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Original article

Early cytokine response of gnotobiotic piglets to *Salmonella enterica* serotype Typhimurium

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Abstract – Cytokine response against *Salmonella* Typhimurium is traditionally studied in conventional animals. Germ-free animals, however, enable to study response against infection without background effect of other microorganisms. Plasma and ileal inflammatory cytokines in germ-free piglets orally infected with virulent LT2 strain or, with a non-virulent SF1591 rough mutant were quantified by ELISA. In plasma and ileal washes, IFN-γ levels significantly increased in both infected groups. TNF-α and IL-18 were mostly missing in plasma 24 h after infection. In the ileum, IFN-γ, TNF-α, and IL-1β were induced mainly by the virulent strain, whereas IL-18 was induced in highest quantity by non-virulent *Salmonella*. These data confirmed an important role of IFN-γ, as well as other inflammatory cytokines in early stage of salmonellosis.

***Salmonella* Typhimurium / gnotobiotic piglet / cytokine**

Résumé – La réponse précoce en cytokines contre *Salmonella enterica* Typhimurium chez des porcelets gnotobiotiques. La réponse en cytokines contre *Salmonella* Typhimurium est traditionnellement étudiée chez les animaux conventionnels. Les animaux sans germes, cependant, permettent d'étudier la réponse à l'infection sans avoir le bruit de fond dû à d'autres micro-organismes. Les cytokines inflammatoires du plasma et de l'iléon chez des porcelets sans germes, infectés oralement par la souche LT2 ou avec un mutant non virulent de SF1591, ont été quantifiées par ELISA. Dans le plasma et dans les lavages de l'iléon, les taux d'IFN-γ ont augmenté significativement chez les animaux des deux groupes infectés. Le TNF-α et l'IL-18 étaient présents en faibles quantités dans le plasma 24 heures après infection. Dans l'iléon, l'IFN-γ , le TNF-α et l'IL-1β ont été induits principalement par la souche virulente, alors que l'IL-18 a été induite en plus grande quantité par les salmonelles non virulentes. Ces données confirment le rôle important de l'IFN-γ et des autres cytokines inflammatoires dans les stades précoces de la salmonellose.

***Salmonella* Typhimurium / porcelet gnotobiotique / cytokine**

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1. INTRODUCTION

Salmonella enterica serotype Typhimurium (hereafter denoted ST)-induced enteritis is an increasing problem in man in developed countries, and a major problem affecting growing pigs in several parts of the world [5]. In mice, this microorganism is accepted as an experimental model for human typhoid fever. Studies in other animals are, however, less frequent. An infectious model of non-typhoid salmonellosis is therefore required.

After oral ingestion and colonisation of the small bowel, ST penetrates the ileum epithelium, moves into the mesenteric lymph nodes and circulation and is rapidly cleared in splenic macrophages and hepatic Kupffer cells [18]. In the pig (and also in ruminants and cats), salmonellae are cleared also by pulmonary intravascular macrophages [19, 29]. *Salmonella* can survive within macrophages that cope with infection before an acquired immune response is generated. Anti-bacterial mechanisms of macrophages are governed by pro-inflammatory cytokines (reviewed recently by Lalmanach and Lantier [15] and Trebichavský [26]). Experiments presented in this paper describe systemic and local cytokine response of germ-free piglets in the early stage of *Salmonella* enteritis.

Germ-free piglets represent a unique experimental model of infection studies for two reasons. First, newborn piglets lack maternal antibodies and only traces of immunoglobulins produced prenatally are present in their sera. Second, endogenous intestinal microflora limiting bacterial multiplication is absent. *Salmonella* microorganisms multiply freely in the gut and therefore infectious dose is several orders of magnitude higher than the dose applied and incomparable to the counts reached in conventional animals [6]. The immune system of germ-free piglets is immature and therefore these animals are highly susceptible to both virulent and non-virulent salmonellae. The aim of this study was to describe a real cytokine response to oral infection with *Salmonella* without the unpredictable effect of gut microflora.

2. MATERIALS AND METHODS

2.1. Animals

Thirty germ-free (GF) piglets of miniature Minnesota-derived breed were obtained by hysterectomy of gilts on the 110th day of gestation under halothane/oxygen anaesthesia (approx. term of delivery of miniature gilts is 112 days of gestation). Piglets were reared in sterile positive-pressure fibreglass isolators and fed by autoclave-sterilised condensed milk and water mixed with a mineral and vitamin supplement [16]. They were checked repeatedly for absence of bacterial contamination by culturing rectal swabs aerobically and anaerobically, and were then used at the age of one week. Piglets were divided into three groups as following: (i) eleven non-infected germ-free piglets (GF), (ii) eleven piglets orally infected with virulent ST (LT2), (iii) eight piglets orally infected with non-virulent ST (SF1591).

2.2. Bacterial strains and conditions of infection

Bacteria were described previously [6]. Briefly, the LT2 strain was streptomycin-resistant mutant highly virulent for GF piglets. The SF1591 was a stable rough mutant of Ra chemotype with a deletion in the His locus and with complete polysaccharide of lipopolysaccharide (courtesy of Dr. O. Lüderitz, Max-Planck Institute for Immunobiology, Freiburg in Breisgau, Germany).

Bacteria were freshly prepared on agar (Immuna, Šarišské Michalany, Slovakia), diluted in PBS, measured at 550 nm and their 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. Four piglets (two infected with LT2 and two infected with SF1591) were sacrificed under halothane anaesthesia 6 h after infection, all other infected piglets were sacrificed 24 h after infection. Experiments were approved by the Ethical Committee of the Institute according to the rules of the Animal Protection Act.

2.3. Plasma collection and ileum washes

Peripheral blood was obtained by heart puncture of piglets under halothane anaesthesia and was collected in a syringe with sodium citrate as an anticoagulant. Blood samples were centrifuged at 400 *g* and 4 °C for 10 min. Ileum washes were performed by washing of a terminal part of the small bowel (the whole ileum and part of the jejunum in length of 40 cm) using 2 mL of Dulbecco's PBS (phosphate buffered saline) (PAA Laboratories, Linz, Austria) supplemented by a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). They were centrifuged at 800 *g* and 4 °C for 20 min, and additionally filtered through 0.2 μm nitrocellulose filter (Sartorius, Goettingen, Germany). All samples were immediately frozen and kept at –70 °C until use.

2.4. Cytokine quantification

Pig IFN-γ, TNF-α, IL-1β and IL-10 ELISA kits (Biosource International, Inc., Camarillo, CA, USA) were used. Their sensitivities in 1:2 diluted samples were at least: 15 pg·mL^{-1} for IFN-γ, IL-10 and TNF-α and 50 pg·mL^{-1} for IL-1β. The IL-18 levels were measured with the sensitivity of 30 pg·mL^{-1} [20]. All cytokine ELISA measurements were performed in the Multiskan RC ELISA reader and Genesis Lite software was used for calculation of cytokine concentrations (Thermo Labsystems Oy, Helsinki, Finland). Values under the detection limit of ELISA systems were calculated as 0 pg·mL^{-1}.

2.5. Statistical analysis

Statistical analysis was performed by one way ANOVA (Student-Newman-Keuls Method) using Graph Pad InStat version 3.05 software (GraphPad Software, Inc., San Diego, CA, USA). Results are presented as mean ± SEM.

3. RESULTS

No significant change in cytokine levels occurred 6 h after infection. All described results were obtained from piglets 24 h after infection and their germ-free counterparts.

3.1. Plasma

Plasma IFN-γ that was negligible in germ-free piglets (11 ± 6 pg·mL^{-1}, $n = 11$) increased significantly 24 h after infection with non-virulent SF1591 or virulent LT2 *Salmonella* strains (2680 ± 727 pg·mL^{-1}, $n = 6$, $P < 0.05$ and 6461 ± 1081 pg·mL^{-1}, $n = 9$, $P < 0.001$, respectively). A significant difference was found also between the virulent strain *vs.* non-virulent strain ($P < 0.01$) (see Fig. 1). TNF-α and IL-18 were missing or low levels were occasionally detected in plasma of all groups (data not shown). Plasma levels of IL-1β in germ-free piglets (95 ± 51 pg·mL^{-1}), piglets infected with the rough mutant (123 ± 79 pg·mL^{-1}), and piglets infected with the virulent strain for 24 h (202 ± 100 pg·mL^{-1}) were not significantly different. Also plasma levels of IL-10 were higher after infection with the virulent strain but not statistically significant (256 ± 128, $n = 9$ vs. 134 ± 70 pg·mL^{-1}, $n = 6$).

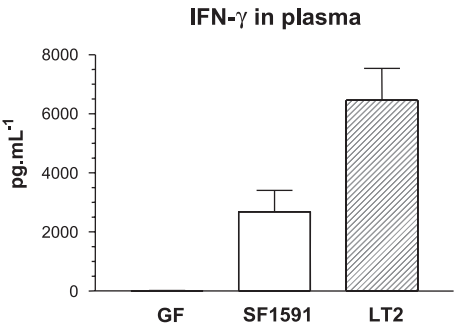

Figure 1. IFN-γlevels in blood plasma. GF: germ-free piglets; SF1591: piglets infected with non-virulent *Salmonella* for 24 h; LT2: piglets infected with virulent *Salmonella* for 24 h.

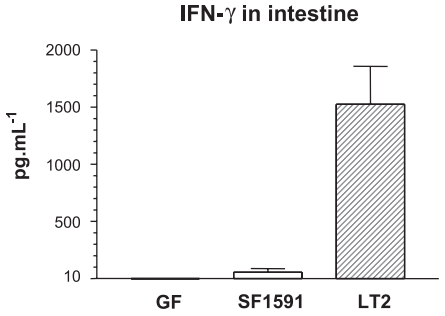

Figure 2. IFN-γ levels in intestinal washes.

3.2. Ileum washes

Highly significant differences in IFN-γ levels were found (Fig. 2) between piglets infected with virulent salmonellae (1526 ± 331 pg·mL^{-1}, n = 6) and germ-free piglets (0 pg·mL^{-1}, n = 8, $P < 0.001$) and between piglets infected with virulent salmonellae and piglets infected with non-virulent salmonellae (55 ± 32 pg·mL^{-1}, n = 3, $P < 0.001$). TNF-α levels (Fig. 3) increased only in piglets infected with the virulent strain (188 ± 66 pg·mL^{-1}, $P < 0.01$). IL-18 levels (Fig. 4) were higher after infection with the rough mutant than after infection with the virulent strain (1472 ± 442, $P < 0.001$ vs. 704 ± 165 pg·mL^{-1}, $P < 0.05$) in comparison to the germ-free group (123 ± 30 pg·mL^{-1}), and both groups

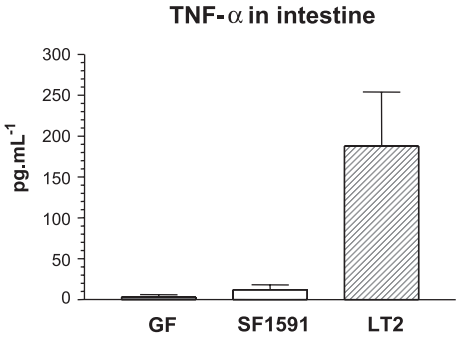

Figure 3. TNF-α levels in intestinal washes.

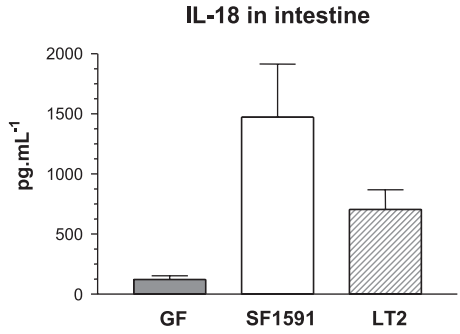

Figure 4. IL-18 levels in intestinal washes.

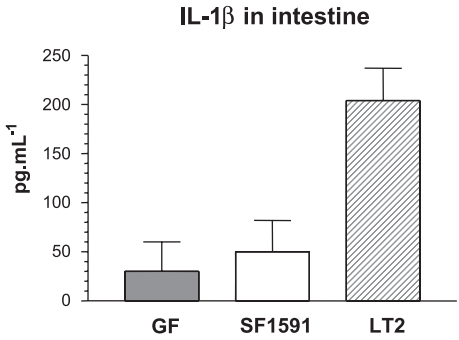

Figure 5. IL-1β levels in intestinal washes.

of infected piglets significantly differed ($P < 0.05$). IL-1β levels (Fig. 5) in piglets infected with virulent strain were significantly higher (204 ± 33 pg·mL^{-1}, $P < 0.01$) than in germ-free piglets (30 ± 30 pg·mL^{-1}) and piglets infected with non-virulent strain (50 ± 32 pg·mL^{-1}, $P < 0.05$). IL-10 levels were negligible in all piglets.

4. DISCUSSION

In the early stage of *Salmonella* infection, macrophage activation by cytokines such as interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α) seems to be a prerequisite for the destruction of bacteria [9, 21]. Activated macrophages produce reactive oxygen and nitrogen intermediates and other biologically active compounds. Recently, we have found that the virulent LT2 strain but not the SF1591 rough mutant of *Salmonella* Typhimurium (ST) induced the synthesis of nitrogen metabolites in infected germ-free piglets [28]. The LT2 and SF1591 ST were cultivated from the small and large intestine 6 h postinfection. Only the virulent strain was found in circulation at this time. Both bacteria were found in the gut, spleen, liver and blood in comparable counts 24 h postinfection [27].

In this study, we have found that plasma levels of IFN-γ significantly increased in all piglets 24 h after infection with ST. Elsewhere, in ileum lavages, the levels of IFN-γ and TNF-α reported here increased only in piglets infected with the virulent strain. Infection with the rough mutant did not influence the levels of IFN-γ in the ileum. Involvement of IFN-γ in the resistance of mice to *Salmonella* infection is well documented. Infection of mice with *Salmonella* induces IFN-γ and its neutralisation in the initial phase of infection leads to impaired resistance [17].

In contrast, TNF-α was not found in the plasma of infected piglets. One of the possible explanations could be its very fast turnover as described by Jesmock et al. [11] in *E. coli*-infected pigs. Balaji et al. [4] found increased serum TNF-α levels in pigs 6 h and later after inoculation of ST. However, the lack of plasma TNF-α was described in septicemic calves [23], mice [14] and weaned pigs infected with ST [25]. The increase of both cytokines, TNF-α and IFN-γ in ileal washes observed after infection with the virulent strain was caused by local secretion and was not correlated to plasma levels. Such a large difference between the effect of virulent and non-virulent salmonellae was not observed in ileum levels of other cytokines. One should realise that cytokine secretions also reflect time-dependent dynamic changes that differ according to the type of cytokine.

It is believed that IL-10 plays an important role in the functioning of regulatory T cells that control inflammatory responses towards intestinal antigens [3] and the dysregulation of IL-10 is connected with inflammatory bowel disease [1, 13]. IL-10 and IL-1 display opposite effects, the former being an inhibitor of IL-1 synthesis by macrophages. The levels of IL-1β and IL-10 were low in all gnotobiotic piglets infected with ST. Similarly, Jotwani et al. [12] found low plasma IL-1 in mice infected with ST. However, plasma levels of IL-10 were higher in piglets infected with the virulent strain. This is in accordance with results of Pie et al. [24] who have found enhanced IL-10 secretion in Nramp1 susceptible mice and have suggested that IL-10 was not involved in protection against ST but rather reflected the severity of the disease.

Interleukin-18, a novel cytokine that is an important inducer of IFN-γ, contributes to the clearance of ST [7]. In addition to stimulating IFN-γ synthesis, IL-18 also exhibits inflammatory effects and its neutralisation protects mice infected with ST against the effect of LPS and TNF [22]. We have found high levels of IL-18 in ileum lavages but not in the plasma of infected

piglets. Foss et al. [8] have recently described an infection of intestinal mucosa with *Salmonella* Choleraesuis that resulted in a decrease in the size of the IL-18 protein, probably due to the cleavage of the pre-IL-18 by caspase-1. This brings additional proofs of the importance of IL-18 in mucosal tissues and its role in the immune response to invading pathogens.

Cytokine network is very sensible to changes in gut microflora. We have accidentally observed absence of ileal IL-18 and high increase of ileal IL-1β when one isolator with piglets infected with the rough mutant of ST was contaminated from environment (unpublished results). Such unexpected but striking rapid change could be caused by bacterial substances [10] and release of cytoplasmic stores. The opposite change of IL-18 and IL-1ß levels could be caused by the fact that both cytokines share an identical signalling pathway and compete for the enzyme (IL-1β converting enzyme) that ensures their processing from a precursor form [2]. The germ-free pig represents therefore a reproducible and microbiologically defined model with all limitations of immunological immature animal model.

The pig provides a number of inflammatory models that act as a bridge between commonly used laboratory rodents and humans. The main result of the study is to show a difference of behaviour of cytokine network (in a reproducible gnotobiotic state) to infection of two *Salmonella* Typhimurium strains and the findings confirmed the usefulness of gnotobiotic piglets as a model of in vivo response to *Salmonella.*

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