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STUDY ON THE ADHERENCE AND THE INVASIVENESS OF A *SALMONELLA GALLINARUM* STRAIN IN CHICKENS

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Summary

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Following a laparotomy, a 5 cm ligated ileal loop was created in salmonella-free 2 month-old female broiler chickens and then, it was inoculated with 0.2 mL 1×10^9 *Salmonella Gallinarum* organisms. By min 30 and 60 post inoculation, bacteriological investigations of viscera and intestines were performed. By min 30 and hours 1, 2, 4, 6 and 8 post inoculation, specimens from the ileal loop were submitted to a histological light microscopic and scanning electron microscopic (SEM) examination. The pathogen was reisolated as early as min 30 and later, by min 60 from viscera and intestines. Pathohistologically, a hypercellularity of propria and a considerable number of migrating pseudoeosinophils among the epithelium of the mucosa were observed. Distinct bacterial cell clusters were observed only withing the intestinal lumen, using the Warthin-Starry method. There were also aggregates of necrotic cells (pseudoeosinophils) in the centre of intestinal lumen. They were pyknotic, with fragmented nuclei.The SEM study revealed adhesions of a big number of bacterial cells on *bursa Fabricii*. Lymphoid cells were most probably the infection atrium under natural conditions. Our results showed that when an intestinal loop was prepared, *S. Gallinarum* migrated through the intestinal mucosa layers and entered the circulation, thus causing a systemic infection. At the same time, the products of *S. Gallinarum* were responsible for the exudative cellular inflammatory morphological changes of intestinal wall. An apoptosis was also present, most probably due to bacterial enterotoxin.

Key words: adhesiveness, apoptosis, invasiveness, *Salmonella Gallinarum*

INTRODUCTION

The earliest host−pathogen interactions of many bacterial infections occur on a molecular level. Thus, at the boundary between host's and pathogen's cells, a complex cascade of interactions is triggered (Darwin and Miller, 1999). Salmonellosis is also characterized by a similar pattern of development. Most commonly, it occurs via an alimentary pathway with a resultant bacterial colonization of viscera. The first important moment in such cases is the contact of salmonellae with the intestinal epithelium that induces brush border changes, known as ruffles (Darwin and Miller, 1999; Desmidt *et al.*, 1998). They are observed about a minute following the adhesion of bacteria to cells (Francis *et al.*, 1992). Entering into the epithelial cells, many bacteria are being included into membrane-associated vacuoles, where they survive and replicate (Mulvey *et al.*, 1998). Salmonellae were observed into free macrophage-like cells in the vascular lumen of vessels in *lamina propria*, from where they disseminate toward other tissues (Desmidt *et al.*, 1998). Pascopella *et al.*, (1995) showed that *Salmonella Typhimurium* strains entered murine M-cells with a characteristic ruffling of cellular surface. *S. Gallinarum*, adapted to birds, did not exhibit such a property. In an ileal loop of rabbit, salmonellae were shown to adhere to microvilli by hour 3 after the infection and they were found in epithelial cells and *lamina propria* by hour 7, simulataneously with an acute inflammation (Darwin and Miller, 1999). The ability of *S. Typhimurium* to invade *in vitro* mammalian cells corresponds to its ability to pass through the intestinal epithelium in rabbits. Those findings are in the basis of modern concepts for molecular interactions of salmonellae with hosts (Darwin and Miller, 1999; Wilson *et al.*, 2000).

Yabunchi *et al.* (1986) isolated *S. Typhimurim* inoculated in murine ileal loops from mesenteric lymph nodes, spleen and liver 30 min following the inoculation. It was also observed that a *Salmonella* strain could internalize a HELA cells monolayer and replicate intracellularly. Carter and Collins (cited by Darwin and Miller, 1999) were the first to report that invasive *S. Typhimurium*, applied orally to mice, adhere primarily in the region of lymphatic follicles of small intestine Peyer's patches.

Following an oral infection of 3-weeks-old chickens with *S. Typhimurium*, Barrow *et al.* (1988) isolated bacteria more frequently from the intestinal content rather than from intestinal wall and conclude that neither flagellar antigens nor the manose-sensitive haemagglutination or the carriership of a 85-kilobases plasmid were essential for colonization and suggested that a microbial factor, yet unidentified, was necessary. Only Zhou *et al.* (1995) showed via scanning electron microscopy (SEM) the ability of *S. Pullorum* to adhere to the ileal epithelium of 2-days-old chickens after oral application.

Bibliographic data for the intricate mechanisms of adhesion, invasion and colonization of *S. Gallinarum* strains, causing infection are still inadequate.

Therefore, our aim was to study the dynamics of bacterial invasion of gastrointestinal tract and viscera as well as the morphological changes of intestinal wall occurring in an experimental *S. Gallinarum* infection in chickens.

MATERIALS AND METHODS

Two-months-old female broiler chickens were used. Prior to the experiment, the carriership of *Salmonella* was excluded on the basis of examination of cloacal specimens and the lack of antibodies against fowl typhoid and pullorosis was evidenced by a serological study of blood sera. After a 48 hour fasting, each chicken was premedicated with 0.2 mL 1% atropine sulfate s.c. and 10 min later, anaesthetized locally with 1% procaine plus adrenaline. A laparotomy was performed, 5 cm ligated ileal loops were prepared and 0.2 mL 1×10^9 *Salmonella Gallinarum* organisms were inoculated within. Afterwards, three inoculated birds per interval were euthanazed by both min 30 and 60 and specimens from inoculated areas of the ileum and from other viscera and intestines were aseptically collected. The specimens were cultivated via enrichment and inoculated on brilliant green phenol red agar. The identification of isolated salmonellae was done serologically and biochemically according to routine methods.

Another group of chickens of the same age, similarly treated, served for obtaining samples from the ileal loops by min 30 and hours 1, 2, 4, 6 and 8 after the inoculation with *S. Gallinarum*. The samples were fixed in 10% neutral formaldehyde, dehydrated in ascending ethanol series and cleared in xylene. Cross-sections (5−8 µm) were stained with haematoxylin-eosin and by the method of Warthin-Starry.

A third group of chickens of the same age were inoculated with 0.5 mL 1×10^9 *Salmonella Gallinarum* organisms in *bursa Fabricii*. The viscera and intestines of three inoculated birds were bacteriologically investigated by min 30 and those of another three − by min 60. The bursae were fixed in 4% glutaraldehyde in phosphate buffer, dried to the critical point, sputtered with gold under deep vacuum and studied on SEM under a magnification of ×500 − ×5000.

RESULTS

S. Gallinarum, inoculated into the ileum and *bursa Fabricii* was reisolated from viscera and intestinal sections (Table 1).

Pathohistologically, there were no considerable morphological changes in intestines of chickens 30 min and 60 min post inoculation. Two hours after the infection, there was a hypercellularity of propria and multiple migrating pseudoeosinophils among the epithelial cells of mucosa. Bacterial cell clusters were found out only within the intestinal lumen by the method of Warthin−Starry (Fig. 1).

The pathological changes in chickens, examined by hour 4 and 6 post inoculation, were similar: multiple necrotic cells (pseudoeosinophils) aggregated in the centre of the intestinal lumen. They were pyknotic and with fragmented nuclei. The vessels of propria and epithelium were infiltrated with pseudoeosinophils. Multiple bacteria were present among the cellular debris within the lumen. There were no bacteria near to the epithelial surface.

The changes in birds 8 hours following the inoculation were similar. The infiltration of the propria and the epithelium with pseudoeosinophils was considerably more intensive and it was present in edematous submucosa, muscles and subserosa as well (Fig. 2).

In all studied cases by hour 2 following the inoculation, a decreased argentophily of proximal third of intestinall villi was observed as well as pyknosis accompanied with fragmentation of intestinal mucosa epitheial cells.

Table 1. Isolation of *S. gallinarum* from viscera and intestine of chickens following inoculation in ileum or *bursa Fabricii*

Time post inoculation (hours)	Inoculation in:	Isolation from:				
		Liver	Spleen	Duodenum	Ileum	Caecum
0.5	ileum	+	+	−	+	+
1.0		+	+	+	+	+
0.5	*Bursa*	−	−	−	−	−
1.0	*Fabricii*	−	+	−	+	−

+ (−) the applied *S. Gallinarum* strain is (is not) isolated.

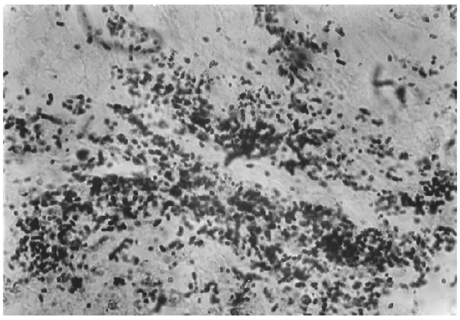

Fig. 1. Bacterial cells into the lumen of ileum 2 hours after inoculation with *S. Gallinarum*. Warthin-Starry staining, × 100.

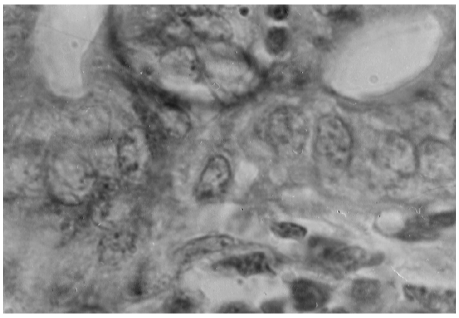

Fig. 2. Infiltration of ileal propria with pseudoeosinophils 8 hours after inoculation with *S. Gallinarum*. H/E, × 100.

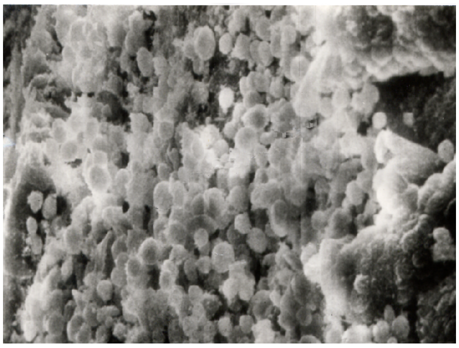

Fig. 3. Bacterial cells, adhered on the epithelial surface of *bursa Fabricii*. SEM, × 5000.

The SEM study showed an adhesion of a big number of bacterial cells on the epithelial surface of *bursa Fabricii* (Fig. 3).

DISCUSSION

Our data about the penetration of *S. Gallinarum* through the intestinal wall and the invasion of viscera and large intestine are in accordance with the results of other authors in other *Salmonella* serovars and other animal species (Darwin and Miller, 1999). Living *S. Gallinarum* bacteria were observed into the cytoplasm of single cells of the propria 4−6−8 hours after the inoculation. Although many heterophils migrated towards *lamina propria* and the intestinal lumen, as stated also by Desmidt *et al.* (1998), there were no bacteria in heterophils. Their presence in viscera and the intestinal content however showed that they penetrated through deep tissues to the systemic circulation and caused generalized infection. Microorganisms reached the liver and the spleen 30 min after the inoculation overcoming the physical, cellular and secretory antibacterial mechanisms of defence in gastrointestinal tract. It is accepted that under natural conditions, the primary site of host colonization is commonly the gastrointestinal tract and especially the ileum, where salmonellae adhere to the cells of mucosa, having surmounted the acid pH of the stomach and the influence of biliary salts in the duodenum (Darwin and Miller, 1999). Such an adhesion of *S. Gallinarum* strains on intestinal mucosa of infected chickens was not observed in our study, but only upon the epithelial surface of *bursa Fabricii*. Lymphoid cells are most probably the infection atrium under natural conditions. This suggestion is in accordance with the findings that the lymphoid cells of avian gastrointestinal tract possess M−cells whose principal function is the transport of intact particles, bacteria, viruses etc. and that play an important role in pathogenicity of salmonellae in mammals (Darwin and Miller, 1999). On the other hand, our experiment gave an evidence that after the creation of intestinal loop, *S. Gallinarum* passed through the layers of intestinal mucosa and entered the general circulation, infecting the duodenum, ileum, caecum, liver and spleen. This correponds to data that pathogenic salmonellae carry an invasion gene, determining the production of a protein from a special type III (Darwin and Miller, 1999). It is probably used by bacteria for their penetration through the intestinal wall and for infection of liver and spleen through the circulation. Furthermore, via their products, *S. Gallinarum* provoke exudative-cellular inflammatory morphological changes in the intestinal wall. An oedema of submucosa, musculature and subserosa was present, with a profuse infiltration of propria and ileal epithelium with heterophils. A fragmentation of pseudoeosinophilic and epithelial cells nuclei was observed, most probably due to toxin-induced apoptosis. This process occurred 4−6 hours after the inoculation, similarly to the findings of other investigators (Bosman *et al.*, 1996; Toledano *et al.*, 1999). Ayala *et al.* (1997) also reported T and B apoptosis and granulocytic apoptosis in response to polymicrobial sepsis. The lipopolysaccharide (LPS) also induced apoptosis in blood and epithelial cells (Frey and Finlay, 1998; Karanashi and Amano, 1998; Warny and Kelly, 1999). Other authors (Tsuji *et al.*, 2000) found out that enterotoxin could be responsible for apoptosis in lymphoid cells. In a previous study of ours, it was proved that *S. Gallinarum* produces an endotoxin that is the principal pathogenic factor in an experimental model of fowl typhoid (Kokosharov, 2000). Later, an enterotoxic production of *S. Gallinarum* strains was established (Kokosharov, 2002). LPS is accumulated in considerable amounts by day 4 after the infection, whereas enterotoxin production occurs more early. In the present experiment, the intestinal loop was filled with fluid by hour 8 − an evidence for the presence of enterotoxin. Therefore, *S. Gallinarum* enterotoxin was probably the cause for the apoptosis in blood and epithelial cells. The problem however requires further studies because apoptosis is a principal mechanism for elimination of normal and pathologically changed cells (Bosman *et al.*, 1996), thus belonging to systemic mechanisms of defence.

Histologically, heterophils were found not only into *lamina propria*, but they pass through the intact epithelial crypts and accumulate into the intestinal lumen. Similar observations were made also by Chansoriya *et al.* (1993) who suggested that the cellular response of chickens to inflammatory stimuli was stereotypic − initially, heterophils migrate into the tissues, followed by other blood cells in the later stages.

Our results extend the knowledge about the mechanisms of adhesion and invasion of *S. Gallinarum* in avian intestines and would be helpful in the elaboration of strategies for control of fowl typhoid.

CONCLUSIONS

When an intestinal loop was created, *Salmonella Gallinarum* passed through the layers of intestinal mucosa and enters the circulation, causing a systemic infection.

Salmonella Gallinarum caused exudative-cellular inflammatory morphological changes of intestinal wall.

An apoptosis of pseudoeosinophils

and epithelial cells of intestinal wall was present.

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