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Effects of LPS, lipid A and polysaccharide from adapted strains of *Escherichia coli* on human leucocyte activity

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Polysaccharide and lipid A are responsible for the wide-ranging pharmacological activity of bacterial lipopolysaccharides (LPS). The alterations in LPS structure result in various effects on different functions of the target cells. The effects of LPS substructures, the polysaccharide (P) and lipid A (L) from *E. coli* on the innate mechanisms of human leucocytes were examined and compared in this study. Incubation of leucocytes with LPS and L and P analogues (1 and 100 μg/ml) enhanced their biological activity in dependence on their structure. These results showed that LPS was a less active immunomodulator of leucocytes than L and P analogues isolated from *E. coli* strains adapted to antimicrobial agents.

1. Introduction

The lipopolysaccharide (LPS) of wild type strains consists of a lipid A region (L) and a polysaccharide region (P). The activation of leucocytes by LPS is known to involve a mechanism where it binds to a leucocyte specific CD14 membrane receptor (Alexander and Rietschel 2001; Akashi et al. 2003). Other reports have dealt with the process of internalization in the membrane (Lenschat et al. 1999; Kutuzova et al. 2001).

High amounts of LPS induce production of mediators leading to manifestation of sepsis. At low concentrations LPS leads to release of mediators with immunostimulatory effects (Lenschat et al. 1999; Alexander and Rietschel 2001).

The L part of LPS plays the essential role in leucocyte activation (Lenschat et al. 1999; Muroi and Tanamoto 2002). Despite various studies of P, the function and structures responsible for its observed biological activity have not been unequivocally identified, even when stimulated in combination with L (Rietschel et al. 1994; Lenschat et al. 1999; Alexander and Rietschel 2001). However, Lebbar et al. (1986) asserted that P at high concentrations is as potent as LPS in terms of biological activity. On the other hand, natural L from bacteria as well as several modifications of its natural structure gives rise to a broad spectrum of biological effects in various eucaryotic organisms both *in vivo* and *in vitro* (Schromm et al. 1998, 2000; Matsuura et al. 1999; Dubničková et al. 2003; Kuželová et al. 2006).

Our experiments were performed with analogues obtained from the native strain of *E. coli* and strains adapted to 1-(methyldodecyl)trimethylammonium bromide (ATDBr) and 1-(methyldodecyl)dimethylamine oxide (ATDNO) to test their effect on human leucocyte activities. ATDBr and the structurally similar ATDNO were synthetized as membrane active antimicrobial agents in an attempt to find useful disinfectants (Kopecká-Leitmanová et al. 1989). At subinhibitory concentrations these compounds are able to modulate various biological activities of bacteria, as well as affecting the metabolic activities of human leucocytes (Čupková et al. 1988; Ferenčík et al. 1990; Hoštacká et al. 1994, 1995; Majtánová et al. 1996). *E. coli* strains adapted to ATDNO or ATDBr differed significantly from the native strain in terms of the fatty acid profiles of L, which caused a decrease in outer membrane permeability due to a reduction of its fluidity (Bukovský et al. 1991; Dubničková et al. 2006). In view of these data, we examined the ability of adaptation changes in the LPS' and in L and P, to modify their immunomodulatory activity.

2. Investigations and results

The leucocytes were incubated with LPS (lipopolysaccharide) and its components L (lipid A) or P (polysaccharide) isolated from native *E. coli* (1) and two resistant strains adapted to the amine oxide (ATDNO) (2) and to the quaternary ammonium salt (ATDBr) (3). Tables 1–3 show the activity of samples for each biological effect in the presence of 1 μg/ml LPS or L or of 100 μg/ml P for certain functions of human leucocytes.

Table 1 shows the results of experiments in which LPS, L and P were compared in their ability to affect the phagocytic activity of leucocytes. Contrary to LPS, its components L and P significantly increase this activity. When relative activities of the phagocytic index were compared, L (relative activity about 119%) displayed a significant decrease of leucocyte engulfment while P was almost twice as great (about 200%), the relative activity of the control sample being taken as 100%.

The results for microbiocidal activity are presented as the decrease/increase of the number of colony forming units (CFU) after 60 min exposure of the bacteria to pretreated

Table 1: Phagocytic index (PI) of human leucocytes treated by LPS or L (c = 1 μg/ml) or by P (c = 100 μg/ml)

Sample	Phagocytic index
Control no.1	3.6 ± 0.6
LPS1	4.0 ± 0.8 NS
LPS2	4.5 ± 1.0 NS
LPS3	4.1 ± 0.8 NS
Control no.2	5.4 ± 0.5
L1	7.0 ± 0.4**
L2	6.3 ± 0.8*
L3	6.4 ± 0.4**
Control no.3	4.0 ± 0.6
P1	6.8 ± 1.5**
P2	7.5 ± 1.5**
P3	9.4 ± 1.7***

The LPS or L or P marked with:
1 – isolated from native strain of *E. coli*
2 – isolated from strain of E. coli adapted to ATDNO
3 – isolated from strain of E. coli adapted to ATDBr
Statistical significance:
* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

leucocytes in comparison with the control sample (100% of living cells). Leucocytes exposed to LPS and L inhibited the growth of *Staphylococcus aureus* particulary when LPS3 (43% decrease of living cells), L3 (25% decrease of living cells) and P1-3 (about 20% decrease of living cells) were used (Table 2). Bactericidal activity against *E. coli* (Table 2) was stimulated after treatment of leucocytes with LPS1 (47% decrease of living cells), LPS3 (29%), L2 (96%), P2 and P3 (about 70%). Thus, these six compounds enhanced this activity, but after treatment with L3 (118% increase in number of living cells) and also P1 (69%) the activity was reduced (Table 2). Interestingly, only L1 from the *E. coli* native strain significantly modulated (27 % increase of living cells) the candidacidal activity of human cells (Table 2).

The production of lysozomal enzymes is a common response of leucocytes to infection. The lysozyme activity was clearly increased only in the presence of analogues P2 (by 125%) and L3 (by 53%), while the other analogues reduced this activity (by about 60%) in comparison with controls (Table 3). Peroxidase activity was significantly stimulated by L3 (51%) from the *E. coli* strain adapted to ATDBr and this effect was more marked compared with L1 (increase by 5%) and P1 (15%) or P2 (11%) (Table 3). Interesting results were obtained by comparing the lysozyme reduction with peroxidase stimulation. Pretreatment of human leucocytes *in vitro* with P2 and L3 stimulated the cells to release lysozyme (Table 3), while LPS and other L samples significantly suppressed this activity. Moreover, peroxidase activity was stimulated after the cells were exposed to LPS and to its substructures (Table 3).

3. Discussion

In order to show the relationship of structure and immunomodulation, LPS and substructures (P, L) analogues were compared with leucocyte activities obtained *in vitro*. The activity of the polysaccharide (P) moiety from analogues of LPS were examined at a 100 μg/ml dose, at which plant and yeast polysaccharides have an immunoactive effect on peripheral human leucocytes (Popov et al. 1999). The comparison of LPS and L (1 μg/ml) activities with P at the different doses used was possible, because Lenschat et al. (1999) found no dose effect of LPS and L during interaction with leucocytes.

Maximum phagocytic activity was induced by P3 (from the strain adapted to ATDBr). In comparison, Masihi et al. (1986) showed that phagocytosis of peritoneal macrophages was enhanced as much as 7 to 10 fold by L. The plant polysaccharide studied by Popov et al. (1999) enhanced the peripheral cell phagocytic index (1.5 times). These and our present results indicate that both L and P markedly enhance the phagocytic index.

The microbiocidal activity of leucocytes is effected by the production of cationic peptide molecules of different sizes and structures, which have tendencies to select one or more targets in microbial cell membranes during their interaction (Hancock et al. 2002; Peschel 2002). This selection causes differences in leucocyte activity against individual microorganisms. As was demonstrated, at least one of the bactericidal peptides produced by leucocytes is able to bind to the LPS of Gram-negative bacteria by electrostatic interactions. Modification of the LPS structure (in Gram-negative bacteria) or of teichoic acid (in Gram-positive bacteria) often make the bacteria resistant to the bactericidal activity of leucocytes (Gunn 2001; Peschel 2002). Accordingly, these mechanisms can explain the differences in bactericidal activity on *E. coli* or *S. aureus* in the case of leucocyte exposure to the analogues.

As was shown in Table 2, only L1 inhibited the microbicidal activity of human cells against *C. albicans*. Similar re-

Table 2: Microbicidal activities of human leucocytes against *Staphylococcus aureus* (S.a.), *Escherichia coli* (E.c.) and *Candida albicans* (C.a.) of human leucocytes treated by LPS or L (c = 1 μg/ml) or P (c = 100 μg/ml)

Sample and conc.	Number of surviving cells		
	S.a. × 10^5/mL	E.c. × 10^5/mL	C.a. × 10^3/mL
Control no. 1	967 ± 194	626 ± 144	395 ± 85
LPS1	761 ± 120 NS	331 ± 59***	333 ± 89 NS
LPS2	930 ± 32 NS	691 ± 166 NS	486 ± 149 NS
LPS3	554 ± 63**	444 ± 85*	486 ± 102 NS
Control no. 2	935 ± 111	689 ± 193	561 ± 80
L1	696 ± 277 NS	573 ± 77 NS	710 ± 101*
L2	696 ± 253 NS	28 ± 26***	632 ± 58 NS
L3	702 ± 179*	1500 ± 160***	570 ± 120 NS
Control no. 3	947 ± 48	61 ± 9	388 ± 77
P1	677 ± 103***	103 ± 22***	428 ± 82 NS
P2	800 ± 105*	13 ± 8***	456 ± 74 NS
P3	756 ± 156*	17 ± 13***	453 ± 97 NS

Abbreviations see Table 1

Table 3: Metabolic activities of human leucocytes treated by LPS or L (c = 1 μg/ml) or P (c = 100 μg/ml)

Sample and conc.	Lysozyme activity Difference in absorbation (410 nm) between 0–20 min	Peroxidase activity Difference in absorbation (490 nm) between 0–10min
Control no. 1	0.012 ± 0.004	0.478 ± 0.017
LPS1	0.006 ± 0.003*	0.481 ± 0.019 NS
LPS2	0.010 ± 0.006 NS	0.485 ± 0.012 NS
LPS3	0.006 ± 0.003*	0.555 ± 0.008***
Control no. 2	0.055 ± 0.003	0.323 ± 0.006
L1	0.037 ± 0.002***	0.339 ± 0.004***
L2	0.038 ± 0.006***	0.319 ± 0.002 NS
L3	0.084 ± 0.001***	0.488 ± 0.001***
Control no. 3	0.004 ± 0.003	0.217 ± 0.015
P1	0.003 ± 0.002 NS	0.249 ± 0.018*
P2	0.009 ± 0.002*	0.241 ± 0.007**
P3	0.007 ± 0.002 NS	0.235 ± 0.013 NS

Abbreviations see Table 1

sults were found after leucocyte stimulation with whole outer membrane extracts obtained from an *E. coli* strain adapted to ATDNO (Bukovský et al. 1998).

Experiments with LPS from cells adapted to ATDNO and its substructures supported the conclusion that the biological activity of L2 was somewhat lower than that of P2. These results are not in agreement with reports showing that L is the essential structural compound of LPS (Henricks et al. 1983; Muroi and Tanamoto 2002). It suggests that the P part of LPS might have a wider biological activity than has been postulated before.

It has also been confirmed that adaptation of the native *E. coli* strain to antimicrobial compounds modifies the fatty acid profile of lipid A analogues (Dubničková et al. 2006). It therefore appears that alterations in chemical composition could modify the interaction of LPS and their substructures with leucocytes, as has been found recently with L analogues (Dubničková et al. 2003).

Further insight into the structural influence of L types was gained from the findings that LPS3 and L3 were more potent than their equivalents from the native strain of *E. coli* and from strains adapted to ATDNO. On the other hand, LPS2 (from these strains) did not modulate any biological activity of leucocytes, although the P2 analogue was the most potent modulator in our study.

In conclusion, we consider that both L and P modulated about 67% of all biological activities in this study. P was the most effective stimulator (stimulating 11 biological activities of all those tested, while L stimulated 8 of them). But on the other hand, L is a more potent inhibitor of the biological activities in this study (inhibiting 4 biological activities). In summary, this study confirmed that substructures of LPS provided about 30% greater biological efficiency than the whole molecule (Tables 1–3). Similar results show that P from LPS was as potent as the whole endotoxin in IL-1 induction, but at a million-fold higher concentrations (Lebbar et al. 1986). Participation of P the biological effects of the whole LPS can be explained by its binding to the cell membrane of leucocytes. Popov et al. (1999) proposed that CD 14 receptor participates in leucocyte activation by plant polysaccharides. For all that, and on the basis of our results, free P (as well as the L substructure) has the capacity to interact with leucocytes through one of the known mechanisms. This suggests that L and P should also be candidates for further immunological investigation and structural optimization.

4. Experimental

4.1. Isolation of LPS, lipid A (L) and polysaccharide (P)

The strain of *Escherichia coli* ATCC 11229 was sensitive to 1-(methyldodecyl)dimethylamine oxide (ATDNO) (at a concentration of 0.82 mmol/L) and to 1-(methyldodecyl)trimethylammonium bromide (ATDBr) (0.62 mmol/L). The LPS was obtained from bacteria by the phenol/water extraction method according to Westphal and Jann (1965). It was next purified by ultracentrifugation at 100 000 × g for 2 h and treated with RNAse and DNAse, and finally with proteinase K (Sigma, USA), (Rodriguez-Carvajal et al. 2001). The L and P were hydrolyzed from LPS (Keleti and Lederer 1974) from the native strain (1) of *E. coli* (analogues LPS1, P1, L1), from the strain (2) adapted to ATDNO (analogues LPS2, P2, L2) and from the strain (3) adapted to ATDBr (analogues LPS3, P3, L3).

4.2. Isolation of human leucocytes

Human leucocytes from 6–8 healthy volunteers (OHT-Derer's Hospital, Bratislava, Slovakia) were isolated and purified by HistoPaque-1077 (Sigma, USA) according to Boyum et al. (1968). In the next step, they were cultivated for 18 h exposed to concentrations of lipid A according to Bukovský et al. (1998). Control samples contained human leucocytes that were not affected by modulators.

4.3. Phagocytic index

The phagocytic index was determined microscopically. A suspension of 100 μl of human leucocytes (2×10^6 cells/ml) was incubated for 1 h at 37 °C with 50 μl of heat inactivated *Streptococcus faecalis* cells (5×10^8/ml) (Bukovský et al. 1998). Wright's staining of the suspension was performed according to the conventional methodology. The number of engulfed bacteria were counted for 100 leucocytes per coverslip.

4.4. Microbicidal activity

The microbicidal activity of disintegrated human leucocytes was determined with strains of *Staphylococcus aureus* MAU 2958, *Escherichia coli* ATCC 11229 or *Candida albicans* SC 1539. One-day cultures of *S. aureus* grown on blood agar and *E. coli* grown on Endo agar and a 2-d culture of *C. albicans* cultured on Sabouraud's agar were adjusted to $OD_{540} = 0.35$ (all media were from Imuna, Slovakia). Bacterial suspensions were resuspended at a concentration of $1 : 10^5$ cells/ml. The suspension of *C. albicans* was resuspended at a concentration of $1 : 10^4$ cells/ml. Aliquots of 150 μl of *E. coli*, 100 μl of *C. albicans* or 75 μl of *S. aureus* were added to 100 μl of ultrasonically disintegrated leucocytes (18 kHz, 10 s). The mixtures were incubated for 1 h at 37 °C. Aliquots of the mixtures were cultivated on solid media (bacteria – 1 day at 37 °C on blood agar or Endo agar, yeasts 2 days at 25 °C on Sabouraud's agar). The numbers of microbes killed were calculated from the decrease in colony forming units (CFU) (Bukovský et al. 1998).

4.5. Metabolic activity

The ultrasonically disintegrated leucocytes (18 kHz, 10 s) were centrifuged (3900 RPM, 10 min, 4 °C). The supernatant (150 μl) was mixed with 50 μl of a suspension of *Micrococcus luteus* ATCC 4698 ($OD_{410} = 0.8$) in phosphate buffer (pH = 6.2, 0.07 mol/l KH_2PO_4 and 0.07 mol/l $Na_2HPO_4 \times 12\,H_2O$) or 50 μl of peroxidase substrate (benzene-1,2-diamine 5 mg/10 ml, freshly diluted H_2O_2 100 μl/10 ml and 0.1 M tribasic sodium citrate dihydrate in distilled H_2O, pH 5.0). The lysozyme activity was measured spectrophotometrically ($\lambda = 410$ nm) in 96 wells/plate over a 20-minute period. The peroxidase reaction was stopped after 20 min with H_2SO_4 (50 μl, 4 mol/l) and changes in A_{490} were measured (MR 5000 spectrophotometer, Dynatech).

4.6. Statistical analysis

The results are presented as means of experimental values ± standard deviations and as relative activities (%). Statistical comparisons were made based on repeated measurements (4–6 parallels) by Student's t test; a value $p < 0.05$ was regarded as statistically significant.

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