L-Rhamnose inhibits proliferation of murine splenocytes by the lipopolysaccharide and polysaccharide moiety of *Shigella dysenteriae* type 1 lipopolysaccharide

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SUMMARY

The induction of proliferation of murine splenocytes by lipopolysaccharide (LPS) of *Shigella dysenteriae* type 1 and its polysaccharide (PS) and lipid A fractions was investigated. The LPS-induced proliferation reached a maximum at a concentration of 30 ng/ml. The PS and lipid A induced proliferation of murine splenocytes at similar concentrations. Preincubation of murine splenocytes with varying concentrations of L-rhamnose blocked LPS- and PS-induced proliferation in a dose-dependent manner. The lipid A-induced stimulation, on the contrary, was not affected by preincubation of the cells with L-rhamnose. These data suggest that activation of splenocytes by LPS and PS is mechanistically different from that induced by lipid A and is presumably involved in the specific recognition of carbohydrate structures on LPS and PS.

INTRODUCTION

Lipopolysaccharide (LPS), a class of glycolipids unique to the outer membrane of Gram-negative bacteria, modulates the activity of a wide range of cells of the immune system.[1] The LPS-induced proliferation of B lymphocytes[2] and differentiation into antibody-secreting plasma cells[1,3] has been well documented during the last decade. Since the stimulatory effect of LPS on splenocytes could be reproduced by lipid A, it was concluded that the immunomodulatory activity of LPS is associated with the lipid A part of the molecule.[4-7] The structural features that affect the biological activity of LPS have been defined.[8] Moreover, LPS-binding proteins with molecular masses of 73 000 MW[9,10] and 40 000 MW[11] have been identified in murine lymphocyte membranes. Binding of LPS to these putative membrane receptors is believed to be mediated by specific recognition of the lipid A part of LPS molecule.[9-11] Despite overwhelming evidence supporting a pivotal role for lipid A in determining the biological activity of LPS, the stimulation of splenocytes by the polysaccharide (PS) part of LPS[12,13] has suggested that lipid A might not be the sole structural entity of LPS recognized by splenocytes.

In this paper, we have studied the proliferation of murine splenocytes by LPS of *Shigella dysenteriae* type 1, as well as the PS and lipid A fractions prepared chemically from the LPS. The inhibitory effect of L-rhamnose, a constituent of the → 3)-α-L-

Rhap(1 → 3)α-L-Rhap(1 → 2)α-D-Galp(1 → 3)α-D-GlcpNAc(1 → repeating unit of the O-antigen of *S. dysenteriae* type 1 LPS,[14] on LPS-, PS- and lipid A-induced proliferation of murine splenocytes was also studied in order to detect any possible difference in specificity of recognition of splenocytes by these moieties.

MATERIALS AND METHODS

Materials
Enzymes were from Sigma Chemical Co. (St Louis, MO). Group- and type-specific antisera of *Shigella* spp. were from Difco Laboratories (East Molesey, UK), and antibiotics, fetal bovine serum and culture media were from Gibco Laboratories (Grand Island, NY).

Bacteria
Shigella dysenteriae type 1 strain N120 used in this study was a local clinical isolate. The strain was identified by biochemical tests and agglutination by group- and type-specific antisera.

Isolation of LPS
LPS was isolated from acetone-dried cells of *S. dysenteriae* type 1, grown overnight in trypticase soy broth at 37°, with shaking by the phenol–water extraction method.[15] The crude LPS was dispersed in phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7·4) and digested sequentially with DNase (50 μg/ml), RNase (50 μg/ml) and proteinase K (100 μg/ml) to remove contaminating DNA, RNA and protein, respectively. The LPS was exhaustively dialysed against water, recovered by sedimentation at 100 000 g, suspended in water and lyophilized.

Analytical methods

Protein was estimated by a modified Lowry method, using bovine serum albumin as standard.[16] Neutral sugar was quantified by the phenol–sulphuric acid assay method, using D-galactose as a standard.[17] Phosphate was estimated by the ammonium molybdate-ascorbic acid method, using NaH_2PO_4 as standard.[18] Ester content was determined by the hydroxamic acid method.[19]

Preparation of lipid A and PS

Lipid A was prepared by mild acid hydrolysis of LPS, as described by Galanos et al.[20] Briefly, 100 mg of lyophilized LPS was incubated with 1% acetic acid at 100° for 2 hr and the precipitated lipid A was recovered by centrifugation at 3000 g for 15 min. The lipid A was subsequently washed three times with water to remove undegraded LPS and PS, dried under reduced pressure and dissolved by gently warming in 10 mM KOH, a treatment which did not degrade lipid A chemically.

Following removal of lipid A, the pH of the supernatant was immediately raised to 7·0 by addition of NH_3, and PS recovered by lyophilization. Crude PS (42 mg) was dissolved in 3·5 ml of 0·1 M ammonium acetate buffer, pH 7·2, and subjected to gel filtration on a column (38 × 1·6 cm) of Sephadex G-50. Eluents were monitored for neutral sugar by the phenol–sulphuric acid method.[17] Fractions eluting in the void volume represented undegraded PS, and were pooled and lyophilized. Contaminating LPS and lipid A were finally removed from the PS fraction by anion-exchange chromatography on a column (20 × 1·6 cm) of DEAE–Sephacel (Pharmacia, Uppsala, Sweden) pre-equilibrated with 0·05 M ammonium acetate buffer, pH 7·4.[21] The PS fraction eluted unretarded during elution of the column with the equilibrating buffer and was finally recovered by lyophilization.

Animal

BALB/c mice, originally obtained from Jackson Laboratories (Bar Harbor, ME), were prepared in the animal care facility of the National Institute of Cholera and Enteric Diseases, Calcutta, India. Six-week-old female mice were killed for isolation of splenocytes.

Culture and proliferation assay of murine splenocytes

Spleen was removed aseptically from mice and transferred to RPMI-1640 culture media supplemented with penicillin and streptomycin (100 U/L) and 10% fetal bovine serum. Splenocytes were isolated by gently teasing the spleen with a syringe and passed through a nylon mesh to obtain a single-cell suspension, which was then depleted of erythrocytes.[22] The number of viable cells was determined by the trypan blue exclusion method.[23] 2×10^5 cells/well/100 μl of culture media was then seeded in 96-well round-bottomed tissue culture plates (Nunc, Rosklide, Denmark) at 37° in a 5% CO_2 humidified atmosphere. Varying concentrations of LPS, lipid A or PS of S. dysenteriae type 1 were added per well and the total volume of culture media adjusted to 200 μl per well. Lipid A, used in a ng range, remained soluble in the medium. 2×10^5 splenocytes/well/200 μl media was then incubated for 54 hr, after which each well was pulsed with 1 μCi [³H]thymidine (specific activity 1800 mCi/mM) for the next 18 hr. After 72 hr of splenocyte culture, cells were harvested with a PHD harvester (Cambridge,

Table 1. Analysis of neutral sugar, phosphorus and ester linkage in LPS, PS and lipid A of S. dysenteriae type 1

LPS fraction	Neutral sugar*	Phosphorus*	Ester linkage[†]
LPS	46	1	0·6
PS	73	ND	0
Lipid A	6	1·4	1·02

* Estimated as percentage (w/w).
[†] Colour yield in hydroxamic acid assay[19] for ester linkage by a 10 mg/ml solution at 520 nm.
ND, not detectable.

MA) onto glass fibre filters, washed and dried. [³H]thymidine uptake by the cells was determined by counting in a liquid scintillation counter.

Inhibition of murine splenocyte proliferation by L-rhamnose

2×10^5 splenocytes/well/200 μl of culture media containing varying concentration of L-rhamnose was preincubated for 2 hr at 37° in a 5% CO_2 humidified atmosphere. After 2 hr, 96-well culture plates were centrifuged at 1000 r.p.m. for 10 min. Culture media containing L-rhamnose was removed and 200 μl of fresh media containing 100 ng/ml of LPS, lipid A or PS was added to 2×10^5 cells/well. Cells were then incubated, harvested and [³H]thymidine uptake determined as described above.

RESULTS

Purity of LPS, PS and lipid A

The LPS preparation was found to be free of detectable amounts of protein. Table 1 shows the relative amounts of phosphorus, hexose sugars and ester linkages in LPS, PS and lipid A preparations. The absence of detectable amounts of phosphorus and ester linkages in PS preparations indicated that they were essentially free of lipid A and undegraded LPS. The presence of 6% by weight of neutral sugars in lipid A, which is ideally a disaccharide of N-acetyl D-glucosamine acylated to hydroxy fatty acids,[14] suggested that the lipid A preparation contained some hexose sugars, presumably belonging to the core oligosaccharide region.

Inhibitory effect of L-rhamnose on LPS-induced proliferation of murine splenocytes

The LPS from S. dysenteriae type 1 stimulated proliferation of murine splenocytes in the concentration range of 2–125 ng/ml (Fig. 1a). Maximal stimulation was observed at and above 30 ng/ml of LPS. When murine splenocytes were preincubated with varying concentrations of L-rhamnose and subsequently treated with LPS at a concentration of 100 ng/ml, there was a sharp decrease in the proliferation of the cells (Fig. 1b), suggesting L-rhamnose-dependent recognition of LPS by murine splenocytes. Preincubation of splenocytes with several other sugars, including D-mannose, D-galactose, L-fucose, N-acetyl D-glucosamine and N-acetyl D-galactosamine, did not affect the cellular response to LPS (data not shown).
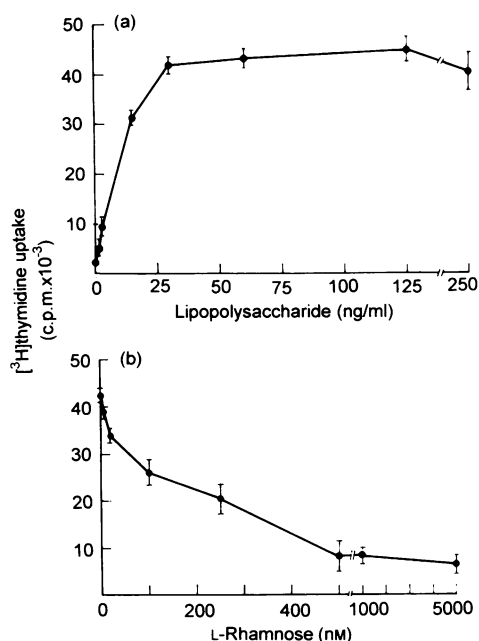
Figure 1. (a) Varying concentrations of LPS of *S. dysenteriae* type 1 were used for inducing [³H]thymidine uptake by murine splenocytes. (b) Effect of preincubation of murine splenocytes with varying concentrations of L-rhamnose on LPS (100 ng/ml) -induced [³H]thymidine uptake.

Inhibitory effect of L-rhamnose on PS-induced proliferation of murine splenocytes

Similar to the LPS, the PS fraction stimulated proliferation of murine splenocytes at a concentration above 2 ng/ml, and reached a maximum at about 30 ng/ml (Fig. 2a). The
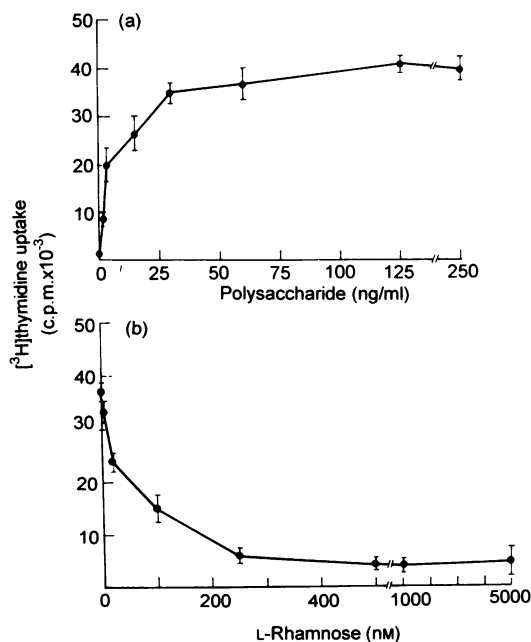

Figure 2. (a) Varying concentrations of PS of *S. dysenteriae* type 1 were used for inducing [³H]thymidine uptake by murine splenocytes. (b) Effect of preincubation of murine splenocytes with varying concentrations of L-rhamnose on PS (100 ng/ml) -induced [³H]thymidine uptake.
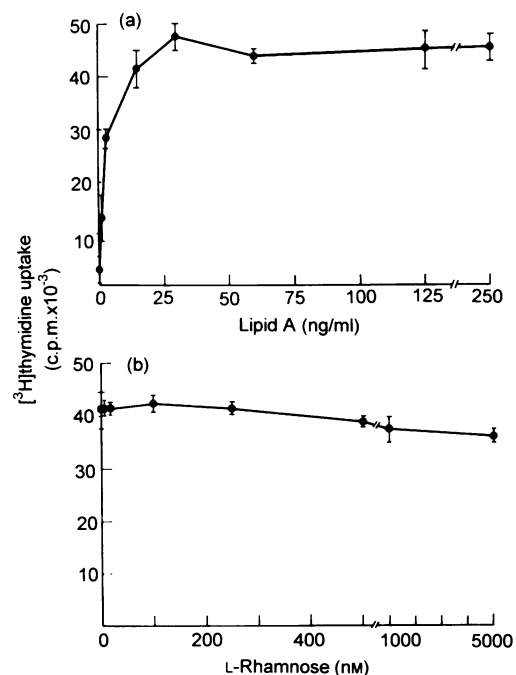

Figure 3. (a) Varying concentrations of lipid A of *S. dysenteriae* type 1 were used for inducing [³H]thymidine uptake by murine splenocytes. (b) Effect of preincubation of murine splenocytes with varying concentrations of L-rhamnose on lipid A (100 ng/ml) -induced [³H]thymidine uptake.

proliferation of splenocytes declined sharply with preincubation of the cells with L-rhamnose (Fig. 2b).

Inhibitory effect of L-rhamnose on lipid A-induced proliferation of murine splenocytes

Similar to LPS and PS, the lipid A fraction of *S. dysenteriae* type 1 LPS stimulated the proliferation of murine splenocytes (Fig. 3a). In contrast to LPS- and PS-induced proliferation, however, the lipid A-induced proliferation of cells was unaffected by preincubation with L-rhamnose (Fig. 3b), suggesting L-rhamnose-independent recognition of lipid A by the splenocytes.

DISCUSSION

We studied the proliferation of murine splenocytes by LPS of *S. dysenteriae* type 1, as well as by the PS and lipid A fractions derived from the LPS. As reported for LPS of other bacteria,[2] we found that LPS of *S. dysenteriae* type 1 profoundly stimulated the murine splenocytes. Besides LPS, lipid A and PS also stimulated the splenocytes. The stimulation of splenocytes by LPS and PS could be blocked by L-rhamnose. *Shigella flexneri* 2a, *S. boydii* 11 and *S. sonnei* LPS and PS had similar effects on splenocyte proliferation, which could be inhibited by L-rhamnose (unpublished observations). In contrast, the lipid A stimulation of splenocytes could not be blocked by L-rhamnose, suggesting that the LPS and PS stimulation of splenocytes was mechanistically different from that of stimulation by lipid A.

In aqueous dispersion, LPS exists as a multimolecular aggregate or micelle due to its amphiphilic nature. In such

micelle, the hydrophilic PS domain of LPS is exposed in the aqueous environment and orientated favourably to interact with the animal cell surface. The hydrophobic lipid A is embedded in the core and restricted from interacting with the cell surface. Moreover, the strong cohesive interaction between lipid A fatty acid chains in LPS micelle, as measured by electron spin resonance studies of spin-labelled phospholipid–LPS mixtures,[24] suggests that the dissociation of the micelle into monomers with exposure of the lipid A region in aqueous milieu is extremely unlikely. Thus, LPS action might first involve specific recognition of carbohydrate structures on the PS moiety by lymphocyte surface receptors.

The lipid A-induced stimulation of murine splenocytes suggests a process involving recognition of putative lipid A receptors[3] or intercalation of lipid A by diffusion into the protein–phospholipid bilayer of the lymphocyte membrane, which can not be blocked by L-rhamnose. The mechanistic differences in recognition of LPS, PS or lipid A could possibly arise from the way these molecules arrange themselves in aqueous dispersion and interact with splenocytes.

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