Plasma lipopolysaccharide level and enterocyte brush border enzymes in gnotobiotic piglets infected with *Salmonella typhimurium*

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ABSTRACT: Gnotobiotic piglets were orally infected either with the virulent LT2 strain or the non-pathogenic SF1591 rough mutant of *Salmonella enterica* serotype Typhimurium. They were sacrificed 6 or 24 h after the infection. All piglets infected for 24 h developed systemic infection with an increase of plasma lipopolysaccharide. Infection with the virulent strain caused a significant decrease ($P < 0.001$) of gamma-glutamyl transpeptidase (GGT) activity in the enterocyte brush border of both the jejunum and ileum, infection with the rough mutant caused a decrease of GGT activity in the ileum only. The activities of other brush border enzymes (lactase, sucrase, glucoamylase, alkaline phosphatase and dipeptidylpeptidase IV) did not change significantly after infection.

Keywords: swine; gnotobiotic piglet; *Salmonella typhimurium*

Infections with *Salmonella* are a serious medical and veterinary problem. Enteritis is the most common form of salmonellosis. Ingested bacteria penetrate through the intestinal epithelium and invade host cells, especially macrophages. If local immune mechanisms are insufficient to limit infection to intestinal mucosae and Peyer's patches, infection can spread systemically to mesenteric lymph nodes and other organs.

Host resistance to *Salmonella* relies initially on the production of inflammatory cytokines (reviewed by Trebichavský, 1999; Eckmann and Kagnoff, 2001; Mastroeni, 2002). We have recently described a cytokine response of gnotobiotic piglets to oral *Salmonella* infection (Šplíchal *et al.*, 2002). Bacterial lipopolysaccharide represents one of the most potent inducers of inflammatory cytokines.

Gnotobiotic piglets with defined gut microflora are a unique animal model for studies of host interactions with enteric pathogens (Barrow *et al.*, 2001). It is known that enterocyte response to chemokine is a prerequisite for a fast migration of neutrophils that protect gut mucosae infected with *Salmonella* (Santos *et al.*, 2002). In the course of infection with virulent *Salmonella typhimurium*, enterocyte membrane is damaged by the lipid peroxidation leading to a loss of cell viability (Mehta *et al.*, 1998a). Thus, enzyme activity of enterocytes could extend our understanding of one of the pathological mechanisms that occur in the gut epithelium infected with *Salmonella*.

Brush border enzyme activities are an important characteristic of normal intestinal epithelial function. The development of digestive enzymes may be influenced by factors of external environment, mainly by bacterial association. To our knowledge, enzymes of *Salmonella* infected enterocytes have not been studied to a significant extent. In this study, we have used gnotobiotic piglets for measuring of plasma lipopolysaccharide and activities of specific

Supported by the Grant Agency of the Czech Republic (Grant No. 524/01/0917), by the Grant Agency of the Academy of Sciences of the Czech Republic (Grant No. A5020101) and by Institutional Research Concept No. AVOZ5020903.

enterocyte brush border enzyme activities after infection with *S. typhimurium.*

MATERIAL AND METHODS

Animals

Thirty six germ-free piglets of miniature Minnesota-derived breed were delivered by hysterectomy, held in sterile isolators and fed autoclave-sterilised milk used for feeding gnotobiotic piglets (Mandel, 1997). Twenty six piglets were infected in the age of one week either with virulent LT2 strain of *Salmonella* Typhimurium or with the SF1591 rough mutant of *Salmonella* Typhimurium and were kept in fibreglass isolators for next 6 hours (six piglets infected with LT2 strain and six piglets infected with SF1591 mutant) or 24 hours (eight piglets infected with LT2 strain and six piglets infected with SF1591 mutant). Ten piglets served as germ-free non-infected controls.

Bacterial strains, culture media and growth conditions

Bacteria were described previously (Dlabač *et al.*, 1997). Briefly, the LT2 strain was highly virulent for germ-free piglets but of a low virulence for conventional piglets. The SF1591 was a stable rough mutant of Ra chemotype with a deletion in the His locus and with complete polysaccharide content of lipopolysaccharide (courtesy of Dr. O. Lüderitz, Max-Planck Institute for Immunobiology, Freiburg in Breisgau, Germany). The latter microorganism was non-pathogenic but highly penetrate in germ-free piglets and protective against subsequent infection of piglets with virulent LT2 strain.

Bacteria were freshly prepared (24 h at 37°C) on meat-peptone agar (the base for the blood agar No. 2, Immuna, Šarišské Michaľany, Slovakia) and diluted in PBS. The optical density of bacterial suspension was measured at 550 nm and the dose was calculated from a calibration curve. Piglets were infected by nipple-feeding with milk diet containing the bacteria at a dose of 10^8 CFU per piglet. Animals were sacrificed by exsanguination under halothane anaesthesia 6 or 24 hours after oral infection. Experiments were approved by the Ethical Committee of the Institute according to the rules of the Animal Act.

Plasma collection

Peripheral blood was collected in a syringe with sodium citrate as an anticoagulant. Blood samples were centrifuged at 800 g and 4°C for 10 min. All plasma samples were immediately frozen and kept at –70°C until use.

Plasma levels of bacterial lipopolysaccharide

Plasma LPS was measured in all experimental piglets by a Kinetic-QCL limulus amebocyte lysate kit (Bio Whittaker, Walkersville, MD, USA). The Kinetic-QCL Reader with Kinetic-QCL Software, Kinetic-QCL Reagent, LAL Reagent Water, *E. coli* O55:B5 endotoxin standards and disposable endotoxin-free dilution tubes were used according to manufacturer's instructions.

Preparation of enterocyte brush-border membrane vesicles (BBMV)

Brush-border membranes were prepared from small bowel scrapings of 24 gnotobiotic piglets (ten germ-free piglets and fourteen piglets infected for 24 hours) essentially according to Kessler *et al.* (1978). Briefly, the jejunum and the ileum were flushed with ice-cold saline and the mucosal layer was gently scraped off. BBMV were obtained by calcium precipitation in the cold for 20 min using solid $CaCl_2$ added to the homogenate (1:100 in 50 mmol/l mannitol, 2 mmol/l TRIS) in a final concentration of 10 mmol/l. The homogenate was left to stand in the cold (4°C) for 20 min and then centrifuged (2 000 g, 15 min) to spin down cell organelles. The supernatant was centrifuged (100 000 g, 1 h, 4°C) and the pellet was resuspended in 0.5% Zwittergent 3–14 detergent (Calbiochem, USA) in 10 mmol/l KCl. After membrane solubilization and centrifugation (100 000 g, 30 min, 4°C), the supernatant was used for further examination.

Enzyme activity measurements

Following brush border enzymes were chosen as representative members of two groups – digestive enzymes and peptidases that are very sensitive to bacterial association. Enzyme activities were

determined as previously described (Kozáková *et al.*, 2001). Briefly, **lactase** (EC 3.2.1.23/62/108), **sucrase** (EC 3.2.1.48/10) and **glucoamylase** (EC 3.2.1.20/3) activities were determined with 50 mmol/l lactose (Kraml *et al.*, 1972) and 50 mmol/l sucrose or 12 g/l starch as substrates (Kolínská and Kraml, 1972), respectively. Alkaline phosphatase (EC 3.1.3.1) activity was determined with 0.1 mol/l 4-nitrophenyl phosphate as substrate in Tris-HCl buffer pH 8.5. Liberated 4-nitrophenol was measured at 405 nm using the TestKit (Lachema, Czech Republic). The activity of **dipeptidyl peptidase IV** (DPP IV, EC 3.4.14.5) was determined with 1.4 mmol/l glycyl-L-proline 4-nitroanilide (Sigma) in 66 mmol/l Tris-HCl buffer pH 8. The reaction was stopped with 1 mol/l sodium acetate buffer pH 4.2 and the released 4-nitroanilide (donor substrate) was measured at 405 nm (Nagatsu *et al.*, 1976).

The activity of **γ-glutamyl transpeptidase** (GGT, EC 2.3.2.2) was determined with 7 mmol/l 5-L glutamic acid 4-nitroanilide (donor substrate), 0.1 mol/l glycylglycine pH 8.2 (acceptor substrate) and 0.1 mol/l NaCl (activator). Released 4-nitroaniline was measured at 405 nm (Thompson and Meister, 1976). Enzyme activities were expressed in nkat/mg protein.

Total protein concentration in BBMV was determined according to Lowry using bovine serum albumin (Serva, Germany) as standard.

Statistical analysis

The results were expressed as means ± SEM. Statistical analysis was performed using SigmaStat (Jandel Corporation). Multiple-comparison procedures were made by One-Way Analysis of Variance (ANOVA) with the Student-Newman-Keuls method.

RESULTS

Plasma levels of lipopolysaccharide

The concentrations of LPS (bacterial lipopolysaccharide) in plasma confirmed a rapid translocation of bacteria in gnotobiotic piglets (Table 1). The mean levels of LPS were higher after the infection with the virulent LT2 strain than after the infection with non-pathogenic SF1591 mutant of *Salmonella typhimurium.* The difference between both strains, however, was not statistically significant.

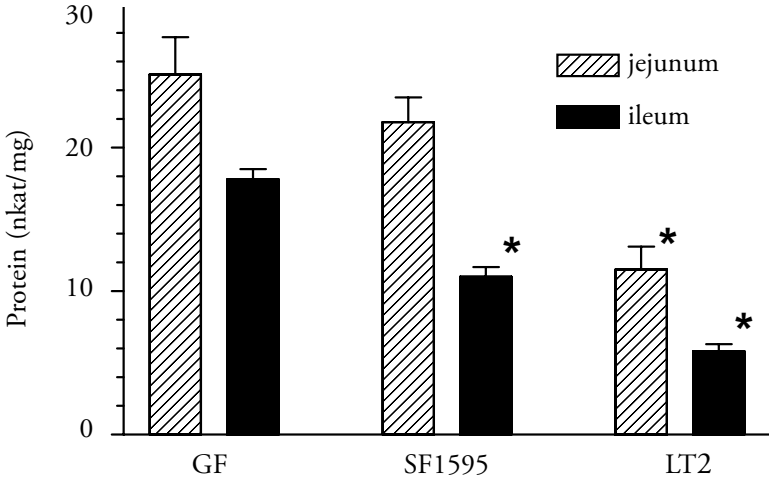
Brush border enzymes in the small bowel of gnotobiotic piglets

The specific activities of glucoamylase and sucrase were low in both the jejunum and ileum of 7-day-old gnotobiotic piglets. In comparison with germ-free control, specific activities of lactase, dipeptidylpeptidase IV and alkaline phosphatase were not significantly affected with *Salmonella* association (Table 2). On the other hand, *Salmonella* infection caused a marked reduction of GGT activity. This decrease was highly significant ($P < 0.001$) in both the jejunum and ileum after colonization with the virulent strain and in the ileum also after infection with the rough mutant (Figure 1).

Table 1. Concentrations of lipopolysaccharide in plasma of gnotobiotic piglets infected with *Salmonella typhimurium*

Animal groups	LPS level in plasma in EU/ml ± SEM	
	6 h	24 h
Germ-free controls*	0.9 ± 0.1	0.9 ± 0.1
S. typhimurium SF1591 infected	6.5 ± 2.9	46.7 ± 42.7
S. typhimurium LT2 infected	10.2 ± 4.2	64.0 ± 36.5

*The mean of all non-infected controls. The difference between non-pathogenic SF1591 mutant and virulent strain and the difference between controls and animals infected for 6 h are not statistically significant



GF = germ-free piglets
SF1591 = piglets infected for 24 hrs with R mutant
LT2 = piglets infected for 24 hrs with virulent strain
mean levels ± SEM, *$P < 0.001$

Figure 1. Effect of oral infection with *Salmonella typhimurium* on γ-glutamyltranspeptidase (GGT) activity in enterocyte brush border membrane vesicles of gnotobiotic piglets

Table 2. Effect of oral infection with *Salmonella typhimurium* on specific activities of enterocyte brush border enzymes (nkat/mg protein) in pig jejunum and ileum

	GA	S	LAC	DPP	AP
Jejunum					
GF	0.4	1.8	27.4	5.6	19.1
SF1591	0.3	0.4	18.5	5.8	20.8
LT2	0.3	0.9	27.2	6.3	18.6
Ileum					
GF	0	0.6	1.4	3.9	6.5
SF1591	0	0.4	1.4	5.8	9.6
LT2	0	0.1	3.2	4.6	5.9

GF = germ-free piglets
GA = glucoamylase
LAC = lactase
S = sucrase
DPP = dipepti dylpeptidase IV
AP = alkaline phosphatase
SF1591 = piglets infected for 24 hrs with R mutant
LT2 = piglets infected for 24 hrs with virulent strain

DISCUSSION

Gnotobiotic piglets are immunologically immature animals that are very sensitive to colonization with bacteria (Rejnek *et al.*, 1968; Barrow *et al.*, 2001). Both microorganisms used in this study colonized the whole gastrointestinal tract of orally infected germ-free piglets as early as six hours after infection and caused septicaemia one day after the infection (Trebichavský, 2000). These results correlated with the present finding of bacterial lipopolysaccharide in circulation of all infected piglets. We recently found that virulent LT2 strain of *Salmonella typhimurium* increased the level of IFN-γ, TNF-α and IL-1β in the intestinal washings of monoassociated piglets 24 h but not 6 h after infection (Šplíchal *et al.*, 2002). These cytokines that are produced in some intestinal inflammatory diseases affect the expression of important brush border membrane markers such as sucrase-isomaltase (Ziambaras *et al.*, 1996). No effect of infection with *Salmonella typhimurium* was observed

on specific activities of intestinal disaccharidases. Similarly, Kaouass *et al*. (1997) did not detect any effect on sucrase activity in neonatal rats injected with TNF-α.

Significant decrease of gamma-glutamyl transpeptidase (GGT) activity after infection of gnotobiotic piglets with salmonellae can be partly explained by the stimulation of immune signalling molecules and by increased cellular renewal. The decrease of GGT activity of enterocyte brush borders was observed also in the jejunum of gnotobiotic mice after the association with *Bifidobacterium bifidum* for eleven days (Kozáková *et al*., 2001). The decrease of GGT activity in infected pig small bowel was, however, more rapid and was greater after infection with the virulent strain than after infection with the rough mutant and it was greater in the ileum – the site of *Salmonella* penetration, than in the jejunum. Therefore, changes of GGT activity correlated with the infection. The GGT participates in homeostasis of glutathione – one of the most abundant thiols in mammalian tissues that plays an important role in detoxifying free radicals and combating oxidative stress. The mucosal pathology of *Salmonella* may in part be due to the excessive production of reactive oxygen species. Mehta *et al*. (1998b) found a significant decrease of glutathione reductase activity and enterocyte-reduced glutathione in enterocytes isolated from ligated loops with live *Salmonella typhimurium*.

Studies of biochemical changes that occur in gut mucosae infected with virulent salmonellae help us to understand the interactions between this enteric pathogen and the host and to elucidate the mechanisms of *Salmonella* enteritis.

Acknowledgements

We thank Marie Zahradníčková, Jarmila Jarkovská, Jaroslava Štěpařová and Alena Smolová for their excellent assistance.

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Received: 02–10–29
Accepted after corrections: 02–12–05

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