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Exopolysaccharides from *Lactobacillus rhamnosus* RW-9595M stimulate TNF, IL-6 and IL-12 in human and mouse cultured immunocompetent cells, and IFN-γ in mouse splenocytes

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Abstract — Exopolysaccharides (EPS) from *Lactobacillus rhamnosus* RW- 9595M have been prepared from bacterial cultures, isolated, concentrated, fractionated and tested in vitro for their possible modulating properties on mouse splenocytes from the C57Bl/6 and BALB/c strains, on the murine RAW 264.7 macrophage-like cell line and on human Peripheral Blood Mononuclear Cells (PBMC) from a total of 14 healthy donors. A first step of EPS fractionation was attempted, using membranes with different molecular weight cut-off. Fractions were as follows: F1: >1000 kg·mol^{-1}; F2: 1000–100 kg·mol^{-1}; F3: 100–10 kg·mol^{-1}; F4: <10 kg·mol^{-1}. Total EPS, as well as F1, appeared slightly mitogenic in both mouse splenocytes and human PBMC in 2–3 d cultures, and F3 also exhibited such a property on human PBMC. Unfractionated concentrated ("total") EPS, as well as F1, elicited TNF, IL-6 and IL-12 p40 both in the mouse and human cells, in 6 h and 24 h cultures, with important variability depending on the cell source.
In 24 h cultures, total EPS or F1 elicited bio-active IFN-γ in both C57Bl/6 and BALB/c splenocytes, and this IFN-γ secretion was sustained until at least 3 d of culture. In human PBMC, no IFN-γ production was observed despite high IL-12p40 secretion.
These results suggest the possibility of enhancing the immune system through EPS from lactic acid bacteria, in individuals responsive to such a stimulus.

***L. rhamnosus* / exopolysaccharide / mouse / human / cytokine / interferon gamma**

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Résumé — Productions de TNF, IL-6, IL-12 et IFN-γ chez des cellules immunocompétentes traitées avec des exopolysaccharides du *Lactobacillus rhamnosus* RW-9595M. Différence entre les réponses de cellules de sang périphérique humain et de splénocytes de souris. Les exopolysaccharides (EPS) du *Lactobacillus rhamnosus* RW- 9595M ont été préparés à partir de cultures bactériennes, isolés, concentrés, fractionnés et testés in vitro quant à leur potentiel immunomodulateur sur des splénocytes de souris C57Bl/6 et BALB/c, sur la lignée macrophagique murine RAW.264.7 et sur des cellules mononuclées du sang humain provenant de 14 donneurs. Nous avons fractionné ces EPS en fonction des tailles moléculaires, avec des membranes filtrantes de différentes porosités. Les fractions correspondent aux tailles suivantes : F1 : > 1000 kg·mol^{-1}, F2 : 1000–100 kg·mol^{-1}, F3 : 100–10 kg·mol^{-1} et F4 : < 10 kg·mol^{-1}. Les EPS concentrés, non fractionnés, ainsi que F1, se sont avérés légèrement mitogènes sur des splénocytes de la souris, ou des cellules sanguines humaines, cultivées 2–3 jours. Dans le dernier cas, F3 manifeste également cette propriété. Les EPS non fractionnés, ainsi que F1, sont capables de provoquer la production de TNF, IL-6, IL-12p40 après 6 ou 24 h de culture, l'origine des cellules étant importante pour l'intensité de la réponse.
Après 24, 48 et 72 h de culture, les EPS non fractionnés, ainsi que F1, ont provoqué la production d'interféron gamma par des splénocytes de souris C57Bl/6 et BALB/c.
Au contraire, dans le cas des cellules sanguines humaines, aucune production d'interféron gamma n'a pu être observée, en dépit d'une bonne réponse en IL-12p40.
Ces résultats suggèrent que les EPS de bactéries lactiques peuvent stimuler certaines fonctions immunitaires chez des sujets aptes à répondre.

***L. rhamnosus* / exopolysaccharide / souris / humain / cytokine / interferon gamma**

1. INTRODUCTION

Genera *Lactobacillus*, *Lactococcus* and *Bifidobacterium* are often referred to as "Lactic Acid Bacteria" (LAB) or dairy-related microorganisms. LAB have been shown to contribute to the yogurt consumer's health by their capacity to stimulate the immune system, especially by eliciting or reinforcing production of cytokines such as TNF, IL-6, IL-12, IL-18 and IFN-γ [22, 27, 30, 31, 45] with possible implications in anticancer defence [49]. In addition, several gut bacteria-derived polysaccharides are also well known potent stimulators of the immune system, most of them being combined to residues of lipidic (lipopolysaccharides) or peptidic (peptidoglycans) nature [17]. Thus, stimulation of the immune system through food-added bacteria or bacterial products has been the focus of active research in the recent years [43]. In particular, stimulation of the specific "cellular" component of the immune system, the "Th1 response" which results in the resistance against most infectious agents and reduces the manifestations of allergy [26] has been particularly focused on. The "Th1 response" is characterized mostly by IFN-γ. Not surprisingly, IFN-γ production, as well as the production of IFN-γ-eliciting cytokines such as IL-12 and IL-18, have therefore been considered as an important criterion in the evaluation of the immunostimulating capabilities of LAB [22, 31, 43, 45]. Besides bacteria, lipo-polysaccharides and peptidoglycan, it has been recently shown that "T cell dependent" responses can also be triggered by "simple" polysaccharides of bacterial origin, but it seems that such a property is unusual among them [21].

On the other hand, the dairy product industry considers the use of polysaccharides released by some lactic bacteria, the exopolysaccharides (EPS), for their capacity to bring about some rheological qualities to yogurt [10, 25, 48]. EPS produced by LAB have been recently reviewed [38] and some preliminary results suggest that some EPS might be used not only for their rheological properties but also for putative health-promoting properties, which would include anti-tumor and immunostimulatory

actions. In the human, EPS originating from bacteria associated with some pathologies have been found to elicit some pro-inflammatory cytokines/chemokines [3].

In this work we studied the possibility, for EPS of *lactic* origin, to elicit cytokines, both of the "pro-inflammatory" and of the "T cell-dependent" kinds. EPS produced by a highly productive *Lactobacillus rhamnosus* strain, RW-9595M [12] have been tested. These EPS exhibited varying abilities to stimulate pro-inflammatory cytokines in mouse splenocytes as well as in peripheral blood mononuclear cells (PBMC) from several healthy human donors. Because the capacity of eliciting IFN-γ production seemed to us of particular interest, and knowing that such a production is genetically regulated, two different strains of mice (C57Bl/6 and BALB/c), well known for their differential IFN-γ responses in some pathological situations [24] were comparatively tested for their cytokine responses. PBMC from several healthy human donors were also screened for their cytokine responses.

EPS-stimulated splenocytes from both murine strains were able to produce high IFN-γ responses, whereas PBMC from none of 14 human donors did so. However, under in vitro stimulation with EPS, these PBMC were able to elicit significant amounts of IL-12, a pro-inflammatory cytokine well known for its IFN-γ-inducing capacity. Thus, there seems to be a potential, in these EPS, to help a "Th1" type of immune response which would favor both anti-infection and anti-allergy reactions. Of course these reactions will have to be monitored in vivo, in further studies.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

The exopolysaccharide (EPS)-producing strain of *Lactobacillus rhamnosus* RW-9595M was provided by Denis Roy from the Food Research and Development Centre (Agriculture Canada and Agri-Food Canada, St-Hyacinthe, Quebec, Canada). *L.rhamnosus* was subcultured in 20 mL of Lactobacilli MRS broth and incubated anaerobically for 48 h at 37 °C. Stock cultures were stored at –40 °C in BHI (Difco) broth in 15% (v/v) of glycerol.

2.2. EPS preparation and fractionation

L. rhamnosus RW-9595M was subcultured twice in MRS broth (Difco Laboratories, Detroit, MI, USA), at 37 °C for 18 h under anaerobic conditions. The bacteria were then inoculated 1% v/v in 1.5 litre fermenters (Biostat M, Braun, Melsungen, Germany), filled with Basal Minimum Medium as described in [16]. Growth occurred at 37 °C during 24 h with an agitation of 100 rpm. The pH was automatically adjusted to 6.0 with NH_4OH 7N. EPS were isolated according to Cerning [6]. The culture broth was heated at 100 °C for 15 min to inactivate glycohydrolases.

The culture broth was centrifuged at 17 700 *g* for 30 min at 4 °C and the bacteria were discarded. The supernatant was mixed with 95% ethanol (1:3) and left overnight at 4 °C to precipitate the EPS. These were collected by centrifugation at 14 300 *g* for 30 min at 4 °C, then dissolved in distilled water. The solution was dialysed against distilled water for 24 h at 4 °C, with periodical changing of water, then freeze-dried. EPS were isolated by three successive additions of 10% trichloroacetic acid (TCA) precipitations followed by centri- fugation at 4620 *g* for 15 min at 4 °C. The EPS solution was dialysed against distilled water for 5 d, at 4 °C, with daily changing of water, then freeze-dried.

Protein content was measured according to Bradford [5] using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) and no trace of proteins was

detected. The isolated EPS preparation was freeze-dried. The molecular weight of EPS was determined by size exclusion gel permeation chromatography (GPC) using a Varian Vista 5500 High-pressure liquid chromatography (HPLC) (Varian Inc., Sunnyvale, CA, USA) coupled with a Varian Auto Sampler, Model 9090, with a standard U.V. detector set at 210 nm. Two Supelco Progel TSK PWH (7.5 mm × 7.5 cm) and TSK GMPW (7.5 mm × 30 cm) guard columns (Supelco, Sigma Aldrich, Canada Ltd, Oakville, ON, Canada), followed by two Waters UltraHydrogel analytical columns (2 000 and 500, 7.5 mm × 30 cm; Waters Corporation, Mississauga, ON, Canada) were used for molecular weight determination. Chromatography was performed on 0.5% EPS, at the rate of 0.5 mL·min^{-1}, with an aqueous eluant (0.02 mol·L^{-1} phosphate buffer, pH 7.0 containing 0.10 mol·L^{-1} NaCl). A molecular weight calibration curve was established using a set of dextran and dextran-blue molecular weight markers ranging from 5 to 2000 kg·mol^{-1} (Polymer Standard Service USA Inc., Silver Spring, MD, USA). The EPS were filtered on 0.45 μm nylon membrane filters (VWR Nalge, Mississauga, ON, Canada) prior to GPC analysis.

EPS preparation was dissolved in deionized water (1.15 mg·mL^{-1}) and fractionated by successive filtrations, under a pressure of 55–60 psi, using membranes of different molecular weight cut-off: 1000 kg·mol^{-1} and 100 kg·mol^{-1} (Omega, Pall Filtron, Calgary, AB, Canada) and 10 kg·mol^{-1} (Diaflo, PM10, Amicon, Beverly, MA, USA). Four fractions were obtained: F1: >1000 kg·mol^{-1}; F2: 100–1000 kg·mol^{-1}; F3: 10–100 kg·mol^{-1} and F4: <10 kg·mol^{-1}, and confirmed by size exclusion chromatography as described above. Each of these fractions was freeze-dried. A stock solution (500 ng·mL^{-1}) of each of these fractions was sterilised by filtration on 0.22 μm filters, aliquoted and kept at –20 °C.

2.3. Mice

Female C57BL/6 and BALB/c mice (Charles River Canada, St-Constant, QC, Canada) were used for the splenocyte stimulations. They were kept in our facilities in specific pathogen free (SPF), temperature-, humidity- and light cycle-controlled conditions. All animals were sacrificed by cervical dislocation at the age of 2–4 months.

2.4. Splenocytes culture

Splenocytes were aseptically prepared and suspended in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), HEPES, glutamine and gentamycin, and placed in 24-well plates (5×10^6 cells·mL^{-1}). Splenocytes were stimulated, in quadruplicate, with EPS preparations at different concentrations (2 500–10 000 ng·mL^{-1}) and their cytokine responses were assessed in either the culture supernatants or in the mRNA preparations. Positive controls consisted of stimulation with either LPS (100 ng·mL^{-1}, lot# 87114062, Sigma, St-Louis, MO, USA) or ConA (5 μg·mL^{-1}, Sigma). The plates were then incubated at 37 °C, under a 5% CO_2 atmosphere and saturated humidity, for either 6 h (TNF) or 24 h (IL-6, IL-12 and IFN-γ). In IFN-γ production kinetic studies, splenocytes were stimulated as above and incubated for 6, 24, 48 or 72 h. After incubation, cells were collected by centrifugation (520 *g*, 7 min) and kept frozen at –80 °C for further RNA extraction. The supernatants were aliquoted and stored at –20 °C for cytokine determinations.

2.5. Peripheral Blood Mononuclear Cells (PBMC)

PBMC were prepared from the blood of healthy donors as in [1]. In brief, mononuclear cells were isolated on a Ficoll-Paque gradient from heparinized blood. They were incubated at the

concentration of 2×10^6 cells·mL^{-1} and tested for their cytokine responses as for the mouse splenocytes.

2.6. RNA isolation

RNA from splenocytes or PBMC was extracted using the Trizol LS reagent (Gibco BRL, Grand Island, NY, USA) as lysing agent, followed by the use of the RNA-Tack Resin (Biotecx Laboratories Inc., Houston, TX, USA). RNA concentration in each sample was measured by spectrophotometry at 260 nm and the concentration was adjusted to 0.1 μg·mL^{-1}.

2.7. RT-PCR

RT reaction was performed on the RNA samples to obtain cDNA, using the Moloney murine leukemia virus reverse transcriptase (MulV, USB Corporation, Cleveland, Ohio, USA). These cDNA samples were submitted to PCR in order to determine the expression of the IFN-γ gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, a "housekeeping gene", was used as a reference. The primers were: mouse IFN-γ: 5'CAA GTG GCA TAG ATG TGG AA 3' / 5' CTG GAC CTG TGG GTT GTT 3' [7]; human IFN-γ 5'GGT TCT CTT GGC TGT TAC TGC C/ 5'GTT GGA CAT TCA AGT CAG TTA CC 3' [34], and mouse or human GAPDH**:** 5' CCT TCA TTG ACC TCA ACT ACA T 3' / 5' CCA AAG TTG TCA TGG ATG ACC 3' [9]. Samples were amplified for 35 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min with a final extension at 72 °C for 7 min. The PCR products were separated on a 2% agarose gel and visualised with ethidium bromide.

2.8. Assessment of cell proliferation

Cell proliferation was measured, for total EPS and for EPS fractions at different concentrations, using the MTT colorimetric method [44]. Working in 96-well plates, 50 μL of 5×10^6 cells (splenocytes or PMBC) /mL were put together with 50 μL of EPS preparations and incubated for various periods of time. After incubation, 100 μL of MTT (0.1 mg·mL^{-1}, Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) were added and cells were incubated overnight at 37 °C. Supernatants were discarded, 100 μL of dimethylformamide-sodium-dodecylsulfate were added to the cells, and the plates were incubated for another 5 h at 37 °C. Absorbances were measured at 570 nm on a plate reader (Easy Reader EAR 400 AT, SLT-LabInstruments, Grödig, Austria).

2.9. Cytofluorometry

Splenocytes were prepared as above and incubated with EPS preparations for either 24, 48 or 72 h. They were incubated with a fluorescein-conjugated goat affinity purified $F(ab')_2$ fragment to mouse IgG (Lot: 00974, ICN Pharmaceuticals, Inc., Aurora, OH, USA) diluted 1:150, which principally marks B lymphocytes. Samples were then washed with the "FACS medium" (PBS pH 7.4 enriched with FCS 1% and NaN_3 0.5%) and suspended in 500 μL of the FACS medium containing 1% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ, USA). Cytofluorometry was performed on an EPICS XL-MCL cytofluorometer (Beckman Coulter, Hialeah, FL, USA). Splenocytes undergoing division were selected on the basis of their larger size (as determined by the forward scatter intensity), and the proportions of fluorescein-positive cells were then determined in this zone.

2.10. Cytokine determinations

Cytokine contents in the supernatants of splenocytes were determined from pooled quadruplicate cultivation wells, for each experimental point.

Bioassays for TNF and for IL-6 were done as in [47]. In brief, for TNF,

actinomycin D-treated L-929 cells were incubated for 16–20 h with supernatants dilutions and stained with crystal violet. One unit was defined as the reciprocal of the dilution giving 50% cytotoxicity. For IL-6, the IL-6-dependent B9 cell line was incubated with supernatant dilutions for 4 d, and B9 cell proliferation was assessed by MTT. Absorbance was read at 540 nm and one unit was defined as the amount inducing half-maximal absorbance. The IFN-γ bio-assays were done on the supernatants according to Migliorini et al. [32] in 96-well plates, using 100 µL/well of a 2×10^5 cells·mL^{-1} suspension of RAW 264.7 cells (ATCC T1B71). After an incubation of 18–24 h, the supernatant was discarded. Fifty µL of both LPS (20 ng·mL^{-1}) and supernatants were added to the cells, before incubating them at 37 °C for 24 h. Fifty µL of the supernatants were put into a new 96-well culture plate, as well as the $NaNO_2$ dilutions used for tracing the standard response curve. Then, 100 µL of "Griess Reagent" (equal amount of the two following solutions: 0.1% naphthylethylene diamine dihydrochloride in H_2O and 1% sulphanilamide in 5% H_3PO_4) was added. Finally, absorbances were measured at 540 nm. At several occasions, we validated the technique by neutralising the IFN-γ activity in the bio-assay, using a rat anti mouse IFN-γ antibody (10 µg·mL^{-1}; Pharmingen, Mississauga, ON, Canada).

ELISA kits were used, according to the manufacturer's instructions, for other cytokines determinations: human and mouse IL-12, p40 (R and D Systems, Minneapolis, MN, USA) with minimum detectable doses of 15 pg·mL^{-1} and 4 pg·mL^{-1}, respectively; human IFN-γ (R and D Systems or BioSource International, Camarillo, CA, USA), with a minimum detectable dose of 8 pg·mL^{-1}.

2.11. Statistical analysis

Variance analysis, Student's *t* and Mann-Whitney tests were used in paired-comparison with negative cell controls [41]. In the cell proliferation determinations, for each independent experiment, mean (from at least 8 wells) absorbance values were compared between the "control" and the LPS-, EPS- or F3-added groups. Taking the "control" group value as "100%", experimental groups values were expressed as a percentage of the control. Taking the standard-deviations of each group into account, groups were compared between each other by the Student's *t* test. For cytofluorometry measurements, splenocytes were stimulated in duplicate (the wells were pooled for the measurements) and 10 000 cells were counted for each experimental point. All mouse cytokine determinations were done on the basis of 5 independent splenocyte stimulations, and tests were performed in quadruplicate. For the IFN-γ production kinetics, 3 independent experiments were performed, and each of them represented a pool of 4 culture wells.

3. RESULTS

3.1. Preliminary characterization of the EPS

The HPLC profile (Fig. 1) shows that the molecular mass of EPS of this preparation exhibits at least two principal peaks corresponding to approximately 200 kg·mol^{-1} and 2 000 kg·mol^{-1}. Elemental analysis revealed the absence of nitrogen, and ipso facto of protein. The total EPS fraction appears of high purity and homogeneity by NMR spectroscopy. The sugar composition of the EPS produced by *L. rhamnosus* RW-9595M used in this work, as determined on the hydrolysate by HPLC and by gas chromatography of the alditol acetates, is the following: rhamnose, 4 ; glucose, 2 ; and galactose, 1 (M-R van Calsteren, manuscript in preparation).

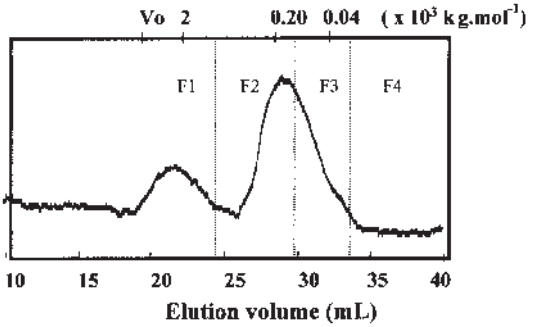

Figure 1. HPLC profile of isolated concentrated EPS. Starting from this initial preparation, fractions were eventually obtained by membrane filtration, as indicated by the dotted lines: F1, F2, F3 and F4 refer to the fractions mentioned in text.

3.2. Mitogenic properties of EPS

The growth response, assessed by the MTT method, of splenocytes from C57 Bl/6 mice cultured with the EPS fractions is shown on Table I. The responses can be clearly observed after an incubation time of 48 h. Considering the control (cells cultured alone) as 100%, the most significant results were obtained with F1 at the concentration of 5 000 ng·mL^{-1}. This is close to what is obtained with LPS, a long known mitogen for mouse B cells [50]. As similar cell number increases were obtained in both F1-treated and LPS-treated samples, we postulated that these increases were possibly linked to B cell proliferation. Indeed, IgG-labelled cells (principally B cells) represent up to 71.1% of the large size cells (principally dividing cells) stimulated with F1 at 5 000 ng·mL^{-1}. For all time points and all concentrations, treatment with F1 results in an overall effect situated between that of the positive (LPS) and negative controls. For the 48 h time point and for the concentration of 5 000 ng·mL^{-1}, F1 treatment gives a result very comparable to that obtained with LPS (Fig. 2). On the other hand, F2 and F3 were less efficient and F4 was inactive (not shown).

Mitogenic properties of F1, F2 and F3, in comparison with those of LPS [28], were tested also on human PBMC. They all exhibited some mitogenic properties, depending on the donor (not shown). Table II examplifies the comparable mitogenic capacities of F3 and LPS, on PBMC from 7 donors.

Table I. Cellular growth of splenocytes from C57Bl/6 mice stimulated with each EPS fraction, as determined by the MTT method.

Fraction designation (kg·mol^{-1})	Concentration (ng·mL^{-1})	Mean cellular growth (%)[a]
F1 (>1 000)	5 000	130.8***
	10 000	121.5**
F2 (100–1000)	5 000	121.6*
	10 000	125.9*
F3 (10–100)	5 000	112.6*
	10 000	114.3*
F4 (<10)	5 000	96.2
LPS	100	132.7***

[a] Time in culture: 48 h.
Comparison with control by the *t* test: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. All values are expressed considering cell control as 100% (n= 6–8 for each experiment). For 5 000 ng·mL^{-1}: 3 independent experiments combined; for other concentrations: 2 independent experiments combined.

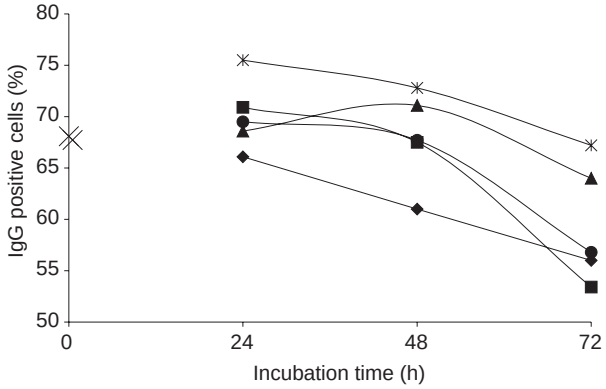

Figure 2. Percentage of IgG positive cells, as determined by flow cytofluorometry, in a population of large size C57Bl/6 splenocytes, in the presence of medium alone, of LPS or of F1 at different concentrations.
(◆) medium alone, (*) LPS 100 ng·mL^{-1}, (■) F1 2 500 ng·mL^{-1}, (▲) F1 5 000 ng·mL^{-1}, (●) F1 10 000 ng·mL^{-1}.

Table II. Cellular growth of human PBMC stimulated with F3, as determined by the MTT method.

Time in culture (h)	Donor designation	LPS 500 ng·mL^{-1} (%)	F3 3 000 ng·mL^{-1} (%)
36	AE	109	110
	AF	122**	120*
48	AA	96[a]	155[b,]*
	AG	119**	111*
	AH	97	107
	AJ	130*	123
	AK	115**	108
60	AE	115	117
	AF	217***	150***
	AH	113	111
	AJ	123	118
	AK	98	125***

Comparison with control by the *t* test: * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. All values are expressed considering the cell control as 100% (n = 8 for all experiments).
[a] LPS : 100 ng·mL^{-1} instead of 500 ng·mL^{-1}
[b] F3 : 2 000 ng·mL^{-1} instead of 3 000 ng·mL^{-1}.

3.3. Proinflammatory responses to EPS

We found that total EPS and F1, but not F2 and F3 (not shown), were able to induce some mouse cells to secrete TNF after 6 h of culture, and this capacity depended on the origin. Splenocytes from C57Bl/6 did not respond, whereas those from BALB/c

responded mildly (from 18.5 to 19 units·mL^{-1}). In contrast the RAW 264.7 cells, which are cultured murine macrophage-like cells and were used as typical TNF secretors, responded strongly to EPS or F1 stimulation (838 to 2 228 units·mL^{-1}).

On the other hand, after 24 h of culture, total EPS and F1 also elicited IL-6 secretion by splenocytes. Figure 3 shows that the response of C57Bl/6 splenocytes to F1 is always statistically different from the unstimulated controls ($p \leq 0.01$, *t* test, for all F1 concentrations). One single experiment was also performed using BALB/c mice splenocytes. These mice responded apparently stronger than did the C57Bl/6 (Fig. 3). The IL-12p40 responses to F1 were also tested in a single experiment (Fig. 3). In both strains IL-12 responses were modest, but the basal IL-12p40 activity in the supernatant was relatively high.

PBMC from up to 14 donors were cultured with total EPS for 6 h (TNF) or 24 h (IL-6, IL-12p40) and cytokine production was assessed. Compared to unstimulated PBMC, EPS- and LPS-activated PBMC secreted significant amounts of TNF-α ($p \leq 0.001$, Mann-Whitney test). PBMC from all donors exhibited IL-6 responses to both EPS and LPS. As for the IL-12p40 production, all of the 7 donors tested responded to total EPS, with one donor (BF) not responding to LPS. The difference between controls and EPS-treated groups is always statistically significant ($p \leq 0.001$, Mann-Whitney test). Table III shows the results obtained with the 7 donors who were all tested for these three cytokines.

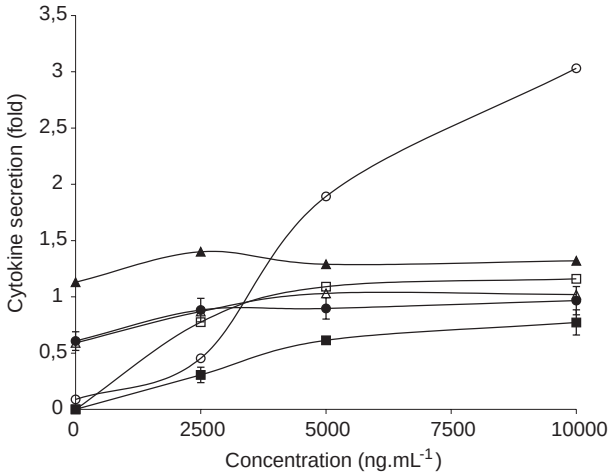

Figure 3. Cytokines present in the supernatants of 24 h cultured splenocytes, as a function of the F1 concentration.
(●/○) IL-6 bioassay, (▲/△) IL-12p40 ELISA, (■/□) IFN-γ bioassay.
The results are expressed as "-fold", where "1-fold" is the numerical value given by the positive controls (LPS for IL-6 and IL-12p40, Con A for IFN-γ). In the different experiments, "1-fold" represents 111.9–115.1 units·mL^{-1} in the case of IL-6, 76.6–121 pg·mL^{-1} in the case of IL-12p40, and 10.3–10.4 mmol·L^{-1} of NO in the case of IFN-γ. Splenocytes were from either BALB/c (open symbols) or C57Bl/6 mice (black symbols).
Bar = standard deviation.

Table III. TNF, IL-6 and IL-12 productions in the supernatants of PBMC from 7 donors, after 6 h (TNF) or 24 h (IL-6, IL-12) of culture with medium alone (MA), or with EPS- or LPS-added medium. Determinations were made with bio-assays (TNF and IL-6) or ELISA (IL-12).

Donor#	TNF[a]			IL-6[a]			IL-12[b]		
	MA	EPS[c]	LPS[d]	MA	EPS[c]	LPS[d]	MA	EPS[c]	LPS[d]
BA	0	99	799	0	2 448	6 422	0	233	1 085
BC	0	360	810	0	11 600	449	0	93	521
BF	0	73	24	40	9 554	7 043	0	93	0
BJ	0	59	0	0	3 080	9 554	0	153	98[e]
AA	0	184	338	55	3 420	2 065	0	530	341
AC	0	197	311	64	2 372	3 218	0	923	429
AE	0	62	256	389	81 920	62 950	0	981	Not done

[a] In arbitrary units·mL^{-1}.
[b] In pg·mL^{-1}.
[c] 10 000 pg·mL^{-1}.
[d] 100 ng·mL^{-1}.
[e] Stimulated with 5 µg·mL^{-1} of Con A instead of LPS.

3.4. IFN-γ responses to EPS

After a 24 h culture, significant ($p \leq 0.01$, Mann-Whitney test) IFN-γ production was observed with C57Bl/6 splenocytes in response to different concentrations of F1. In a single experiment, BALBC/c splenocytes responded in a similar fashion to this stimulus (Fig. 3).

In addition to the bioassay validations described in "Materials and methods", IFN-γ response specificity was also checked by RT-PCR. Both F1 and total EPS were able to stimulate the IFN-γ gene expression, as well as did ConA (Fig. 4a).

A kinetics study showed that IFN-γ production did not occur before 6 h, and even

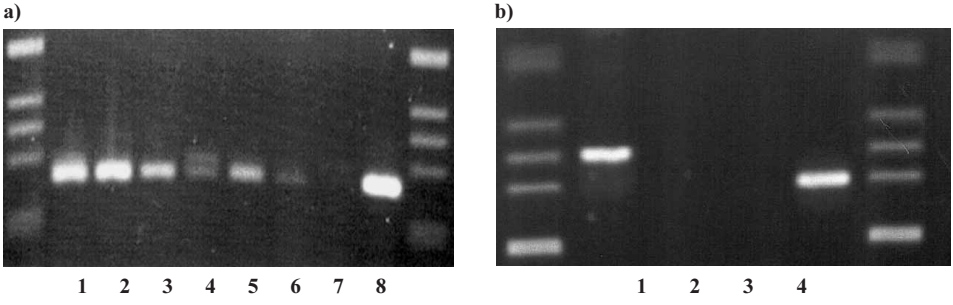

Figure 4. IFN-γ gene expression from: (**a**) C57Bl/6 mouse splenocytes stimulated with different F1 concentrations (lane 1: 2 500 ng·mL^{-1}; lane 2: 5 000 ng·mL^{-1}; lane 3: 10 000 ng·mL^{-1}) or total EPS concentrations (lane 4: 2 500 ng·mL^{-1}; lane 5: 5 000 ng·mL^{-1}; lane 6: 10 000 ng·mL^{-1}). Lane 7, no stimulation (negative control). Lane 8, stimulation with ConA, 5 µg·mL^{-1} (positive control). (**b**) Human PBMC stimulated with: lane 3: total EPS,10 000 ng·mL^{-1}; lane 4: ConA, 5 µg·mL^{-1} (positive control). Lane 2: no stimulation (negative control). Amplification of the GAPDH reference gene is shown in lane 1.

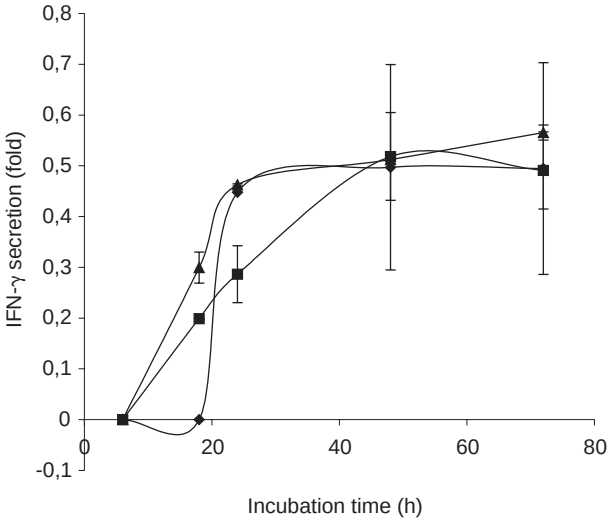

Figure 5. IFN-γ secretion kinetics by C57Bl/6 splenocytes stimulated with 3 different concentrations of F1, as a function of time in culture.
(◆) 2 500 ng·mL^{-1}, (■) 5 000 ng·mL^{-1}, (▲) 10 000 ng·mL^{-1}.
Three independent experiences were performed. Values are expressed as "-fold" taking the ConA positive controls as "1-fold". "One fold" values were comprised between 13.6 mmol·L^{-1} and 57.2 mmol·L^{-1} of NO, based on a bioassay. Bar = standard deviation.

18 h in culture in the case of the lower F1 concentration, and reached a plateau between 48 and 72 h , regardless of the F1 concentration (Fig. 5).

With the human PBMC, none of the 14 donors tested exhibited any convincing IFN-γ response to either total EPS or F1, at the concentrations which were effective in the case of the mouse splenocytes (not shown). Also, human IFN-γ gene expression was not modified by EPS, under similar conditions (Fig. 4b).

4. DISCUSSION

Polysaccharides from various sources have long been known to be adjuvant or stimulators of the immune system. Most polysaccharides are complex and appear as combined to various lipidic, peptidic or polar groups. However "pure" polysaccharides, as the EPS we prepared from *L. rhamnosus* RW-9595M appear to be, have also been reported to exert immunomodulating activities including IFN-γ stimulation [14, 20]. Some immunomodulating properties of polysaccharides have been found to depend on stereochemistry [33], molecular size [21] or number of sugar residues implicated [19].

In the present report, it appears that F1, which corresponds to EPS of the highest molecular weights (>1000 kg·mol^{-1}), can stimulate cytokine production, whereas fractions corresponding to lower molecular size seem less capable of doing so, at least in the mouse. However, further EPS preparations, and more discriminate separations based on the molecular weight, are certainly necessary to establish a clear relationship between molecular weight and immunomodulating capacity, both in the mouse and in the human.

Polysaccharides are well known mitogens for a variety of cells. For instance

LPS is a B lymphocyte mitogen in the mouse [50] and, in the presence of monocytes, a T lymphocyte mitogen in the human [28]. EPS from *L.rhamnosus* also exhibited mitogenic properties on mouse (Tab. I), human (Tab. II) swine and bovine cells (Lessard, unpublished). EPS from *Halomonas Eurihalina* were recently reported to influence the non-specific proliferation of human PBMC [36]. In the present work EPS were also found to stimulate proinflammatory cytokines in mouse and human, in a fashion comparable to LPS.

The present study therefore seems to suggest some shared properties between LPS and EPS from *L.rhamnosus* RW-9595M, which appear as slightly mitogenic and able to stimulate the production of proinflammatory cytokines. However, in the case of EPS, we observe a striking difference between the responses of mouse splenocytes on the one hand, and human PBMC on the other hand, in the IFN-γ response. Preliminary studies suggest that PMBC from swine cannot be elicited by EPS preparations to secrete IFN-γ, either. However, both swine and bovine PBMC exhibited a dose-dependent PGE2 response to stimulation with EPS (Lessard, unpublished). Presently we do not know whether such a difference stems from the *species,* or from the *tissue* origin, of the cells stimulated with the EPS. The absence of IFN-γ response of the human PBMC, as compared to the murine splenocyte response, could result from a lack of co-signal or co-factor. IL-12, however, does not seem to be a good candidate for such a critical role of co-signal, because a clear IL-12 response to EPS stimulation was actually found in the human (Tab. III), in contrast to a less pronounced response in the mouse, where a relatively high IL-12p40 concentration was observed even in the absence of EPS stimulation (Fig. 3). However, in the present work, IL-12 response was assessed by the IL-12p40 secretion, which certainly indicates a response but does not necessarily means that the response consists in functional IL-12. Bioactive IL-12 is in a heterodimeric form (IL-12p70) and an excess of IL-12p40 may be inhibitory. Therefore, the IL-12 response observed here must be taken cautiously, as for its biological signification. But since IL-12 is a key actor in the production of IFN-γ as well as in other important functions [40] we consider that the IL-12 response of human PBMC to EPS reflects an encouraging response capacity of the human to the immunostimulating capacity of EPS. However, additional factors, such as IL-2 [4] or IL-18 [11] for instance, should also be considered in future investigations, to try to explain the difference in IFN-γ responses between the two systems.

These immunomodulating polysaccharides exert their action through cell membrane receptors whose nature, density and cellular distribution contribute to the response, but presently we can only speculate on the nature of the receptor(s) incriminated in the findings reported here. Microbial products can be recognised by several kinds of macrophage receptors, including the "mannose receptor" (MR) [13, 23]. Rhamnose is 6-deoxy-L-mannose and the EPS of *L. rhamnosus* are rich in rhamnose. We thus consider that a MR family receptor might be involved in the immunostimulatory action of EPS, including presentation to T lymphocyte [37] with possible subsequent IFN-γ production. In addition, rhamnose-containing polysaccharides, such as rhamnose glucose polymers, are ligands for both CD14 and CD11b cell receptors. "CR3" (CD11b/CD18), a receptor expressed on phagocytes, NK cells and some cytotoxic T cells, could play a role since it has been reported to recognize also mannose-containing zymosan in soluble form [46] which corresponds to the presentation form of the EPS studied in this report. However, in the case of rhamnose/glucose polymer mediated TNF production, it seems that CD14 only, and not CD11b,

plays a functional role [42]. A recent report also suggests some involvement of "CR4" (CD11c/CD18) in the cytokine response [8]. Galectins, a large family of animal lectins [2] are also able to bind to oligomannosides [15]. In the case of CD14 ligands of glycolipidic nature, it seems that the "Toll Like Receptor" (TLR) family [39] could possibly deserve consideration in trying to explain some of our observations: it has been recently shown that different TLR family members possess different cell signalling capabilities, in the presence of ligands of different natures [29]. However, recent findings suggest that TLR signalling is very complex and that some responses are in fact due to lipoprotein contaminants present in commercial preparations of the glycolipids, rather than to the glycolipid itself [18]. In any case, the exact nature of the receptor(s) incriminated in the presently reported activity, as well as their density and cellular distribution, is totally unknown.

But the ultimate question, as to the human response to EPS, is of course the response of the gut associated lymphoid system, in vivo, for which the present work gives no answer. From the present results, we can however speculate that yogurt-associated EPS are potential actors in the health improvement observed among yogurt-consuming people. From a more practical view point, these preliminary results could be useful in the choice of LAB to use as a supplement to a "probiotic" yogurt. We have compared some immunomodulating properties of intact, irradiation-inactivated, bacterial preparations from the EPS-producing *L. rhamnosus* RW-9595M strain and of its closely related, non-producing counterpart *L. rhamnosus* ATCC-9595 strain. Bacteria were used at several concentrations. It appears that both strains are equally able to elicit IFN-γ, in experimental conditions similar to those described in this article. However, in the case of the ability to secrete the pro-inflammatory cytokine TNF, the EPS-producing RW-9595M strain exhibits a comparatively much less ability than the non-EPS-producing strain (Chabot, unpublished). Since TNF is often associated with gut inflammation [35], which is also associated with an excessive IgE production driven by Th2-type lymphokines**,** it seems therefore to us more appropriate to select the EPS-producing strain RW-9595M*,* rather than the original ATCC-9595, as an additive to yogurt, for the consumer's well-being.

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