



RESEARCH COMMUNICATION

Bacterial colonization and endotoxin activity during experimental acute fowl typhoid in chickens

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ABSTRACT

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Bacterial colonization and endotoxin production were investigated before and after experimental *Salmonella gallinarum* infection in 8-week-old female broiler chickens.

These parameters were assayed by means of colony forming units test (CFU) and the Limulus Amebocyte Lysate test (LAL), respectively. Birds were infected *per os* with $1,5 \times 10^9$ CFU/ml of wild strain of *S. gallinarum* isolated from a dead hen. Approximately $1,5 \times 10^2$; $1,3 \times 10^2$ and $1,2 \times 10^2$ CFU of *S. gallinarum* were recorded from 1 g of liver, 1 g of spleen and 1 ml of blood from the chickens on day 1 post infection. By day 4 corresponding data were $3,7 \times 10^4$; $4,8 \times 10^3$ and $1,1 \times 10^3$ respectively and on day 7 10^5 CFU were present in all three specimen types. The liver and spleen of dead birds were contaminated with more than 10^7 CFU per g. The endotoxin from *S. gallinarum* was found to have an activity of 1,5; 12,0 and 15,0 endotoxin units (EU)/ml on day 1, 4 and 7 after infection, respectively. No endotoxin activity was established in the blood of the control group (before infection) by the LAL test. This is the first time the connection between the amount of live *S. gallinarum* in the blood, liver and the circulating level of endotoxin in the blood during the infectious stage of experimental acute fowl typhoid, has been demonstrated.

Keywords: bacterial colonization, endotoxin, fowl typhoid, *Salmonella gallinarum*

INTRODUCTION

Septicaemia is a leading cause of morbidity and mortality among hospitalized human patients. It has been estimated that septic shock causes 100 000 deaths in the U.S.A. alone (Parilo 1993; Schlether, Heine, Ulmer & Reitschel 1995). The prime initiator of Gram-negative bacterial septic shock is endotoxin, a lipopolysaccharide (LPS) compound of the bacterial outer membrane which may be released in a bacteraemia (Morrison & Bucklin 1996). It has been clearly established that the LPS of *Salmonella* spp. strains is critical for the establishment of disease and that it plays multiple roles in an infection of a host (Nnalue & Lindberg 1990; Jones, Nichols, Gibson, Sunshine & Apicella 1997; Garcia, Del Portillo, Stein & Finlay 1997). The presence of a large amount of

LPS in the bloodstream leads to dramatic pathophysiological reactions such as fever, hypotension, leukocytosis, disseminated intravascular coagulation (DIC) and multi-organ failure (Schlether *et al.* 1995). LPS was detectable in 34 of 38 patients suffering from culture positive Gram-negative bacteraemia (Bhanumathy, Ong, Yong, Parasakthi, Koh, Slow & Bosco 1995) and in another study no patients survived with circulating endotoxin level > 12 pg/ml (Pfeiffer, Ehrhardt & Kretzchanar 1996).

Fowl typhoid is one of the most important poultry diseases and occurs worldwide (Shivaprasad 1997). Nothing is known about the endotoxin activity in the blood of poultry suffering from experimental acute typhoid, or the connection between the amount of live *S. gallinarum* in the blood and internal organs and the circulating endotoxin level. An attempt was made to address this lack of information.

MATERIALS AND METHODS

Birds

Twenty eight-week-old female *Salmonella*-free broiler chickens were used in this study. The chickens received an antibiotic-free diet and water *ad libitum*.

Limulus Amebocyte Lysate endotoxin quantitation assay

The standardized endotoxin activity of LPS was determined by using a Limulus Amebocyte Lysate (LAL) gel clot kit [Sigma Chemical Company, St Louis, USA (E-Toxate)]. The assay was performed according to the manufacturer's recommendations. Briefly, a series of standards was prepared from stock endotoxin solution each time an assay was performed. Nine standards were prepared in tubes as tenfold dilutions containing endotoxin concentrations ranging from 400,0 EU/ml to 0,015 EU/ml. A negative control was also prepared. Reconstituted LAL reagent was added to each tube after all the dilutions had been prepared. The rack in which the tubes were placed was vigorously shaken and the tubes incubated for 1 h at 37 ± 1 °C in a waterbath. A positive test was indicated by the formation of a solid gel clot that did not collapse upon inversion of the tube. The endpoint of the assay was defined as the lowest concentration of endotoxin to yield a positive result.

$$\text{Endotoxin (EU/ml)} = \frac{1}{\text{Dilution of positive sample}} \times \text{Positive endotoxin standard}$$

Experimental procedure

Salmonella gallinarum wild strain isolated from a dead hen was stored in Dorset medium at 4 °C. The isolate was recovered by inoculating a small portion of the stock onto blood agar medium and inoculat-

ing the agar overnight at 37 °C. Bacterial suspensions in normal saline, each containing a concentration of $1,5 \times 10^9$ colony forming units (CFU) of the bacterium, were prepared and one of these was delivered into the crop of each of the experimental chickens. Before (control group) and at 1, 4 and 7 day intervals after inoculation, respectively three chickens from control and infected groups and dead infected birds were sacrificed during which process 1 ml of blood, 1 g of liver and 1 g of spleen from each of them were taken. The specimens of liver and spleen were each homogenized in 10 ml of saline. Each of these was then subjected to serial tenfold dilution to 10^{-8} using normal saline. Colonization was assessed by counting the CFU's after culturing 0,1 ml from the different dilutions on brilliant green phenol red agar medium for 16 h at 37 °C. In addition, 2 ml of blood for LAL testing was drawn from three chickens before and at day 1, 4 and 7 after infection into sterile pyrogenic-free tubes containing 50 units of free heparin. For removal of the LAL inhibitor in the plasma, it was diluted 1:10 in endotoxin-free water and heated for 10 min at 70 °C in a waterbath (Pitkin, Chugani, Chenette, Shen & Du Monlin 1996). Each of the tests was performed twice.

RESULTS

Approximately $1,5 \times 10^2$; $1,3 \times 10^2$ and $1,2 \times 10^2$ CFU of *S. gallinarum* were recorded from 1 g of liver, 1 g of spleen and 1 ml of blood from the chickens which were slaughtered on day 1 post infection (Table 1). By day 4, the corresponding figures were: $3,7 \times 10^4$; $4,8 \times 10^3$ and $1,1 \times 10^3$ respectively, and on day 7 10^5 CFU were present in all three specimen types. The internal organs (liver and spleen) of dead birds were contaminated with more than 10^7 CFU per g. The endotoxin from *S. gallinarum* was found to have an activity of 1,5; 12,0 and 15,0 EU/ml on day 1, 4 and 7 days after infection (Table 1), respectively.

TABLE 1 Quantitative bacterial counts in the blood, liver and spleen and plasma endotoxin levels in chickens with experimental acute fowl typhoid

Index	Unit	Days post infection				
		0	1	4	7	Dead
No. of bacteria in: Blood	cfu/ml	0	$1,2 \times 10^2$	$1,1 \times 10^3$	$4,1 \times 10^5$	N D
Liver	cfu/g	0	$1,5 \times 10^2$	$3,7 \times 10^4$	$6,3 \times 10^5$	$2,4 \times 10^7$
Spleen	cfu/g	0	$1,3 \times 10^2$	$4,8 \times 10^3$	$2,6 \times 10^5$	$2,1 \times 10^7$
Plasma endotoxin level	EU/ml	0	1,5	12,0	15,0	N D

N = 3 in each group
 cfu = Colony forming units
 EU = Endotoxin units
 N D = No data

At a concentration of approximately 10^3 – 10^5 CFU/ml of *S. gallinarum* in the blood or in 1 g of liver and spleen and an activity of endotoxin of 12,0–15,0 EU/ml all the birds developed signs of systemic toxicity, including loss of appetite, a drooping attitude, anaemia and shrunken combs (data not shown). Those that had received $1,5 \times 10^9$ *S. gallinarum* developed bacteraemia on 1, 4 and 7 day post infection. The plasma level of endotoxin was approximately proportional to the level of bacteraemia and of bacterial colonization of the liver and spleen of infected birds.

DISCUSSION

In the present study, we observed that an isolated pathogen, *S. gallinarum*, rapidly colonized, multiplied and persisted 1–7 days in the blood, liver and spleen of the inoculated chickens. The endotoxin activity increased from 1,5 EU/ml on day 1 to 12,0–15,0 EU/ml on days 4 and 7 post infection, respectively. All control birds (before infection) did not show any endotoxin activity by the LAL method. This is in accord with the statement of Arditi, Kabat & Yogev (1993) that the increased levels of endotoxin are attributed to the relative increase in microbial biomass. Total plasma levels of endotoxin are approximately proportional to the level of bacteraemia (Corrigan & Kiernat 1979). The latter authors concluded that host defense mechanisms clear bacteria without release of significant amounts of endotoxin perhaps as a result of the intracellular lysis of bacteria in tissues other than blood. Buxton & Davies (1963) found substantial amounts of endotoxin in the tissues of birds dying from experimental fowl typhoid. Their results are to be expected in a disease which develops as a severe generalized infection.

Prins, Van Deventer, Knijer & Speelman (1994) and Garcia *et al.* (1997) have reported that during a Gram-negative infectious process, microbial constituents degrade and release biologically active LPS. LPS interacts with specific membrane receptors on host inflammatory cells and this interaction leads to the secretion of mediator molecules (Leeson & Morrison 1994). Lopnow, Brade, Rietschel & Flad (1997) have found a correlation between the concentration of LPS and cytokine production, resulting in the dramatic pathophysiological reactions which occur in animals suffering from *Salmonella* bacteraemia (Kluger 1991; Collins 1996). The results in the present study demonstrate for the first time the connection between the production of endotoxin in the blood and the colonization of *S. gallinarum* in blood, liver and spleen during experimental acute fowl typhoid. A favoured hypothesis to explain the high mortality of poultry with experimental acute fowl typhoid is the lysis of bacterial cells and thus the release a large amounts of endotoxin into the circulation, an event leading to intractable shock and death. These macromolecules have been recognized as

microbial toxins capable of eliciting a lethal response in experimental birds. Administration of purified LPS from *S. gallinarum* reproduce remarkably well many of the clinical, haematological and pathophysiological responses observed in poultry with experimental acute fowl typhoid (personal observation). Blood endotoxin levels have been shown to correlate rather precisely with an adverse outcome. A maximal mortality in the infected birds occurred on 4–7 days post infection (data not shown), when the endotoxin levels were maximal in the blood. The results provide strong additional support for the concept that endotoxin from *S. gallinarum* is an important and highly relevant factor in the pathogenesis of fowl typhoid.

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