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Effects of *Salmonella enterica* serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens

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ABSTRACT

Although vaccination of poultry is a suitable method to limit human food borne gastroenteritis caused by *Salmonella* (*S.*), the immune mechanisms responsible for a longer lasting protection against *Salmonella* infection in birds are not completely understood. To reveal unique protection-related immune parameters, day-old chicks were vaccinated with a commercial live *S.* Enteritidis vaccine and challenged with wild-type *S.* Enteritidis 147N at day 56 of life. The bacterial cell count was determined in gut and liver, while the immune cell composition and cytokine gene expression patterns were analysed by immunohistochemistry and quantitative real-time RT-PCR in caecum samples.

The presented data suggest that the vaccine-elicited immune protection against the *Salmonella* wild-type infection was rather related to the bacterial count in gut mucosa and liver than to the colonisation in gut lumen. The higher number of *Salmonella* wild-type organisms found in caecal wall and liver of the non-immunised compared to immunised birds after challenge correlated with a more pronounced gene expression rate for IL-8, LITAF, iNOS, IL-12 and IFN-γ. In contrast, immunised birds exhibited higher amounts of $CD8^+$ T cells as well as IgA than the non-immunised chickens after *S.* Enteritidis 147N infection in caecum.

The results demonstrated a distinctive immune reaction pattern of previously vaccinated compared to non-vaccinated chickens upon *S.* Enteritidis wild-type challenge.

1. Introduction

Salmonella (*S.*) spp. is one of the major causes of food-borne illnesses in humans. In Germany, more than 52,000 cases of human salmonellosis were reported per annum and, among them, about 60–70% were caused by *S.* Enteritidis, and 20–25% by *S.* Typhimurium [40]. Particularly, human *S.* Enteritidis infections have become a world-wide problem. The most important source is poultry-derived food, mainly eggs and egg-products, but also chicken meat [41]. Indeed, about 30% of the laying hen farms were found *Salmonella*-positive in Germany [34]. Especially, *S.* Enteritidis phage type 4 is the most frequently isolated type from humans as well as poultry livestock [42]. To address this problem, comprehensive control programmes are being implemented with poultry. Beside improved management systems and consequent veterinary hygiene regimes in poultry livestocks, *Salmonella*-eradication programmes particularly include the application of attenuated live vaccines of *S.* Enteritidis [44] and *S.* Typhimurium [23]. The few registered *S.* Enteritidis and Typhimurium live-vaccine strains commercially available for use in poultry in Europe are either auxotrophic double-marker mutants derived through chemical mutagenesis or have been developed on the basis of the principle of metabolic drift mutations [19]. It is thought that live vaccines have some advantages over killed vaccines as they stimulate both humoral and cell-mediated immunity. However, the efficacy of live vaccines is variable and not always satisfactory. Humoral immune mechanisms seem not to play an essential role in clearance of primary infection as well as long-term protection [4], and a better understanding of the specific immunological interrelations leading to an effective protection is of great importance.

Most of the present knowledge on immunity to salmonellosis originates from investigations in mice using a typhoid-like model with *S.* Typhimurium. However, the resulting systemic disease is not very comparable with the rather asymptomatic colonisation of most non-host-adapted *Salmonella* serovars in poultry [19]. Nevertheless, under certain conditions some non-typhoid infections may cause severe clinical signs and mortality also in birds [19]. Thus, *Salmonella* infections of day-old chicks with

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non-host-adapted *Salmonella* serovars may lead to severe morbidity and high mortality [15,21]. In fact, cell-mediated immune mechanisms are of special importance for resolution of *Salmonella* infections in both mice and chickens presumably because *Salmonella*e survive and replicate within macrophages and, therefore, require activation of macrophages for clearance [4,31]. In poultry, a distinct T-cell answer and rapid expression of various cytokines in response to oral *Salmonella* infection of newly hatched and older chickens have been described [2,5,6,8,25,47]. However, how far these reactions can contribute to development of *Salmonella*-specific immunological memory and in what way immunised chickens do effectively respond upon *Salmonella* challenge remains unclear. Specific protection is only ensured by a powerful memory immune answer. Immunological memory has been defined as the faster and stronger immune response of an animal that follows re-exposure to the same antigen [18]. A prerequisite seems to be the emergence of specific memory T-cells. It has been shown that memory T cells express characteristic surface antigens as CD45RO in humans and CD44 in mice, and respond functionally different from those of naive cells in several ways. After re-encounter with the same antigen, memory cells can rapidly expand to a higher number and generate a population of effectors with highly protective capacity. Compared to naive T cells with an only restricted cytokine repertoire, memory CD4 and CD8 cells are able to secrete a larger quantity of T-cell cytokines in higher amounts and in shorter time. Moreover, they can be polarised to produce special cytokine patterns for specific functions in immune defence. In mice, splenic CD4 and CD8 T cells gained the capacity to secrete IFN-γ in response to *Salmonella* re-stimulation [36]. Secondary infection of birds with *S*. Typhimurium was restricted to the intestine and of shorter duration than primary infection [6]. Additionally, the rapid expression of a MIP-family chemokine and interleukine-6 was found to be accompanied by an influx of lymphocytes in chickens [48]. A more expeditious recruitment of $CD8^+$ and γδ T cells in caecum was shown after *S*. Enteritidis challenge of previously infected birds [9].

In this study, we analysed the effectiveness of a commercially available live *Salmonella* vaccine and intended to reveal possible distinct immune reaction patterns of naive and *Salmonella*-vaccinated chickens after exposure to the *Salmonella* wild-type strain 147N. For this purpose, we examined the commercially available live vaccine in terms of its capability to invade the caecal mucosa and to induce an immune reaction. After infection with the *S*. Enteritidis wild-type strain 147N, we studied the protective effect of the live vaccine concerning both the *Salmonella* wild-type invasion of gut and liver as well as colonisation in caecal lumen in connection with the elicited immune response as cellular recruitment and immune gene expression profile in gut.

2. Materials and methods

2.1. Experimental animals

At the facilities of the Friedrich-Loeffler-Institute, Jena, Germany, the specific pathogen-free (SPF) White Leghorn chickens were hatched from eggs obtained from Charles River Deutschland GmbH, Extertal, Germany. Commercial feed (in powder form without antibiotics or other additives) and drinking water were both available ad libitum. Experimental and control groups were kept in separate rooms. The cleaning and feeding regimens effectively prevented cross-contamination throughout the course of the experiment. The animal test was performed in accordance with the German Animal Protection Act (registration number: 04-01/01).

2.2. Experimental design, bacterial strains and microbiology

The commercial live-attenuated *Salmonella* Enteritidis vaccine strain (SE-LV), Salmovac SE (Impfstoffwerk Dessau-Tornau GmbH, Germany), was used for the oral immunisation of 57 chicks at their first day of life (henceforth named vaccinated chicks). Further 75 chicks remained non-treated. The group administered the SE-LV was booster-immunised at day 14 of life. The viable count of the attenuated SE-LV was $1–2 \times 10^8$ colony forming units (CFU) per bird. At day 56 of life 18 vaccinated and 18 non-vaccinated chickens were infected orally using a nalidixic acid-resistant variant (N) of the wild-type strain *Salmonella* Enteritidis 147 (SE 147N) at a dose of 2×10^8 CFU/bird (henceforth named vaccinated/challenged chickens and non-vaccinated/challenged chickens, respectively). Another control group was neither vaccinated nor infected (henceforth named non-treated chickens). All strains used were stored in the Microbank system (PRO-LAB Diagnostics, Ontario, Canada) at −20 °C. Oral administration was performed by instillation into the crop of the birds using a syringe with an attached flexible tube. The volume of bacterial suspension used was 0.1 ml/bird. The *Salmonella* suspensions for immunisation and infection were cultivated by shaking (18 h at 37 °C) in nutrient broth (SIFIN, Berlin, Germany). Doses were estimated by measuring extinction at 600 nm against a calibration graph determined for the strains used, and subsequently confirmed by plate counting on nutrient agar (SIFIN). Bacterial counts of SE 147N in liver and caecal content were estimated using a standard plating method as described previously [33]. Briefly, homogenised organ samples or caecal content were diluted in phosphate-buffered saline, plated on deoxycholate-citrate agar (SIFIN) supplemented with sodium nalidixate (50 μg/ml) to detect the bacterial organisms and incubated at 37 °C for 18–24 h.

2.3. Flow cytometry

For analysis of changes in T-cell composition upon *Salmonella* vaccination and infection heparinised blood of each individual animal (three per group and day) at day (d) 2, 3, 6, 8, 10, 13, 14, 16, 20, and 50 after vaccination (dpv; vaccinated and non-treated group) and at 6 h and day 1, 2, 4, 7, and 9 after infection (hpi; dpi) at day 56 of life (vaccinated/challenged, non-vaccinated/challenged and non-treated group) was mixed with 3% hetastarch (Sigma Immuno Chemicals, St. Louis, USA) at a ratio of 1:2 and centrifuged at $65 \times g$ for 10 min to allow erythrocytes to sediment. The cells of the supernatant were used for flow-cytometrical analysis of blood lymphocyte as described [9]. 2×10^5 isolated leukocytes were incubated with the FITC-labelled monoclonal antibody TCR1 (TCR-1) and RPE-conjugated CD8α (CT-8) or with FITC-labelled monoclonal antibody TCR2 (TCR-2) and RPE-conjugated CD8α (all from Southern Biotechnology Associates, Eching, Germany) for 30 min in the dark. After washing the cells, aliquots of 20,000 cells per sample were analysed using a FACSCalibur (BD Bioscience, Heidelberg, Germany) equipped with a 15 mW, 488 nm argon ion laser and the percentages of positively stained cells calculated by the CellQuestPro 4.0.2 software (BD Bioscience).

2.4. Immunohistochemistry

To study the *Salmonella* invasion and immune cell influx into the gut mucosa, frozen sections were prepared from caecum of each individual animal (three per investigation time and group) at 2, 3, 6 and 13 days after vaccination of day-old chicks (dpv; vaccinated and non-treated group) as well as at 6 h and days 1, 2, 4, 7 and 9 after infection (hpi; dpi; vaccinated/challenged,