widely assumed that MS is an autoimmune disease mediated by CD4+ T-cells of the T helper 1 subset. The current therapies available for MS are non-specific, may have toxic side effects or can not be used in long term treatment. Therefore, antigen-specific therapies to silence or delete autoreactive T-cells are preferred. In animal models for MS (EAE, experimental autoimmune encephalomyelitis), induction of peripheral T cell tolerance can be achieved by injection of large doses of soluble myelin antigens intravenously or intrathymically (Goss *et al.*, 1994; Khoury *et al.*, 1993; reviewed by Liblau *et al.*, 1997). Also systemic administration of altered peptide ligands (APL) or MHC-II-peptide complexes can suppress EAE in an antigen-specific manner (reviewed by Liblau *et al.*, 1997). For use in humans a disadvantage is that some of these therapies need to be tailored to the HLA haplotype of individual patients. During disease T cell responses against more epitopes of the myelin will develop (epitope spreading), which further complicates these approaches.

A promising antigen-specific therapy is the induction of peripheral T-cell tolerance by mucosal administration of autoantigen (mucosal tolerance). Three main mechanisms have been described; anergy, deletion and active suppression. When anergy (non-responsiveness of autoreactive T-cells) (Karpus *et al.*, 1996; Whitacre *et al.*, 1991) or deletion (Chen *et al.*, 1995a) are the mechanisms of peripheral T-cell tolerance induction, the autoantigen needs to be known. This can be circumvented when active bystander suppression is induced (e.g. al-Sabbagh *et al.*, 1994; Anderton and Wraith, 1998; Higgins and Weiner, 1988; Miller *et al.*, 1991). A local Th2/Th3 (e.g. Chen *et al.*, 1994) environment or a suppressive CD8+ T-cell subset (Lider *et al.*, 1989; Chen *et al.*, 1995b) can suppress the inflammatory autoimmune response. Mucosal tolerance by oral and nasal administration has been achieved with several different myelin antigens in distinct EAE models (e.g. Anderton and Wraith, 1998; Bitar and Whitacre, 1988; Higgins and Weiner, 1988; Li *et al.*, 1998).

In the Lewis rat, EAE can be induced by immunisation with guinea pig spinal cord homogenate (gpSCH), myelin basic protein (MBP) or proteolipid protein (PLP) emulsified in complete Freund's adjuvant (CFA). The dominant encephalitogenic epitope of MBP in the Lewis rat is $MBP_{72\text{-}85}$ (Hashim, 1977; Mannie *et al.*, 1985). EAE induced with this peptide is mediated by CD4+ T lymphocytes. A second MBP epitope that is encephalitogenic for Lewis rats has been localized to residues 86-98 (Offner *et al.*, 1989; Sun *et al.*, 1992). However, this peptide is only weakly encephalitogenic requiring higher doses to elicit clinical signs (Gould and Swanborg, 1993). This secondary encephalitogenic peptide of MBP in the Lewis rat is the major encephalitogenic MBP peptide in SJL/J mice, but 100% disease incidence is hard to achieve, even with high peptide doses (Kono *et al.*, 1988; Sakai *et al.*, 1988). EAE in SJL/J mice can be induced by immunization with SCH (e.g. al-Sabbagh *et al.*, 1996), MBP (e.g. Fritz *et al.*, 1983) or $PLP_{139\text{-}151}$ (e.g. Laman *et al.*, 1998a).

In all these models oral tolerance induction leading to reduction of the disease burden was demonstrated. Of increasing interest is the induction of mucosal tolerance by nasal application of autoantigen. Several groups have now reported that nasal administration of autoantigens or peptides derived from self as well as foreign antigens can induce peripheral T cell tolerance, thereby protecting animals from the induction of autoimmune disease (Dick *et al.*, 1994; Li *et al.*, 1998; Liu *et al.*, 1998; Metzler and Wraith, 1993; Staines *et al.*, 1996; Tian *et al.*, 1996). Nasal tolerance induction has not been reported yet in MBP_{72-85} EAE in Lewis rats, the rat model used in this study. Some studies have shown that the nasal route is more effective than the oral route, requiring significantly lower antigen doses (Ma *et al.*, 1995; Metzler and Wraith, 1993). The mechanisms by which nasal administration of soluble antigens suppress autoimmunity are poorly understood. A number of studies has suggested that nasal administration of soluble antigens can lead to peripheral T-cell tolerance by processes of active suppression, such as immune deviation (i.e. shift from Th1 to Th2 response) or induction of regulatory TGF-ß producing Th3 cells (al-Sabbagh *et al.*, 1996a; Bai *et al.*, 1997; Li *et al.*, 1998; Ma *et al.*, 1996; Tian *et al.*, 1996). Li et al. showed that, like in oral tolerance, dose-dependent mechanisms are involved in nasal tolerance induction. Nasal administration of 5 doses of only 6µg gpMBP led to complete prevention of EAE in Lewis rats, probably due to an active suppression mechanism. In contrast, nasal tolerance induction by administration of 600µg gpMBP probably induced anergy (Li *et al.*, 1998).

Mucosal administration of autoantigens is very effective in animal models in preventing autoimmune disease, but in humans chronic disease often has been established. In some experimental autoimmune models it is possible to treat ongoing disease (Anderton and Wraith, 1998; Benson *et al.*, 1999). The efficacy of these treatments is crucially dependent on dosing and scheduling (Meyer *et al.*, 1996). For several autoimmune diseases in humans therapy by oral administration of antigen has been attempted. Although some trials were promising, no clear positive results were obtained (e.g. Barnett *et al.*, 1998; Weiner *et al.*, 1993; Nussenblatt *et al.*, 1997). For MS, one of the problems is the fact that the autoantigen is still unknown, a problem that does not exist in animal models. Another major problem is the purity and the amount of antigen that needs to be obtained. In the human MS trial, bovine myelin was used. From animal studies it is known that heterogeneous antigen mixtures such as myelin are less effective in inducing oral tolerance than single protein antigens such as MBP (Benson *et al.*, 1999). In addition, very high doses are required in oral tolerance induction, partially due to breakdown of antigen in the stomach. In order to overcome a number of these problems, we use genetically modified lactobacilli that secrete the antigen locally in the gut. The use of these recombinant lactobacilli is probably safer than purified myelin, because there is no risk of administering viruses or prions co-isolated with myelin. A panel of recombinant *Lactobacillus* strains was constructed which secrete myelin proteins and peptides, including human and guinea pig MBP and $PLP_{139-151}$ (Maassen *et al.*, 1999a). cDNAs of a number of

encephalitogenic myelin proteins and peptides were cloned, because encephalitogenic antigens have been demonstrated to be efficient tolerogens (e.g. Liu *et al.*, 1998; Karpus *et al.*, 1996).

Lactobacilli are Gram-positive lactic acid bacteria which are frequently used in dairy products because of their health promoting effects such as the non-specific enhancement of the immune response (adjuvanticity), control of intestinal infections, control of serum cholesterol levels and anti-carcinogenic activity (Bloksma *et al.*, 1979). Oral or nasal administration of these diverse species of lactic acid bacteria with the generally regarded as safe (GRAS) status is cost-effective and simple (Pouwels *et al.*, 1996). Since individual *Lactobacillus* strains are clearly distinct, strain selection is very important. We have chosen *L. casei* for recombinant autoantigen expression, because this strain possibly favors tolerance induction in the gut by inducing TGF-ß and IL-10 expression (Maassen *et al.*, 1999b), while the bacterium itself is not immunogenic and does not enhance the humoral immune response to exogenous protein antigen in a non-specific manner (Maassen *et al.*, 1999c).

Our data show that *Lactobacillus* recombinants can prevent EAE by oral and nasal administration, and that in at least one model, nasal administration enhances EAE.

Materials and methods

Animals

Female Lewis rats of approximately 175 gram were obtained from Charles River/ The Broekman Institute, Someren, The Netherlands. Female SJL/J mice (8-12 weeks) were obtained from the Erasmus University Rotterdam, The Netherlands. All animals were kept under filtertop hoods in a DII facility with free access to pelleted food and acidified water (pH 2.8). Experiments were performed according to regulations in the Dutch law on animal experimentation.

Recombinant lactobacilli

For the induction of tolerance by oral or nasal administration of recombinant lactobacilli in SJL/J mice or Lewis rats, a combination of the following vectors was used; pLP402-gpMBP, pLP402-hMBP, pLP402-MBP86 (pLP402-peptide), pLP402-gpMBP/u, pLP402-hMBP/u, pLP402-MBP72/u, pLP403-MBP72/u, pLP402-PLP139/u, pLP402/u and pLP402-Ha/u (pLP402-peptide2/u). All pLP402 vectors secrete heterologous protein. The pLP403 vector retains the heterologous protein intracellularly. The basic *E. coli/Lactobacillus* shuttle vectors and the general construction method have been described by Maassen *et al.* (1999a). All pLP400/u vectors express a fusion protein with the marker enzyme ß-glucuronidase (ß-gluc) from *E. coli*. Vectors were transformed to *L. casei* (ATCC 393).

gpMBP = guinea pig myelin basic protein, hMBP = human MBP, MBP86 = $MBP_{86\text{-}98}$ (VHFFKNIVTPRTP), MBP72 = $MBP_{72\text{-}85}$ (QKSQRSQDENPV), PLP139 = proteo-lipid protein 139-151 (HCLGKWLGHPDKF), Ha = hemagglutinin peptide 111-120.

Culturing of recombinant lactobacilli

For the oral administration of recombinant lactobacilli, the cells were prepared as described below. One liter (rat experiments) or 500 ml (mouse experiments) of mMRS containing 1% mannitol and erythromycin (Maassen *et al.*, 1999a) was inoculated at 1:200 with a stationary phase culture of the recombinant *Lactobacillus* strains and cultured without aeration at 37°C till an OD_{690} of 1.0 was reached. For all recombinants used in this study the highest level of heterologous gene expression was approximately at the optical density of 1.0. The cells were harvested and washed twice with PBS and once with 0.2M $NaHCO_3$. The cells were resuspended in $NaHCO_3$ to a volume of 12 ml (rat) or 3 ml (mouse). A small volume was plated out to calculate the number of colony forming units (CFU) orally administered.

For the induction of tolerance by nasal administration, extracts of recombinant lactobacilli were used. The cells were grown as described above, harvested and washed with PBS. The extracts were made by sonicating the cells in PBS as described before (Maassen *et al.*, 1999a). The soluble fraction was used for intranasal administration.

Intranasal tolerance induction

Rats received either 80µl of *Lactobacillus* extracts (≈320µg total protein), synthetic MBP_{72-85} (100 µg or 200 µg), purified guinea pig MBP (Deibler *et al.*, 1972)(100µg or 200µg) or PBS divided over two nostrils. Nasal administration took place at day -15, -10 and -5. At day 0, EAE was induced (Prakken *et al.*, 1997).

In order to determine the dose dependent effect of nasal administration of $PLP_{139-151}$ in $PLP_{139-151}$ induced EAE, groups of 3 mice received 50µg, 100µg or 200µg $PLP_{139-151}$ in PBS 10 and 5 days prior to induction of EAE. To study the effect of nasal administration of *Lactobacillus* extracts on $PLP_{139-151}$ induced EAE and spinal cord homogenate (mSCH) induced EAE, groups of 3 to 6 mice received either 20µl of *Lactobacillus* extracts or PBS, divided over two nostrils. The mice received 10 nasal applications from day -10 till day -1 ($PLP_{139-151}$ induced EAE and SCH induced EAE), or at day -10 and -5 ($PLP_{139-151}$ induced EAE). At day 0, EAE was induced.

Oral tolerance induction

Rats or mice were fed one strain of recombinant lactobacilli or $NaHCO_3$ buffer daily for 20 days (from day –10 till day 9). The cells were prepared as described above. The rats received approximately 2.10^{11} lactobacilli in 2 ml with a gastric syringe, while the mice received approximately 5.10^{10} lactobacilli in 500µl. At day 0, EAE was induced.

EAE induction in Lewis rats

EAE was induced by subcutaneous immunisation in the hind footpads with in total 70µg MBP_{72-85} emulsified in Difco's incomplete adjuvant with 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Clinical disease was monitored daily from day 6 onward by weighing the rats, and by grading symptoms of

paralysis using an internationally accepted clinical scoring scale ranging from 0 (no signs) till 5 (death)(see Laman *et al.*, 1998a).

EAE induction in SJL mice
Two mouse EAE models were used in this study. In the first model EAE was induced by subcutaneous immunisation with 50µg synthetic $PLP_{139-151}$, in the other model the induction of EAE was performed with 1.8 mg spinal cord homogenate prepared from BALB/c mice (mSCH). On day 0, 200µl of an emulsion of 9 parts homogenate in $NaHCO_3$ with 11 parts Difco's complete adjuvant containing 1 mg/ml *M. tuberculosis* H37Ra (Difco) was divided over two flanks. At day 1 and 3, animals received intravenous injections with 10^{10} heat-killed *Bordetella pertussis* bacteria (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Weight and symptoms of paralysis were monitored from day 6 onward as described above.

ELISA
Serum was collected every 7 days and tested in ELISA for the presence of antibodies against myelin proteins and peptides as a measure of T cell and B cell stimulation and tolerance induction. Plates were coated with 5µg/ml antigen in PBS (50µl/well) overnight at 4°C. Non-specific antibody binding was blocked by incubation with 0.2% gelatin in PBS (50µl/well) for 1 hour at 25°C. Subsequently the plates were incubated with dilutions of serum of treated animals and preimmune sera to correct for background reactivity for one hour at room temperature. For the detection of IgG antibodies specific for the diverse myelin antigens in rat, alkaline phosphatase-labelled goat anti-rat IgG (Sigma Chemical, La Jolla, CA) was used. For the detection of IgG antibodies specific for $PLP_{139-151}$, gpMBP, hMBP, MBP_{86-98} or mSCH in mouse sera, alkaline phosphatase-labelled goat anti-mouse IgG (KPL, Gaithersburg, MD) was used. For the detection of $PLP_{139-151}$ specific IgG1 or IgG2a antibodies, rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibodies (ICN Immunobiologicals, Costa Mesa, CA) were used respectively, followed by 1 hour incubation with alkaline phosphatase-labelled swine anti-rabbit Ig antibodies (Dako A/S, Glostrup, Denmark). At several timepoints after addition of the substrate paranitrophenyl phosphate, the absorbance was read at 405nm.

Statistics
In the EAE experiments, the disease burden of each animal was calculated in percentages of the mean disease burden of the control group which received intranasally PBS or orally $NaHCO_3$ only within the same experiment. The disease burden per treatment over several experiments was determined by calculating the mean disease burden of all animals that had received the same treatment in percentages. In order to determine whether differences reached significance, statistical analysis was performed using a single factor ANOVA, followed by calculating the least significant difference.

Results

Exacerbation versus inhibition of EAE in Lewis rats by intranasal administration of MBP protein or peptide

To investigate whether it was possible to induce tolerance in Lewis rats by intranasal administration of myelin antigen, guinea pig MBP (gpMBP) and the immunodominant synthetic peptide MBP_{72-85} were administered intranasally before induction of EAE. The protein and the peptide were administered in two doses of 100µg or 200µg at 15, 10 and 5 days before induction of EAE with MBP_{72-85}. The disease burden of each animal was expressed as percentage of the mean disease burden of the control group which intranasally received PBS only within the same experiment. The disease burden per treatment over 3 experiments was determined by calculating the mean disease burden of all animals which had received the same treatment (*Figure 1*). Intranasal administration of the MBP peptide 72-85 partially ameliorated EAE in a dose dependent manner. When 100µg peptide was administered per application, the disease burden was reduced with 56%. A significant reduction of EAE with 85% compared to the control group was found when 200µg MBP_{72-85} was given intranasally ($p<0.05$). In contrast, when the whole MBP protein was administered intranasally, an enhancement of EAE was found. The increase of the disease burden was comparable for both doses applied (100µg =171%, 200µg =155%), and this was significant for the 100µg dose ($p<0.05$).

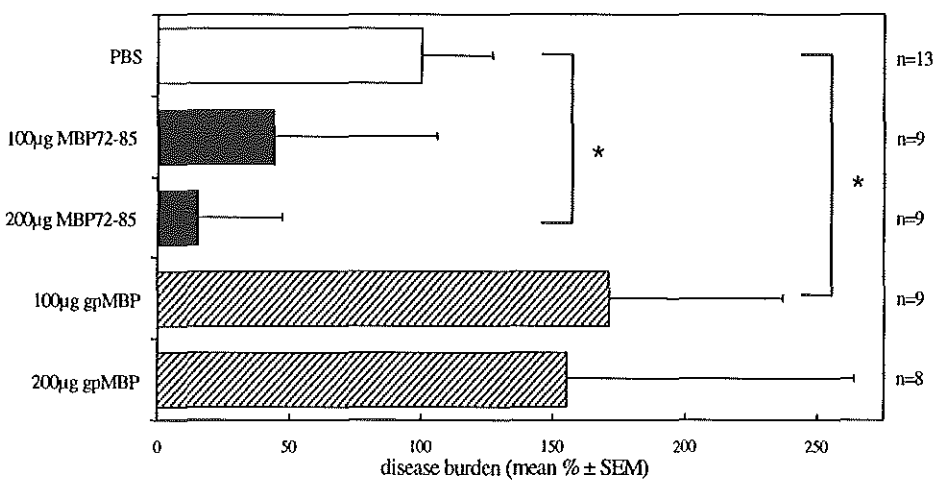


Figure 1: ***Exacerbation versus inhibition of EAE in Lewis rats by intranasal administration of MBP protein or peptide***

At day -15, -10 and -5 Lewis rats intranasally received 100µg or 200µg MBP peptide 72-85 (black bars) or gpMBP protein (gray bars). At day 0 EAE was induced with MBP_{72-85}. The disease burden of each animal was expressed as a percentage of the mean disease burden of the control group which received intranasally PBS only within the same experiment. The disease burden per treatment over 3 experiments was determined by calculating the mean disease burden of all animals which had received the same treatment. n= number of animals per treatment

* = $p<0.05$ compared to control group which nasally received PBS (white bar)

Intranasal tolerance induction in Lewis rats by Lactobacillus *extracts containing myelin antigens*

Prior to intranasal application of recombinant lactobacilli extracts containing myelin antigens for tolerance induction, expression of myelin antigens by the recombinants was analyzed. The constructs pLP402-MBP72/u and pLP402-gpMBP/u secrete MBP_{72-85} peptide and gpMBP protein fused to ß-gluc, respectively. The construct pLP402/u, which secretes ß-gluc only, was used as a negative control. Expression of these heterologous proteins by *L. casei* was confirmed by immunoblotting with anti-ß-glucuronidase antibody (Maassen *et al.*, 1999a; results not shown). The heterologous protein gpMBP fused to ß-gluc was also detected with anti-gpMBP antibody (Maassen *et al.*, 1999a). The fusion protein MBP_{72-85}/ß-gluc was also demonstrated with anti-MBP_{72-85} antibody (results not shown). Soluble fractions of recombinant lactobacilli were administered intranasally 3 times, at day -15, -10 and -5 prior to EAE induction with MBP_{72-85} on day 0. The mean disease burden was calculated as a percentage of the mean disease burden of the control group which received PBS intranasally (*Figure 2*). Intranasal application of *Lactobacillus* extracts of the control recombinant pLP402/u did not affect the EAE course, as expected. Intranasal pretreatment with *Lactobacillus* extracts of pLP402-gpMBP/u reduced the mean disease burden

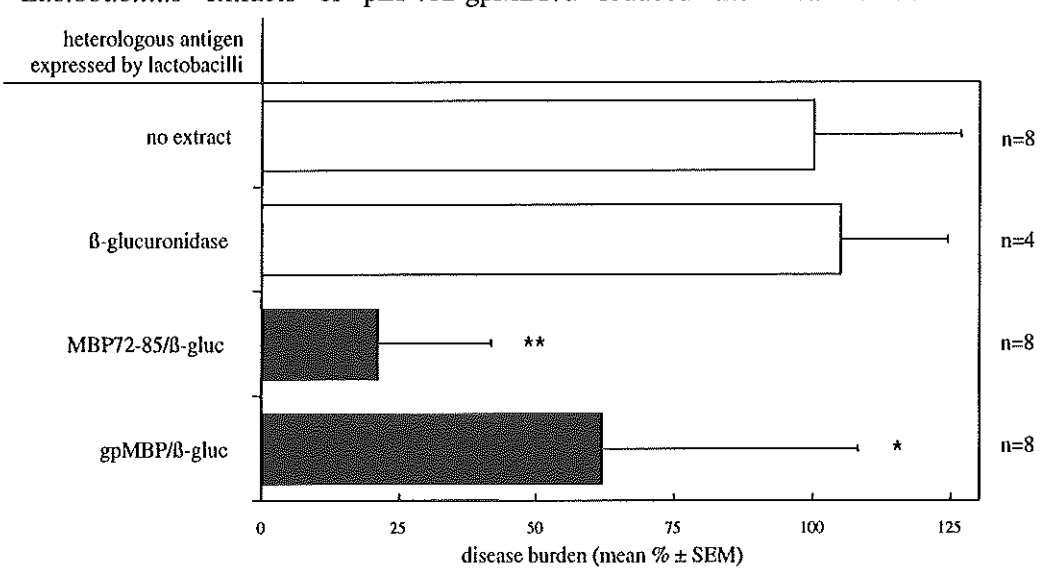


Figure 2: ***Reduction of EAE after intranasal administration of*** **Lactobacillus** ***extracts containing myelin antigens in Lewis rats***

At day -15, -10 and -5 Lewis rats intranasally received 80µl *Lactobacillus* extract containing approximately 320µg protein. The *Lactobacillus* contained expressed heterologous myelin antigen fused to ß-gluc (black bars) or ß-gluc only (white bar). At day 0 EAE was induced with MBP_{72-85}. The disease burden was expressed as a percentage of the mean disease burden of the control group which intranasally received PBS only. The results of 2 experiments are shown. n=number of animals per treatment

* = $p<0.05$ compared to both control groups (white bars), **= $p<0.01$ compared to both control groups (white bars)

significantly when compared to either of both control groups (PBS and pLP402/u) (both $p<0.05$). When *Lactobacillus* extracts containing MBP_{72-85} were applied intranasally, EAE was even further ameliorated. The mean disease burden was reduced with almost 80% ($p<0.01$) of the PBS treated group (*Figure 2*).

Oral tolerance induction in Lewis rats by recombinant lactobacilli expressing MBP72-85 intracellularly or extracellularly

Three recombinant *Lactobacillus* strains were used to test whether oral administration of live lactobacilli expressing myelin antigens could prevent EAE. In addition to the strains used in the intranasal tolerance experiments pLP402-MBP72/u (secretion of MBP_{72-85} fused to ß-gluc) and the control strain pLP402/u (secretion of ß-gluc), the strains with pLP402-gpMBP, which secretes guinea pig MBP protein and pLP403-MBP72/u, which intracellularly retains the MBP_{72-85} peptide fused to ß-gluc, were used. Approximately 2.10^{11} cells were orally administered per animal daily from day -10 to day 10. At day 0 rats were immunized with MBP_{72-85} to induce EAE. When lactobacilli expressing ß-gluc only (pLP402/u) was administered orally, only 3 out of 5 rats developed EAE, but no significant difference was found in day of onset, mean maximum score or mean disease burden, when compared to the control group who had received buffer ($NaHCO_3$) orally (*Figure 3*). Oral administration of pLP402-gpMBP had no effect on EAE, but when lactobacilli expressing the MBP peptide 72-85

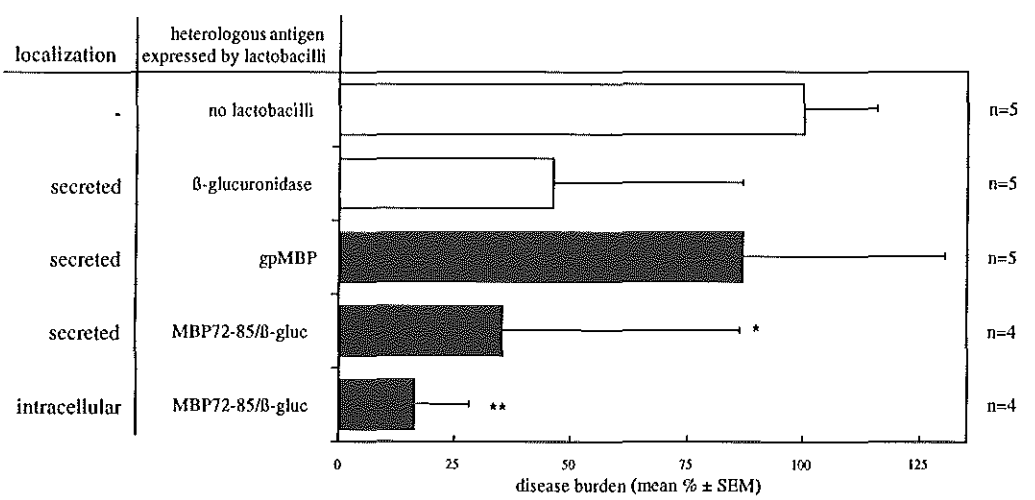


Figure 3: ***Oral tolerance induction in Lewis rats by recombinant lactobacilli expressing MBP_{72-85} intracellularly or extracellularly***

Groups of 5 rats received orally 2.10^{11} live recombinant lactobacilli expressing myelin antigens for 20 days (day -10 till 10). The vectors used were designed for intracellular expression or secretion of the heterologous antigen. EAE was induced with MBP_{72-85} at day 0. The disease burden was expressed as a percentage of the mean disease burden of the control group which received orally $NaHCO_3$ only (white bar).*=$p<0.05$ compared to the control group which received orally $NaHCO_3$, **=$p<0.01$ compared to the control group which received orally $NaHCO_3$ only and $p<0.05$ compared to the group fed gpMBP.

intracellular or secreted (pLP402-MBP72/u and pLP403-MBP72/u), EAE was significantly inhibited (*Figure* 3). The mean disease burden of animals treated with pLP402-MBP72/u was significantly reduced to 35% compared to the $NaHCO_3$ buffer control group ($p<0.05$). A further reduction was achieved by oral administration of pLP403-MBP72/u. The mean disease burden was only 16% of the buffer control group ($p<0.01$). This reduction was also significant compared to the group orally treated with pLP402-gpMBP ($p<0.05$) (*Figure* 3).

Intranasal administration of Lactobacillus *extracts containing MBP inhibits EAE in SJL/J mice*

In order to determine whether EAE could be inhibited by intranasal administration of *Lactobacillus* extracts containing myelin antigens in SJL/J mice, two EAE models were used. EAE was induced with $PLP_{139-151}$ or with SCH isolated from BALB/c mice. To optimize the model for modulation experiments a dose response analysis with 0-800µg $PLP_{139-151}$ was performed previously (Laman *et al.*, 1998a). 50µg of peptide induced an EAE of medium severity (mean maximum score 3.3) with 100% incidence, which could effectively be modulated by immunotherapy with antibodies against CD40L and CD44. Anderton and Wraith (1998) already demonstrated that it was possible to prevent $PLP_{139-151}$ induced EAE by nasal administration of 100µg $PLP_{139-151}$. In this study, three different doses were tested for their ability to prevent EAE. 50µg, 100µg or 200µg $PLP_{139-151}$ were administered intranasally at day –10 and –5 prior to EAE induction. A clear reduction in mean disease burden was observed,

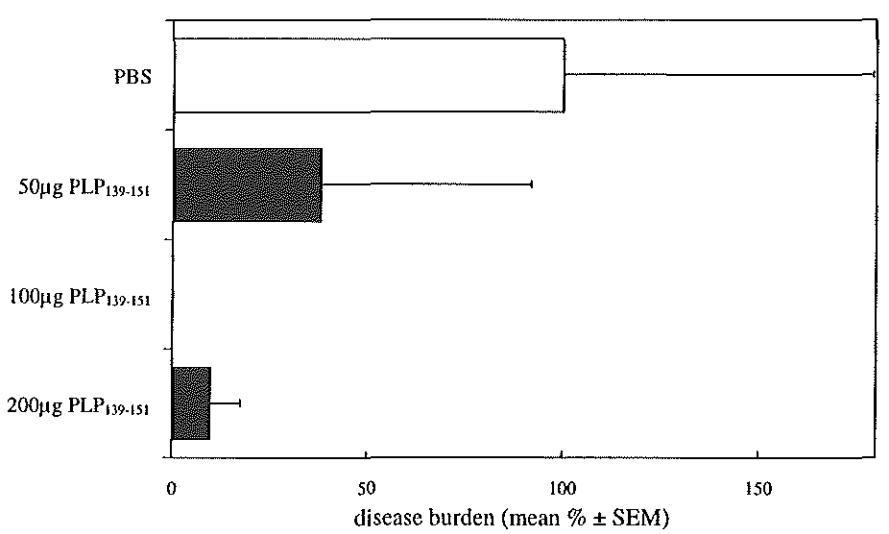


Figure 4: ***Intranasal tolerance induction with $PLP_{139-151}$ in SJL/J mice***
Groups of 3 mice received different doses (0, 50µg, 100µg or 200µg in PBS) of $PLP_{139-151}$ intranasally at days –10 and –5. At day 0, EAE was induced with 50µg $PLP_{139-151}$. The disease burden was expressed as percentage of the mean disease burden of the control group which received no $PLP_{139-151}$ intranasally.

which was dose dependent (*Figure* 4). Due to the small groups (n=3) these differences did not reach significance. Fifteen days after EAE induction, relatively high $PLP_{139-151}$ specific IgG2a levels were detected in the control group which had received PBS intranasally. The groups which showed reduced EAE after intranasal application of $PLP_{139-151}$ (50µg or 200µg), had relatively high levels of IgG1 $PLP_{139-151}$ specific antibodies. The group treated with 100µg $PLP_{139-151}$ had diminished levels of $PLP_{139-151}$ specific antibodies. No correlation could be determined between the antibody levels and disease course.

Mice intranasally received *Lactobacillus* extracts containing $PLP_{1391-151}$ fused to ß-gluc (pLP402-PLP139/u) or $PLP_{139-151}$ only (pLP402-PLP139) daily from day -10 to day 10 prior to induction of EAE with $PLP_{139-151}$ or at day -10 and day -5 prior to EAE induction. *Lactobacillus* extracts containing a hemagglutinin peptide fused to ß-gluc (pLP402-peptide2/u) were used as negative control. None of these intranasal treatments had any effect on the disease course (data not shown).

In contrast, when mSCH was used to induce EAE, intranasal administration of *Lactobacillus* extracts reduced the disease burden (*Figure 5*). The *Lactobacillus* strain secreting MBP_{86-98} was considered as a negative control, because this peptide could not be detected by immunoblotting. The recombinant is further referred to as pLP402-peptide. *Lactobacillus* extracts containing pLP402-peptide, pLP402-hMBP

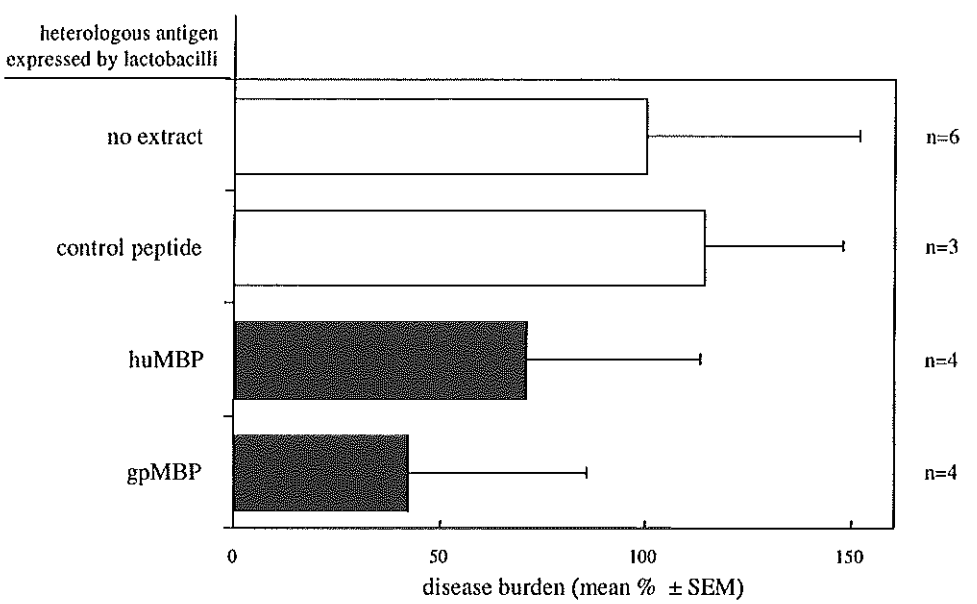


Figure 5. ***Intranasal administration of*** **Lactobacillus** ***extracts containing MBP inhibits EAE in SJL/J mice***

Groups of mice received intranasally *Lactobacillus* extracts containing heterologous myelin antigens at day –15, -10 and -5. At day 0, EAE was induced with msch. The disease burden was expressed as a percentage of the mean disease burden of the control group which did not receive *Lactobacillus* extracts. n= number of animals per group.

(human MBP) or pLP402-gpMBP (guinea pig MBP) were administered daily 10 days before EAE induction. No effect was found with pLP402-peptide treatment. Extracts with human MBP partially inhibited EAE (34% reduction of the mean disease burden) and extracts with guinea pig MBP showed further reduction of EAE (58% reduction), but both effects did not reach significance (*Figure 5*).

No correlation was found between the reduction in EAE disease course and the antibody response, since no specific antibody responses could be detected against mSCH, $PLP_{139-151}$ or MBP in any of the tested mouse sera as was also demonstrated by al-Sabbagh *et al.* (1996b).

Oral administration of recombinant lactobacilli expressing myelin antigens inhibits EAE in SJL/J mice

In order to determine whether oral administration of live lactobacilli expressing myelin antigens can prevent EAE in SJL/J mice, six recombinant *Lactobacillus* strains were tested. Three *Lactobacillus* strains with and three strains without ß-gluc were used. The strains without ß-gluc pLP402-hMBP (secreting hMBP) and pLP402-gpMBP (secreting gpMBP) were tested for their EAE reducing capacity,

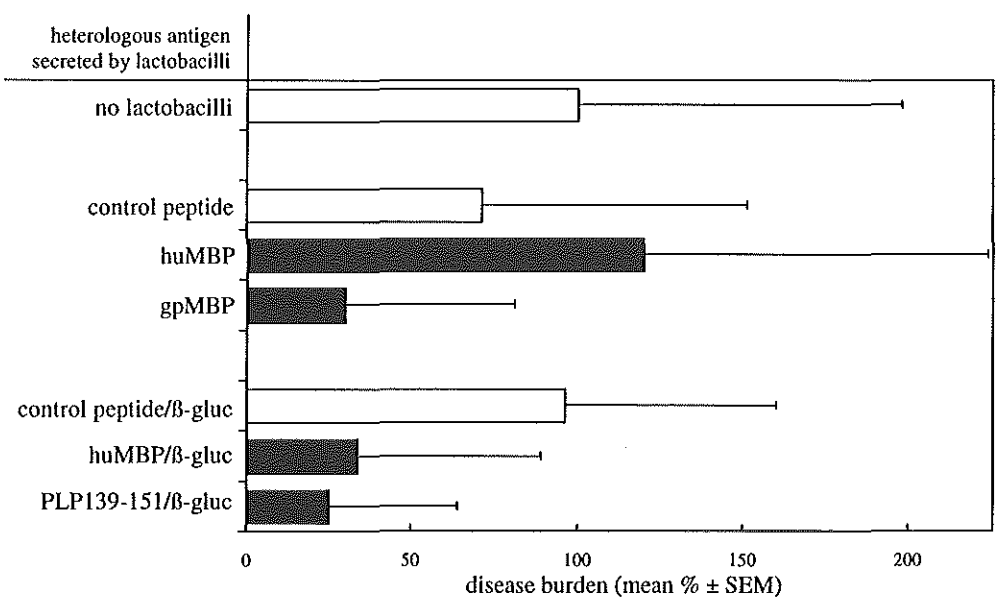


Figure 6: ***Oral administration of recombinant lactobacilli expressing myelin antigens inhibits EAE in SJL/J mice***

Groups of 5 mice were fed recombinant lactobacilli containing secretory vectors for the expression of myelin antigens. Approximately 5.10^{10} live lactobacilli were administered for 20 days, from day –10 till day 10. At day 0, EAE was induced with mSCH. Lactobacilli expressing myelin antigens fused to ß-gluc and without ß-gluc were tested for their effect on EAE. Lactobacilli expressing an irrelevant peptide fused to ß-gluc and lactobacilli expressing a control peptide were considered as controls for heterologous fusion proteins and non-fused heterologous proteins respectively. The disease burden was expressed as a percentage of the mean disease burden of the control group which did not receive lactobacilli.

whereas pLP402-peptide was used as negative control. The ß-gluc containing *Lactobacillus* strains are pLP402-hMBP/u (secreting hMBP fused to ß-gluc) and pLP402-PLP139/u (secreting $PLP_{139-151}$ fused to ß-gluc) and the control pLP402-peptide2/u (secreting control peptide fused to ß-gluc). Approximately 5.10^{10} live lactobacilli per animal were orally administered daily from day -10 to day 10. At day 0, EAE was induced with spinal cord homogenate. As expected, lactobacilli expressing the irrelevant peptide-ß-gluc fusion protein had no effect on EAE course (*Figure 6*). Although none of the tested recombinant lactobacilli had a significant effect on EAE, strong reductions in the mean disease burden were found after oral administration of pLP402-gpMBP (70% reduction), pLP402-PLP139/u (75% reduction) and pLP402-hMBP/u (66% reduction)(*Figure 6*). *Lactobacillus* recombinants expressing human MBP without ß-gluc slightly enhanced EAE (20% increase of disease burden). Small reductions in disease burden were determined with the control recombinants pLP402-peptide (29% reduction) and pLP402-peptide2/u (4%)(*Figure 6*).

Similar to intranasal administration of recombinant lactobacilli, no correlation was found between disease reduction and antibody responses. Except for one mouse, orally untreated, which showed a low response against mSCH, no specific antibody responses occurred against any of tested myelin antigens, as was also demonstrated by al-Sabbagh *et al.* (1996b).

Antibody responses after intranasal tolerance induction in rats

In all rat experiments the animals were immunized with MBP_{72-85} to induce EAE. In none of the animals an anti-MBP_{72-85} antibody response could be detected, despite the fact that T-cell priming was effective as evidenced by a 100% incidence in control groups. In mucosally untreated animals which were immunized with MBP_{72-85}, no MBP specific antibodies could be detected. This gave us the opportunity to investigate whether intranasal application of gpMBP induced gpMBP specific systemic antibodies. Only in animals with an enhanced disease burden after intranasal treatment with gpMBP, IgG antibodies specific for gpMBP could be detected in serum 14 days after EAE induction. No gpMBP-specific antibody responses could be detected in any of the rats which mucosally received lactobacilli expressing MBP or MBP peptide.

Discussion

General

In this study we have demonstrated nasal as well as oral tolerance in a rat EAE model induced by MBP_{72-85}, which was not previously used for mucosal tolerance induction. In the same model we also showed enhancement of disease after nasal administration of purified gpMBP. Further, we showed that nasal as well as oral administration of recombinant lactobacilli expressing myelin antigens could effectively reduce EAE.

Mucosal administration of low antigen doses can prime instead of tolerize
The MBP_{72-85} induced rat EAE model was not previously used for mucosal tolerance experiments. In another rat EAE model induced with gpMBP complete prevention of EAE was demonstrated with 5 nasal doses of either 6μg or 120μg of purified gpMBP (Li *et al.*, 1998). Five nasal administrations of 120μg of the immunodominant epitope MBP_{68-86} were slightly less effective in preventing EAE (Liu *et al.*, 1998). Here we show that comparably high doses of MBP_{72-85} peptide reduced MBP_{72-85} induced EAE to the same degree as was demonstrated in gpMBP induced EAE. In contrast, EAE induced by MBP_{72-85} was enhanced by nasal administration of gpMBP, although it has been demonstrated that it is possible to induce oral tolerance with intact protein (MBP and PLP) in peptide ($PLP_{140-159}$) induced EAE (al-Sabbagh *et al.*, 1994). It is known that the dose and administration regimen is crucial for the induction of mucosal tolerance. Oral administration of low doses can enhance disease, as was demonstrated by Meyer *et al.* (1996). Also feeding of very low doses of OVA appears to prime rather than tolerize the immune response, resulting in enhanced DTH responses (Lamont *et al.*, 1989). Based on molarity, an approximately 15 times lower number of the MBP_{72-85} epitope was present in nasally administered gpMBP, compared to synthetic MBP_{72-85} peptide. In our experiments the amount of nasally administered gpMBP equalled 6,7μg and 13,3μg MBP_{72-85} peptide, indicating that much lower doses were administered than the 100μg synthetic MBP_{72-85} peptide that not even completely prevented EAE induction after nasal administration. However, this dose related explanation is not consistent with the findings that low doses (5 times 6μg gpMBP) can prevent disease in a different EAE model (induced with gpMBP) in the Lewis rat (Li *et al.*, 1998). Possibly, the fact that gpMBP harbours more T cell and B-cell epitopes has influenced the tolerizing properties of gpMBP. Also other myelin components which were retained in the purified MBP fraction could have affected the immune response. Benson *et al.* (1999) have demonstrated that heterogeneous antigen preparation such as myelin are less effective in inducing tolerance than single antigens (e.g. MBP).

MBP_{72-85} does not contain a B-cell epitope for the Lewis rat
No peptide specific antibody response could be detected after subcutaneous immunization of Lewis rats with MBP_{72-85} (containing a T-cell epitope). Gould and Swanborg (1993) have demonstrated strong IgG antibody responses in Lewis rats against MBP_{67-85} (YGSLPQKSQRSQDENPV). These apparent contradictory results may be explained by the additional residues of the MBP_{67-85} peptide that could result in a B-cell epitope. Hashim *et al.* (1986) showed that the MBP peptide SQRSQDEN, which residues are also present in our MBP peptide 72-85, was able to induce weak antibody responses in Lewis rats. In accordance with the results of Gould and Swanborg (1993), elongation of the peptide by three or six naturally found residues at its N-terminal end, strongly increased the immunogenicity of the peptide (Hashim *et al.*, 1986). Also elongation at the C-terminus with the residues VHF increased

antibody levels (Fritz *et al.*, 1979). These results indicate that the peptide used in our EAE model contains only part of the residues of two distinct B-cell epitopes, therefore hardly evoking an antibody response. Consequently, the MBP specific antibody response detected after nasal administration of whole gpMBP and subcutaneous immunisation with MBP_{72-85} is probably only due to the nasally applied MBP.

Presentation of recombinant myelin antigens to the mucosal immune system
According to the general dogma, mucosal administration of soluble antigens leads to systemic T-cell tolerance whereas particulate antigens can induce local and systemic humoral and cellular responses (e.g. Benson *et al.*, 1999; Fairweather *et al.*, 1990). Therefore, we expected that oral administration of lactobacilli secreting soluble antigens would be more effective in reducing EAE than lactobacilli that retain the antigen intracellularly. Contrary to this expectation, lactobacilli that expressed MBP_{72-85} fused to ß-gluc intracellular appeared to reduce EAE further than lactobacilli secreting the peptide-fusion protein. Interpretation of these findings is hampered by the general lack of insights into behaviour of *Lactobacillus* strains in the gut, as well as cellular uptake and processing of lactobacilli and their intracellular or secreted antigens.

When antigens are presented by APCs without or with low levels of costimulatory molecules, anergy of CD4+ T cells can be induced. Increasing evidence is emerging that also dendritic cells can act as tolerogenic APCs in the mucosal immune system priming for Th2 responses (Viney *et al.*, 1998; Kalinski *et al.*, 1997). It has been proposed that such dendritic cells can sustain the induction of T-cell tolerance to soluble proteins or non-invasive micro-organisms, such as lactobacilli. Active immunity is generated only when the DCs are confronted with potentially harmful antigens in the context of appropriate secretion of cytokines or other inflammatory signals.

gpMBP is less effective than MBP_{72-85} in reducing MBP_{72-85} induced EAE
In this study, nasal administration of gpMBP enhanced MBP_{72-85} induced EAE. However, nasal administration of gpMBP (fused to ß-gluc) expressed by lactobacilli did reduce EAE significantly, but less effectively than when lactobacilli expressing MBP_{72-85} fused to ß-gluc were used. Comparable results were obtained by oral administration of lactobacilli expressing gpMBP or MBP_{72-85}, although in those experiments gpMBP was not fused to ß-gluc. In contrast to the experiments performed with purified gpMBP and synthetic peptide, equimolar amounts of recombinant gpMBP and MBP_{72-85} both fused to ß-gluc were administered. This was deduced from immunoblots that showed approximately equal amounts of ß-gluc per unit weight of total *Lactobacillus* protein, indicating equal numbers of molecules of heterologous antigen. This was true for all secretory vectors and not for vectors which retain the heterologous protein intracellularly. Therefore, the difference in inhibition

of EAE can not be explained by a difference in dose. Conformational differences between recombinant MBP_{72-85} and gpMBP both fused to ß-gluc, partially due to fusion to ß-gluc, could render the immunodominant epitope less accessible in the gpMBP recombinant than in the MBP_{72-85} recombinant. Differences in susceptibility to proteases can also play a role.

Beneficial effects of lactobacilli on tolerance induction

Although it was demonstrated that $PLP_{139-151}$ induced EAE can be prevented by nasal administration of the same peptide, we were unable to prevent disease by nasal or oral application of *Lactobacillus casei* secreting $PLP_{139-151}$ fused to ß-gluc (data not shown). The applied nasal dose of recombinant $PLP_{139-151}$ (±1µg) was much lower than that used to prevent EAE with synthetic peptide (100µg, Anderton and Wraith, 1998). However, in the other nasal experiments also lower doses of recombinant antigen were able to prevent EAE than those necessary to prevent EAE with synthetic or purified antigen. For instance, in rats significant reduction of EAE was demonstrated with *Lactobacillus* extracts containing approximately 1µg MBP_{72-85} or 10µg gpMBP. This indicates that the presence of *Lactobacillus* antigens had a positive effect on disease reduction, even though administration of lactobacilli itself did not have any effect on the disease course (*Figure 2*, lactobacilli expressing ß-gluc). In a previous study, we demonstrated that wild type administration of *L. casei* did not influence EAE disease course (Maassen *et al.*, 1998b). Also in SJL/J mice, nasal administration of low dosages of hMBP and gpMBP (both approximately 3µg) partially prevented EAE, although the reduction was not significant.

Also in the oral tolerance experiments lactobacilli may have additional beneficial effects in the reduction of EAE. Effective oral tolerance can be achieved by oral administration of multiple doses of 1 mg gpMBP (5 times, Miller *et al.*, 1992a) up to four doses of 5 mg gpMBP in gpMBP induced EAE in Lewis rats (Whitacre *et al.*, 1991). When peptide containing the MBP residues 72-85 was used for peripheral T-cell tolerance induction, 4 oral administrations of 1.25 mg peptide were necessary to prevent EAE induced with the same peptide (Javed *et al.*, 1995). In this study Lewis rats which were fed lactobacilli containing approximately 375µg gpMBP or 25µg MBP_{72-85} for 20 days showed significantly reduced signs of EAE. Even though the cumulative amount of recombinant gpMBP might be more than necessary to prevent EAE by oral administration with purified gpMBP in a 20-day administration regimen, this recombinant *Lactobacillus* strain was not able to suppress EAE by this administration regimen. The cumulative amount of MBP_{72-85} peptide was still 10-fold lower than the one used to suppress MBP_{68-88} induced EAE with MBP_{68-88}. This implies that the presence of lactobacilli has additional beneficial effects. The results in the SJL/J mice are more difficult to interprete. The orally administered doses of recombinant antigens in lactobacilli containing secretory vectors in SJL/J mice were approximately 6µg of the peptides and 95µg of the MBP proteins, administered for 20 days. The cumulative amount of MBP is similar to the cumulative amount used to

(partially) prevent myelin induced EAE (al-Sabbagh *et al.*, 1994; Miller *et al.*, 1992a). Therefore it can not be concluded that in this case lactobacilli have an additional beneficial effect. As there are no published reports on oral tolerance induction in myelin or SCH induced EAE by MBP or PLP peptides, no definite conclusions about the contribution of lactobacilli can be drawn.

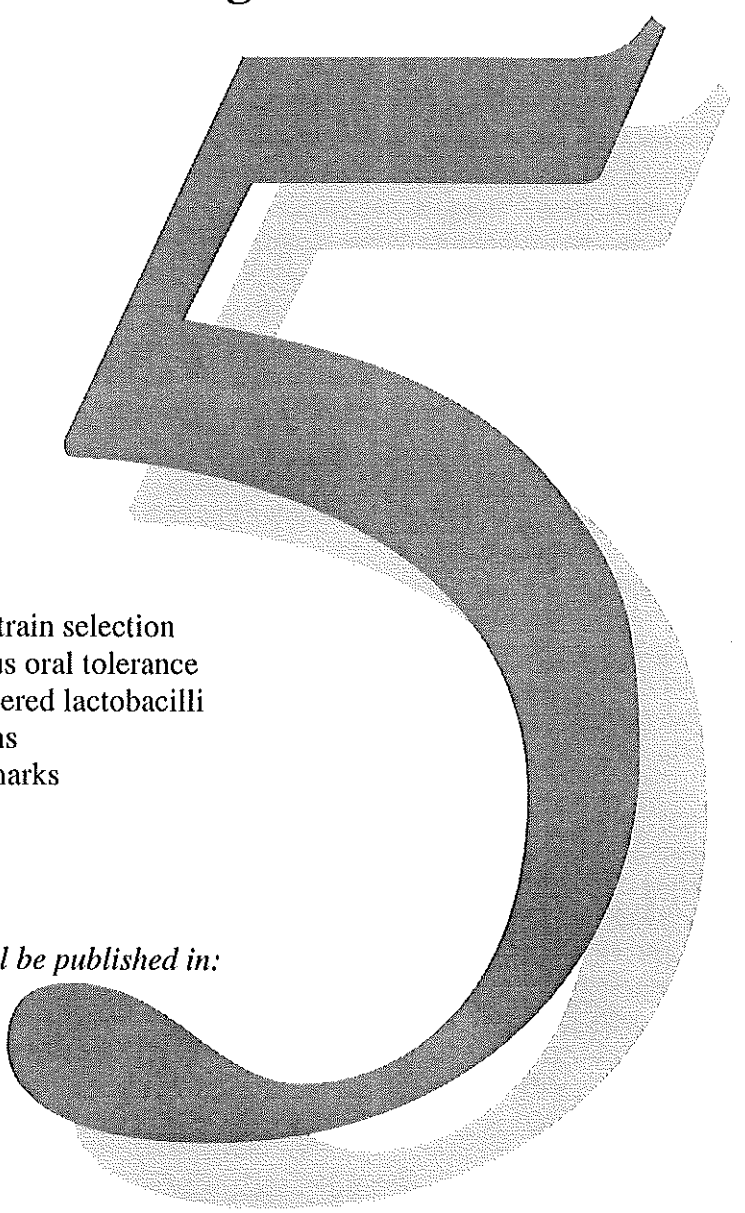
The higher efficacy in tolerance induction by oral administration of lactobacilli retaining MBP_{72-85} fused to ß-gluc intracellularly, could simply be the result of the higher expression level of this heterologous protein (approximately 3 times higher than secretory MBP_{72-85} fused to ß-gluc). There is currently no obvious explanation for the different effects found with lactobacilli expressing hMBP fused to ß-gluc and lactobacilli expressing hMBP without ß-gluc. Conceivably, it could be that different conformation renders the recombinant hMBP more susceptible to proteases, possibly affecting an important epitope.

Comparison of the results obtained by nasal and oral tolerance induction suggests that the efficacy of the nasal route is higher than the oral route, requiring lower doses of antigen, which is in accordance with the current view derived from studies with soluble antigens (Ma *et al.*, 1995; Metzler and Wraith, 1993). It has to be kept in mind that the form in which the antigens administered was different for nasal versus oral administration (soluble extracts versus particulate live bacteria).

Concluding remarks

This study has provided 'proof of principle' that EAE can be reduced by nasal as well as oral administration of recombinant lactobacilli expressing myelin antigens. Tolerance induction may be improved by optimizing expression, dose and administration regimen. The importance of the dose in mucosal tolerance induction was demonstrated by the enhancement of EAE disease burden by nasal administration of gpMBP in Lewis rats.

Summarizing discussion



Part of this chapter will be published in:
Vaccine

5.1 Introduction

Multiple sclerosis is a T-cell dependent autoimmune disease causing chronic inflammation of the central nervous system. The cause of MS and the autoantigen(s) involved are still unknown. Consequently, the currently available therapies for this severe disease are all non-antigen-specific. Antigen-specific therapy was effective in the experimental animal model EAE by oral administration of myelin antigens inducing peripheral T-cell tolerance (e.g. al-Sabbagh *et al.*, 1994; Benson *et al.*, 1999). However, oral administration of myelin failed as a treatment of MS patients (Fukaura *et al.*, 1996). The purity, the heterogeneity of myelin proteins and degradation of antigen in the stomach could be among the causes of this failure. To circumvent large-scale purification of myelin antigens and to avoid infection with prions and viruses, recombinant proteins produced by microorganisms could be a solution. This way also homologous (human) protein antigens can be used. In the human trials, bovine myelin was given orally since it was practically impossible and considered unethical to administer purified human brain antigens. Additional advantages for local delivery of myelin antigens are provided by using non-pathogenic bacteria as a delivery system, such as *Lactobacillus* strains. Oral administration of these recombinant Gram-positive bacteria circumvents antigenic degradation in the stomach. The large genus *Lactobacillus* provides the opportunity to select a *Lactobacillus* strain with properties that may increase peripheral T-cell tolerance induction.

These studies provide 'proof of principle' that mucosal administration of recombinant *Lactobacillus* strains expressing myelin antigens can reduce EAE. Further studies could potentially develop this approach for treatment of MS in man.

5.2 *Lactobacillus* strain selection

General Lactobacillus properties

The genus *Lactobacillus* consists of many species and strains (Klein *et al.*, 1998). Several of these strains are used as probiotics, which are defined as 'mono- or mixed cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microflora' (Havenaar *et al.*, 1992). A main probiotic effect is the enhancement of immune responses against enteric pathogens, preventing diarrhoea (reviewed by Salminen *et al.*, 1996). The extent to which *Lactobacillus* strains have probiotic activity and the mechanisms of probiotic activity are clearly different between individual *Lactobacillus* strains. *Lactobacillus* strains transformed to express antigens from pathogens or autoantigens for use as oral vaccine or therapeutic in autoimmune disease respectively, do not necessarily have to possess probiotic activities. Examples of criteria for *Lactobacillus* strain selection for use as recombinant oral vaccines or recombinant mucosal therapeutics are listed in *Table 1*. All mentioned properties are strain dependent and may influence the efficacy of vaccination or tolerance induction. However, the impact of each individual property is difficult to predict and requires empirical evaluation.

Adherence and persistence of lactobacilli

Adhesion of lactobacilli to the mucosa enhances the persistence of the strains in the gastro-intestinal tract (and possibly aids in colonization) but as a result may also facilitate uptake by the body. Longer persistence also increases the opportunity to locally produce and secrete recombinant heterologous antigen, which is important for both tolerization and vaccination purposes. Persistence is *Lactobacillus* strain and host dependent. Shaw *et al.* have demonstrated that the persistence of different *Lactobacillus* strains in BALB/c mice varies between 3 and 30 days (personal communication). In mice, niche colonization of the gut by orally administered lactobacilli is unlikely to happen because of competition with the existing microflora. A promising approach to increase persistence is cloning the gene encoding the S-layer protein from *L. acidophilus* into the *Lactobacillus* strain chosen for a certain application (Pouwels *et al.*, 1998), since this protein is thought to be involved in adherence to the mucosa (Coconnier *et al.*, 1992; Schneitz *et al.*, 1993).

Table 1: ***Criteria for*** **Lactobacillus** ***strain selection***

General properties:
Non-adhering ◄——► Adhering
Non-persisting ◄——► Persisting ◄——► Colonizing
Low intrinsic immunogenicity ◄——► High intrinsic immunogenicity
Properties affecting the immune system:
Induction of cytokines in gut (proinflammatory ◄——► non-inflammatory)
No adjuvanticity ◄——► High adjuvanticity
Growth phase dependent skewing of T-cell pathways (Th1 ◄——► Th2)
Proinflammatory properties of cell wall components (low ◄——► high)
Properties in relation to heterologous antigen expression:
Suitable for transformation (yes/no)
Expression level (low ◄——► high)
Efficacy translocation system (low ◄——► high)

Intrinsic immunogenicity of lactobacilli

We have indications that the intrinsic immunogenicity (i.e. ability to elicit an adaptive immune response against *Lactobacillus* antigens) after oral administration of the *Lactobacillus* strains is positively related to the intrinsic adjuvanticity of the *Lactobacillus* strain. Orally administered *Lactobacillus* strains that enhance the antibody response against a parenterally immunized antigen, evoke an antibody response against themselves, in contrast to non-adjuving *Lactobacillus* strains (unpublished observation and in chapter 2.3). This is consistent with data that an immunogenic *Lactobacillus* strain induces higher antibody responses against the expressed heterologous antigen than a non-immunogenic *Lactobacillus* strain (Shaw *et*

al., personal communication). Exactly how the immunogenicity of *Lactobacillus* strains is related to persistence is currently unknown.

The composition of the cell wall is strain- and growth phase-dependent (Logardt and Neujahr, 1975; Wicken *et al.*, 1982; Knox and Wicken, 1978). Probably, the differences in cell wall composition are related to the persistence and intrinsic immunogenicity of individual *Lactobacillus* strains (Knox and Wicken, 1978). Since it is known that peptidoglycan, a major cell wall component, can influence cytokine expression *in vitro* (reviewed by Henderson *et al.*, 1996), it is assumed that the differential effects of various *Lactobacillus* strains on the immune system are at least partially due to differences in cell wall composition.

Lactobacillus properties affecting the immune system

Intrinsic adjuvanticity and cytokine inducing properties of lactobacilli

Several of the effects which lactobacilli may have on the immune system as mentioned in *Table 1* were investigated in this thesis for a proper selection of *Lactobacillus* strains. From the species *L. plantarum,* two strains were tested for cytokine profiles in the gut and adjuvanticity after oral administration. No differences were detected between those two strains. Adjuvanticity, here defined as enhancement of the specific humoral response against a parenterally administered antigen, appeared to be dependent both on the *Lactobacillus* strain as well as on the mouse strain. Analysis of the antibody subclasses of the induced antibodies revealed that the strain with the strongest adjuvanticity, *L. reuteri*, induced relatively high antigen specific IgG2a levels when compared to specific IgG1 (chapter 2.2). In mice, IgG2a specifically mediates opsonization and complement activation. IgG2a reflects activation of Th1 cells which can activate macrophages and may provide help to cytotoxic T-cells. Direct and indirect evidence of activation of Th1 cells is provided by a strong induction of TNF-α, IL-1ß and IL-2 positive cells in the gut villi of mice fed *L. reuteri* and immunized parenterally with Chikungunya virus. Since IL-2 is produced by proliferating Th1 cells, IL-2 induction may be considered as direct evidence of activation of Th1 cells. TNF-α can be produced by Th1 cells, but it is more likely that this cytokine is produced by macrophages as is IL-1ß (Arai *et al.*, 1990). The induction of IL-1ß and probably TNF-α indicates that macrophages are activated. Transformation of this particular *Lactobacillus* strain with viral antigens containing B-cell and T-cell epitopes might induce protective immunity by activating humoral as well as cell-mediated immunity upon oral administration. Multiple administrations of large numbers of these bacteria theoretically may have adverse effects due to the inflammatory response they can evoke, but we did not find indications of damage to the mucosa after oral administration of *L. reuteri* or *L. brevis* (cf Bloksma *et al.*, 1979).

Growth phase dependent effects of lactobacilli in BALB/c mice

As was demonstrated in the Th1 biased SJL/J mouse strain, the growth phase of the individual *Lactobacillus* strains can differentially affect the IgG1/IgG2a ratio (chapter 2.3). Stationary phase cultures of *L. casei* and *L. murines* induced high ratios of IgG1/IgG2a. The tendency to induce Th2 cytokines after oral administration of log phase cultures in BALB/c mice (chapter 2.2) may be further increased by oral administration of stationary phase cultures of recombinant *L. casei* or *L. murines*. It appeared that after oral administration of stationary phase cultures of *L. casei*, the total IgG response specific for the i.p. administered antigen was significantly increased ($p<0.05$) compared to the control group and the group fed log phase cultures (unpublished observation). However, oral administration of stationary phase cultures of *L. casei* only slightly increased the IgG1/IgG2a ratio (unpublished observation). The increase of the IgG1/IgG2a ratio in BALB/c mice probably was not as pronounced as in SJL/J mice due to the relatively high IgG1/IgG2a ratio of the control group that did not receive lactobacilli and of the group fed log phase cultures in this Th2 biased mouse strain. For vaccination purposes *L. casei* and probably also *L. murines* can therefore best be used in stationary phase.

Intrinsic adjuvanticity of the *Lactobacillus* strain: An advantage or disadvantage for the induction of peripheral T-cell tolerance?

The advantage of a *Lactobacillus* strain with high adjuvant activity for the use in vaccination is clear. Whether adjuvanticity is an advantage or disadvantage in oral tolerance induction is not that obvious. Therefore it is necessary to point out that we want to achieve peripheral T-cell tolerance in a Th1 mediated autoimmune disease by mucosal administration of autoantigen. Oral co-administration of the systemic adjuvant LPS with MBP enhanced T-cell tolerance in EAE (Khoury *et al.*, 1990). Oral administration of MBP or insulin conjugated to cholera toxin subunit B (CTB) was also found to enhance T-cell tolerance in EAE and diabetes respectively (Bergerot *et al.*, 1997; Sun *et al.*, 1996). This latter findings present an apparent paradox, because CTB is also used to increase the efficacy of mucosal vaccines (e.g. Dertzbaugh and Elson, 1993). The resulting responses of CTB conjugates in tolerance induction as well as in vaccination are closely related to the induction of Th2-associated cytokines and antibody isotypes (IgG1)(reviewed by Williams *et al.*, 1999).

The occurrence of T-cell tolerance without B-cell tolerance is a comparable paradox. The antibody responses to soluble antigens are generally more difficult to suppress than T-cell responses (B-cell versus T-cell tolerance)(Husby *et al.*, 1994; Mowat *et al.*, 1982). However, it remains controversial how local mucosal immune responses to orally administered soluble antigens are generated. Some reports suggest that suppression of systemic immunity is accompanied by local secretory IgA production (Challacombe and Tomasi, 1980; Husby *et al.*, 1994), but others have reported that intestinal IgA antibody production is reduced by oral administration of soluble antigen (Kelly and Whitacre, 1996). A possible explanation for this apparent

contradiction is the fact that different mechanisms of T-cell tolerance can be induced. It can be reasoned that when the mechanism of oral tolerance induction is based on active suppression involving TGF-ß and/or immune deviation towards Th2, a local IgA response may be increased, since TGF-ß and the Th2 cytokine IL-5 are involved in isotype switching to IgA as well as in IgA production. Alternatively, TGF-ß is known to inhibit Th1 as well as Th2 responses, suggesting reduction of antibody responses in addition to suppression of cell-mediated immunity (Higgins and Weiner, 1988; Lider *et al.*, 1989). Induction of T-cell anergy as the main mechanism of oral tolerance induction may lead to inactivation of antigen specific B-cell responses as well. Consistent with this hypothesis are the findings of Kelly and Whitacre (1996), who found reduced numbers of IgA antibody forming cells in Peyer's patches in experiments where an anergy inducing protocol in EAE was used. Also IgG antibody responses were reduced in tolerized animals in which anergy is the main mechanism of peripheral T cell tolerance (Inada *et al.*, 1997; Melamed and Friedman, 1994; Mowat *et al.*, 1996). When T-cell tolerance is due to immune deviation towards a Th2 response, in addition to a possible increase in IgA, a reduction of the specific IgG2a response against the orally administered antigen can be observed as well as an increase in the antigen specific IgG1 response. This is supported by findings of Bai *et al.* (1997), Staines *et al.* (1996) and Tian *et al.* (1996), who demonstrated increased levels of IgG1 and/or reduced levels of IgG2a after mucosal administration of autoantigen. Active suppression was the main mechanism of tolerance in these experimental models of autoimmunity (Bai *et al.*, 1997; Staines *et al.*, 1996; Tian *et al.*, 1996). In contrast, Ke and Kapp (1996) reported suppression of both IgG1 and IgG2a responses, and this suppression could be adoptively transferred with CD8+ T cells.

All these data together imply that adjuvanticity can be advantageous in mucosal tolerance induction dependent on the mechanism of tolerance induction. When immune deviation towards Th2 is preferred as the mechanism of mucosal tolerance, a *Lactobacillus* strain with high adjuvanticity could be advantageous, with the restriction that IgG2a production (in mice) will not be enhanced.

Orally administered lactobacilli affect EAE disease course

Some of the properties of lactobacilli affecting the immune system appeared to be growth phase dependent. Different growth phases may also account for the different results obtained when *L. casei* was orally administered before induction of EAE. In chapter 2.1 enhancement of EAE was shown after oral administration of stationary phase cultures of *L. casei*. In experiments using log phase cultures of *L. casei* (Maassen *et al.*, 1998b) or end-log-phase cultures of *L. casei* transformed with an irrelevant antigen, no effect on EAE could be demonstrated (chapter 4.2). The enhancement of EAE by orally administered *L. casei* was surprising and not in accordance with the results obtained with respect to the growth phase dependent effects on IgG1 and IgG2a. However, we and others have demonstrated that oral

administration of *L. casei* can induce proinflammatory cytokines such as TNF-α, IL-12 and IFN-γ (Maassen *et al.*, 1999b; Matsuzaki *et al.*, 1998; Tejada-Simon *et al.*, 1999). These seemingly contradictory results could also be due to the number of CFUs administered. Approximately 10^{10} (or 5.10^{10} in the case of control recombinant) lactobacilli were orally administered of *L. casei* log phase cultures, whereas only 10^{8} cells of stationary phase cultures were orally administered (chapter 2.1). Low doses of stationary phase cultures of *L. casei* may have been presented by professional APCs such as dendritic cells with high levels of co-stimulatory molecules such as B7-1, leading to activation of T cells. High doses of log phase cultures may have led to presentation of *Lactobacillus* antigens by naïve B cells which outnumber professional APCs (Brandtzaeg *et al.*, 1999). Moreover, there are indications that presentation by naïve and memory B cells can result in down-regulation of the T-cell response by engagement of B7 with CTLA-4, transiently expressed on activated T-cells (Alegre *et al.*, 1998; Samoilova *et al.*, 1998).

Noteworthy are the effects of orally administered log phase cultures of *L. murines*, *L. reuteri* and *L. plantarum NCIB* on EAE disease course in SJL/J mice. *L. plantarum* did not affect EAE, *L. reuteri* increased the severity of EAE and *L. murines* significantly reduced EAE (Maassen *et al.*, 1998b). Possibly EAE can be further reduced by oral administration of *L. murines,* since stationary phase cultures of this *Lactobacillus* strain induce relatively more IgG1, reflecting activation of Th2 cells, without enhancing the overall humoral response. We do not have results in SJL/J mice which support or oppose these findings. However, the results in BALB/c mice are consistent with the observations in EAE. Log phase *L. reuteri* enhanced the antibody response by inducing relatively high levels of IgG2a and induced inflammatory cytokines in the gut villi, properties that could be related to enhanced EAE disease course. The tendency to induce TGF-ß and the induction of relatively high levels of IgG1, could be cause of the disease limiting effect of *L. murines*.

Selection of a *Lactobacillus* strain for oral tolerance induction in EAE

The strain of choice for use in mucosal tolerance induction in EAE, based on the results obtained in chapter 2, (without taking into account the effects of heterologous antigen expression), would be *L. murines* in stationary phase. This choice is based upon the reduction of EAE, the skewing towards Th2 when stationary phase cultures were used, no (strong) induction of inflammatory cytokines and the tendency to induce TGF-ß by wild type *L. murines*. The second choice would be log phase *L. casei,* mainly for the same reasons as *L. murines*. Log phase culture is preferred since it lacks the enhancing effects of stationary phase cultures of *L. casei* on EAE. Another advantage of *L. casei* is the existing experience in industrial production.

Lactobacillus *and vector properties in relation to heterologous antigen expression*

Not only the intrinsic characteristics of *Lactobacillus* strains influence the efficacy of tolerance induction, also properties in relation to transformation may play a role. The

ability to genetically transform the *Lactobacillus* strain is of utmost importance for the use of recombinant lactobacilli as oral vaccine or therapeutic. Despite considerable effort, we were not able to transform our *Lactobacillus* strain of choice, *L. murines*. Gram-positive bacteria in general are difficult to transform due to their peptidoglycan layer, in contrast to the easy to transform Gram-negative bacteria, such as *E. coli*. The difference in cell wall composition of *Lactobacillus* strains requires specific electroporation settings for each individual strain, and no settings are yet known for *L. murines*.

A series of expression vectors was developed which are segregationally stable in many *Lactobacillus* strains (Pouwels *et al.*, 1996 and chapter 3.1). These plasmids allow expression of the antigen of choice at three defined cellular locations under control of a constitutive or regulatable promoter. The antigen can be expressed either in the intracellular compartment, anchored to the cell wall, or secreted into the environment. A continuous presence of soluble antigen locally at the gut mucosa was thought to be the most efficient way to induce tolerance, because oral administration of purified soluble antigens, in contrast to most particulate antigens, induced hyporesponsiveness in previous studies (e.g. Benson *et al.*, 1999; Fairweather *et al.*, 1990; Melamed *et al.*, 1996). In order to achieve high levels of soluble protein, vectors that secrete myelin antigens were chosen for oral tolerance induction purposes. Attempts to clone $PLP_{139\text{-}151}$ and human MBP behind the constitutive *ldh* promoter in a secretion vector failed. Although the vector without heterologous antigen was stable, insertion of cDNA of the myelin antigens caused segregational instability in *E. coli*. cDNA from $PLP_{139\text{-}151}$ and other myelin antigens were successfully cloned in secretion vectors containing the regulatable *amy* promoter. In addition, cDNA from $MBP_{72\text{-}85}$ was cloned in an expression vector under control of the regulatable *amy* promoter which retained the antigen intracellularly.

The expression level of an intracellularly expressed antigen is much higher than the same antigen expressed to be secreted (both under control of the *amy* promoter). In *amy* controlled vectors resulting in secretion of the heterologous antigen, the expression levels of antigen per colony forming unit increased during growth, with an optimum at the end of log phase and a decrease in stationary phase. This has implications for the choice of growth phase of recombinant lactobacilli for use in oral tolerance induction. Based on the highest expression level we have chosen to use *L. casei* recombinants grown till end-log-phase for oral tolerance experiments. In contrast to mid-log phase cultures, no enhancing effects on EAE disease course were found after oral administration of end-log phase cultures of *L. casei* expressing an irrelevant antigen (chapter 4.2).

The expression level of heterologous protein is not solely dependent on the expression vector. It appeared that the expression levels varied between *Lactobacillus* strains when genetically transformed with the same vector. Strain dependent differences were also found when the amount of secreted heterologous protein was analyzed. This could be due to differences in the efficacy of the translocation systems.

In addition to the continuous translocation of antigen, an inefficient translocation system could be the cause of the presence of heterologous antigen at the cell surface of *L. casei* recombinants containing a secretory expression vector (chapter 3.1).

5.3 Intranasal versus oral tolerance

Three mechanisms have been described for the induction of oral tolerance in autoimmune disease; active suppression, anergy and deletion of auto-reactive T-cells. In general, active suppression mechanisms are induced with (multiple) low doses of antigen (see introduction, section 1.4). Anergy and deletion were reported to result from high (single) dose administrations (Chen *et al.*, 1995a; Whitacre *et al.*, 1991). These dose dependent mechanisms have also been described for nasal tolerance induction (Li *et al.*, 1998). The doses necessary to induce the different mechanisms via the nasal route are much lower than via the oral route. Approximately ten fold higher doses are necessary to induce tolerance by oral administration (Ma *et al.*, 1995; Metzler and Wraith, 1993). The reason of this more efficient tolerance induction via the nasal route is not known. Orally administered soluble antigens may be degraded in the acidic environment of the stomach, decreasing the local antigenic dose at the gut mucosa. But also a more rapid uptake of antigen, uptake by other cells or drainage to functionally different lymph nodes after nasal administration may be responsible for this difference. Wolvers *et al.* (1999) have demonstrated that superficial cervical lymph nodes, the nose-draining lymph nodes, are necessary for nasal tolerance induction. Removal of these lymph nodes abrogates nasal tolerance induction. Tolerance can be restored by transplantation of the superficial cervical lymph nodes but not by replacing them with peripheral lymph nodes, indicating that intrinsic properties of the draining cervical lymph nodes are important in nasal tolerance induction. Removal of Peyer's patches does not abolish oral tolerance induction (Enders *et al.*, 1986). Whether oral tolerance can be induced in animals without lymph nodes is interesting in order to elucidate the function of these lymph nodes in oral tolerance (Fu *et al.*, 1997).

Dosing appears to be important in nasal as well as in oral tolerance induction. The low zone and high zone tolerance as demonstrated after a second s.c. or i.p. immunization with antigen do not occur after mucosal administration. Low zone and high zone tolerance have been extensively investigated for humoral responses against various parenterally administered antigens with low and high doses respectively. A broad dose range between low and high induces humoral immunity. After mucosal administration of soluble antigen enhancement of the response occurs at the very low doses (*Figure 1*)(Lamont *et al.*, 1989). It has been demonstrated that (very) low doses of antigen can enhance EAE severity (Bai *et al.*, 1998; Meyer *et al.*, 1996). Low doses are associated with the induction of active suppression mechanisms and very high dosages can lead to anergy and possibly deletion. However, it is important to consider that all of these tolerance mechanisms may coexist and are not mutually exclusive. Which mechanism predominates depends on the frequency of antigen administration

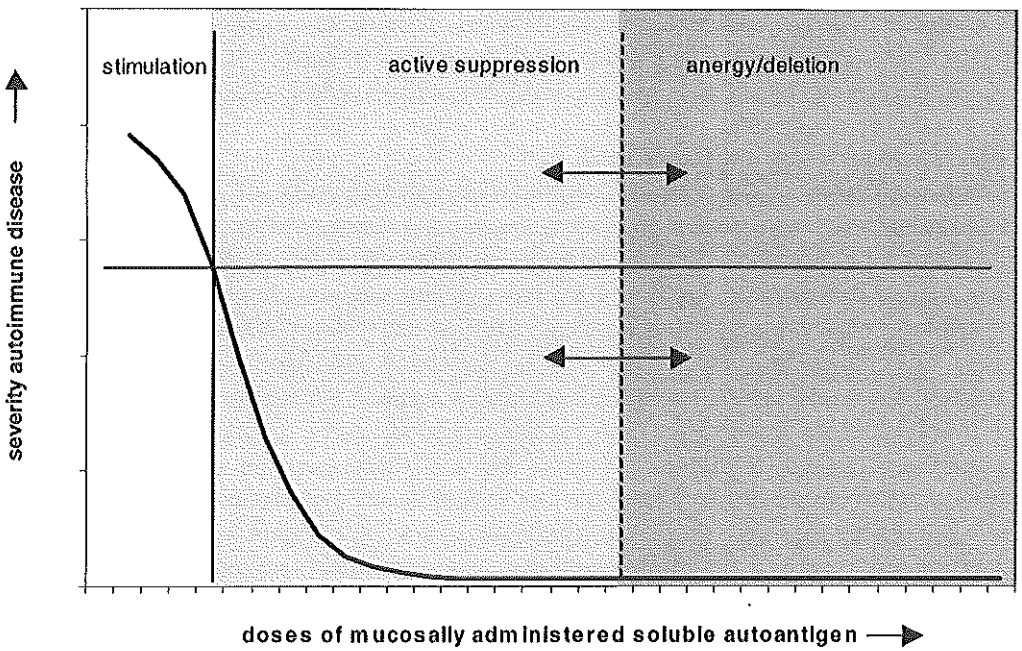


Figure 1. ***Dose dependent effects of antigen on mucosal tolerance induction***
Mucosal administration of soluble antigens induces peripheral T cell tolerance, in particular of the Th1 subset. In this figure the dose dependent effects of mucosally administered soluble antigen on a Th1 mediated autoimmune disease are depicted. The horizontal line in the middle of the graph indicates the mean disease severity in animals which did not receive autoantigen mucosally. Mucosal administration of very low doses of autoantigen can enhance disease severity (left part). Higher doses of autoantigen probably suppress disease via active suppression (middle part). Very high doses of autoantigen suppress disease via the induction of anergy or deletion (right part). The three mechanisms of tolerance induction are dose dependent but not mutually exclusive as indicated by the arrows.

in addition to the dose. The dose range determining the mechanism of T-cell tolerance is different per peptide, protein or antigenic mixtures administered, and in addition dependent on the mucosal route, host and state of disease (prevention or treatment).

When mucosal routes are used to apply therapeutics in order to restore tolerance towards autoantigens or allergens, it is important that no adverse effects occur. From *Figure 1* it can be deduced that very low doses need to be avoided, but it is hard to predict what minimal dose will be effective. Taking into account that tolerance induction occurs after intranasal administration at far lower doses compared to oral administration, it can be argued that the risk of stimulating the immune response is higher via the oral route (chapter 4.2). Especially when antigen availability is a limiting factor, nasal application can be preferred. Antigen availability is no longer an issue when recombinant lactobacilli are used. In our experiments the soluble fraction of *Lactobacillus* extracts, without cell wall components, was used, because the effect of induction of intranasal tolerance is unknown. It appeared that the presence of

soluble *Lactobacillus* components enhanced tolerance induction, because much lower doses of recombinant antigen were effective as compared to the amount of synthetic peptide used to decrease EAE. Although the EAE reducing effects can be achieved by intranasal administration of lower doses than for equal reduction after oral administration, the oral route may be preferable. Since it was reported that maintenance of oral tolerance requires continuous antigenic presence, daily oral intake of recombinant lactobacilli could be a suitable strategy. It is not likely that the dose administered will be too high to induce oral tolerance. However, when bystander suppression is desired as the mechanism of T cell tolerance, theoretically the administered dose can be too high, resulting in the induction of anergy. Lactobacilli do not persist in the nose or in the normally sterile lungs, and negative effects of multiple nasal administrations of large numbers of live lactobacilli cannot be ruled out.

It has been demonstrated that the nasal route also can be used for vaccination purposes. Nasal application of live lactobacilli expressing an immunogenic bacterial antigen intracellularly induced antigen specific IgG and IgA responses (Shaw *et al.*, manuscript in preparation). The efficacy of this route for vaccination could be due to the particulate nature of the lactobacilli, according to the current dogma that the soluble versus particulate nature of the antigens is at least partially responsible for the induction of T cell tolerance versus immunity respectively.

5.4 Orally administered lactobacilli

Persistence

After oral administration most protein antigens are proteolytically digested in the stomach and the rest of the gastro-intestinal tract. Cytoplasmic presence in lactobacilli protects the antigen against this degradation. From all known probiotic lactic acid bacteria *Lactobacillus* strains were chosen because of their live passage of the acidic environment of the stomach, in contrast to *Lactococcus* species. Live (attenuated) bacteria are more efficient inducers of immunity than dead bacteria (Kaul and Ogra, 1998). This live passage could therefore be an advantage for the use of recombinant lactobacilli as oral vaccines. In addition, this live passage allows persistence and subsequent *in vivo* expression of the heterologous antigen. In order to determine the importance of persistence/colonization in oral tolerance induction, experiments could be performed in germ-free mice. A disadvantage is that those mice have small Peyer's patches with quiescent B cell follicles through the lack of interaction with the indigenous microflora (Cebra *et al.*, 1998). Alternatively, deliberately colonized adult mice with a simple mixture of bacterial strains (gnotobiotic mice) including the wild type form of the chosen *Lactobacillus* strain as the only *Lactobacillus* strain introduced, can be used (Cebra, 1999). By treating those mice with huge amounts of recombinant lactobacilli, a competition will take place, possibly resulting in colonisation by the recombinant strain. Another method is to remove the present microflora physically or by treatment with antibiotics before the start of treatment with recombinant lactobacilli. This latter method could also be applied in humans.

In vivo *expression of heterologous proteins*
A non-persisting *Lactobacillus* strain was used in the mucosal tolerance experiments described in chapter 4.2. It is known that *L. casei* passes the gastrointestinal tract in two to three days. The dividing rate of the used recombinant lactobacilli under optimal conditions *in vitro* is around 2.5 hours in log phase. Since the growth conditions *in vivo* are not optimal (e.g. limited availability of nutrients), it is unlikely that the recombinant lactobacilli will multiply in the gastro-intestinal tract. There are two related arguments to assume that no *de novo* synthesis of heterologous protein under control of the regulatable *amy* promoter will occur when the lactobacilli do not multiply *in vivo*. First, under control of the *amy* promoter, the highest rate of heterologous antigen synthesis is observed in log phase cultures. In stationary phase the level of heterologous protein expression is decreased. Second, the regulatable *amy* promoter is de-repressed in the presence of a carbon source other than glucose, with the prerequisite that the *Lactobacillus* strain is able to ferment the particular sugar. *In vitro*, the stationary phase is entered when the carbon source is exhausted, resulting in repression of the *amy* promoter. When the *Lactobacillus* strain manages to multiply *in vivo*, glucose will be the most probable carbon source. As a result, the *amy* promoter will remain repressed and no *de novo* synthesis will take place. However, in case of a colonized recombinant *Lactobacillus* strain, feeding sugar that results in derepression of the *amy* promoter could provide an *in vivo* regulatable system of myelin protein expression. Since the constitutive *ldh* promoter is not dependent on the carbon source, synthesis of heterologous proteins under control of this promoter is more likely to happen *in vivo*.

Mechanisms of tolerance induction
Even when orally administered recombinant lactobacilli are able to secrete heterologous proteins, it will be only trace amounts, because we assume that *de novo* synthesis of heterologous antigen will not occur *in vivo*. The same is true for the surface associated heterologous proteins from a secretory vector that could be detected by flow cytometry (chapter 3.1). The proteins associated with the cell wall at the moment of administration probably will be (partially) degraded. This implies that in the gastro-intestinal tract *Lactobacillus* strains secreting heterologous antigen are comparable to *Lactobacillus* strains expressing the heterologous antigen intracellularly. Both *Lactobacillus* strains expressing the heterologous MBP_{72-85} peptide fused to ß-glucuronidase, intracellularly or secreted, were able to reduce EAE. How this MBP peptide was presented to the immune system is not known. Two possible ways of antigen uptake and presentation can be distinguished. First, the antigen is released from the cytoplasm of the lactobacilli upon death and cytolysis in the gut lumen. This results in soluble antigen, which can be taken up by epithelial cells of the gut villi and probably presented to CD8+ intraepithelial lymphocytes (IEL), leading to T-cell tolerance induction (Yamamoto *et al.*, 1998). Although lactobacilli are quite resistant to proteolytic digestion (bacteria in log phase probably are more

susceptible to enzymatic lysis than stationary phase bacteria (Logardt and Neujahr, 1975)) eventually also these bacteria will be lysed. Another pathway is the uptake of lactobacilli via the Peyer's patches, which has been demonstrated by Claassen *et al.* (1995). When the second mechanism is the main mechanism of antigen presentation, the question remains why particulate structures containing autoantigen were able to induce T-cell tolerance. Possibly adjuvanticity and cytokine induction play a role.

In the dome of the Peyer's patch the lactobacilli probably will be taken up by APC. Studies have revealed that conventional APC, abundantly present throughout the intestinal wall, PPs and MLNs are likely to contribute to the induction of tolerance (Mowat and Viney, 1997). APCs isolated from the lamina propria of normal mice, preferentially induce tolerance when transferred to naïve animals after stimulation with antigen *in vitro* (Harper *et al.*, 1996). Although the nature of these APCs is unknown, recent studies implicate a role for dendritic cells in tolerance induction. Administration of the growth factor Flt3 ligand to mice expands the number of DCs in the intestine and other lymphoid organs. This increase in number of DCs enhanced the susceptibility to tolerance induction by feeding OVA (Viney *et al.*, 1998). These findings are in contrast with the common assumption that DCs have a constitutive ability to activate T cells. It seems that the phenotype of DC is tissue dependent. The DC recruited to the intestine by Flt3 ligand express only low levels of B7-1 or CD40 costimulatory molecules, supporting the view that intestinal DC may normally be in a resting state without the ability to prime T cells. It has been proposed that DCs are important regulators of the mucosal immune response (Mowat and Viney, 1997). The induction of T-cell tolerance to soluble proteins or non-invasive microorganisms is supported by DC. However, when confronted by potentially harmful antigens accompanied by appropriate cytokines or other inflammatory signals, active immunity can be stimulated. When the low immunogenicity and non-invasive character of the *Lactobacillus* strains used is considered, induction of anergy via DC expressing low levels of costimulatory molecules could be a possible mechanism for oral tolerance induction. When presentation of antigen by DC leads to the priming of Th2 cells, active suppression mechanisms could be underlying T-cell tolerance induction. The increase of IL-10 and TGF-ß in gut villi after oral administration of *L. casei* suggests that active suppression occurs as a tolerance induction mechanism (chapter 2.2). Whether this is the case can not be deduced from our data, although the reduction of EAE by oral administration of MBP_{72-85} in a spinal cord homogenate induced EAE could be due to bystander suppression (chapter 4.2). When oral administration of MBP_{72-85} induced regulatory cells that secreted the appropriate cytokines, the inflammatory response against various, anatomically co-localized myelin antigens present in the spinal cord homogenate could be suppressed. This bystander suppression allows oral treatment with antigens which are present at the site of inflammation without the need to identify the initiating autoantigen(s)(reviewed by Weiner, 1997).

5.5 Future directions

In this thesis it has been demonstrated that lactobacilli can be genetically engineered to express myelin antigens, and that mucosal administration of some of these recombinant lactobacilli reduced the severity of the T-cell mediated experimental autoimmune disease EAE. In order to improve this method of mucosal tolerance induction several directions can be followed. Approaches that could lead to enhanced T-cell tolerance induction are discussed below.

Genetic engineering

Since it does not seem important that the tolerogenic myelin antigen is secreted by the lactobacilli, the myelin antigens can be expressed in vectors that express the antigen intracellularly, leading to increased expression levels. By choosing a vector containing the constitutive *ldh* promoter, the expression levels are further increased. Constructing vectors with even stronger promoters than *ldh*, such as the promoter of the S-layer protein, would lead to an additional increase in expression level (Savijoki *et al.*, 1997). In case of heterologous peptide expression, using a vector containing repeated cDNA sequences of the peptide increases the number of epitopes per microbe. Another method to achieve this, is the use of superoxide dismutase (SOD). This protein is expressed as a tetramer, allowing 4 peptides to be expressed in one molecule.

Design of a possible future experiment

The design of an experiment to improve tolerance induction by recombinant lactobacilli could be as follows. *L. reuteri* and *L. murines* containing vectors for the intracellular expression of MBP_{72-85} and a control peptide of hemagglutinin (Ha) both fused to ß-glucuronidase are used. These *Lactobacillus* strains are of special interest because they have the most extreme properties with respect to modulation of the induction of cytokines in the gut, EAE severity and IgG1/IgG2a ratio. The expression is under control of the *ldh* promoter, in order to express as much antigen as possible. The maximum dose that can be handled practically is approximately 2.10^{11} CFU per oral administration. Ha is used as a control antigen, because it is an (immunogenic) peptide. Parenteral challenge with the same peptide will reveal whether B cell activation occurs. In order to be able to detect the effects on antibody levels, the rat EAE model induced with gpMBP will be used. After determining the persistence of *L. murines* and *L. reuteri*, two oral administration regimens will be used to determine the importance of the maintenance of antigen. Animals will be fed antigen from day -2 till day 2, or from day -6 throughout the experiment. When EAE can be prevented by recombinant lactobacilli expressing MBP_{72-85} fused to ß-gluc, adoptive transfer experiments to determine whether active suppression mechanisms underlie the induction of tolerance can be designed.

Therapy in established EAE

In humans recombinant lactobacilli expressing myelin antigens must function as a therapeutic in established disease. Since we demonstrated prevention of disease in

animal models, it remains to be shown that oral administration of recombinant lactobacilli can have beneficial effects in a chronic disease model. Possibly this can be achieved by simple administration of recombinant lactobacilli after onset of EAE. However, it is known that it is difficult to tolerize an ongoing vigorous Th1 response, therefore co-administration of for instance anti-IL12 (Marth *et al.*, 1996) or lactococci expressing TGF-ß could enhance the efficacy of tolerance induction. Alternatively, suppression the total immune response prior to mucosal treatment with recombinant lactobacilli to induce tolerance may increase the efficacy as well. The immune system can be suppressed by for instance corticosteroids or blocking the interaction of T-cells, B-cells and APC by injection of antagonist anti-CD40 or anti-CD40L monoclonal antibodies (Gerritse *et al.*, 1996; Laman *et al.*, 1998a).

Potential application in humans

In this thesis we demonstrated proof of principle that recombinant lactobacilli expressing myelin antigens can partially prevent EAE. Before we can apply this approach to humans with MS, we have to make sure that it is possible to treat chronic EAE in animals. It also has to be elucidated whether active bystander suppression is the main mechanism of T-cell tolerance induction. If not, this would be an obstacle for use in the heterogeneous human MS population. When it appears to be possible to treat chronic disease mediated by regulatory T-cells, several approaches can be followed to use recombinant lactobacilli in MS patients.

When *Lactobacillus* strains can be identified which are commensals in a large part of the (European/American) population, such strains can be characterized for properties such as intrinsic immunogenicity, adjuvanticity and transformation suitability after isolation. Factors responsible for adhesion and persistence can be important but may be altered by *in vitro* culturing (Pouwels *et al.*, 1998). Alternatively, *Lactobacillus* strains can be isolated from individual MS patients. A *Lactobacillus* strain against which no antibodies can be detected in the patient and which can be transformed could be a useful strain for that particular patient.

The efficacy of peripheral T-cell tolerance induction by oral administration of recombinant lactobacilli may be enhanced when the microflora is removed physically or by treatment with antibiotics. This should be studied in animal models first.

In addition, no harmful effects are reported so far after oral intake of lactobacilli. When a study reveals that (daily intake) of industrial *Lactobacillus* strains do not negatively affect the MS course, such a strain could also be used after extensive *in vitro* evaluation.

Recombinant lactobacilli can only be used in humans when the vectors are food grade. Food grade vectors are characterized by the absence of an antibiotic resistance marker and by the presence of a nutritional selection system, which allow bacteria containing such vector to be distinguished from the indigenous microflora *in vitro*. Such food grade vectors are currently under development. *In vitro*, lactobacilli containing vectors with an antibiotic resistance gene will loose the vector after a

number of cycles when cultured without selection pressure. Since *in vivo* no selection pressure is present for lactobacilli containing a food grade vector, the lactobacilli probably will discard the vector after a number of cycles. Therefore it is also very unlikely that bacterial strains of the host will inherit the vectors.

5.6 Concluding remarks

The aim of this thesis was to prevent EAE by oral administration of recombinant lactobacilli expressing myelin antigens. However, since the genus *Lactobacillus* is very large and diverse in genetic and biochemical properties, we first studied some of the effects lactobacilli can have on the immune system. The large diversity within the *Lactobacillus* genus opens opportunities to increase the desired effects by choosing an appropriate strain. However, the demonstrated strain and growth phase dependent differences cautions that not all *Lactobacillus* strains have beneficial effects on human health.

Based on the effects of *Lactobacillus* strains and the analysis of the recombinant lactobacilli, we have chosen *L. casei* grown till end-log phase for mucosal tolerance experiments. We succeeded in reducing EAE disease burden by nasal administration of soluble recombinant *Lactobacillus* extracts and by oral administration of live recombinant lactobacilli. These results are 'proof of principle' that it is possible to reduce EAE by mucosal administration of recombinant lactobacilli. Oral administration of recombinant lactobacilli expressing myelin antigen can therefore be a promising antigen specific approach for the treatment of multiple sclerosis.

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Samenvatting voor niet-ingewijden

Multiple sclerose (MS) is een chronische aandoening van het centrale zenuwstelsel. In het centrale zenuwstelsel van MS patienten zijn meerdere ontstekingshaarden aanwezig en wordt de myelineschede rondom de zenuwbanen afgebroken. Het wordt aangenomen dat MS een autoimmuunziekte is. Dat betekent dat het immuunsysteem lichaamseigen eiwitten (autoantigenen) aanvalt, in dit geval myeline eiwitten. Normaliter bestaat er tolerantie voor lichaamseigen eiwitten, maar blijkbaar is die tolerantie niet meer aanwezig in MS patienten. Het is daarom van belang om opnieuw tolerantie te verkrijgen voor de myeline eiwitten. Hiertoe kunnen wij gebruik maken van het immuunsysteem in het maag-darm kanaal (een onderdeel van het mucosale immuunsysteem)(mucosa = slijmvlies). Tegen voedseleiwitten wordt normaliter geen sterke immuunrespons opgewekt, omdat dat tot een continue ontsteking zou leiden en tot beschadiging van de darmwand. Het mucosale immuunsysteem is dus een natuurlijke plaats voor tolerantie-inductie. Het autoantigeen aanbieden aan het mucosale immuunsysteem, dus via de orale route (mond), maar ook via de nasale route (neus) zou tot tolerantie kunnen leiden voor het autoantigeen. Orale en nasale toediening van autoantigenen remt ziekteverschijnselen in diermodellen voor verschillende autoimmuunziekten. Ook in het diermodel voor MS, experimentele autoimmuun encefalomyelitis (EAE) kan orale en nasale toediening van myeline eiwitten de ziekteverschijnselen voorkomen.

Om de efficiëntie van de inductie van orale tolerantie te verhogen maken wij gebruik van genetisch gemodificeerde lactobacillen die het autoantigeen kunnen uitscheiden in de darm. Lactobacillen zijn Gram-positieve bacteriën die van nature voorkomen in de menselijke darm. Omdat ze in het algemeen als veilige microorganismen worden beschouwd (GRAS-status), zijn ze uitermate geschikt als aanbieder van het autoantigeen.

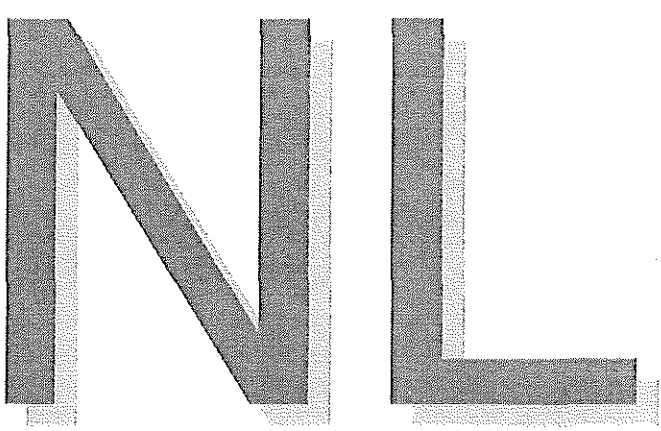
Het is bekend dat melkzuurbacteriën zoals lactobacillen gezondheids-stimulerende eigenschappen kunnen bezitten. Dit komt onder meer doordat verschillende *Lactobacillus* stammen in staat zijn om het immuunsysteem te moduleren. Dit geldt echter niet voor alle stammen. Om de inductie van tolerantie te stimuleren en niet tegen te werken, is het van belang om de meest geschikte *Lactobacillus* stam te kiezen. Daarom hebben wij van verschillende *Lactobacillus* stammen diverse immuunsysteem modulerende eigenschappen na orale toediening bestudeerd. Ten eerste is onderzocht of verschillende *Lactobacillus* stammen intrinsieke adjuvant activiteit bezitten, dat is de mogelijkheid om de antilichaam respons tegen een geïnjecteerd eiwit te verhogen. Van de acht onderzochte stammen, bleken *L. reuteri*, *L. brevis* en *L. fermentum* intrinsieke adjuvant activiteit te bezitten. Cytokinen, uitgescheiden door cellen van het immuunsysteem, worden beschouwd als belangrijke regulatoren van de immuunrespons. Cytokine profielen in de darmen werden bestudeerd na orale toediening van lactobacillen. Twee van de *Lactobacillus* stammen die een adjuverende activiteit vertoonden, bleken cytokinen te induceren die

betrokken zijn bij ontstekingsreacties (Th1). Twee andere *Lactobacillus* stammen, *L.casei* en *L. murines* leken juist onstekings-remmende cytokinen te induceren, TGF-ß en IL-10 (Th2). Deze laatste twee stammen zouden mogelijk geschikt zijn voor de inductie van orale tolerantie nadat ze getransformeerd zijn vectoren die coderen voor myeline eiwitten.

Niet alleen *Lactobacillus* stam afhankelijke effecten op het immuunsysteem werden gevonden, ook de groeifase waarin de lactobacillen zich bevinden bij de orale toediening bleek van invloed te kunnen zijn op het immuunsysteem. Het antilichaam-isotype subklasse IgG2a een reflectie is van een ontstekings-inducerende Th1 respons en IgG1 een reflectie is van een Th2 respons die mogelijk betrokken is bij T-cel tolerantie inductie. Wij hebben gevonden dat de IgG1/IgG2a ratio verhoogd is wanneer stationaire culturen van *L. casei* of *L. murines* oraal werden toegediend. De ratio was verlaagd wanneer log fase culturen oraal aan muizen werden toegediend. Dit impliceert dat, afhankelijk van de groeifase, lactobacillen de Th1/Th2 balans differentiëel kunnen beinvloeden. Op grond van deze gegevens hebben wij gekozen voor *L. casei* als drager van de myeline antigenen voor de inductie van orale tolerantie.

Extracten van getransformeerde *L. casei* die myeline basisch eiwit (MBP)(een myeline eiwit) of een klein stukje hiervan (MBP peptide MBP_{72-85}) tot expressie brengen, werden intranasaal toegediend aan Lewis ratten, voordat EAE werd geinduceerd. Beide *L. casei* transformanten remden de ziekte significant. Ook orale toediening van dezelfde recombinanten, maar dan levend, verminderde de ziekteverschijnselen significant. Ook orale en nasale toediening van lactobacillen die myeline antigenen tot expressie brengen, aan SJL muizen lijkt tot vermindering van EAE te leiden.

Uit dit onderzoek is dus gebleken dat het mogelijk is om met oraal en nasaal toegediende recombinante lactobacillen die myeline antigenen tot expressie brengen, EAE te remmen. Verder onderzoek is nodig naar mogelijkheden voor deze vorm van therapie voor MS.



Dankwoord

Eindelijk is het dan zover, het proefschrift is goedgekeurd. Maar voor het naar de drukker kan moet eerst nog het meest gelezen onderdeel geschreven worden, het dankwoord. Ik wil beginnen met iedereen te bedanken die direct of indirect heeft bijgedragen aan de tot stand koming van dit boekje, op de werkvloer, erboven, in de sporthal, op het terras, aan het thuisfront of waar dan ook.

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Thanks !!

Abbreviations

AA	adjuvant arthritis
AChR	acetylcholine receptor
AIA	antigen-induced arthritis
amy	α-amylase gene
APC	antigen presenting cell
APL	altered peptide ligand
a.u.	arbitrary unit
ß-gluc	ß-glucuronidase
CII	type II collagen
CFA	complete Freund's adjuvant
CFU	colony forming units
CGG	chicken gamma globulin
CIA	collagen induced arthritis
CNS	central nervous system
CTB	cholera toxin subunit B
DAS	disability scale
DC	dendritic cell
DTH	delayed type hypersensitivity
EAE	experimental autoimmune encephalomyelitis
EAMG	experimental autoimmune myasthenia gravis
EAN	experimental autoimmune neuritis
EAT	experimental autoimmune thyroiditis
EAU	experimental autoimmune uveoritinitis
EGC	experimental granulomatous colitis
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate-conjugated
GAD65	glutamate decarboxylase peptide
GALT	gut associated lymphoid tissue
GRAS	generally regarded as safe
gpMBP	guinea pig myelin basic protein
Hig	hamster immunoglobulin
HLA	human leukocyte antigen
hMBP	human myelin basic protein
HRP	horseradish peroxidase
IDDM	insulin-dependent diabetes mellitus
IEL	intraepithelial lymphocyte
IFA	incomplete Freund's adjuvant
IFN	interferon
IL	interleukin
IRBP	interphotoreceptor retinol binding protein
i.p.	intraperitoneal
i.v.	intravenous
kDa	kilodalton
KLH	keyhole limpet haemocyanin
ldh	L-(+)-lactate dehydrogenase gene
LP80	*Lactobacillus plantarum 80*
Mab	monoclonal antibody
MBP	myelin basic protein
MCS	multiple cloning site
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MRI	magnetic resonance imaging
MS	multiple sclerosis
mSCH	mouse spinal cord homogenate
NIDDM	non-insulin-dependent diabetes mellitus
NRS	normal rabbit serum
OD	optical density
orf	open reading frame
ori	origin of replication
OVA	ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PLP	proteolipid protein
PP	Peyer's patches
PVC	polyvinyl chloride
rMBP	rat myelin basic protein
rt-PCR	reverse transcriptase polymerase chain reaction
S-Ag	S-antigen
SDS	sodium dodecyl sulphate
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper (cell)
TNBS	trinitrobenzene sulfonic acid
TNF	tumour necrosis factor
TNP	trinitrophenyl
TTFC	tetanus toxin fragment C

Curriculum vitae

Kitty Maassen werd op 11 juni 1968 geboren te Oss. In 1986 behaalde zij het VWO-B diploma aan het Liemers College te Zevenaar. In datzelfde jaar begon zij met de studie Biologie aan de Universiteit Utrecht. Als hoofdvakken werden stages gelopen bij de vakgroep Moleculaire Microbiologie (Dr. M. Kleerebezem) en de vakgroep Medische Microbiologie (Drs. J.J. Cornelissen en T.A.M. Oosterlaken). In augustus 1992 behaalde zij het docteraal examen. Als vrijwilligster heeft zij enkele maanden gewerkt bij de vakgroep Farmacognosie aan de Universiteit Utrecht (Dr. K. Beukelman). Van september 1993 tot en met oktober 1994 heeft zij als assistent in opleiding gewerkt bij de vakgroep Toxicologie aan de Rijksuniversiteit Leiden (Dr. J.H. Meerman). Van november 1994 tot en met oktober 1998 was zij werkzaam als onderzoeker in opleiding bij de afdeling Immunologie van de Erasmus Universiteit Rotterdam. In die periode werd, onder begeleiding van Drs. J.D. Laman en W.J.A. Boersma en Prof.dr. E. Claassen, het in dit proefschrift beschreven onderzoek uitgevoerd bij de divisie Immunologische- en Infectieziekten van TNO Preventie en Gezondheid te Leiden.

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