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Neutrophil Cellular Responses to Various *Salmonella typhimurium* LPS Chemotypes

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1. Introduction

The first line of defense against invading bacteria is provided by the innate immune system, and polymorphonuclear leukocytes (PMNL) contribute to bacterial clearance by uptake and intracellular killing of microbes. Lipopolysaccharides (LPS, endotoxin), a major component of the outer membranes of Gram-negative bacteria, is shed into the environment and acts as a highly potent proinflammatory substance. About 15—25% of the bacterial surface in *Salmonella typhimurium* was found to be covered by LPS (Mühlradt et al., 1974). LPS initiates the cascade of pathophysiological reactions called endotoxin shock. LPS released from Gram-negative bacteria induces a strong priming of superoxide production (Guthrie et al., 1984) and facilitates the rapid elimination of the bacteria. However, an excessive activation of neutrophils could be self-destructive in septic shock. A number of mediators, such as cytokines, nitric oxide and eicosanoids, are responsible for most of the manifestations caused by LPS. The toxic and other biological properties of LPS are due to the action of endogenous mediators, which are formed following interaction of LPS with cellular targets (Galanos & Freudenberg, 1993). Biological activities of LPS have been well established, but some uncertainty remains regarding to the responses to various LPS chemotypes.

LPS are phosphorylated glycolipids that possess complex chemical structures (Müller-Loennies et al., 2007). LPS are composed of covalently linked structural domains: lipid A, an oligosaccharide core, and O- polysaccharide (or O- antigen) (Raetz & Whitfield, 2002). Lipid A is the minimal biologically active unit of LPS and is thus called the 'endotoxic principle' of LPS. The full chemical structures of lipid A from *E. coli* and *Salmonella enterica* serovar Typhimurium (S. Typhimurium) were identified in 1983, and the similarity of their structures was proved (Takayama et al., 1983; Alexander & Rietschel, 2001, review). Lipid A

is the hydrophobic portion of the molecule. The hydrophilic polysaccharide portion may be further subdivided into the O-specific and the core oligosaccharide. Bacteria which contain an O- polysaccharide have a smooth colony appearance when grown on agar plates and therefore this type of LPS is referred to as smooth(S)-type LPS. The outer parts of LPS (O-polysaccharide) interact with the host immune system. Westphal and al. established that the O-polysaccharide component contained the serologically active determinants (the species-specific bacterial O-antigen) (Westphal, 1978; Westphal & Luederitz, 1961). Currently, based on O-antigens (O-polysaccharides), *Salmonella* strains have been classified into over 50 serogroups (Fitzgerald et al., 2007).

The presence of O-antigen in LPS is irrelevant for bacterial invasion of epithelial cells; in contrast, a core structure is necessary for adhesion and subsequent entry of S. Typhimurium into epithelial cells (Bravo et al., 2011). Mutant bacteria (rough mutants) produce LPS with short oligosaccharide chains but not O- polysaccharide. Chemical analysis of LPS from such *Salmonella* mutants distinguished Ra from Re chemotypes: Ra describes the largest core structure and Re was assigned to the smallest core structure. LPS from rough mutants, so-called Ra, Rb, Rc, Rd and Re LPS, mainly differ in the length of the core oligosaccharide, while the lipid-A portion is assumed to be identical. The chemical structures of *Salmonella* LPS have been investigated in many details (Olsthoorn et al., 1998; Perepelov et al., 2010).

Neutrophil-mediated innate host defense mechanisms include phagocytosis of bacteria. Upon activation, polymorphonuclear leukocytes (PMNL, neutrophil), produce signicant amounts of leukotriene B4 (LTB4) in addition to several cytokines and inammatory mediators, and thus recruit other neutrophils to the site of inammation. LTB4 is one of the most potent chemotactic compounds produced in macrophages and neutrophils (Toda et al., 2002). Stimulation of leukotriene B4 synthesis in PMNLs plays a role in stimulation of phagocytosis and bacterial killing (Mancuso et al., 2001). The key enzyme of LT synthesis in neutrophils is 5-lipoxygenase (5-LO), which metabolizes arachidonic acid (AA), first to 5S-hydroperoxyeicosatetraenoic acid (5-HPETE), and then to leukotriene A4 (LTA 4) (Samuelsson, 1983). Unstable LTA4 intermediate is converted to 5S,12R-dihydroxy-6,14-cis-8,10-trans-eicosatetraenoic acid (leukotriene B4, LTB4) and (non-enzymatically) to its isomers. The 5-LO metabolite LTB4 is a proinflammatory mediator that activates neutrophils, thus changing their shape and promoting their binding to endothelium by inducing the expression of cell-adhesion molecules. The localization of leukocytes to the site of inflammation results in endothelial and other tissue damage, i.e. metabolites of 5-LO contribute to the multiple organ injury and dysfunction during inflammatory process (Collin et al., 2004; Cuzzocrea et al., 2003; 2004). Any modulation of the activity of PMNL is a potential cause of the altered immune response to infection. The phagocytosis of microorganisms by PMNL is enhanced by LPS. And though *Salmonella*-LPS related complications have been successfully blunted with 5-LO inhibitors (Matera et al., 1988; Altavilla et al., 2009), little is known about phagocytosis and 5-LO products regulation by LPS chemotypes.

Effects of structurally different LPS types upon neutrophil functions were examined. Ruchaud-Sparagano et al. (Ruchaud-Sparagano et al., 1998) investigated the mechanisms of LPS action by examining the effect of smooth and rough chemotypes of LPS in stimulating neutrophil beta2 integrin activity and fMLP-induced respiratory burst. They reported just kinetic differences in the action of rough LPS and smooth LPS: rough LPS acts more rapidly

than S-LPS to cause functional alterations in neutrophils. Similar results were obtained on neutrophils in whole blood: again just kinetic difference was observed between R- and S-LPS in the expression of cell surface receptors CD11b and CD11c on neutrophils (Gomes et al., 2010). Nevetheless, the rough mutant as well as S LPS differ in some distinct physico-chemical properties. Due to these differences, it was found a lower fluidity of S LPS chemotype than Ra and Re mutants (Luhm et al., 1998). It was established that the bioactivity of LPS was dependent on the length of their core oligosaccharides, and endotoxin-induced cytokine secretion decreased with decreasing sugar moiety (and increasing fluidity) in the order S ≥ Ra>Rc>Re LPS (Luhm et al., 1998). Comparative evaluation of the endotoxic properties of LPS preparations by using the LAL assay showed that endotoxic activity of the rough Re mutant SL1102, the rough Ra mutant TV119, and the smooth strain SH4809 of *Salmonella* Typhimurium increased in the order S < Ra < Re (Shnyra et al., 1993).

When neutrophils were challenged with *Salmonella minnesota* smooth-strain and rough-strain mutants (Ra, Rb2, RcP-, Rd1P- and Re) as well as with lipid A, in the case of luminol-dependent chemiluminescence (respiratory burst), lipid A was the most potent stimulus, with the response decreasing as molecular complexity increased, with S- LPS equally potent as Ra LPS (Pugliese et al., 1988). An oxygen-independent system in the antimicrobial effects of neutrophils is also sensitive to LPS chemotype. As the carbohydrate content of the mutant LPS decreased, the bacteria became less resistant to the oxygen-independent bactericidal activity of neutrophils (Okamura & Spitznagel, 1982). Based on these data, one can conclude that there are qualitative as well as quantitative effects of the carbohydrate moieties of LPS. We report here that various LPS forms from *Salmonella typhimurium* bacteria significantly differ in their ability to influence adhesion, phagocytosis as well as formation of 5-LO products, and reactive oxygen and nitrogen species in human neutrophils.

2. Materials and methods

Zymosan A from *Saccharomyces cerevisiae*, lipopolysaccharides from *Salmonella* enterica serovar Typhimurium (the source strain for smooth form is ATCC 7823, rough strains from *Salmonella* typhimurium TV119 (Ra mutant) and SL1181 (Re mutant)), *N*ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), staurosporine from *Streptomyces sp.* were from Sigma (St. Louis, MO, USA and Steinheim, Germany). *S.* Typhimurium virulent strain C53 was a kind gift of Prof. F. Norel (Pasteur Institute, France) (Kowarz et al., 1994). Bacteria were grown in Luria–Bertani broth and washed twice using physiological salt solution with centrifugation at 2000 *g*. The concentration of the stock suspension was 1×10^9 CFU/mL. The bacteria were opsonized with 5% fresh normal human serum (NS) from the same donor whose blood was used for preparation of neutrophils. NS was prepared by clotting and centrifugation of fresh whole blood at room temperature. In some experiments, the NS was decomplemented by heat inactivation for 30 min at 56°C (heat inactivated serum, HIS). Nitrate/Nitrite fluorometric assay kit was from Cayman Chemical (Ann Arbor, MI, USA). Ficoll-Paque was purchased from Pharmacia (Uppsala, Sweden). Human serum albumin, fraction V (HSA) was from Calbiochem (La Jolla, CA, USA). Hepes and *o*-phenylenediamine were from Fluka (Deisenhofen, Germany). Phosphate buffered saline (PBS) was purchased from Gibco (Paisley, UK, Scotland, UK). Dextran T-500 was from Pharmacosmos (Holbaek, Denmark). High-pressure liquid chromatography (HPLC) solvents were purchased from Chimmed (Moscow,

Russia). Prostaglandin B2 was from Cayman Chemical Company (Ann Arbor, USA). Hank's balanced salt solution (with calcium and magnesium but without phenol red and sodium hydrogen carbonate, HBSS), HBSS modified (without calcium, magnesium, phenol red and sodium hydrogen carbonate), Dulbecco's PBS (with magnesium, but without calcium), cytochrome *c* from horse heart were purchased from Sigma (Steinheim, Germany).

2.1 Human neutrophil and red blood cell (RBC) isolation

PMNLs were isolated from freshly drawn EDTA-anticoagulated donor blood by standard techniques, as previously described (Sud'ina et al., 2001). Leukocyte-rich plasma was prepared by sedimentation of RBCs with 3% dextran T-500 at room temperature. Granulocytes were purified by centrifugation of leukocyte-rich plasma through Ficoll-Paque (density 1.077 g/mL) followed by hypotonic lysis of the remaining RBCs. PMNLs were washed twice with PBS, resuspended at 10^7/mL (purity 96–97%, viability 98–99%) in Dulbecco's PBS containing 1 mg/mL glucose (without $CaCl_2$), and stored at room temperature. RBCs were isolated from EDTA-anticoagulated donor blood by sequential centrifugation (at 1100 rpm) and washing with PBS. After three washes, the cells were resuspended at 2.7×10^9/mL in PBS and stored at room temperature.

2.2 Preparation of collagen-, fibronectin- or HUVEC-coated surfaces

Plastic tissue-culture 24-well plates (Corning Incorporated, Corning, NY, USA) were coated with 75 μg/ml type I collagen or 15 μg/ml fibronectin for 24h. Prior to use, the protein coated surfaces were washed, incubated for 1 h in PBS with 0.1% human serum albumin, and then thoroughly washed with PBS. Human umbilical vein endothelial cells (HUVEC), passages 1–3, were maintained in medium 199 containing 10% fetal calf serum (FCS), 3.5 units/ml heparin (Fluka, Deisenhofen, Germany), 50 μg/ml endothelial cell growth factor (ICN, Ohio, USA), 10 U/ml penicillin and 10 mg/ml streptomycin. The cells were passaged using trypsin-EDTA solution (500 BAEE units trypsin and 180 mg EDTA/ml in PBS), and seeded on 24-well plates (Galkina et al., 2004). One day before the experiments, the monolayers were washed and medium was replaced with the same medium containing 2% FCS, rather than 10 %.

2.3 Preparation of lipopolysaccharides (LPS) solutions and opsonized zymosan (OZ)

Lipopolysaccharides from *Salmonella enterica* serovar *typhimurium* were solubilized in PBS (1 mg/ml) by vortexing, heated in a water bath to 60°C for 30 min, cooled to room temperature, and subjected to one more cycle of heating to 60°C and cooling to room temperature. Zymosan A particles from *Saccharomyces cerevisiae* were suspended in PBS and boiled for 5 min. After cooling to room temperature, the prepared suspension was washed with PBS and opsonized by adding 20-30 % freshly prepared autologous human normal serum for 30 min at 37°C, washed 3 times with PBS and resuspended in the Hank's balanced salts medium containing 10 mM Hepes (HBSS/Hepes).

2.4 PMNL adhesion assay

Myeloperoxidase activity was used to measure PMNL attachment under static conditions to collagen or HUVEC adsorbed on to plastic surfaces. For measuring PMNL adhesion, HUVECs grown in 24-well plates were washed once with HBSS. PMNLs (10^6/well) were

added to a coated 24-well culture plate in 500 µl of HBSS/Hepes medium. After 30 min of incubation with or without the additives in a CO_2 incubator at 37°C to allow neutrophil adherence, wells were washed twice with 500 µl of PBS solution for removal of non-adherent PMNLs. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate, as described (Schierwagen et al., 1990; Sud'ina et al., 1998). A solution (300µl) of 5.5mM *o*-phenylenediamine and 4mM H_2O_2 in buffer (67mM Na_2HPO_4, 35mM citric acid and 0.1% Triton X-100, pH5) was added to each well, and after 5 min the reaction was stopped by the addition of an equal volume of 1M H_2SO_4. Standard dilutions of PMNLs with or without tested compounds were used for calibration.

2.5 Phagocytosis experiments

PMNLs (5×10^6/ml) were placed into 6-well plates (2 ml/well) containing collagen- of fibronectin-coated coverslips for 30 min of incubation with tested compounds. Then 0.25 mg/ml of opsonized zymosan (OZ) was added for another 5 min. The cells were gently washed with PBS, and then fixed for 30 min in HBSS medium modified, with 10 mM HEPES and 2.5% glutaraldehyde. After gentle washing with PBS, the samples were examined by phase contrast microscopy. The number of OZ particles ingested was counted and the data were expressed as a phagocytic index, which was derived by multiplying the portion of PMNLs containing at least one ingested target by the mean number of phagocytosed targets per positive PMNL. Data were obtained from ~ 100 cells per coverslip.

2.6 Scanning electron microscopy

Cells were fixed for 30 min in 2.5% glutaraldehyde, postfixed for 15 min with 1% osmium tetroxide in 0.1 M cacodylate (pH 7.3), dehydrated in an acetone series, critical-point dried with liquid CO2 as the transitional fluid in a Balzers apparatus, sputter-coated with gold–palladium, and observed at 15 kV with a Camscan S-2 (Tescan, USA) or JSM-6380 (JEOL, Germany) scanning electron microscope.

2.7 Nitrite measurement

Nitric oxide, derived from the conversion of L-arginine to L-citrulline, reacts with molecular oxygen to form nitrite and nitrate (Moncada & Higgs, 1993). NO production was measured as total nitrite concentration in the sample after enzymatic conversion of nitrate to nitrite by nitrate reductase. A highly sensitive fluorometric assay for nitrite measurements, which is based on the acid-catalyzed ring closure of 2,3-diaminonaphtalene (DAN) with formation of highly fluorescent product 2,3-aminonapthotriasole in the presence of nitrite, was used to probe PMNLs for NO production (Nath & Powledge, 1997). For this purpose, PMNLs (2×10^7/ml) were incubated with compounds tested for 30 min, then OZ was added for the next 30 min, reaction was stopped by centrifugation (400g, 10 min) and supernatant was filtered though 10 000 Mr cutoff microcentrifuge filters (Millipore corporation, USA) at 14 000g for 30 min at room temperature. The ultrafiltration step was necessary to remove any trace amounts of zymosan particles and hemoglobin which may be present in PMNL samples due to red cells contamination, which strongly interferes with the fluorescent measurements (Misko et al., 1993). Nitrite measurements in the prepared supernatants were performed in triplicate using Nitrate/Nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI,

USA) according to manufacturer's protocol at excitation and emission wavelengths of 360 and 430 nm, respectively, by plate reader Infinite 200 (Tecan Group Ltd., Mainz, Germany). All compounds added to PMNLs were tested for their autofluorescence within the spectrum region in the assay buffer.

2.8 Superoxide measurement

PMNL incubations on collagen- and fibronectin-coated surfaces were performed as described for PMNL adhesion assay. 50 μM cytochrome c, tested compounds and 300 u/ml superoxide dismutase (SOD), were added (as indicated) to the medium prior to the cells. The plates were incubated at 37 °C for 30 min, then OZ was added or not for another 30 min. The incubation was stopped by cooling to 4°C, and cytochrome c reduction was measured as the increase in Δ550/535 (the change in the ratio of absorbances at 550 and 535nm). Reduction of 10 μM cytochrome c produced an increase in Δ550/535 of 0.18 absorbance unit.

2.9 Assay of reactive oxygen species

The formation of active oxygen by neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA), LPS chemotypes and OZ was monitored by measuring luminol-enhanced luminescence as described earlier (Sud'ina et al., 1991). Chemiluminescence was monitored in a 1251 LKB luminometer, using 1 μM luminol. Measurements were made every 5 min over a 30 min period at 37°C.

2.10 Incubations for studies of arachidonic acid (AA) metabolism and leukotriene (LT) synthesis

PMNLs suspension (2×10^7 cells) was incubated in 6 ml HBSS/Hepes medium at 37 °C with or without agents tested for 30 min, and then stimulated by the addition of opsonized Salmonella (OS) or OZ for 20 min. The incubations were stopped by addition of an equal volume of methanol at -20°C with prostaglandin B2 (PGB2) as an internal standard. The samples were stored at -20°C. The denatured cell suspension was centrifuged (at 2000 rpm), which yielded supernatants designated as water/methanol extracts.

2.11 Lipoxygenase product analysis

The water/methanol extracts were purified by solid-phase extraction using C18 Sep-Paks (500mg), which was conditioned first with methanol, then with water. 5-LO metabolites were extracted with 1.4 ml methanol, the samples were evaporated, redissolved in 35 μl methanol/water (2:1) and chromatographed by reversed-phase HPLC. The purified samples were injected into a 5 μm Nucleudur C18 column (250 mm×4.6 mm; Macherey-Nagel, Dueren, Germany). The products were eluted at 0.7 ml/min in a linear gradient from 30 to 100% solvent B: the eluents consisted of methanol/acetonitrile/water/acetic acid/triethylamine in the ratios (solvent A) 25/25/50/0.05/0.08 and (solvent B) 50/50/0/0.05/0.04, and elution was monitored using a UV detector at 280 nm and 238 nm. Products of the 5-LO pathway that were measured included leukotriene B4 (LTB4), 5-hydroxyeicosatetraenoic acid (5-HETE), 20-hydroxy-LTB4 (ω-OH-LTB4) and iso-LTB4 [5(S),12(S,R)-dihydroxy-all-trans-eicosatetraenoic acids], identified by their co-elution with

authentic standards. The respective extinction coefficients and their ratios to that of the internal standard were used to quantify products.

2.12 Statistics

Statistical analysis was performed using the Student's t-test. Statistical significance was assumed, where probability values of less than 0.05 were obtained. Results are reported as mean ± SD of the data of at least three independent experiments.

3. Results and discussion

Neutrophils are professional phagocytes and the first line of defense of innate immune system at bacterial challenge (Borregaard, 2010). Circulating lipopolysaccharides released from bacteria may activate neutrophils. LPS elicit wide spectra of biological responses in human body. When activating an immune response, they may produce pathologically imbalanced immune response, - that is why they are called "endotoxins". Depending on a dose, they may stimulate antigen-specific immune response, and they are added as accompanying agents in vaccination (Baldridge et al., 1999; Gereda et al., 2000). High levels of endotoxins cause endotoxic shock (Rietschel et al., 1996). LPS induce numerous cellular signals. In the focus of the current work, we should stress on NO formation and leukotriene synthesis. Superproduction of NO in sepsis results in the disturbed blood flow (Li & Forstermann, 2000), which originates from contractile disfunction of smooth muscle cells and impaired PMNL chemotaxis (Lopez-Bojorquez et al., 2004). LPS interaction with leukocytes signal to phosphorylation of phospholipase A2 and arachidonic acid (AA) release from cell membranes (Doerfler et al., 1994). AA and its methabolites are the compounds with high biological activity.

In this work, we investigated effects of *Salmonella* enterica serovar typhimurium LPS species of various chemotypes (from deep rough Re mutant consisting of the lipid A and the KDO (3-deoxy-D-manno-oct-2-ulosonic acid) residues, rough Ra mutant with complete core, and S form) on cellular responses of human PMNLs. LPS chemotype structures are schematically presented in Fig.1.

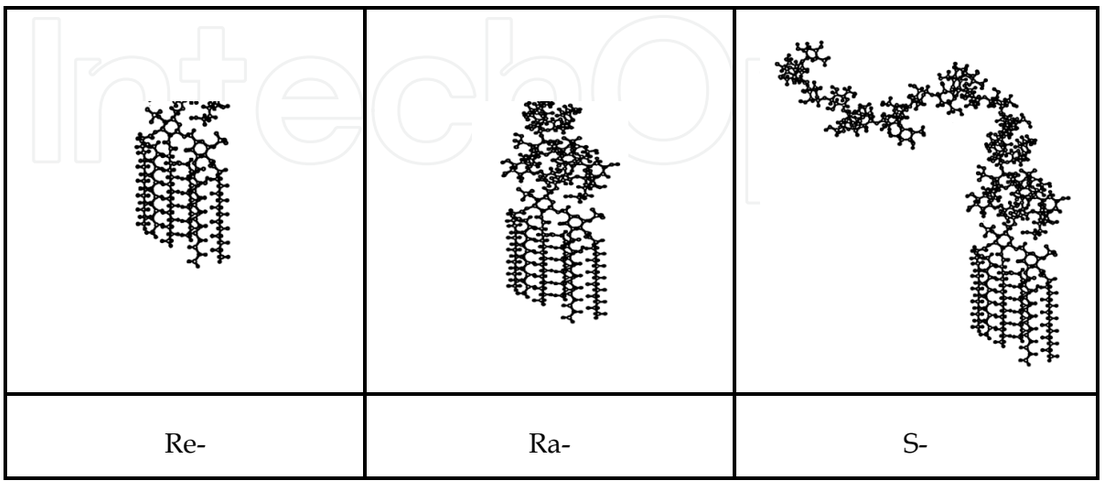
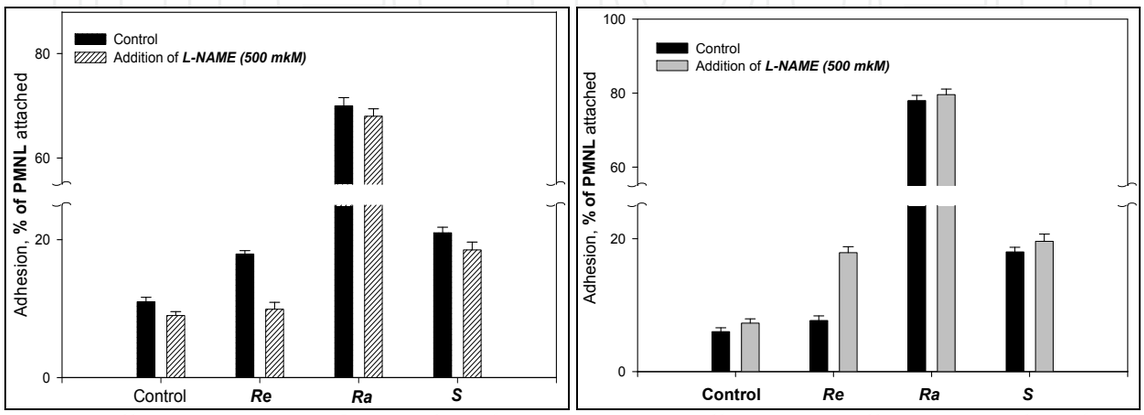


Fig. 1. Schematic presentation of LPS molecules: Re-, Ra- and S-LPS.

3.1 Neutrophil adhesion

Regulation of neutrophil adhesiveness is generally considered to be a key element in the development of inflammatory reactions. Neutrophils are known to spread on a protein-coated surface, a process that has been interpreted as "frustrated" phagocytosis. To elucidate whether Salmonella LPS of various chemotypes selectively influenced the number of adherent neutrophils, an adhesion study was performed. PMNL adhesion to collagen-coated surface was crucially increased by Ra LPS (Salmonella LPS from Ra mutant TV119). The effect was slightly lower on the surfaces coated by endothelial monolayer (Fig.2).



PMNL adhesion to HUVEC-coated surfaces PMNL adhesion to collagen-coated surfaces

Fig. 2. PMNL attachment to HUVEC- and collagen-coated plastic surface determined as described in Methods after 30 min incubation without (control) or with various LPS forms, and 500 μM L-NAME. Cell attachment has been expressed as a percentage of PMNLs adhered in relation to the total number of PMNLs added.

Priming of PMNLs with the LPS chemotypes induced cell activation including NO and superoxide release, as well as an increase in intracellular calcium concentration. The experiments with nonselective NOS inhibitor L-NAME at 500 μM demonstrated that only Re LPS (Salmonella LPS from Re mutant SL1181) was sensitive to NOS inhibition (Fig.2). The antioxidant agent diphenileleiodonium (DPI) that inhibits NADPH oxidase-mediated ROS formation, and also inhibits other flavo-enzymes such as NO synthase and xanthine oxidase (Wind et al., 2010), did not affect LPS-induced PMNL attachment (data not shown). LPS- induced intracellular calcium concentration varied in the order Ra ≥ S > Re (Zagryazhskaya et al., 2010). Taking into account the slight sensitivity of PMNL attachment to NO synthesis inhibitors, we can propose that the divalent cation requirements for the Mac-1 and LFA-1-dependent processes of adhesion (Graham & Brown, 1991; Wright & Jong, 1986) may limit the role for NO and superoxide in the specificity of these LPS chemotypes in PMNL adhesion, in the serum-free medium.

The addition of heat inactivated normal serum (HIS) markedly decreased neutrophil adhesion, but the selective prominent increase of the neutrophil attachment induced by Ra LPS chemotype was evident (Fig.3). It has been published that serum enhanced LPS-induced production of nitric oxide in J774.1 and BAM3 macrophage-like cell line (Ohki et al., 1999). Human serum albumin is known to increase iNOS expression in the lung of rats (Jakubowski et al., 2009). In our assay 500 μM L-NAME partially reversed the effect of

serum (Fig.3), which supported the hypothesis that nitric oxide mediates smoothing out the specificity of various LPS chemotypes in the presence of serum. NO is known to reduce adhesion molecules expression on neutrophils (Kubes et al., 1991; 1994; Banick et al., 1997; Kosonen et al., 1999), and we observed decreased attachment in the presence of serum (Fig.3). We propose that in the serum-containing medium, along with other factors, NO also plays a role in the specificity of LPS chemotypes in PMNL adhesion.

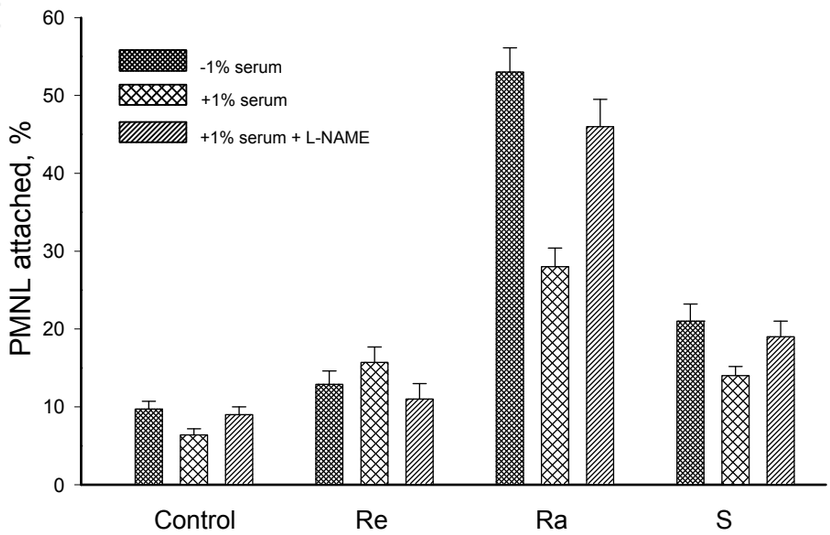


Fig. 3. Effect of serum on PMNL attachment, induced by LPS chemotypes. PMNLs ($5x10^5$/well) were added to a collagen-coated 24-well culture plate in 500 µl of HBSS/Hepes medium, without (control) or with 1% serum, 5 µg/ml LPS and 500 µM L-NAME. After 30 min of incubation in a CO2 incubator at 37 °C to allow leukocyte adherence, wells were washed twice with 500 µl of PBS solution, for removal of non-adherent PMNLs. The extent of adherence was measured after the addition of detergent and a myeloperoxidase substrate, and has been expressed as a percentage of PMNLs adhered in relation to the total number of PMNLs added.

3.2 NO and ROS formation in neutrophils

NO release is an important endogenous regulatory mechanism of inflammatory response. Human neutrophils were evaluated for their ability to generate nitric oxide more than 30 years ago (Schmidt et al., 1989; Wright et al., 1989). Nitric oxide synthase (NOS) enzymes in neutrophils were characterised, and the data on the expression of NOS isoforms are contradictory (Amin et al., 1995; Carreras et al., 1996; Greenberg et al., 1998; Cedergren et al., 2003; de Frutos et al., 2001; Molero et al., 2002; Saini et al., 2006; Chatterjee et al., 2007; 2008). The level of NO synthesis in PMNLs is comparable to one in endothelial cells, and therefore contributes significantly to the amount of NO in circulation (Miles et al., 1995; Wright et al., 1989). It is proposed that NO synthesis in neutrophils is of great physiological significance, as it modulates neutrophil function at sites of inflammation. NO participates in activation of a newly described mechanism of immune defense as formation of neutrophil extensions (cytonemes), when neutrophils do not phagocyte, but bind bacteria extracellularly (Galkina et al., 2009).

Lipopolysaccharides are well known for their ability to elicit the release of NO from eukaryotic cells including macrophages, neutrophils, and endothelial cells (Jean-Baptiste, 2007; Titheradge, 1999; Tsutsui et al., 2009). Endotoxemia is often associated with increased NO (Evans et al., 1993; Szabo et al., 1993; Gomez-Jimenez et al., 1995). NO is a unique "messenger". The biological half-life of NO is rather long - several seconds (Lancaster & Ignarro, 2002), and this molecule easily passes through cell membranes, and can interact with transition metals forming nitrosyl complexes and influencing activity of many enzymes (Korhonen et al., 2005). A reaction of particular biological relevance is the reaction of NO with superoxide with the formation of OONO- (peroxynitrite, PN) (Beckman et al., 1990). Concentration of superoxide increases up to 0.1 μM during inflammatory responses (Zweier et al., 1989), but the spectrum of reactive oxygen/nitrogen species depends on the balance of NO and superoxide within the local chemical environment (Jourd'heuil et al., 1999; 2001).

Conditions for production and release of NO in human PMNLs are still largely unknown. We recently published a paper on the influence of various LPS differing in their chain length on NOS activity in opsonized zymosan stimulated human PMNLs. We observed significant difference between Re and Ra forms of S. Typhimurium LPS in the capacity to induce NO release: Re LPS was twice more potent than Ra LPS (Zagryazhskaya et al., 2010). It is known that LPS activates protein kinase C (PKC) in macrophages and PMNLs. Increased PKC activity may inhibit NOS activity and staurosporine was shown to reverse this inhibition (Muniyappa et al., 1998). Therefore, we tested if staurosporine (St), the nonselective PKC inhibitor, could influence the specific LPS effects on NO synthesis, observed in our studies. The results are presented in Fig. 4.

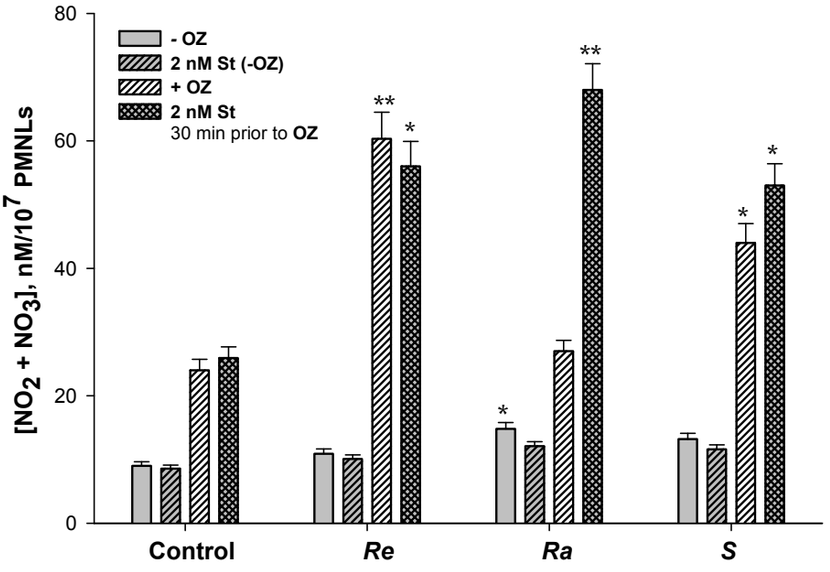


Fig. 4. Modulation of nitrite production in PMNLs by staurosporine. PMNLs (2×10^7/ml) were incubated for 30 min at 37 °C in the presence or absence of additives, (as specified): 5 μg/ml of different LPS forms, 2 nM staurosporine (St) and then stimulated for 30 min with 2 mg/ml OZ. * $P < 0.05$ vs corresponding control. ** $P < 0.01$ vs corresponding control.

Staurosporine (1-2 nM) inhibited a small increase in nitrate/nitrite level, produced by Ra and S LPS in the absence of OZ, slightly increased NO production caused by OZ alone, and partially reversed NO synthesis in OZ-stimulated PMNLs, primed by various LPS. Ra LPS form, which produced the minimal increase in NO synthesis (and even decrease in some experiments), caused the maximal NO production in the presence of staurosporine, Re and S LPS were less active. L-NAME, NOS inhibitor, significantly decreased NO production in the presence of St (data not shown) indicating staurosporine influence on NOS activity in human PMNLs, primed with LPS. These experiments demonstrated significant difference in NO production between LPS species and confirmed the role for NO in the specificity of LPS chemotypes.

LPS-priming of phagocytic leukocytes leads to nicotinamide adenine dinucleotide (NADPH) oxidase activation and potent generation of reactive oxygen species (ROS) upon stimulation (Curnutte & Babior, 1974; Drath & Karnovsky, 1975), and this process is often referred to as the respiratory burst. We determined the capacity of various LPS species to modulate superoxide anion (O2-) production measured as cytochrome c reduction, as well as ROS production measured as luminol-dependent chemiluminescence, in PMLNs prior to or without their activation by OZ. The most potent O2- production was detected in Ra-primed cells in which we observed approximately 5-fold increase in the production level detected in control cells (Zagryazhskaya et al., 2010). It is noteworthy that activation of the cells with OZ dramatically increased O2- generation in both LPS-primed and control cells, while relative values of the LPS effects were diminished. In luminol-dependent chemiluminescence, again, the efficacy pattern Ra > S > Re was found (Fig. 5). Ra LPS was the most potent chemotype in ROS and O2- release from LPS-treated PMNL.

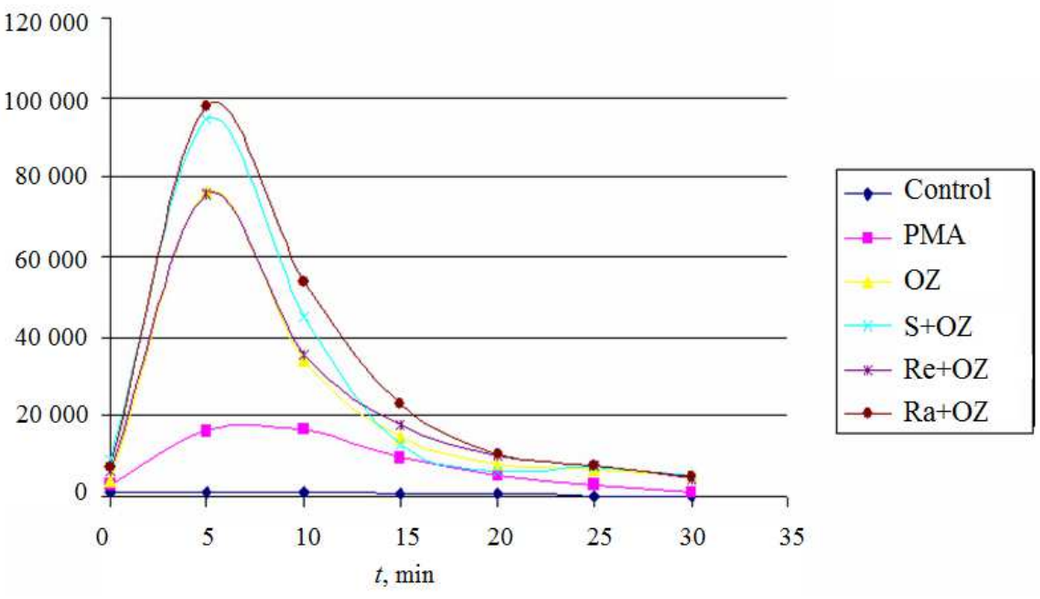


Fig. 5. Effect of various LPS forms on ROS formation in PMNLs, as recorded by luminol-enhanced luminescence (y-axis, arbitrary units). PMNLs were incubated at 37 °C in the presence or absence of additives, as specified: 100 nM PMN, 5 μg/ml of different LPS forms, 0.2 mg/ml OZ.

The addition of heat inactivated normal serum (HIS) decreased superoxide release, but the effect of Ra LPS chemotype was still maximal (Fig.6). Supposedly, serum increased LPS-induced NO release, which neutralized superoxide and resulted in smoothing out the specificity of various LPS chemotypes (Fig.6.).

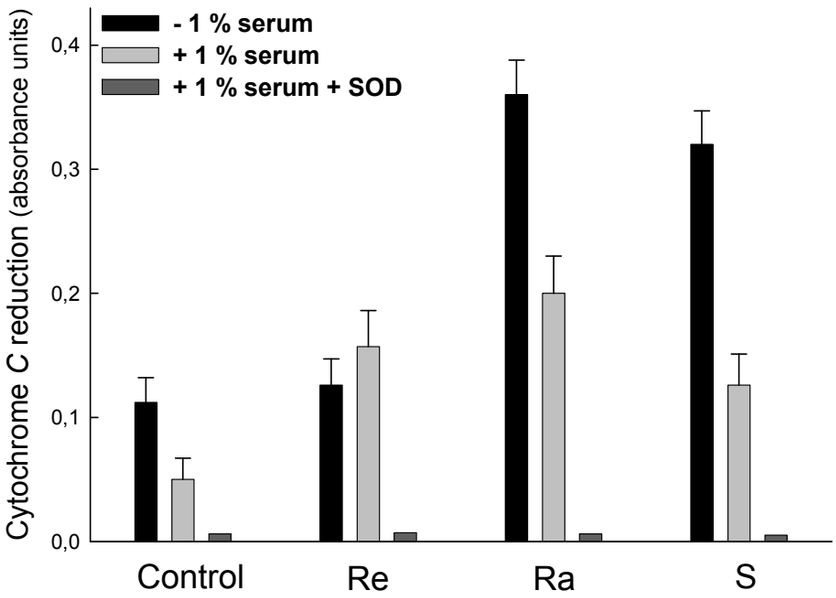


Fig. 6. Effect of serum on LPS-induced superoxide production in human PMNLs. 10^6 PMNLs/ml were incubated for 30 min at 37 °C without (control) or with 5 µg/ml LPS on a collagen-coated surface, in the medium without (- 1% serum) or with 1 % serum (heat inactivated human serum). When indicated, 300 u/ml superoxide dismutase (SOD) were added. Superoxide production was measured as cytochrome c reduction, as described in Methods.

3.3 NO levels dictate the signaling pathway to phagocytosis and LT synthesis in PMNL

We addressed the role for NO in phagocytosis of opsonized zymosan (OZ) influenced by various LPS chemotypes. OZ was prepared by incubating zymosan particles (dried cell walls of Saccharomyces cerevisiae) with autologous serum. Using phase-contrast microscopy we determined the phagocytic index in the cells exposed to 1 µg/ml of LPS species (Re, Ra, S) from *Salmonella* enterica serovar typhimurium for 30 min prior to OZ addition (for additional 5 min). The role for NO in distinct effects of various LPS chemotypes is clearly evident in phagocytosis of OZ by PMNL (Fig. 7).

Ra LPS mutant caused maximal increase in the index as compared to the control measurement. The S- and Re- forms were less effective than the Ra-form and the resulting pattern of their efficacy can be presented as Ra >S >Re.

In Fig. 8 scanning electron microscopy photos illustrate phagocytosis of OZ by untreated PMNLs (Control + OZ) and cells preincubated for 30 min with 1 µg/ml Ra LPS (Ra LPS + OZ). Scanning electron microscopy studies revealed that LPS-treated neutrophils engulfed simultaneously more particles of opsonized zymosan (OZ) than control cells (Fig.8).

We investigated how the LPS forms augment neutrophil phagocytosis in the presence of NOS inhibitor (500 μM L-NAME). L-NAME produced an increase in the effects of Re and S LPS chemotypes and furthermore attenuated the difference between effects of various LPS chemotypes (Fig.7). These data pointed out that the potency of different LPS chemotypes to activate neutrophil phagocytosis is largely due to their ability to induce NO synthesis.

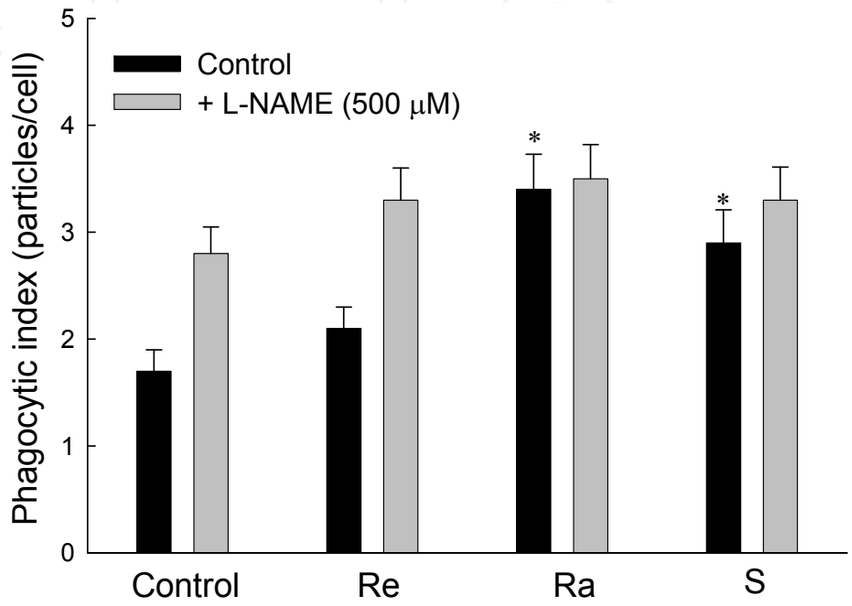
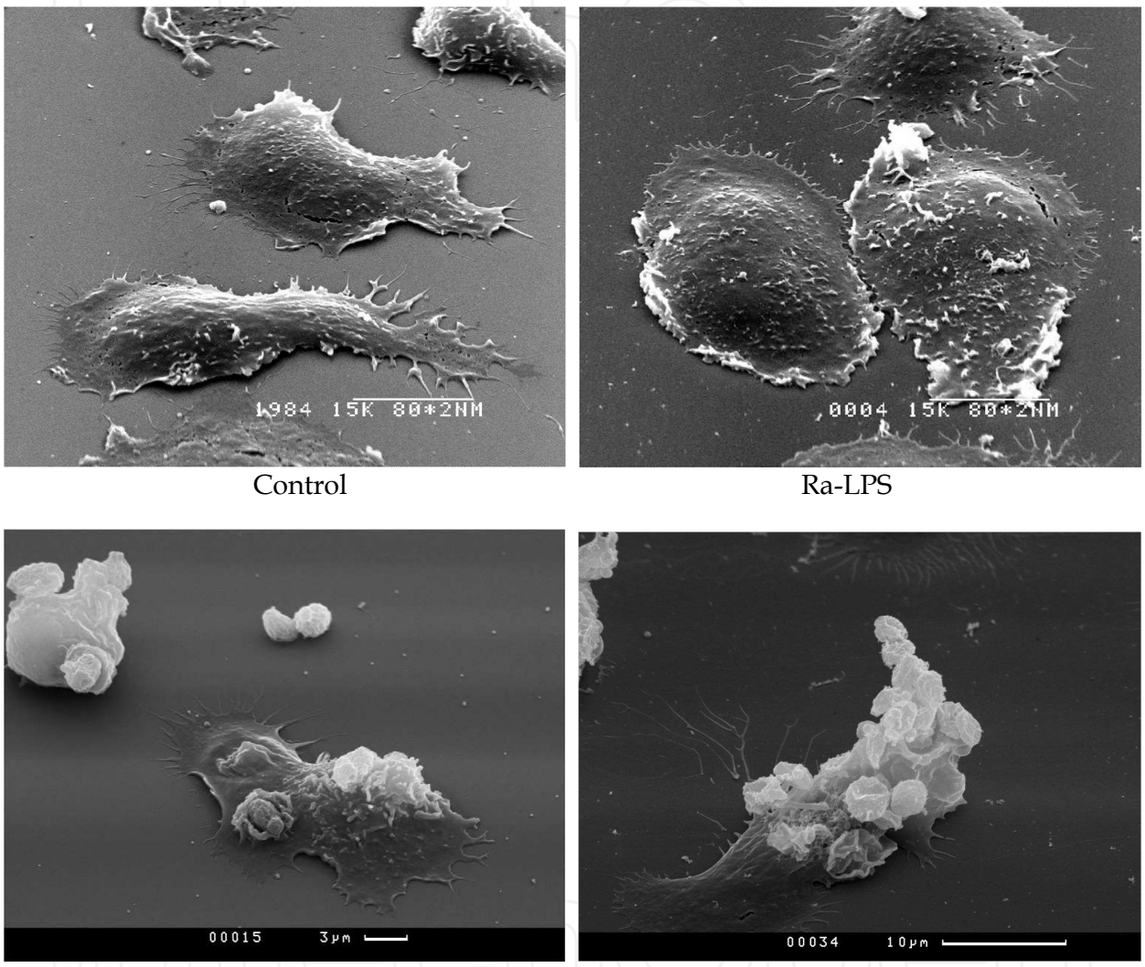


Fig. 7. Effect of LPS chemotypes on OZ uptake by PMNL. Phagocytic index was assessed by light microscopy 5 min after OZ addition to PMNLs, pretreated for 30 min at 37 °C without (control) or with 1 μg/ml of different LPS species and 500 μM L-NAME. * $P < 0.05$ vs corresponding control.

The phagocytosis of zymosan is a good experimental model to study leukotriene synthesis in PMNLs. Leukotrienes constitutes a family of inflammatory mediators, being formed in PMNL during phagocytosis of bacteria or zymosan particles. The opsonization of zymosan with normal serum resulted in enhanced activation of LT synthesis in PMNLs (Fig.9). LPS-priming of neutrophils further increased LT synthesis, with the effect increasing in the order Re < S < Ra LPS, as we published recently (Zagryazhskaya, et al., 2010). 100 μM and 500 μM L-NAME decreased the specificity of LPS chemotypes (Zagryazhskaya, et al., 2010).

In contrast, 10 μM diphenileleiodonium chloride (DPI) emphasized the effects of Re and S LPS (Fig 10). The antioxidant agent diphenileleiodonium inhibits NADPH oxidase-mediated ROS formation, and also inhibits other flavo-enzymes such as NO synthase and xanthine oxidase (Wind et al., 2010). In our experiments, inhibition of NO release attenuated the specificity of LPS chemotypes, but when we simultaneously inhibited NO and ROS formation, the chemotypes demonstrated the most prominent variation of their effects on LT synthesis (Fig.10). We suggest that LT synthesis is regulated by various LPS chemotypes via multiple mechanisms, and peroxynitrite is involved in this regulation. The specificity of LPS species is mainly dependent on nitric oxide generation induced by LPS. The published data concerning NO interaction with 5-LO admit various interpretations however, the most

recent findings support the inhibitory effect of NO on 5-LO synthetic capacity (Coffey et al., 2000). Furthermore, we revealed that minimal NO synthesis facilitated OZ uptake, adhesion, LT and O2- production, as it was observed in the cells primed with Ra LPS. As soon as we inhibited NOS with L-NAME, the other LPS forms, Re and S chemotypes, exhibited comparable capacity to stimulate OZ uptake, LT and O2- production.



Control Ra-LPS

Control (+OZ) Ra-LPS (+OZ)

Fig. 8. Scanning electron microscopy of PMNLs untreated (control) or treated with 1 µg/ml Ra-LPS for 30 min (Ra-LPS). OZ uptake in untreated PMNLs (Control + OZ) and PMNLs exposed to Ra-LPS chemotype (Ra-LPS + OZ).

NO can inhibit 5-lipoxygenase directly (Coffey, et al., 2000) and via activation of soluble guanilate cyclase (Coffey, et al., 2008). Peroxynitrite (PN), formed by NO and superoxide, can cause inhibition of 5-LO (Coffey, et al., 2001), as well as 5-LO activation by increasing 'peroxide tone' of the cell (Goodwin et al., 1999; Ullrich & Kissner, 2006). Complex interplay between NO, superoxide and PN is obviously involved in fine regulation of LT synthesis. When we inhibited both NO and PN in incubations with DPI, we revealed huge activation of LT synthesis in Ra- and S- LPS primed cells (Fig 10).

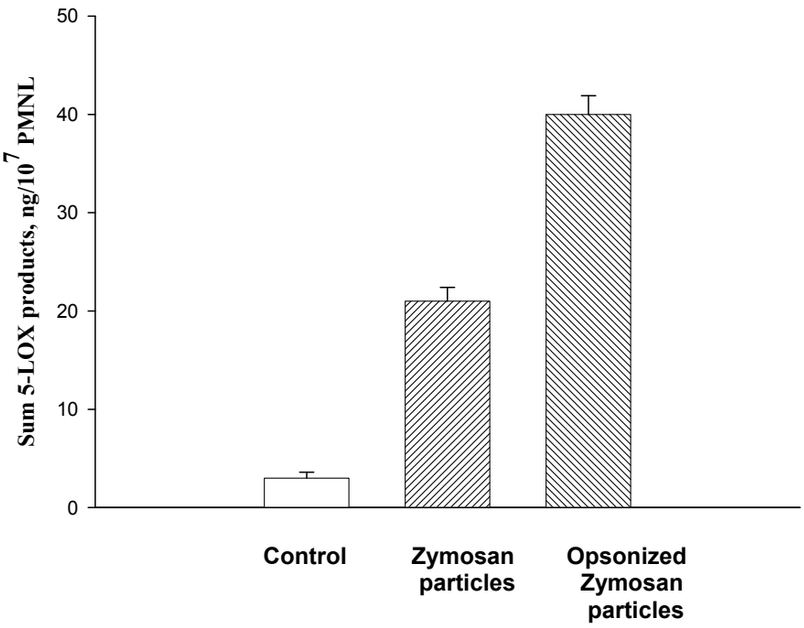


Fig. 9. Opsonized zymosan (OZ) activates LT synthesis in PMNLs. PMNLs suspension (2 × 10^7 cells) was incubated at 37 °C for 30 min, then zymosan particles and opsonized zymosan particles were added where indicated for 20 min. Products of the 5-LO pathway were determined by HPLC, as described in Materials in Methods.

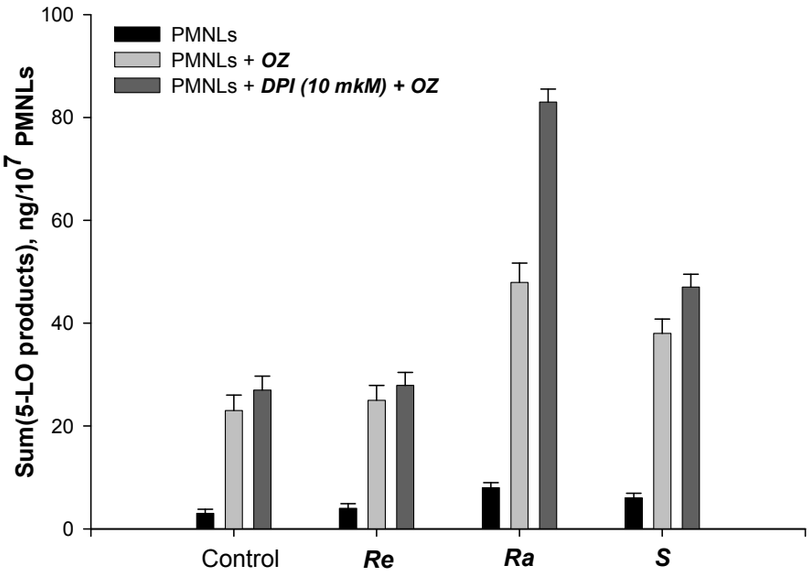


Fig. 10. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes. PMNLs (2 × 10^7) were treated for 30 min at 37 °C without (control) or with 5 μg/ml of various LPS forms and 10 μM diphenileleiodonium chloride (DPI), and then stimulated for 30 min with 2 mg/ml OZ.

Red blood cells are known to consume NO (Romero et al., 2006). In the vascular space, where phagocytes are relatively rare, particles that have been opsonized by complement are

immobilized to the surface of red blood cells for further clearance by phagocytes (Pilsczek et al., 2005). When we added RBC in incubations with neutrophils, we found higher effects of LPS on LT synthesis in the presence of red blood cells. This effect was observed in PMNL interaction with OZ (Fig.11) and with opsonized bacteria (Fig.12). Establishing which mechanisms of NOS and NADPH-oxidase activation and signaling are essential for phagocytosis and 5-LO activation is the next objective.

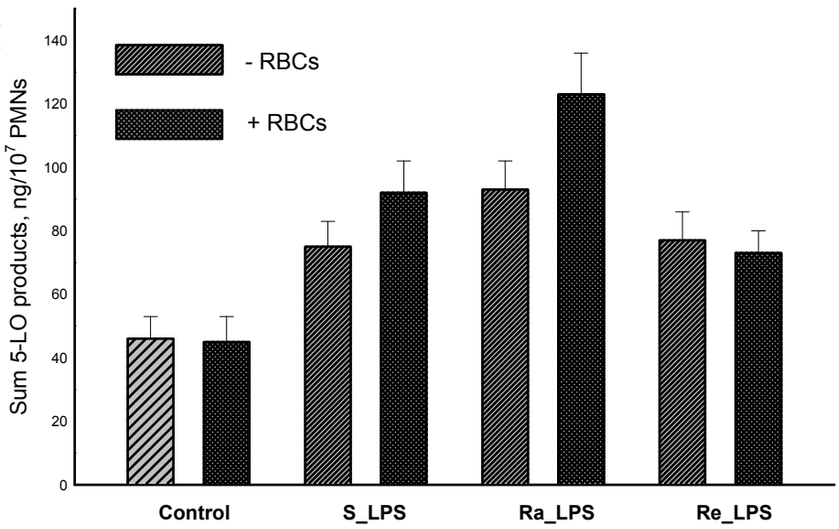


Fig. 11. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes in the presence of red blood cells (RBC). PMNLs (2×10^7) without or with RBC (5×10^7) were preincubated for 30 min without (control) or with 5µg/ml LPS, then stimulated for 20 min with 2mg/ml OZ.

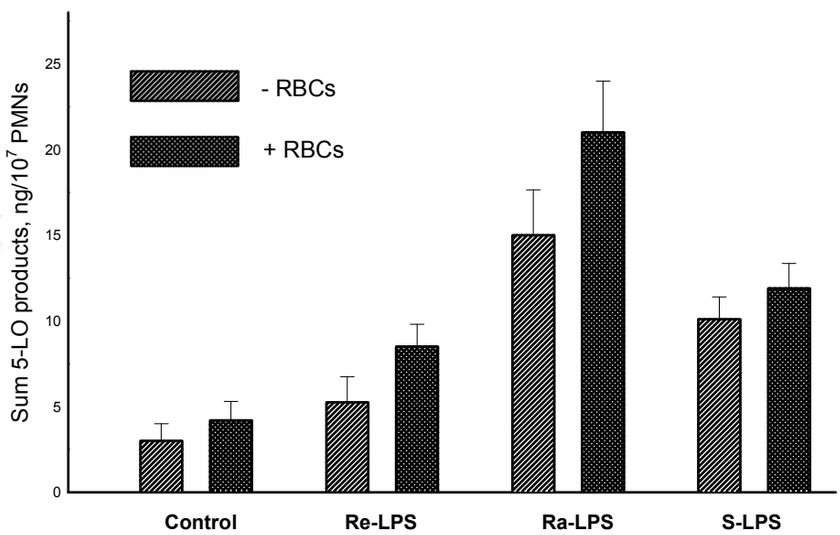


Fig. 12. 5-LO products synthesis in PMNLs is selectively regulated by LPS chemotypes in the presence of red blood cells (RBC). PMNLs (2×10^7) without or with RBC (5×10^7) were preincubated for 30 min without (control) or with 5µg/ml LPS, then stimulated for 20 min with $3x10^8$ OS (opsonized *S. Typhimurium* cells).

4. Conclusion

The regulation of neutrophil adherence, phagocytosis and leukotriene synthesis by various LPS chemotypes from S. Typhimurium has received little attention in scientific literature, which prompted us to this study. We presented data on the regulation of neutrophil cellular responses by three LPS species from *Salmonella enterica* serovar *typhimurium* with different increasing chain lengths, namely Re mutant SL 1181 (lipid A + 2 KDO residues), Ra mutant TV 119 (comprising lipid A and complete core) and S - form smooth LPS which possesses all three main components of endotoxin structure (lipid A, core and O-antigen). Our investigation supports the hypothesis that NO plays a crucial role in regulation of LPS-induced phagocytosis and leukotriene synthesis in neutrophils. High levels of endogenous NO inhibit 5-LO activity and leukotriene synthesis, and erythrocytes constitute an important 'sink' for NO and its product peroxynitrite. When excess NO is consumed by red blood cells, we found distinct and significant priming of neutrophils by LPS chemotypes. We conclude that LPS and red blood cells mediate activation of leukotriene synthesis in PMNL using NO release as intra- and intercellular regulatory mechanism. These data with LPS chemotypes contribute to the understanding of the basic factors involved in the regulation of neutrophil responses to LPS.

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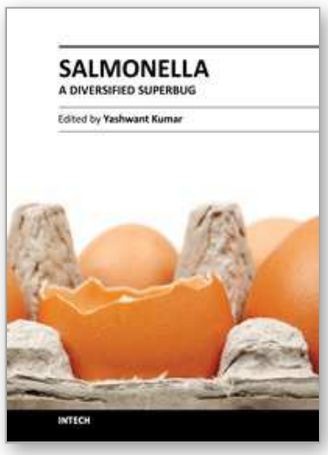

Salmonella - A Diversified Superbug

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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