Folia Microbiol. **48** (4), 543–547 (2003) **http://www.biomed.cas.cz/mbu/folia/**

Activation of Human Leukocytes by Lipid A from *E. coli* Strains Adapted to Quaternary Ammonium Salt and Amine Oxide

M. DUBNIČKOVÁ, M. BUKOVSKÝ, D. MLYNARČÍK

Faculty of Pharmacy, Comenius University, 832 32 Bratislava, Slovakia

Received 4 July 2002
Revised version 20 January 2003

ABSTRACT. The immunomodulatory activities of monophosphoryl lipid A (MLA) and diphosphoryl lipid A analogues obtained from the sensitive strain of *E. coli* and from the resistant strains adapted to a quaternary ammonium salt and an amine oxide were compared. All analogues considerably stimulated the activity of human leukocytes although the analogue from the sensitive strain at a higher concentration significantly suppressed phagocytosis. The MLA analogue exhibited a suppressive effect on the microbicidal activity of human leukocytes against *E. coli* and the peroxidase activity. Adaptation of bacteria to amphiphilic antimicrobial compounds, which is accompanied by chemical changes in their lipid A, only slightly reduced their immunomodulatory activity when compared with the analogue from the sensitive strain. On the other hand, the diphosphoryl analogues were less active than MLA.

Bacterial cell-surface components reveal a large scale of biological activities on the host organism and some of them are modulators of the immunity response. Adaptation of bacteria to various environmental factors (*e.g.*, temperature, antimicrobial compounds) often leads to chemical changes in their cell-surface structures (Van Alphen *et al.* 1979; Pospíšil and Řezanka 1994; Dubois-Brissonnet *et al.* 2001) and modification of their biological activities (Salkowski *et al*. 1998; Matsuura *et al*. 1999).

Quaternary ammonium salts (QAS) and structurally similar amine oxides (AO) are membrane active antimicrobial agents. In subinhibitory concentrations they are able to modulate various biological activity of bacteria (Čupková *et al*. 1988; Hoštacká *et al*. 1994, 1995; Majtánová *et al*. 1996). Moreover, QAS and AO themselves affect the metabolic activities of human leukocytes (Ferenčík *et al*. 1990). A strain of *E. coli* adapted to antibacterial AO significantly differed from the natural sensitive strain in fatty acid and hydroxy fatty acid profiles of lipid A, which caused a decrease in the outer membrane permeability due to reduction of its fluidity (Bukovský *et al.* 1991).

Several factors, such as changes in the disaccharide backbone, structure orientation and the number of phosphate groups, number, order, length and degree of saturation of acyl chains of fatty acids have been reported to influence the biological activity of lipid A variants (Frecer *et al*. 2000). The natural lipid A of *E. coli* is commonly described as a 1,4′-bisphosphorylated 1,6-β linked D-glucosamine disaccharide with acylated amide- and/or ester-linked (*R*)-3-hydroxymyristic acids in 2,3-tetradecanoic acid in positions 2′,2″ and dodecanoic acid in 3′,3″ positions of the glucosamine group (Lüderitz *et al*. 1978). In contrast, structurally related monophosphoryl lipid A (MLA) differs from the natural lipid A by the absence of the phosphate group on the 1 position of glucosamine and β-hydroxyacyl side chain in 3′ position (Hagen *et al.* 1997). Concerning the structure and conformation differences between the natural and MLA analogues of lipid A, several studies confirmed their different effects on the immune system and other biological activitiess (Ikeda *et al*. 1990; Elliot 1998; Matsuura *et al*. 1999; Kuželová *et al*. 1999).

It was of interest to investigate the ability of adaptation changes in this cell-surface component to modify its immunomodulatory activity. Therefore, the lipid A analogues obtained from the sensitive strain of *E. coli* and strains adapted to QAS and AO were tested for their activities on the human leukocytes, in comparison with MLA.

MATERIAL AND METHODS

The strain of *Escherichia coli* ATCC 11229 was adapted to 1-(methyldodecyl)trimethylammonium bromide (ATDBr) (620 mmol/L) and to 1-(methyldodecyl)dimethylamine oxide (ATDNO) (820 mmol/L). Lipid A was isolated according to Westphal and Jann (1965) from the sensitive strain of *E. coli* (analogue C), from the strain adapted to ATDNO (analogue LNO) and from the strain adapted to ATDBr (analogue LBr). MLA from the deep rough (Rd) mutant of *E. coli* F 538 was obtained from the *Sigma*.

Human leukocytes from 6–8 healthy volunteers per one control (no. *1–3* in Tables I–V) (*Hematology and Transfusion Unit, Derer's Clinic and Hospital*, Bratislava) were isolated and purified by HistoPaque-1077 (*Sigma*) according to Boyum (1968). Next, they were cultivated with a competent concentration of lipid A according to Bukovský *et al*. (1998).

Phagocytotic activity and phagocytotic index. A suspension of 100 μL of human leukocytes (2/nL, *i.e*. 2×10^6 cells per mL) was incubated for 1 h at 37 °C with 50 μL of heat-killed *Streptococcus faecalis* (500/nL, *i.e*. 5×10^8 cells per mL). Wright's staining of the suspension was performed by the conventional methodology. Phagocytotic activity was calculated as percentage of the phagocytosing cells per 100 phagocytes; the phagocytotic index was calculated as the average number of *S. faecalis* cells ingested by one phagocyte.

The *microbicidal activity* of disintegrated human leukocytes was determined on strains of *Staphylococcus aureus* MAU 2958, *Escherichia coli* ATCC 11229 or *Candida albicans* SC 1539. A 1-d culture of *S. aureus* on blood agar, *E. coli* on Endo agar or a 2-d culture of *C. albicans* on Sabouraud's agar were adjusted to absorbance $A_{540} = 0.35$ (all the media were from *Imuna*, Slovakia). One-hundred-μL aliquots of *E. coli* (or *C. albicans)* and 75 μL of *S. aureus* were added to 100 μL of ultrasonically disintegrated leukocytes (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 d at 37 °C on blood agar or Endo agar, yeasts 2 d at 25 °C on Sabouraud's agar). The surviving microbes were calculated as colony-forming units (CFU).

Lysozyme and peroxidase (metabolic) activity. The ultrasonically disintegrated leukocytes (18 kHz, 10 s) were centrifuged (65 Hz, 10 min). The supernatant (150 μL) was mixed with 50 μL of the suspension of *Micrococcus luteus* ATCC 4698 in phosphate buffer ($A_{410} = 0.8$) or 50 μL of the peroxidase substrate (1,2-benzenediamine and freshly diluted H_2O_2, pH 5.0). The lysozyme activity was measured spectrophotometrically at 410 nm in 0–20-min period. The peroxidase reaction was stopped after 20 min by H_2SO_4 (4 mL/L) and changes in A_{490} were measured (MR 5000 spectrophotometer; *Dynatech*).

Statistical analysis. The results were analyzed by Student's *t* test; values of $p < 0.05$ were regarded as statistically significant.

RESULTS AND DISCUSSION

In vitro treatment of human leukocytes with various concentrations of lipid A analogues led to distinct changes in their reaction to various stimuli. At a lower concentration diphosphoryl lipid A analogues enhanced twice both the phagocytotic activity and the phagocytotic index of human macrophages (Table I). At higher concentrations, the phagocytosis was suppressed only by C analogue. Phagocytosis of human macrophages was stimulated two- to three-times by MLA analogue. In comparison, Masihi *et al.* (1986) showed that phagocytosis of peritoneal macrophages was enhanced even 7–10-fold by diphosphoryl lipid A and 5-fold by monophosphoryl lipid A.

Microbicidal activity against *S. aureus* cells was significantly stimulated after treatment of human leukocytes with MLA and LBr (Table II). On the other hand, their microbicidal activity against *E. coli* was stimulated only by the C analogue. The other tested analogues reduced the ability of the leukocytes to kill *E. coli*.

As shown in Table III, only C and LNO analogues at higher concentrations stimulated the microbicidal activity of human cells against *C. albicans*. In previous work with the whole outer-membrane extracts obtained from *E. coli* cells adapted to an AO (Bukovský *et al*. 1998), the microbicidal activity of human cells against *C. albicans* was stimulated only by extracts from the sensitive strain of *E. coli*.

The *in vivo* production of lysosomal enzymes is a common response of leukocytes to infection. Pretreatment *in vitro* of the human leukocytes by all the lipid A analogues at 1 mg/L stimulated the cells to release lysozyme (Table IV). Moreover, at this concentration the peroxidase activity was also stimulated after the cells were treated by the analogues (Table V), though MLA at lower concentration significantly suppressed this activity.

Among the various biological effects of lipid A, its immunomodulatory activity is of particular interest not only because of its ability to enhance the immune response. On the other hand, its immunosuppressive activity might serve in protection against sepsis and probably cardiac ischemia–reperfusion injury; this means to decrease the destructive inflammation changes in the tissues (Salkowski *et al.* 1998; Matsuura *et al*. 1999). Therefore, natural lipid A from bacteria as well as several modifications of its natural structure were studied both *in vivo* and *in vitro.*

Table I. Phagocytotic activity and phagocytotic index of human macrophages after treatment with MLA, LBr, C and LNO

Treatment	μg/L	Phagocytotic activity		Phagocytotic index	
		phagocytosing cells, %	relative activity, %	n^a	relative activity, %
Control no. *1*	–	37 ± 13	100	4.3 ± 0.5	100
MLA	0.1	59 ± 14***	159	5.2 ± 0.5***	121
	0.5	94 ± 6***	254	8.9 ± 0.6***	207
	1	89 ± 9***	241	8.5 ± 1.4***	198
	3	50 ± 11***	135	5.0 ± 0.6***	116
LBr	1	80 ± 11***	216	6.5 ± 0.4***	151
Control no. *2*	–	54 ± 4	100	3.8 ± 0.2	100
C	1	89 ± 10***	164	7.3 ± 0.9***	192
LNO	1	72 ± 8**	133	6.0 ± 1.4**	158
Control no. *3*	–	90 ± 4	100	6.6 ± 0.6	100
C	5	77 ± 2***	86	5.3 ± 0.1***	80
LNO	5	84 ± 4	93	6.5 ± 0.6	98
LBr	5	85 ± 5	94	7.7 ± 1.1	116

[a]Number of engulfed particles. $^{**}p < 0.01$, $^{***}p < 0.001$.

Table II. Bactericidal acitivity of human leukocytes against *Staphylococcus aureus* (*S.a.*) and *Escherichia coli* (*E.c.*) after treatment with MLA, LBr, C and LNO

Treatment	μg/L	Concentration of living cells[a]		Cell survival, %	
		S.a.	*E.c.*	*S.a.*	*E.c.*
Control no. *1*	–	1560 ± 130	1100 ± 240	100	100
MLA	0.1	780 ± 130***	1480 ± 160*	50	135
	0.5	676 ± 198***	1100 ± 200	43	100
	1	546 ± 104***	1260 ± 160	35	115
	3	806 ± 135***	1540 ± 120**	52	140
LBr	1	702 ± 179***	1500 ± 160**	45	136
Control no. *3*	–	847 ± 95	1400 ± 560	100	100
C	5	835 ± 141	480 ± 100**	99	34
LNO	5	933 ± 226	1260 ± 100	110	90
LBr	5	658 ± 122*	–	78	–

[a]100/μL, *i.e.* $\times 10^5$ per mL. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

The stimulation process of leukocytes by lipid A involves its binding to the cell and internalization, which is followed by cell activation. The binding is half-saturated at 10–30 μg/L of lipid A (Lentschat *et al.* 1999) but its biological activity varies in a wide range of concentrations (0.3 μg/L to 1 mg/L). Among the tested analogues of lipid A, only MLA at 5 mg/L was without any effect on human monocytes, though it induced full T-cell activation (Ismaili *et al*. 2002). Saha *et al.* (2001) reported that 10 mg/L of MLA induces a significant release of H_2O_2 in primary stimulation of leukocytes.

At the relatively high concentration (100 mg/L), MLA had been reported to be an activator of human leukocytes for calcium mobilization, activation of mitogen-activated protein kinase (MAPK) and for

induction of the NF-κB transcription factor (Ismaili *et al.* 2002). Matsuura *et al*. (1999) reported that the natural lipid A (*E. coli* type) at 1 μg/L affected the ability of human cells to produce the proinflammatory mediators TNF and IL-6.

Table III. Microbicidal activity of human leukocytes against *C. albicans* after treatment with MLA, LBr, C and LNO

Treatment	μg/L	Concentration of living cells[a]	Cell survival %
Control no. *1*	–	780 ± 120	100
MLA	0.1	680 ± 140	87
	0.5	700 ± 160	89
	1	880 ± 100	113
	3	780 ± 260	100
LBr	1	720 ± 160	92
Control no. *2*	–	620 ± 111	100
C	1	624 ± 55	101
LNO	1	544 ± 83	88
Control no. *3*	–	702 ± 146	100
C	5	427 ± 75**	61
LNO	5	422 ± 105**	60
LBr	5	453 ± 102	65

[a]1/mL, *i.e.* $\times 10^3$ per mL. $^{**}p < 0.01$.

Table IV. Lysozyme activity[a] of human leukocytes at 1 and 5 μg/L of lipid A

Treatment	$\Delta A_{410} \times 10^3$	%[c]
1 μg/L		
Control no. *2*[b]	37 ± 3	100
C	40 ± 3	108
LNO	50 ± 8**	135
LBr	48 ± 2***	129
5 μg/L		
Control no. *3*[b]	28 ± 5	100
C	41 ± 9*	146
LNO	36 ± 3*	129
LBr	27 ± 4	96

[a]Difference in absorbance at 410 nm after 0 and 20 min of incubation (ΔA_{410}).
[b]Without lipid A. [c]Relative activity.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Adaptation of *E. coli* cells to amphiphilic compounds leads to changes in the composition of its lipopolysaccharide, particularly of its lipid A portion. Structural analysis of lipid A from *E. coli* adapted to ATDNO (Bukovský *et al.* 1991) revealed a decrease in the content of hydroxy fatty acids. It was supposed that alterations in chemical composition could modify the interaction of lipid A and leukocytes. Nevertheless, as shown here, the changes in lipid A due to adaptation of bacteria to the amphiphilic compounds only slightly reduced its immunomodulatory activity compared with the natural lipid A from untreated cells. On the other hand, all diphosphoryl analogues were less active than the monophosphoryl analogue MLA.

Table V. Peroxidase activity[a] of human leukocytes after treatment with MLA, LBr; C and LNO

Treatment	μg/L	$\Delta A_{490} \times 10^3$	%[b]
Control no. *1*	–	353 ± 2	100
MLA	0.1	199 ± 4***	55
	0.5	296 ± 28*	84
	1	399 ± 17***	113
LBr	1	385 ± 8***	109
Control no. *2*	–	51 ± 3	100
C	1	79 ± 3***	155
LNO	1	72 ± 3***	141

[a]Difference in absorbance at 490 nm after 0 and 10 min of incubation (ΔA_{490}).
[b]Relative activity.
$^{*}p < 0.05$, $^{***}p < 0.001$.

The authors thank the *Slovak Science Grant Agency VEGA* (grant no. 1/0518/03) and the *Comenius University* (grant no. UK/3/2002) for financial support. We also thank Prof. F. Devínsky and Assoc.Prof. I. Lacko (*Department of Chemical Theory of Drugs, Faculty of Pharmacy, Comenius University*, Bratislava) for the gift of quaternary ammonium salt and amine oxide.

REFERENCES

BOYUM A.: Isolation of leukocytes from human blood – further observations. Methylcellulose dextran, and ficoll as erythrocyte aggregating agents. *Scand.J.Clin.Lab.Invest.* **97** Suppl., 31–50 (1968).

BUKOVSKÝ M., MLYNARČÍK D., NAGY A., BELLA J.: Outer membrane alterations in *Escherichia coli* cells adapted to amine oxides. *Acta Fac.Pharm.* **46**, 153–167 (1991).

BUKOVSKÝ M., MLYNARČÍK D., KOŠČOVÁ H.: Change of immunomodulating properties of *Escherichia coli* caused by artificial resistance to amine oxides. (In Slovak) *Farm.Obzor* **57**, 59–68 (1998).

ČUPKOVÁ V., VINTER V., DEVÍNSKY F., LACKO I., MLYNARČÍK D.: Inhibitory effect of 1-methyldodecyldimethylamine oxide and *N*,*N*-bis(dodecyldimethyl)-1,2-ethanediammonium dibromide on the spores of *Bacillus cereus*. *Folia Microbiol*. **33**, 433–439 (1988).

DUBOIS-BRISSONNET F., MALGRANGE C., GUÉRIN-MÉCHIN L., HEYD B., LEVEAU J.-Y.: Changes in fatty acid composition of *Pseudomonas aeruginosa* ATCC 15442 induced by growth conditions: consequences of resistance to quaternary ammonium compounds. *Microbios* **106**, 97–110 (2001).

ELLIOT G.T.: Monophosphoryl lipid A induces delayed preconditioning against cardiac ischemia/reperfusion injury. *J.Mol.Cell.Cardiol.* **30**, 3–17 (1998).

FERENČÍK M., LACKO I., DEVÍNSKY F.: Immunomodulatory activity of some amphiphilic compounds. *Pharmazie* **45**, 695–696 (1990).

FRECER V., HO B., DING L.J.: Molecular dynamics study on lipid A from *Escherichia coli*: insights into its mechanism of biological action. *Biochim.Biophys.Acta* **1466**, 87–104 (2000).

HAGEN S.R., THOMSON J.D., SNYDER S.D., MYERS K.R.: Analysis of monophosphoryl lipid A immunostimulant preparation from *Salmonella minnesota* RP 595 by high-performance liquid chromatography. *J.Chromatogr.A* **767**, 53–61 (1997).

HOŠTACKÁ A., MAJTÁN V.: Enzymic and permeability activity of *Pseudomonas aeruginosa* after treatment with sub-MICs of organic ammonium salts. *Folia Microbiol.* **39**, 197–202 (1994).

HOŠTACKÁ A., MAJTÁN V., HYBENOVÁ D.: Antimicrobial efficacy of quaternary bisammonium salts and the effect of their sub-MICs on *Pseudomonas aeruginosa* virulence factors. *Folia Microbiol.* **40**, 283–287 (1995).

IKEDA S., NISHUMIRA C., MATSUURA M., HOMMA J.Y., KISO M., HASEGAWA A.: Effect of acyl substituents of synthetic lipid A-subunit analogues on their immunomodulating antiviral activity. *Antiviral.Res.* **13**, 327–333 (1990).

ISMAILI J., RENNESSON J., AKSOY E., VEKEMANS J., VINCART B., AMRAOUI Z., VAN LAETHEM F., GOLDMAN M., DUBOIS P.M.: Monophosphoryl lipid A activates both human dendritic cells and T cells. *J.Immunol*. **168**, 926–932 (2002).

KUŽELOVÁ M., BUKOVSKÝ M., SOLČANOVÁ M., KOŠČOVÁ H., ŠVEC P.: The immunomodulatory properties of modificated lipid A-endotoxin A in ischemic/reperfusion injury conditions. (In Slovak) *Československ.Fyziol.* **48**, 153 (1999).

LENTSCHAT A., EL-SAMALOUTI V.T., SCHLETTER J., KUSUMOTO S., BRADE L., RIETCHEL E.T., GERDES J., ERNST M., FLAD H.-D., ULMER J.: The internalization time course of a given lipopolysaccharide chemotype does not correspond to its activation kinetics in monocytes. *Infect.Immun.* **67**, 2515–2521 (1999).

LÜDERITZ O., GALANOS C., LEHMANN V., MEYER H., RIETSCHEL E.T., WECKESSER J.: Chemical structure and biological activities of lipid A's from various bacterial families. *Naturwissenschaften* **65**, 578–585 (1978).

MAJTÁNOVÁ Ľ., MAJTÁN V., HOŠTACKÁ A.: Effects of amphiphilic compounds on metabolic and biological activities of *Salmonella typhimurium*. *Arznem.Forsch./Drug Res.* **46**, 64–67 (1996).

MASIHI K.N., LANGE W., JOHNSON A.G., RIBI E.: Enhancement of chemiluminescence and phagocytic activities by non-toxic and toxic forms of lipid A. *J.Biol.Resp.Mod.* **5**, 462–469 (1986).

MATSUURA M., KISO M., HASEGAWA A.: Activity of monosaccharide lipid A analogues in human monocyte cells as agonist or antagonist of bacterial lipopolysaccharide. *Infect.Immun.* **67**, 6286–6292 (1999).

POSPÍŠIL S., ŘEZANKA T.: Changes in fatty acid branching and unsaturation of *Streptomyces cinnamonensis* as a response to NaCl concentration. *Folia Microbiol*. **39**, 187–190 (1994).

SAHA D.C., BARUA R.S., ASTIZ M.E., RACKOW E.C., EALES-REYNOLDS L.J.: Monophosphoryl lipid A stimulated up-regulation of reactive oxygen intermediates in human monocytes *in vitro*. *J.Leukoc.Biol.* **70**, 381–385 (2001).

SALKOWSKI C.A., DETORE G., FRANKS A., FALK M.C., VOGEL S.N.: Pulmonary and hepatic gene expression following cecal ligation and puncture: monophosphoryl lipid A prophylaxis attenues sepsis-induced cytokine and chemokine expression and neutrophil infiltration. *Infect.Immun.* **66**, 3569–3578 (1998).

VAN ALPHEN L., LUGTENBERG B., RIETSCHEL E.T., MOMBERS C.: Architecture of the outer membrane of *Escherichia coli* K12. Phase transitions of the bacteriophage K3 receptor complex. *Eur.J.Biochem.* **101**, 571–579 (1979).

WESTPHAL O., JANN K.: Bacterial lipopolysaccharides. Extraction with phenol–water and further applications of the procedure, pp. 83–91 in R.L. Whistler, J.N. BeMiller, M. Wolfrom (Eds): *Methods in Carbohydrate Chemistry, Vol. 5.* Academic Press, New York–London 1965.