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**Influence of the lipopolysaccharide structure of *Salmonella enterica* serovar Enteritidis
on interactions with pig neutrophils**

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Abstract

The key process for immune response development is the recognition of bacteria by the immune system of the host based on the sensing of pathogen-associated molecular patterns (PAMP). One of the most important PAMP is the lipopolysaccharide (LPS) molecule, a complex molecule present in the outer membrane of Gram negative bacteria. In this study we were interested in how different parts of the LPS of *Salmonella enterica* serovar Enteritidis are recognized by porcine neutrophils. To this aim, we constructed *S. Enteritidis* mutants with

rfaL and *rfaC* genes disabled in the attachment of the O-antigen and in the synthesis of the inner oligosaccharide core of LPS, respectively. We found that in the absence of serum, both the *rfa* mutants associated with neutrophils and stimulated them for reactive oxygen species (ROS) production significantly more than the wild-type strain. Addition of polymyxin B, which neutralized lipid A, the endotoxic moiety of LPS, effectively decreased the association of the wild-type strain and the *rfaC* mutant with neutrophils, but not the *rfaL* mutant. This indicates that the oligosaccharide core newly exposed on the surface in the *rfaL* mutant, protected from interaction in the wild-type strain by the O-antigen but completely absent in the *rfaC* mutant, may represent a new ligand for porcine neutrophils that cannot be neutralized by polymyxin B.

Keywords

Salmonella, pig, pathogen-associated molecular patterns, lipopolysaccharide, neutrophil

Introduction

After ingestion by susceptible host, *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) multiplies in gut lumen and invades intestinal epithelial cell which start to produce cytokines and chemokines (e.g. IL-8 or TNF α). The cytokine signaling then serve as a signal for recruitment of innate immune system cells, neutrophils in particular, to the site of infection (McCormick et al., 1993; Scharek and Tedin 2007). During primary infection, i.e. in the absence of specific antibodies, immune system cells interact with *S. Enteritidis* by two mutually complementary ways. The first one is based on binding of complement opsonized bacteria by complement receptors present on a surface of professional phagocytes and is aimed primarily to pathogen uptake and inactivation (Underhill and Ozinsky 2002). The second one is focused on a more detailed perception and identification of a pathogen and is

based on the recognition of structural motifs common to many pathogens, i.e. pathogen-associated molecular patterns (PAMP). In the case of *S. Enteritidis*, the most important PAMPs include flagellin recognized by Toll-like receptor (TLR) 5 and lipopolysaccharide (LPS) which is recognized by TLR4 receptor (Gerold et al., 2007).

LPS of Gram- bacteria consists of lipid A, KDO domain, oligosaccharide core and O-antigen (Rickles and Rick 1977). Full sized LPS is synthesized in a step by step manner, addition of each new part of growing LPS being dependent on a particular enzyme, and it is therefore possible to generate single gene mutants which produce LPS of different complexity. Despite the complex nature of LPS, till now, out of the whole LPS molecule only the lipid A has been identified as a TLR4 ligand (Bäckhead et al. 2003, Jerala 2007). The interaction of LPS with TLR4 can be neutralized by antimicrobial peptides present in blood serum, or by polymyxin B, a cationic antimicrobial peptide structurally and functionally similar to those in blood serum (Shimazu et al., 1999, Cruz et al. 2007).

Rough mutants, i.e. the mutants with incomplete LPS can grow under optimal conditions but are affected in the survival under the non-favorable conditions indicating a passive barrier function of distal parts of LPS. When we previously tested *rfaC* and *rfaL* mutants of *S. Enteritidis in vivo* (Karasova et al. 2009), we considered a hypothesis that the unusual heptoses and octoses present in the oligosaccharide core of LPS may serve as a ligand for either the association or recognition by receptors present in the cells of host immune system. If so, these interactions could be detected by experiments with mutants defective in different steps of LPS biosynthesis performed in the presence and absence of polymyxin B. Polymyxin B should neutralize lipid A-TLR4 interaction and the residual interactions would be a function of the remaining parts of LPS present in the mutants. In this study we therefore

tested a mutant with inactivated *rfaL* gene which was unable to attach O-antigen to the core LPS and a mutant with inactivated *rfaC* gene which expressed LPS which consisting of only the lipid A and KDO domain, together with the wild strain for their ability to associate with porcine neutrophils *in vitro* and stimulate them for ROS production. We found that both the *rfa* mutants associated with porcine neutrophils more and stimulated them for a higher ROS production than did the wild type *S. Enteritidis*. Interestingly, polymyxin B treatment decreased the interaction of the *rfaC* mutant with porcine neutrophils to a greater extent than the interaction of the *rfaL* mutant. This indicates, that the oligosaccharide core newly exposed on the surface of the *rfaL* mutant after the removal of O-antigen, but protected from any interaction by the O-antigen in the wild type strain or completely absent in the *rfaC* mutant, may represent a new ligand for porcine neutrophils which cannot be neutralized by polymyxin B.

Material and methods

S. Enteritidis 147 (wild-type strain) was used in this study (Methner et al., 2004). The mutations in the *rfaL* and *rfaC* genes were generated by a one-step PCR inactivation method as described previously (Karasova et al., 2009). All of the strains were transformed with the pFPV25.1 plasmid constitutively expressing green fluorescent protein (GFP). The strains were grown for 16 h in Luria Bertani (LB) media, then washed twice with Dulbecco's phosphate buffered saline (DPBS, Cambrex, IA) and stored in PBS supplemented with 10% glycerol at -70 °C until use. The bacterial concentration was determined both spectrophotometrically and by serial dilutions and plating on LB agar followed by colony counting. The integrity of bacterial cell membranes before and during the experiments was measured by propidium iodide staining followed by flow cytometry.

Peripheral blood was taken from the *vena jugularis* of seven three-month-old pigs. White blood cells (WBC) were isolated independently by hypotonic lysis of the erythrocytes as follows: 5 ml of heparinized blood was mixed with 40 ml of apyrogenic distilled water for 20 s and then the osmotic pressure was normalized with 5 ml of ten times concentrated DPBS. The WBC were washed twice with 1× DPBS and finally resuspended in Hank's balanced salt solution (HBSS, Cambrex, IA) at a concentration of 10^7 per ml.

One million WBC from each pig were separately incubated at 37 °C in 100 µl of HBSS with 10^7 (multiplicity of infection of 10) GFP-expressing wild-type strain or *rfaC* and *rfaL* mutants under serum-free conditions (Underhill and Ozinsky, 2002; Blander and Medzhitov, 2004). In parallel, for blocking lipid A signalling, the WBC were treated for 30 min before and during the infection with all strains (Tohno et al. 2007) by polymyxin B (Sigma Aldrich, Germany) at a concentration of 20 µg/ml.

For the flow cytometry analysis, the cells were resuspended in PBS containing 0.2% gelatine from cold water fish skin (Sigma Aldrich, Germany), 0.1% NaN₃ and 0.63 mM EDTA (Sigma Aldrich, Germany). Neutrophils were gated based on forward and side scatter characteristics (R1, Fig. 1) and GFP positivity was used for the detection of *S. Enteritidis*-positive neutrophils (R2, Fig. 1). The associations were measured at 30 min intervals during the 3 h incubation. Flow cytometry was performed with a FACSCalibur operated by CELLQuest software (BD Biosciences, MD).

The release of reactive oxygen species (ROS) was measured by 8-amino-5-chloro-7-phenylpyridopyridazine (L-012, Wako Pure Chemical Industries, Ltd., Osaka, Japan)-enhanced luminometry. Samples were run in triplicate with a 20 µM concentration of L-012

using a multi-detection microplate reader Synergy™ 2 (BioTek Instruments, VT). Neutrophils were stimulated with opsonized zymosan particles (InvivoGen, CA) as a positive control.

In all of the figures, the mean $\pm$ standard errors are shown. The data obtained were tested for normality by the Kolmogorov-Smirnov test and the homogeneity of the variance among the *Salmonella* strains and treatments was compared by the F ratio test. The significance (P) of the differences between sampling time points for a given strain or treatment was tested by one-way ANOVA followed by Tukey's post hoc tests for comparisons among given time points. The repeated measures model of the two-way analysis of variance (rmANOVA) was used to analyse the influence of *Salmonella* strains, treatments and time on the neutrophil-*Salmonella* association and ROS production followed by Tukey's post hoc tests for the mutual comparison of *Salmonella* types within the given single time point of the follow-up. Correlations between neutrophil-*Salmonella* associations and ROS production were assessed using Pearson's correlation. Differences with a value of $P < 0.05$ were considered statistically significant. Statistica software (StatSoft, Inc., OK) was used for data analyses.

Results

First, we were interested in the time-dependent associations of all *S. Enteritidis* strains used with the neutrophils (Fig. 2). The wild-type strain association gradually increased over the 3 h period. When compared with time 0, significantly more wild-type strain associated neutrophils were recorded after 60 minutes of incubation (Fig. 2 Tab. A). At the end of the experiment, 22% of the neutrophils were associated with the wild-type strain. Mutant *rfaC* exhibited significantly higher associations as early as 30 minutes after the beginning of the

experiment, but *rfaL*, similarly to wild-type strain, at 60 minutes after the beginning of the experiment. In the case of the *rfaC* mutant, the peak of the association was at 60 minutes, when it reached 49% and then remained at the same level (there were no significant differences between time points 30-180 minutes). In the case of the *rfaL* mutant, the associations continuously increased over the 3 hours period, as confirmed by significantly different associations at time points 30 and 180 minutes. At the end of the experiment, 58% of the neutrophils were associated with *rfaL* mutant. The addition of polymyxin B significantly decreased the associations of wild-type *S. Enteritidis* and the *rfaC* mutant with porcine neutrophils when compared to the conditions without polymyxin B, but it had no effect on the association of the *rfaL* mutant with the neutrophils (Fig. 2 Tab. B).

In order to investigate not only the ability of the strains used to bind to neutrophils but also their competence in stimulating them, in the next experiment we determined ROS production by luminometry (Fig. 3). The ROS production of neutrophils stimulated by the wild-type strain gradually increased during the first 150 minutes, although when compared with time 0, significantly higher ROS production was recorded after 90 minutes of incubation (Fig. 3 Tab. A). The *rfaL* and *rfaC* mutants stimulated ROS production more rapidly and to a greater extent than the wild-type strain, both stimulating significantly higher ROS production as early as 30 minutes after the beginning of the experiment when compared with time 0. The maximum ROS production stimulated by both *rfa* mutants was observed 60 minutes after infection, followed by a slow decrease in time points 120-180 minutes for the *rfaL* mutant. In the case of the *rfaC* mutant, no change after time point 60 minutes was observed. The addition of polymyxin B decreased the global ROS production for all strains (Fig. 3 Tab. B), although this decrease was more significant for the wild-type strain and the *rfaC* mutant than for the *rfaL* mutant. Moreover, when the effect of polymyxin B was determined in individual

measurement time points, out of six measurements (not considering time 0), polymyxin B significantly decreased the ROS production stimulated by the wild-type and the *rfaC* mutant four and five times, respectively. However, in the case of the *rfaL*-stimulated ROS production, this was significantly reduced only once, i.e. at 60 minutes after the beginning of the experiment.

When analysing the correlations between the neutrophil-*Salmonella* association and ROS production, a significantly positive correlation was observed for the wild-type strain and the *rfaC* mutant, both with and without polymyxin B (Tab. 1). On the other hand, the same correlation was insignificant for the *rfaL* mutant, regardless of the presence or absence of polymyxin B (Tab. 1). This was most likely caused by maximal ROS production stimulated by the *rfaL* mutant 60 minutes after the beginning of the experiment, whereas the association continuously increased throughout the whole experiment.

Discussion

In this study, we were interested in the interactions between porcine neutrophils and *S. Enteritidis* mutants defective in the synthesis of full sized LPS. Since we were interested in the recognition of *S. Enteritidis* antigens independent of Fc or complement receptors present in the plasma membrane of neutrophils, the study was performed in the absence of serum. Such conditions have been already used for the identification of neutrophil receptors that recognize the surface structures of bacteria (Underhill and Ozinsky, 2002; Blander and Medzhitov, 2004).

The complete structure of LPS present in the wild-type strain acted as an inhibitor of the neutrophil-*Salmonella* association since removal of the O-antigen in the *rfaL* mutant and the

oligosaccharide core in the *rfaC* mutant led to a more intensive association. This masking effect of the O-antigen has also been documented in *Escherichia coli* (Bäckhead et al., 2003), and the ability of neutrophils to bind *S. Enteritidis* was negatively correlated with the complexity of LPS. The dynamic of ROS production was very slow for the wild-type strain but much faster for both mutants, similar to human neutrophils infected with the *S. Typhimurium* 395 MR10 strain (Lock and Dahlgren, 1988). Maximal ROS production was observed at 60 minutes after the interaction of both *rfa* mutants with the neutrophils. Although we do not have any explanation for this observation, we can exclude that maximal ROS production was achieved by the neutrophils since when the opsonized zymosan particles were used for neutrophil stimulation, ROS production ten times higher than that induced by the *rfa* mutants was recorded (data not shown).

Although significantly different associations and ROS induction profiles were observed for the wild-type strain and the *rfaC* mutant, when we analysed the correlation between these two factors, a positive and significant correlation was observed for these two strains. On the other hand, in the case of the *rfaL* mutant, the correlation between neutrophil association and ROS production appeared to be insignificant, indicating a difference in the interactions between the two *rfa* mutants and porcine neutrophils. This difference became even more apparent when the experiments were performed in the presence of polymyxin B. Polymyxin B had a lower effect on both the association and the ROS production by porcine neutrophils induced by the *rfaL* mutant than by the *rfaC* mutant. This indicated that the interaction of the *rfaL* mutant with porcine neutrophils was less dependent on lipid A, and therefore a new ligand absent in the wild-type strain and the *rfaC* mutant had appeared in the *rfaL* mutant. The likely candidates are the core oligosaccharides containing unusual heptose residues. These sugar residues were protected by the O-antigen in the wild-type strain and were absent in the *rfaC*

mutant. We also considered that completely new ligands not related to the LPS structure could appear in the *rfaL* mutant, but similar interactions of human monocytes were described with purified LPS isolated from wild-type *S. Typhimurium* and mutants similar to *rfaC* and *rfaL* in the presence of polymyxin B (Rickles and Rick, 1977). Based on our and Rickles and Rick (1977) observations, it is probable that new PAMP was really represented by the core oligosaccharides of the LPS, rather than by the unmasking of new structures independent of LPS.

Conclusion

When compared with the wild-type *S. Enteritidis*, the *rfaC* and *rfaL* mutants exhibited increased associations with porcine neutrophils and stimulated them for a higher production of reactive oxygen species. Polymyxin B treatment decreased the interactions of the wild-type strain and the *rfaC* mutant with neutrophils more effectively than in the case of *rfaL* mutant, thus showing a lower influence of lipid A recognition on *rfaL* mutant interactions with porcine neutrophils.

Conflict of interest statement

The authors have no financial or personal relationships with other people or organizations that could have inappropriately influenced our work.

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291 Table

292

293 Table 1. Correlations between the neutrophil-*Salmonella* associations and ROS production of
294 the wild-type *S. Enteritidis* (wt), *rfaC* and *rfaL* mutant strains in the absence and presence of
295 polymyxin B (PmxB). Significant correlations and corresponding P values are in bold.

296 Figure captions

297

298 Figure 1. Gating strategy and determination of GFP positivity. R1: gating for neutrophils, R2:
299 gating for GFP positivity of all neutrophils in R1.

300

301 Figure 2. Association of *S. Enteritidis* and its *rfaC* and *rfaL* mutants with the neutrophils and
302 the influence of polymyxin B treatment during 3 h incubation. Circles – wild-type strain,
303 squares – *rfaC* mutant, triangles – *rfaL* mutant, open symbols – interaction without
304 polymyxin B treatment, closed symbols – interactions in the presence of polymyxin B. Time
305 points are separated by vertical dashed lines.

306 A: Table of significance levels of the differences between sampling time points tested by one-
307 way ANOVA; significant levels ($P < 0.05$) are in bold.

308 ¹ Global difference between sampling time points

309 ² Test of differences between sampling times (Tukey's post hoc test): significantly different
310 groups ($P < 0.05$) are marked by different letters ("a" differ from "bc", but not "ab", etc.).

311 B: Table of significance levels of the F tests of repeated measures two-way ANOVA
312 incorporating time and *Salmonella* strains and treatments as the main factors, significant
313 levels ($P < 0.05$) are in bold.

314 ¹ Significance level (P) of F tests for *Salmonella* strains and treatment factors; all significance
315 levels (P) of the F tests for the time factor and interactions were lower than 0.001 and are not
316 shown in the table.

317 ² Tests of differences between *Salmonella* strains and treatments performed separately within
318 each sampling time point (Tukey's post hoc tests).

319

320 Figure 3. Kinetics of ROS production and the influence of polymyxin B treatment during 3 h

321 incubation. Circles – wild type strain, squares – *rfaC* mutant, triangles – *rfaL* mutant, open
 322 symbols – interaction without polymyxin B treatment, closed symbols – interactions in the
 323 presence of polymyxin B. Time points are separated by vertical dashed lines.

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 325 way ANOVA, significant levels ($P < 0.05$) are in bold.

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329 B: Table of significance levels (P) of F tests of repeated measures two-way ANOVA
 330 incorporating time, *Salmonella* strains and treatments as the main factors; significant levels (P
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332 ¹ Significance level (P) of F tests for *Salmonella* strains and treatment factors; all significance
 333 levels of the F tests for time factor and interactions were lower than 0.001 and are not shown
 334 in the table.

335 ² Tests of differences between *Salmonella* strains and treatments performed separately within
 336 each sampling time point (Tukey's post hoc tests).

	Pearson r	P value (two-tailed)
wt	0.965	0.000
wtPmxB	0.855	0.014
<i>rfaC</i>	0.939	0.002
<i>rfaCPmxB</i>	0.782	0.038
<i>rfaL</i>	0.050	0.916
<i>rfaLPmxB</i>	0.171	0.714

Figure1

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Figure2
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A

Strain	One-way global difference ¹	Time point differences (min) ²						
		0	30	60	90	120	150	180
wt	<0.001	a	ab	bc	cd	d	e	e
rfaC	<0.001	a	b	b	b	b	b	b
rfaL	<0.001	a	ae	be	bce	bcd	cd	d
wtPmxB	<0.001	a	a	ab	abc	abc	bc	c
rfaCPmxB	0.718							
rfaLPmxB	<0.001	a	ab	b	b	b	b	b

B

Strains and treatment	Two-way global difference ¹	Time point differences (min) ²						
		0	30	60	90	120	150	180
wt vs. rfaC	<0.001	1.000	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
wt vs. rfaL	<0.001	1.000	0.060	0.002	0.001	0.001	<0.001	<0.001
rfaC vs. rfaL	0.453							
wt vs. wtPmxB	<0.001	1.000	0.970	0.065	<0.001	<0.001	<0.001	<0.001
rfaC vs. rfaCPmxB	<0.001	1.000	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
rfaL vs. rfaLPmxB	0.247							

Figure3
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A

Strain	One-way global difference ¹	Time point differences (min) ²						
		0	30	60	90	120	150	180
wt	<0.001	a	ac	acd	bcd	bd	b	b
rfaC	<0.001	a	b	b	b	b	b	b
rfaL	<0.001	a	c	c	bc	b	ab	ab
wtPmxB	<0.001	a	b	b	ab	ab	b	b
rfaCPmxB	<0.001	a	c	bc	ab	ab	ab	ab
rfaLPmxB	<0.001	a	c	c	b	ab	ab	ab

B

Strains and treatment	Two-way global difference ¹	Time point differences (min) ²						
		0	30	60	90	120	150	180
wt vs. rfaC	<0.001	1.000	<0.001	<0.001	<0.001	0.001	0.009	0.089
wt vs. rfaL	<0.001	1.000	<0.001	<0.001	<0.001	0.042	0.316	0.835
rfaC vs. rfaL	0.043	1.000	0.003	0.002	0.565	1.000	1.000	1.000
wt vs. wtPmxB	<0.001	1.000	1.000	0.370	0.001	<0.001	<0.001	<0.001
rfaC vs. rfaCPmxB	0.001	1.000	0.881	0.004	0.001	0.003	0.006	0.038
rfaL vs. rfaLPmxB	0.030	1.000	0.922	0.015	0.089	0.571	0.888	0.992