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INVOLVEMENT OF CD LYMPHOCYTES IN THE PRODUCTION OF INTERFERON INDUCED BY NON-PATHOGENIC BACTERIA

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SUMMARY

The effects of non-pathogenic bacteria on the production of different cytokines by circulating mononuclear cells from healthy subjects were studied *in vivo* and *in vitro*. Feeding a diet containing fermented dairy products, induced a significant increase of 2-5A synthetase activity in these cells suggesting a production of interferon. *In vitro*, mononuclear cells produced interferon-γ in presence of lactic bacteria like *Lactobacillus acidophilus*, *Streptococcus lactis* and *Streptococcus thermophilus*. *Streptococcus thermophilus* induced also the production of interleukin-1β. All the studied bacteria, including *Bifidobacterium* and *Lactobacillus casei* induced the production of interleukin 6. When incubated in presence of a monocyte depleted lymphocyte population, the production of interferon-γ was reduced by 76% when an anti-CD4 co-receptor was added in the medium, and by 62% in presence of an anti-CD8 co-receptor. The data indicates that non-pathogenic bacteria were susceptible to induce the production of different cytokines depending on the species and that the mechanism involved the two pathways of major histocompatibility complex.

INTRODUCTION

The possibility for non-pathogenic bacteria, to induce or stimulate immune functions, has been poorly studied. It has been suggested however that the gut flora may play a role as shown by higher levels of 2-5A synthetase, a marker of the production of interferon (IFN), in circulating mononuclear cells of conventional mice compared to germfree mice (*Galabru* et al., 1985). Indeed, it has been shown *in vitro*, that non-pathogenic bacteria from the human gut flora are able to induce the production of different cytokines (*Solis Pereyra* and *Lemonnier*, 1993; *Rusch*, 1994). In healthy subjects, both *in vivo* and *in vitro*, we have shown an increased production of IFN induced by bacteria from yoghurt, this effect being due to their walls (*Solis Pereyra* and *Lemonnier*, 1991, 1993). Interestingly, feeding 10^{11} lactic bacteria from yoghurt increased the 2-5A synthetase activity of circulating mononuclear cells from healthy subjects (*Solis Pereyra* and *Lemonnier*, 1991). This would indicate that consuming non-pathogenic bacteria can stimulate the production of IFN and that this production is added to that spontaneously observed in healthy subjects.

In this work, we have compared dif-

ferent species of dairy bacteria on their ability to induce the production of different cytokines by human mononuclear cells. The production of IFN-γ was studied in healthy subjects after a regular ingestion of yoghurt and the involvement of class I and class II histocompatibility systems was investigated.

METHODS

In vivo study

Samples of blood mononuclear cells (BMC), for 2-5A synthetase activity determination, were prepared from blood of 8 healthy subjects who were included in a cross-over protocol during two periods of 15 days each: during which, they were asked either to consume at least one yoghurt per day or no yoghurt. Samples of blood were taken at the end of each period.

2-5 A synthetase activity in BMC

Cell extracts were prepared by resuspending the BMC in 100 µl lysis buffer: 10 mM Hepes buffer pH 7.6, 10 mM KCl, 2 mM Mg (OAc)2, 4 H_2O, 7 mM 2-mercaptoethanol, with 0.5% Nonidet P-40, incubating the suspension at 4°C for 10 min and centrifuging 10 min at 5000 g. The cytosol was used immediately for the enzyme assay.

Cytoplasmic activity of 2-5A synthetase was determined as described previously (*Hovanessian* et al., 1977, *Justensen* et al., 1980). Briefly poly(rI):(rC)-agarose beads (Pharmacia Fine Chemicals, Uppsala, Sweden) were washed with several volumes of 20 mM Tris-HCl pH 8,5 mM Mg(OAc)2, 1 mM dithiothreitol, 25 mM KCl, 10% glycerol, 1 mM EDTA, (buffer D), and 30 µl aliquots were placed in microtiter tubes. Aliquots (100 µl) of cytosol, were mixed with the beads and incubated for 15 min at room temperature. The beads were washed three times with buffer D and all liquid carefully removed. The beads were then incubated with 10 µl reaction mixture: 25 mM Mg(OAc)2, 0.25 mg/ml bovine serum albumin, 12 µg poly(rI):(rC), 7 mM ^{32}ATP (50 mCi/ml > 15 TBq/mmol, Amersham, UK), 0.25 mg/ml creatin kinase, 0.01M creatin phosphate, in 20 mM Tris-HCl buffer pH 8 for 2 hours at 37°C. The reaction was stopped with 20 µl of 50 mM EDTA and 6 µl samples of incubation mixture were spotted onto PEI cellulose plate. The plates were chromatographed for 16 hours in 2 M Tris-HCl, pH 8.6 to separate 2-5 A oligoadenylates from ATP. The radioactive spots were located using X Ray film, cut out, collected in scintillation liquid and counted in the ^{32}P-channel of a scintillation counter. The percentage conversion of ATP to oligoadenylates was calculated as pmoles ATP incorporation/min per 10^7 cells. Tubes containing cell extracts from were BMC stimulated with IFN and tubes containing no cell extract were included in each assay to minimise the variations due to the batch of ^{32}P ATP used or other experimental parameters.

Preparation of cells and cultures

Human blood was drawn in heparin (30 U/ml) from healthy donors, the mononuclear cell fraction (BMC) was obtained by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, NJ) (*Boyum*, 1968). BMC were washed 3 times with phosphate buffered saline and counted. In the second part of this work, IFN-γ was the sole cytokine studied. This cytokine being produced by T lymphocytes, monocytes depleted lymphocytes population (LDM) were obtained by incuba-

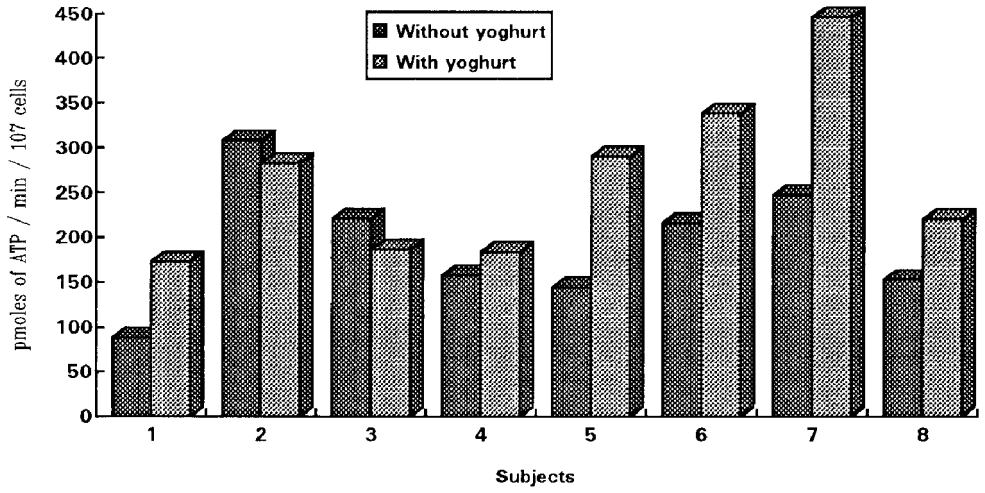

Figure 1: 2-5 synthetase activity of blood mononuclear cells from healthy subjects consuming a diet containing or not at least a yoghurt per day during 2 periods of 15 days (n=8)

tion of BMC with L-leucine methyl ester (Leu-OMe, Sigma, France): Leu-OMe was dissolved in phosphate buffered saline (PBS, Flow Laboratories, Irvine, UK), and filtered through a 0.45 µm filter immediately before use. Cells were suspended in PBS at concentration of $5x10^6$ cells/ml. They were incubated in 17x100 mm polypropylene culture tubes (Becton Dickinson, Rutherford, NJ) with 5 mM Leu-OMe at 22°C for 40 min. When the incubation interval was completed, 10% of foetal calf serum (FCS) was added, and the cells were washed twice with PBS. The population remaining was found to contain 6% residual monocytes as shown by flow cytofluorometry analysis.

Cells were cultured in DMEM medium (Flow Laboratories, Irvine, UK) containing 10% FCS, 200 mM glutamine, 2 g/l sodium bicarbonate, 100 µg/ml streptomycin, 100 U/ml penicillin. The proportion of viable cells before culture was determined by trypan blue exclusion. Samples containing two million BMC or LDM were incubated with 2.10^7 bacteria in a total volume of 1 ml in 24 well plates for 48 hours at 37°C in a humidified 5% CO_2 incubator. 10 µl of ConA (250 µg/ml) and 10 µl of LPS (10 µg/ml) were used as a positive control respectively for IFN-γ and for IL-1β and TNF-α. BMC and LDM were used as negative controls. 10 µg/ml of antibody (anti-CD4, anti-CD8; monoclonal antibodies for CD4 T helper, CD8 T cytotoxic/suppressor, respectively; IgG type, synthesised in mice) were added 1 hour before addition of bacteria and 24 hours after incubation. At the end of the incubation, cell-free supernatants were obtained and stored at -80°C until assayed for cytokines.

Bacterial strains

Streptococcus thermophilus (strain 158) was kindly provided by Centre de Recherche International Daniel Carasso (Plessis-Robinson, France). *Bifidobacterium*, *Lactobacillus acidophilus* and *Streptococcus lactis* and *Streptococcus casei* were obtained from Pilege (Champtoceaux, France). Before use, the bacteria were washed 3 times with DMEM medium without foetal calf serum and antibiotics.

Table 1: IL-1β, IL-2, IL-6, and IFN-γ production by BMC (blood mononuclear cells) in the presence of lactic bacteria for up to 48h

	IL-1β ng/ml	IL-2 U/ml	IL-6 ng/ml	IFN-γ U/ml
BMC	0.59±0.02	14.07±0.17	2.20±0.06	3.46±0.10
BMC + *S. thermophilus*	29.01±1.89	14.00±0.22	72.20±1.96	13.69±0.40
BMC + *Bifidobacterium*	1.02±0.02	13.84±0.24	38.92±0.54	4.14±0.33
BMC + *L. acidophilus*	0.96±0.05	14.22±0.39	46.35±0.80	40.94±1.38
BMC + *S. lactis*	1.37±0.07	14.96±0.38	56.88±1.34	48.10±1.31
BMC + *L. casei*	0.72±0.01	14.42±0.34	14.79±0.52	3.97±0.09

BMC = 2.10^6; bacteria = 2.10^7; n = 6

Cytokine assays

IFN-γ, IL-1β, IL-2, IL-6, and TNF-α were measured by specific immunoradiometric assays (Medgenix, Belgium); the sensitivities of the assays were: >5 pg/ml, >5 pg/ml, >1 U/ml, >6 U/ml and 6 pg/ml respectively. Radioactivity was measured in an automatic gamma counter (LKB-Wallac, Turku, Finland).

RESULTS

Consuming yoghurt for 15 days roughly doubled the levels of 2-5A synthetase found in the circulating BMC of 5 subjects, but had no effect on the 3 others (Figure 1), so that there was a significant increase by 38% (192±24 vs. 265±33, $p<0.05$) when considering all of them.

Streptococcus thermophilus were the only bacteria that induced the production of IL-1β. IL-2 was spontaneously produced by blood mononuclear cells, without any effect by the bacteria. In spite of this, the production IFN-γ was not produced by *Bifidobacterium* nor by *L. casei*. *L. casei* were also the less effective bacteria to induce of IL-6 (Table 1). *S. thermophilus* was able to induce the secretion of TNF-α. Mitogens (ConA and LPS), induced markedly the production of IFN-γ but not that of IL-1β and of TNF-α (Table 2). The addition of an antibody to CD4 co-receptor reduced to 1/4 the production of IFN. Interestingly, the anti CD8 had also an important effect. However, adding the two antibodies together, did not further reduce the production of IFN when compared to that observed with anti CD4 alone (Table 3).

DISCUSSION

The data presented here confirm that the ingestion of yoghurt in healthy subjects was able to increase the level of 2-5A synthetase in circulating mononu-

Table 2: IL-1β, TNF-α, and IFN-γ production by BMC in the presence of *Streptococcus thermophilus*

	IL-1β ng/ml	TNF-α ng/ml	IFN-γ U/ml
BMC	0.52±0.18	0.28±0.05	3.73±0.78
BMC + ConA	0.99±0.35	1.67±0.13	24.4±2.7
BMC + LPS	1.89±0.31	1.91±0.22	13.8±2.5
BMC + *S. thermophilus*	24.8±3.7	9.51±1.14	17.6±2.6

BMC = 2.10^6; *S. thermophilus* = 2.10^7; n = 6

clear cells. This suggests that the intestinal immune system has been stimulated by the ingestion of the non-pathogenic bacteria contained by yoghurt. The observed increase level of 2-5A synthetase suggest an augmentation of a physiological production of IFN in healthy subjects. However, the change observed here was less than the one observed previously (*Solis Pereyra* and *Lemonnier*, 1991). In our previous work, the subjects ingested in the lab a controlled yoghurt enriched in bacteria (2×10^{11}/g) once. Here the subjects were allowed to buy commercial yoghurts of their choice and to consume them at home. Thus a precise control of the quality and quantity ingested was not possible. Nor it was possible to be sure that no dairy products were consumed during the control period. Interestingly, there was a significant increase in the activity of the 2-5A synthetase, indicating that a regular ingestion of yoghurt is compatible with a maintenance of a higher level of the enzyme activity and probably with a higher production of IFN. In agreement with this, was the *in vitro* induction of the production of IFN-γ by *Streptococcus thermophilus*, one of the two bacteria contained in yoghurt.

The mechanism by which consuming of non-pathogenic bacteria could induce the production of IFN is not established. It may be suggested that these bacteria, probably their walls, rather than there cytoplasm or secreted substances (*Solis Pereyra* and *Lemonnier*, 1993), have been recognised by the immune system of the small intestine -a milieu usually poor in microorganisms- as the effect is detectable in blood four hours after feeding (*Solis Pereyra* and *Lemonnier*, 1991). Then, some lymphocytes, may be from the Peyer's patches, were liberated from the intestine and allowed to circulate in the blood as indicated by a higher level of 2-5A synthetase. A nearby mechanism might be involved to explain the spontaneous level of 2-5A synthetase observed in these subjects even then when they were not consuming fermented dairy products. Other dietary substances, like lectins from peas, have been suggested to explain this level of 2-5A synthetase (*Bocci* et al, 1988), but it is more likely that this is the result of a stimulation in the large intestine of the colon flora, as it has been shown that some of these bacteria are able to induce the production of IFN *in vitro* (*Solis Pereyra* and *Lemonnier*, 1993; *Rusch*, 1994). These data suggest that the spontaneous -thus physiological- production of IFN, indirectly detected by the spontaneous activity of the 2-5A synthetase, might be

Table 3: IFN-γ (U/ml) production by LDM (monocyte depleted lymphocyte populations) in the presence of anti-CD4 and anti-CD8 antibodies

		Anti CD4	Anti CD8	Anti CD4 + Anti CD8
LDM	1.71±0.42 (4)	–	–	2.56±0.31 (4)
LDM + *S. thermophilus*	32.2±7.9 (4)	7.83±0.74 (3)	12.2±0.90 (3)	8.18±1.67 (4)

LDM = 2.10^6; *S. thermophilus* = 2.10^7; (): number of subjects

regulated by dietary non-pathogenic bacteria fed regularly.

It is well established that activation and action of different T lymphocytes types, necessitate receptor of T cells (RCT) antigen recognition in association with major histocompatibility complex (MHC, Human Leukocyte Antigen: HLA for human) molecules (*Davis* et al., 1988). $CD4^+$ and $CD8^+$ lymphocytes interact with antigens associated with MHC I and II molecules, respectively (*Bierer* et al., 1989). Models for antigen presentation have divided antigens into two categories: endogenous (such as virus) and exogenous (such as bacteria), which are presented to T cells by class I and class II MHC respectively (*German*, 1986; *Sweetser* et al., 1989, *Morison* et al., 1986). Recently, the separation between these two pathways for presentation was called into question (*Bolonesi*, 1990). *Nuchtern* and co-authors (1990) suggest that Influenza virus proteins can associate not only with MHC I but also with MHC II molecules. The mechanism of presentation of non-pathogenic bacteria have been poorly studied. This study showed that Streptococcus thermophilus could induce IFN-γ production by monocytes depleted lymphocytes population *in vitro* via TCR-CD8-HLAI/TCR-CD4-HLAII complex, as this production was inhibited by anti-CD4 (76% inhibition) and to a lesser extent by anti-CD8 (62% inhibition).

A significant production of IFN was still existing when the antibodies, anti-CD4 and anti-CD8 were both present. This could be due to the stimulation of lymphocytes independently of TCR-CD8-HLAI/TCR-CD4-HLAII complex. This mechanism might be involved either through the cytokine network: a stimulation of other cells, such as B cells and monocytes (not tested in this study), can produce cytokines; or non protein components of *Streptococcus thermophilus* could stimulate immune cells, independently of the HLA system. It has been reported that molecules such as theichoic acid, present in the wall of Gram-positive bacteria, can induce cytokines *in vitro* (*Tufano* et al., 1991). Unfortunately, no data are available on the structure and composition including theichoic acid of the bacteria presented in this paper. All the dairy bacteria tested *in vitro* were not equally efficient to induce the production of cytokines, it is not known if this might be related in part, to differences in the composition of their membranes.

Numerous interactions are known between cytokines (*Reyes* et al., 1986). For example, IFN can stimulate the synthesis of IL-1. This was not observed, in our experiment where high levels of IFN were produced *in vitro* without any production of IL-1 (Table 1). IL-2 was spontaneously produced in vitro at the same level by BMC without any effect of the bacteria. IL-2 is con-

sidered to be necessary for a production of IFN (*Croll* and *Morris*, 1986). In our conditions this production of IFN was not related to that of IL-2, as IFN was not produced in the presence of *Bifidobacterium* and of *L. casei*.

In conclusion, non-pathogenic bacteria used in dairy products seemed to be able to stimulate the production of different cytokines *in vitro*, and also in vivo for IFN. However, the role of this IFN production is not yet clear. It is known, for example that consuming lactic bacteria renders laboratory animals more resistant to Salmonella infection (*De Simone* et al., 1988, *Hitchins* et al., 1985), but the underlying mechanism has not been established. Such a protection to infection, might be compatible with a role of the induction of the production of cytokines in a system of immune surveillance, but this has not been yet demonstrated.

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