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Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers

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Probiotic microorganisms, especially lactic acid bacteria, are effective in the treatment of infectious diarrhoeal diseases and experimental colitis. Although the mechanisms by which these organisms exert their anti-inflammatory effects are largely unknown, immunomodulating effects are suggested. The objective of this study was to examine the effect of a 5-week oral administration of *Lactobacillus rhamnosus* subspecies GG (*Lb.* GG) on the cellular immune response to intestinal microorganisms in ten healthy volunteers. Peripheral blood cells (PB) were stimulated with either 'self' or 'non-self' preparations of faecal samples and isolated *Bacteroides fragilis* group-organisms (Bfg) or *Escherichia coli* (*Esch. coli*), and pro- and anti-inflammatory cytokines (IL-10, IL-4, IL-6, IFN-γ, TNF-α) were measured in the culture supernatant. $CD4^+$ T-lymphocyte activation was determined by measurement of intracellular ATP following lysis of the cells. The activational response of $CD4^+$ T-lymphocytes towards isolated and heat-inactivated intestinal organisms was increased after the probiotic treatment. Additionally, TNF-α, IL-6 and in part IFN-γ cytokine secretion by PB cells following stimulation with whole stool preparations and single members of the flora was significantly decreased, whereas the IL-10 and in part IL-4 cytokine secretion was increased at the end of the study. In contrast, the activational response of $CD4^+$ T-lymphocytes following stimulation with whole 'non-self' intestinal flora was higher than by 'self' intestinal flora, but both responses showed a trend towards a reduction at the end of the study. This study documents a direct effect by *Lb.* GG on the cellular immune system of healthy volunteers and offers a promising tool to investigate systemic immunomodulation due to oral administration of probiotic microorganisms.

Keywords: Probiotic therapy, immune system, Lactobacilli, *Lactobacillus* GG, intestinal microflora, human.

In healthy individuals there seems to be a genetically determined, regulated balance between pro- and anti-inflammatory mediators (Fiocchi, 1998), stimulated by intestinal contents leading to homeostasis, also called physiological inflammation. The immune response, induced by pathogenic and non-pathogenic intestinal microorganisms and food antigens, following transmucosal passage through M cells and other pathways, is characteristic for each antigen (Wahl et al. 1988; Kraehenbuhl & Neutra, 2000). As a result, subsets of T cells (T-helper, T-suppressor, T-regulatory) may be activated and re-circulate throughout the periphery (Rothkotter et al. 1999). In healthy individuals, the 'tolerance' towards non-pathogenic antigens prevents the mucosal immune system from over-responding (Husby, 2000). This normal tolerance could be used to gain access to the immune system with potentially immunomodulating agents, e.g. probiotic bacteria.

T cell response to normal intestinal bacteria or their products may be important in the immunopathogenesis of chronic enterocolitis. Duchmann et al. suggested that the immune system of the healthy individual is tolerant towards its own intestinal flora and that this tolerance might be broken in inflammatory bowel disease (IBD) (Duchmann et al. 1995, 1996b, 1997). It seems, however, that bacteria differ in their capacity to stimulate inflammation. *Bacteroides* sp. especially might play a crucial role in the

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initiation and perpetuation of inflammatory colitis whereas *Esch. coli* sp. are neutral (Rath et al. 1996b).

As early as 1907, probiotics were regarded as health-promoting to the human organism (Metchnikoff, 1907). Later, Lactobacilli and other probiotic organisms were used as adjuvants to treat mainly infectious diarrhoeal diseases and, more recently, also chronic inflammatory intestinal conditions (Gorbach et al. 1987; Oksanen et al. 1990; Hilton et al. 1997; Guandalini et al. 2000; Shanahan, 2000; Vanderhoof, 2000). The mechanisms by which probiotic organisms exert their effects are still largely unknown. Several theories are currently under investigation (Kohashi et al. 1979; Isolauri et al. 1993; Mack et al. 1999; Madsen et al. 1999). While the impact on the intestinal flora might be limited (Venturi et al. 1999; Tannock et al. 2000), there is mounting evidence that Lactobacilli and other probiotic bacteria somehow directly influence the human immune system (Cunningham-Rundles et al. 2000; Erickson & Hubbard, 2000). The regulatory role of cytokines within the immune system, and the impact of probiotic organisms have been studied intensively in recent years, using cell lines and primary cells of both rodents and humans (Nicaise et al. 1993; Miettinen et al. 1996; Marin et al. 1998; Miettinen et al. 1998; Nicaise et al. 1999; Tejada-Simon et al. 1999a, b; Ha et al. 1999; Miettinen et al. 2000; Christensen et al. 2002). There are, however, very few reports on the effects *in vivo* of probiotic bacteria in healthy individuals.

This study examined the effect of oral administration of the probiotic microorganism *Lb.* GG to healthy volunteers on the cytokine secretion profile and T-lymphocyte activation following stimulation with 'self' and 'non-self' intestinal organisms and whole stool preparations.

Materials and Methods

Two groups each of five healthy adult volunteers with no known intestinal disorders and no concurrent antibiotic therapy (six female, four male) aged from 21–43 years ($29{\cdot}9 \pm 2{\cdot}1$ years) were recruited from amongst the staff of the University of Regensburg, Germany. Two groups of five volunteers were investigated 6 months apart, but the cytokine measurement was performed at one time-point to rule out external influences on cytokine secretion. All participants received a daily oral dose of 2×10^9 cfu freeze-dried *Lactobacillus rhamnosus* GG (*Lb.* GG; CAG Functional Foods, Omaha, NE 68110) in capsule form for 35 d. The organism was coated in gelatine to allow its release in the stomach. No special advice on food intake was given. A fresh stool sample was collected 3 weeks prior to the study and again, together with a peripheral heparinized blood sample the day before the first dose was given and on the day following the last dose of *Lb.* GG. The study was approved by the local ethics committee of the University of Regensburg, School of Medicine.

Microbiological procedures

Stool specimens from each volunteer were collected in 10 ml thioglycolate broth on day 1 and day 35 of the trial. Inoculated specimens were homogenized thoroughly, and gross debris was allowed to settle on the bottom and on the surface of the tube for 10 min. Five ml of the supernatant was transferred to another tube and incubated at 75 °C for 45 min. The suspension was then centrifuged at 4000 ***g*** for 5 min, the sediment was washed twice with sterile phosphate-buffered-saline (PBS), and adjusted to an optical density of 1·0 (560 nm; Perkin-Elmer, 63110 Überlingen, Germany), and finally diluted at 1 : 100 and 1 : 1000 in PBS.

To isolate *Esch. coli* and organisms of the *Bacteroides fragilis* group (Bfg), stool samples from each volunteer were collected in thioglycolate broth 3 weeks before the start of the trial. Only one colony was picked from each species of each volunteer. *Esch. coli* was isolated from MacConkey agar plates (Merck, 64293 Darmstadt, Germany), and lactose-positive colonies were further tested for negative citrate and positive SIM reactions (Merck). All ten strains of *Esch. coli* were checked for the absence of the pathogenetic factors *stx* 1, *stx* 2, *hly*, and *eae* by PCR (Paton, 1998). Organisms of the *Bact. fragilis* group were isolated on Bacteroides Bile Aesculin agar plates (Merck), and black colonies were further identified by the Vitek AN card (Vitek Systems, bioMérieux-Vitek Inc., Hazelwood, MO 63042 USA). *Lb.* GG was cultured from freeze-dried powder (CAG) in MRS bouillon (deMan-Rogosa-Sharpe, Difco Laboratories, Becton-Dickinson, Sparks, MD 21152 USA). All bacteria were heat killed at 75 °C for 45 min, washed twice with ice-cold PBS, adjusted to an optical density of 1·0 (560 nm), diluted at 1 : 100 and 1 : 1000 in PBS, and finally stored at −70 °C until use. Aliquots (100 μl) of the heat killed bacterial preparations, cultured on Columbia agar plates (Merck) remained sterile for 48 h. The indigenous ('self') flora corresponds to the blood donor, the 'unrelated' ('non-self') flora was picked at random from other volunteers participating in the study.

Measurement of CD4$^+$ T-lymphocyte responses following stimulation in whole blood assays

Heparinized peripheral blood was taken from each volunteer and the assay was begun within 4 h. The Luminetics assay for T-cell activation (Cylex Inc., Columbia, MD 21045 USA) was used for the stimulation, separation and measurement of activation of peripheral CD4$^+$ T-lymphocytes as described by Sottong et al. (2000). Use of ATP was recently confirmed as a suitable surrogate marker to measure T-lymphocyte proliferation (White et al. 1989; Sottong et al. 2000). Peripheral blood was diluted 1 : 4 in RPMI 1640 (Sigma, 89552 Steinheim, Germany) and different stimuli were added: *Lb.* GG; 'self' and 'non-self' Bfg; *Esch. coli;* whole faecal preparations; and phytohaemagglutinin-M (PHA-M) (Roche Diagnostics

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GmbH, 68305 Mannheim, Germany). Various concentrations of stimuli were tested. Highest stimulatory results were achieved with *Lb.* GG $\sim 2 \times 10^6$ cfu/ml, Bfg $\sim 9 \times 10^3$ cfu/ml, *Esch. coli* $\sim 7{\cdot}5 \times 10^7$ cfu/ml, corresponding to a 1 : 100 dilution, PHA-M 1 μg/ml, and faecal preparations at a 1 : 100 dilution. Stimulation with lower concentrations yielded lower results (data not shown). After 24 h of cultivation in 96-well round bottom plates at 37 °C ambient air, supplemented with 5% CO_2, stimulated $CD4^+$ T-lymphocytes were separated using paramagnetic beads coated with monoclonal antibodies (anti-CD4). Following lysis of the separated cells, the amount of released ATP was quantified by the use of a luciferin-luciferase enzyme system. Luminescence was measured with a top counter (Packard Bioscience GmbH, 63303 Dreieich, Germany). All measurements were performed in triplicate, and the amount of ATP was expressed as mean ± SEM relative light units (rlu) (White et al. 1989).

Cytokine ELISA

Peripheral blood, diluted 1 : 4 in supplemented (2 mM glutamine, 100 IU penicillin, 100 μg/ml streptomycin; Biochrom, 12247 Berlin, Germany) RPMI 1640 (Sigma) was incubated with the above mentioned stimuli for 24 h at 37 °C ambient air, supplemented with 5% CO_2 as described by Hartung et al. (1996). Culture supernatants were collected and kept at −70 °C until assayed at the same time. Cytokine levels in the culture supernatant were quantified using commercially available ELISA kits (EH-IFNG, EH2-IL-6, EH2-TNFA, EH-IL-10, EM-IL-4-2 Endogen-Perbio Science, 53113 Bonn, Germany) based on antibodies against IFN-γ, IL-6, IL-4, TNF-α, and IL-10. Measurements were performed in duplicate and results given as difference of the mean ± SEM.

Statistical analysis

For statistical analysis, Sigma Stat 2.03 (SPSS Inc., Chicago, IL 60606 USA) software was used. To control for normally distributed variables, the Kolmogorow-Smirnow test was used, followed by a paired *t* test to determine if the effect of the treatment on the same individual was significant. If the test for normality failed, a Wilcoxon-signed-rank-test for paired data was performed. A probability of $P<0{\cdot}05$ was regarded as significant, while higher values were regarded as non-significant (NS).

Results

After 5 weeks of daily oral administration of 2×10^9 cfu/ml *Lb.* GG to ten volunteers, lactobacilli indistinguishable from *Lb.* GG were isolated from the faeces of all participants. With the exception of mild abdominal bloating and meteorism in three individuals, no side effects were reported.

Table 1. Activation of peripheral $CD4^+$ T-Lymphocytes following stimulation with sonicates of 'self', to the donor corresponding bowel flora, 'non-self' bowel flora and phytohaemagglutinin (PHA) as a standard stimulus. The amount of ATP released is given in relative light units (rlu)

Values are means ± SEM

	'Self' intestinal flora	'Non-self' intestinal flora	PHA
pre (in 10^{-3} rlu)	52 ± 15	129 ± 46	1198 ± 301
post (in 10^{-3} rlu)	38 ± 5	73 ± 11**	844 ± 127

** $P<0{\cdot}01$ *v.* 'self' intestinal flora

Peripheral $CD4^+$ T-lymphocyte activation

The pattern of $CD4^+$ T-lymphocyte activation prior to and after the study period following stimulation with whole 'self' and 'non-self' faecal samples and PHA as a standard stimulus is summarized in Table 1. Stimulation with 'non-self' faecal samples caused a higher ATP release, corresponding to a higher activational level, than stimulation with 'self' intestinal flora, and the effect was significant at the end of the study ($P<0{\cdot}01$). After the 5-week course of *Lb.* GG, for both the stimulation with 'self' and 'non-self' faecal samples, the amount of ATP release was unchanged.

The activational response of $CD4^+$ T-lymphocytes upon stimulation with sonicates from isolated, single intestinal organisms ('self' and 'non-self' Bfg and *Esch. coli*) and *Lb.* GG is shown in Fig. 1. Whereas the effect of stimulation with 'self' and 'non-self' whole faecal samples was unchanged following 5 weeks of oral *Lb.* GG, the stimulation with isolated microorganisms led to increased released of ATP from lysed $CD4^+$ T-lymphocytes, being significant following stimulation with 'self' Bfg (mean (10^{-3} rlu): 22 ± 8 *v.* 36 ± 6; $P<0{\cdot}001$; Fig. 1A), and 'self' and 'non-self' *Esch. coli* (mean (10^{-3} rlu): 'self' 25 ± 7 *v.* 46 ± 5 and 'non-self' 27 ± 10 *v.* 63 ± 9; $P<0{\cdot}05$; Fig. 1C/D). Stimulation with 'non-self' Bfg resulted in a trend towards increased activation (mean (10^{-3} rlu): 22 ± 6 *v.* 29 ± 4; $P<0{\cdot}10$; Fig. 1B), while there was no significant difference following stimulation with *Lb.* GG (mean (10^{-3} rlu): 42 ± 10 *v.* 60 ± 13; Fig. 1E). There were marked differences between individuals in the activational response of $CD4^+$ T-lymphocytes upon stimulation with different isolated bacterial strains. However, there was no significant difference between the stimulation with 'own' or 'foreign' intestinal organisms, as opposed to stimulation with whole faecal samples.

Cytokine secretion by peripheral blood (PB) cells

The effect of the 5-week course of oral *Lb.* GG on cytokine production, induced by stimulation with PHA, sonicated faecal samples ('self' and 'non-self'), or single intestinal organisms ('self' and 'non-self' Bfg and *Esch. coli*) and *Lb.* GG is shown in Fig. 2. The amount of TNF-α was

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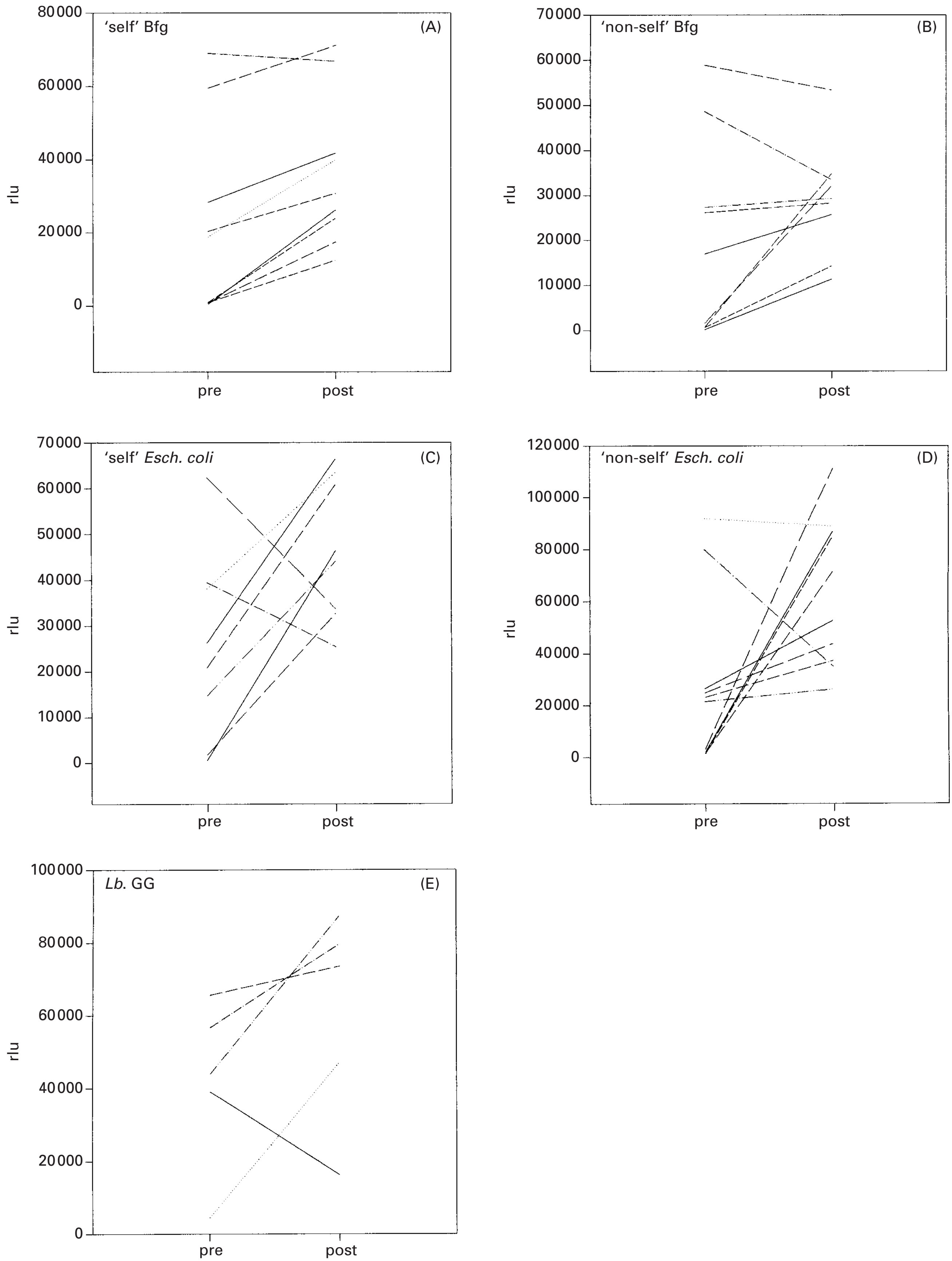

Fig. 1. Activation of peripheral CD4^{+} T-Lymphocytes prior to and following a 5-week course of oral *Lb.* GG upon stimulation with 'self' *Bacteroides fragilis* group organisms (Bfg) (A), 'non-self' Bfg (B), 'self' *Esch. coli* (C), 'non-self' *Esch. coli* (D), and *Lb.* GG (E). The amount of released ATP prior to and after the 5-week study period is given in rlu.

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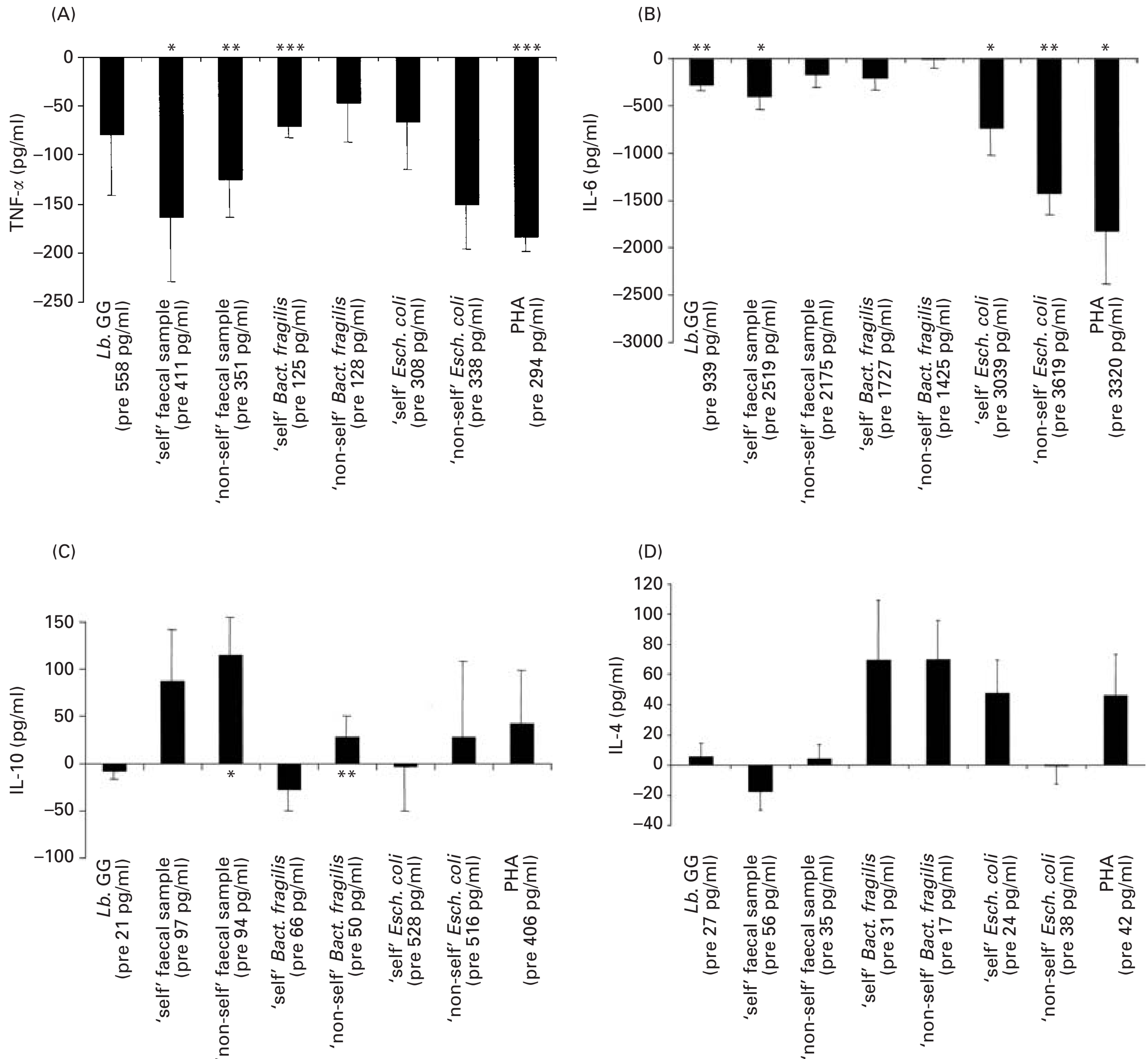

Fig. 2A–D. Difference of the mean cytokine secretion by peripheral blood (PB cells) following a 5-week course of *Lb.* GG administration compared with the beginning of the study, upon stimulation with *Lb.* GG, 'self' and 'non-self' faecal samples, 'self' and 'non-self' Bfg, 'self' and 'non-self' *Esch. coli* and phytohaemagglutinin (PHA). $*P<0{\cdot}05$; $**P<0{\cdot}01$; $***P<0{\cdot}001$ *v.* pre study levels.

significantly decreased following stimulation with PHA ($P<0{\cdot}001$), 'self' and 'non-self' faecal samples ($P<0{\cdot}05$), and 'self' Bfg ($P<0{\cdot}001$) at the end of the study period (Fig. 2A). Moreover the amount of IL-6 secreted by PB cells was significantly decreased at the end of the 5 week course of *Lb.* GG following stimulation with *Lb.* GG ($P<0{\cdot}01$), 'self' faecal samples ($P<0{\cdot}05$), 'self' ($P<0{\cdot}05$) and 'non-self' *Esch. coli* ($P<0{\cdot}01$), and PHA ($P<0{\cdot}05$). A trend towards decreased secretion of IL-6 was noted following the stimulation with 'self' Bfg (Fig. 2B). Furthermore, stimulation with PHA (1134 ± 139 *v.* 664 ± 60 pg/ml; $P<0{\cdot}001$) and 'self' Bfg (28 ± 13 *v.* 8 pg/ml $\pm$ 2 pg/ml; $P<0{\cdot}05$) led to a significant decrease of IFN-γ secretion at the end of the study. In contrast to the above findings, IL-10 production was significantly increased following the stimulation with 'non-self' faecal sample ($P<0{\cdot}05$) and 'non-self' Bfg ($P<0{\cdot}01$) while a trend was seen following the stimulation with 'non-self' faecal sample (Fig. 2C). There was also a trend to increased IL-4 production following stimulation (Fig. 2D).

Discussion

Evidence is accumulating for the benefit of probiotic preparations in the treatment of intestinal disorders (Gorbach et al. 1987; Oksanen et al. 1990; Hilton et al. 1997; Gorbach et al. 1999; Madsen et al. 1999; Isolauri, 2000; Gionchetti et al. 2000; Guandalini et al. 2000; Shanahan, 2000; Vanderhoof, 2000; Schultz et al. 2002). Several mechanisms have been suggested for the effects of probiotics. Although, to begin with, a change in the composition of the indigenous microflora had been expected, only transient alterations were found (Venturi et al. 1999; Tannock et al. 2000). More recently, other possible mechanisms of probiotic action, such as barrier-enhancing effects (Isolauri et al. 1993; Mack et al. 1999) or immunomodulation (Dugas et al. 1999; Neish et al. 2000) have been suggested. The present study gives the first evidence for a direct modulation of the systemic cellular immune response to intestinal microorganisms *in vivo* due to oral administration of the probiotic microorganism *Lb.* GG.

It is assumed that naïve T-lymphocytes, derived from the bone marrow via the thymus, are primed by circulation through the intestinal mucosa and by close contact with intestinal antigens presented by specific antigen-presenting cells in Peyer's Patches and mesenteric lymph nodes. These cells can then circulate through the peripheral blood before homing to the intestine (Groux, 2001). In our experimental setting, we used unfractionated human peripheral blood, which offers the advantages of few preparation artifacts and a more natural cell environment. Our method is regarded as especially suitable for the evaluation of pro- and anti-inflammatory properties of extrinsic stimuli (Hartung et al. 2000). With the use of a whole blood assay, it is unclear which cells are responsible for the release of cytokines but it is known that $CD4^{+}$ T-lymphocytes, peripheral macrophages, and dendritic cells have a role in the production of various cytokines, including supressive cytokines, such as IL-10 (Howard & O'Garra, 1992; de Waal-Malefyt, 1992) and IL-4 (MacDermott, 1996), and pro-inflammatory cytokines, such as TNF-α (Owen-Schaub et al. 1991), IFN-γ (Bogdan, 2000), and IL-6 (Hirano, 1990), depending on the antigenic stimulus. To overcome possible alterations of the cytokine release due to culture handling or interindividual variations, the study population was divided into two groups, receiving treatment 6 months apart. The cytokine measurements, however, were performed at one time point.

We used stimulation assays with heat-treated 'self' and 'non-self' faecal preparations and single bacterial components to measure $CD4^{+}$ T-lymphocyte activation and cytokine secretion by PB. We were able to confirm the previously reported tolerance to 'self' intestinal antigens (Duchmann et al. 1995, 1996a) by demonstrating firstly that stimulation with 'non-self' faecal samples resulted in a higher activation of $CD4^{+}$ T-lymphocytes compared with 'self' faecal samples (Table 1). Secondly, we showed that after 5 weeks of oral *Lb.* GG this effect on $CD4^{+}$ T-lymphocytes was unchanged, while the response to antigens of individual intestinal bacteria ('self' and 'non-self' Bfg and *Esch. coli*) increased (Fig. 1). Thirdly, we demonstrated that the secretion of TNF-α, IL-6 and IFN-γ by PB cells was decreased, while the IL-10 and IL-4 response was increased (Fig. 2), irrespective of the origin of the antigen ('self' or 'non-self').

Inflammatory bowel diseases (IBD), especially Crohn's disease, are thought to be Th-1 mediated diseases, with $CD4^{+}$ T-lymphocytes playing a central role in the pathogenesis (Powrie, 1995; Fuss et al. 1996; van Deventer, 2000). Although the antigens that drive T-cell activation (Schreiber et al. 1991) and clonal expansion in IBD (Probert et al. 1996) still need to be defined, both clinical and experimental evidence strongly incriminate normal luminal bacteria as a source of antigen (Brand et al. 1996; Rath et al. 1996a; Sartor et al. 1996; Schultz, 1997a, b; Yamanaka et al. 1997; Cong et al. 1998). Duchmann et al. (1995) suggested that the immune system of the healthy individual is tolerant towards its own indigenous intestinal flora and that this tolerance might be broken in IBD. In accordance with this suggestion, the present study with healthy volunteers showed $CD4^{+}$ T-lymphocyte activation to be 2·5-fold higher in response to 'non-self' than to 'self' faecal preparations, with the effect being significant at the end of the study period. The discrepancy between the decreased response towards stimulation with whole faecal samples and the increased response towards the isolated individual bacterial strains before and following probiotic treatment, however, points up the complexity of the intestinal microflora and their interaction with the intestinal immune system (Schultz, 1997b). It would be expected that all constituents of the intestinal flora would be needed to form the immune response seen in an individual and that this immune response could not be mimicked by only a few selected microorganisms.

Evidence from animal models for experimental colitis shows that not all bacterial components of the intestinal microflora are equal in their capacity to induce inflammation (Schultz, 1997b). Rath et al. (1996b, 2001) demonstrated the primary role for *Bacteroides sp.* in the induction and perpetuation of experimental colitis in HLA-B27 transgenic rats, while *Esch. coli* was neutral. *Bacteroides sp.* played an essential role in the pathogenesis of carrageenan-induced colitis in guinea pigs (Onderdonk et al. 1981). In the present study, the activational response of peripheral $CD4^{+}$ T-lymphocytes upon stimulation with bacterial sonicates was significantly increased at the end of the study period, irrespective of the source ('self' *v.* 'non-self'). This effect must be attributed to the administration of the probiotic agent, and might indicate a general enhancement of the immune system. A similar effect was noted by Cunningham-Rundles et al. (2000) who found an increased immune response towards vaccinations in HIV-positive children, following the treatment with *Lactobacillus plantarum* 299v.

Subsequently, we measured the cytokine release of PB cells following stimulation. According to results of experiments *in vitro*, lactic acid bacteria induce the production of mainly pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-12, IL-18, and to a lesser extent suppressive cytokines such as IL-10 in PBMC (Miettinen et al. 1996, 1998). A more differential modulation of cytokine expression, however, was shown recently (Christensen et al. 2002). A recent report describes the possibility of anti-inflammatory action of members of the intestinal microflora by inhibition of the inhibitor κB/nuclear factor κB pathway by blockade of IκB-α degradation (Neish et al. 2000). We were able to demonstrate a strong induction of TNF-α, less IL-6 and IFN-γ, and minimal IL-10 and IL-4 secretion in PB cells prior to the study. At the end of the study period, following a 5-week course of oral *Lb.* GG, we noted a significant decrease in the release of IL-6 and TNF-α whereas release of IL-10 and IL-4 was increased. These findings confirm earlier studies, documenting increased IL-10 tissue levels following the clinically effective treatment of pouchitis with a combination of probiotic organisms (Ulisse et al. 2001).

In summary, this study in healthy volunteers demonstrates that oral administration of the probiotic microorganism *Lb.* GG exerts immunomodulatory effects on the systemic immune response towards intestinal organisms, leading to a heightened activational response of peripheral $CD4^+$ T-lymphocytes to intestinal bacterial components. The cytokine profile induced by these organisms is shifted towards an enhanced anti-inflammatory response by a heightened secretion of suppressive cytokines (IL-10, IL-4) and decreased secretion of pro-inflammatory cytokines (TNF-α, IL-6, IFN-γ). This outlines a possible mechanism by which probiotic bacteria may mediate a therapeutic effect. Moreover, immunomodulation of antibacterial immune responses may represent an option for IBD treatment. These results, however, cannot be extrapolated to other probiotic strains. Moreover, if Crohn's disease is a Th-1 driven disease, it has to be shown that treatment with *Lb.* GG is able to shift the cytokine profile into a Th-2 mediated cytokine release, not only in healthy adults but also in those suffering from this disease.

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