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Early Events in the Pathogenesis of Avian Salmonellosis

S. CHRISTINE HENDERSON, DENISE I. BOUNOUS, AND MARGIE D. LEE*

Departments of Medical Microbiology and Pathology, The University of Georgia, Athens, Georgia 30602

Received 16 February 1999/Returned for modification 18 March 1999/Accepted 22 April 1999

Salmonellae are gastrointestinal pathogens of man and animals. However, strains that are host-specific avian pathogens are often avirulent in mammals, and those which are nonspecific are commensal in poultry. The objective of this study was to determine whether host specificity was exhibited by bacterial abilities to invade epithelial cells or resist leukocyte killing. In this study, leukocytes isolated from humans and chickens were used to kill Salmonella in vitro. Both Salmonella pullorum, an avian-specific serotype, and Salmonella typhimurium, a broad-host-range serotype, were sensitive to killing by polymorphonuclear leukocytes isolated from both species. Both serotypes replicated in cells of the MQ-NCSU avian-macrophage cell line. In contrast, S. pullorum was noninvasive for cultured epithelial Henle 407, chicken kidney, chick ovary, and budgerigar abdominal tumor cells. In the bird challenge, however, S. typhimurium rapidly caused inflammation of the intestinal mucosa, but S. pullorum preferentially targeted the bursa of Fabricius prior to eliciting intestinal inflammation. Salmonella serotypes which cause typhoid fever in mice have been targeted to the gut-associated lymphoid tissue. Observations from this study show that S. pullorum initiated a route of infection in chicks comparable to the route it takes in cases of enteric fever.

* Corresponding author. Mailing address: Department of Medical Microbiology, The University of Georgia, Athens, GA 30602. Phone: (706) 542-5778, Fax: (706) 542-5771. E-mail: lecm@calc.vet.uga.edu.

MATERIALS AND METHODS

Bacteria. S. typhimurium SR-11 has been previously described (29). The host-specific avian pathogen S. pullorum x3423 was a gift from Roy Curtiss III (Washington University, St. Louis, Mo.). Cultures were statically grown overnight at 37°C in Luria broth (LB). Confirmation that these isolates contained the invA gene and the virulence plasmid was obtained through PCR with svaC- and invA-specific primers. The 21-mer primers for Salmonella svC and invA gene probes were designed by using published sequences (GenBank accession no. M64295 and M90846, respectively) and Oligo software (National Biosciences, Plymouth, Minn.). The svaC primer sequences were 5’-CGCAAATACATT ACAATA-3’ and 5’-CCCAAACCATCACATTGTG-3’ and were predicted to yield a 669-bp product. The invA primer sequences were 5’-TTGTACGCG TATTTTGACCA-3’ and 5’-CTGACTGCTACCTTGTATG-3’ and were
predicted to yield a 521-bp product. Primers were prepared by the University of Georgia molecular genetics laboratory with the ABI Model 394 DNA synthesizer. Template DNA was isolated from *S. typhimurium* and *S. pullorum* by boiling loopfuls of bacteria for 20 min in water. PCR was conducted by using a PTC-100 model thermocycler (MJ Research, Inc., Watertown, Maine) with denaturation at 95°C for 1 min, primer annealing at 42°C for 1 min, and primer extension at 72°C for 2 min, for a total of 30 cycles. The products were examined by agarose gel electrophoresis for the presence of DNA fragments of the appropriate size. PCR performed with primers in the absence of genomic DNA served as the negative control.

**Leukocyte isolation.** We used 20 specific-pathogen-free White Leghorn chickens, 5 to 8 weeks in age, as avian blood donors. The birds were placed in poultry house floor pens on a 16 h of light/8 h of dark cycle and were provided water and growth ration (The University of Georgia, Athens) ad libitum.

Avian leukocytes were obtained from blood by using a modification described by Brooks et al. of a procedure previously described by Glick et al. (8, 19). Neutrophils were isolated from venous blood samples taken from healthy male and female human volunteers as previously described (15). Whole blood was collected in EDTA-containing Vacutainer tubes (Becton Dickinson, Rutherford, N.J.) from the median cubital vein and then subjected to a discontinuous Ficoll-Hypaque density gradient. Contaminating erythrocytes were lysed with phosphatase-buffered deionized water, and the remaining cells were washed three times in magnesium-free Hanks balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, Mo.) supplemented with 1% fetal bovine serum (FBS) and suspended in phosphate-buffered deionized water before 150,000 g for 1 h. The leukocytes were then resuspended in HBSS and then incubated at 37°C for 2.5 h. The remaining cells were then lysed with 0.1% sodium deoxycholate in LB, and the bacteria were then lysed with distilled water, and surviving bacteria were titered by standard methods on MacConkey agar plates. These experiments were repeated eight times. Macrophage viability was confirmed by exclusion with 0.2% trypan blue dye. Viability confirmation of replication was obtained by Wright's staining after 2.5 h of incubation.

In order to confirm phagocytosis, a double-fluorescence staining technique was used to discriminate between intracellular and extracellular bacteria (14). *Salmonella* was labeled with rabbit anti-serotype B (*S. typhimurium*) or anti-serotype D (*S. pullorum*) antiserum as the primary antibody and goat anti-rabbit fluorescein isothiocyanate-conjugated antisera as the secondary antibody. Epifluorescence microscopy was used to examine the preparations.

**Animal challenge.** Forty-day-old, specific-pathogen-free White Leghorn chicks were used for the animal challenge. The birds were housed in biosafety level 3 Horsfall units at the Southeastern Poultry Research Laboratory (U.S. Department of Agriculture, Athens, Ga.) and provided with water and growth ration ad libitum. Bacteria (10^6 CFU) from a static overnight culture grown in LB were used to inoculate chicks per os prior to food and water access. Three chicks from each challenge group were euthanatized by cervical disarticulation each day for 4 days postchallenge. Large and small intestine en bloc, cecum, bursa, liver, and spleen were aseptically removed from each challenge group, homogenized in 4 ml of PBS, and plated on brilliant green agar. Organisms from the intestines were assayed for virulence in chicks and adults as described by Brooks et al. of a procedure previously described by Glick et al. (8, 19).

**RESULTS**

**TABLE 1. Percent adherence and invasion of salmonellae on cultured human and avian epithelial cells**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Adherence</th>
<th>% Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> y3181</td>
<td>Human</td>
<td>4.23 ± 0.077</td>
</tr>
<tr>
<td></td>
<td>Avian</td>
<td>1.3 ± 0.25</td>
</tr>
<tr>
<td><em>S. pullorum</em> x3423</td>
<td>Human</td>
<td>0.42 ± 0.022</td>
</tr>
<tr>
<td></td>
<td>Avian</td>
<td>0.019 ± 0.006</td>
</tr>
<tr>
<td><em>Escherichia coli</em> HB101</td>
<td>Human</td>
<td>2.35 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Avian</td>
<td>0.002 ± 0.000007</td>
</tr>
</tbody>
</table>

* Percent adherence and invasion were calculated as the percent of inoculum detected after four washes at a multiplicity of infection of 100 bacteria per epithelial cell. The human cells were Henle 407 and the avian cells were the budgerigar abdominal tumor cell line. Results and standard errors were calculated from two experiments with duplicate wells.

**TABLE 2. Percent killing of salmonellae by PMNs isolated from chickens (heterophils) and humans (neutrophils)**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Killing by PMNs from:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhimurium</em> y3181</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>51.57 ± 5.23</td>
</tr>
<tr>
<td><em>S. pullorum</em> x3423</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>77.29 ± 5.03</td>
</tr>
</tbody>
</table>
the *S. pullorum* inoculum was adherent, and only 4% of the attached cells invaded epithelia. In addition, *S. pullorum* was poorly invasive on chick ovary and chick kidney cells with only 0.5 and 0.02% of inoculum recovered from those respective monolayers (data not shown).

**Killing by leukocytes.** The ability of polymorphonuclear leukocytes (PMNs) to kill the isolates is represented in Table 2. Differential staining after density gradient centrifugation confirmed that each leukocyte preparation resulted in greater than 90% purity. Human PMNs were significantly more efficient than heterophils at killing *Salmonella* (*P* < 0.05). *S. pullorum* was significantly more sensitive to PMN killing than *S. typhimurium*, suggesting that the host specificity of this isolate is not dependent on resistance to PMN killing.

**Intracellular survival and replication.** Epifluorescent microscopy confirmed that the bacteria had been phagocytosed by the MQ-NCSU macrophages after 2.5 h of incubation. Surviving organisms detected after the 1-h gentamicin kill step reflect the number of bacteria internalized by the avian cell line macrophages. Table 3 shows data obtained after phagocytosis and the extracellular kill. An increase in the number of detected bacteria at 5 h postphagocytosis indicated bacterial rep-

![Fig. 1. Histopathologic changes and immunohistochemical staining from *S. typhimurium*-challenged chicks, 2 days postchallenge. (A) Section taken from cecum shows marked infiltration of heterophils into the lamina propria and migration through the mucosal epithelium (arrows). Multiple crypt abscesses are identified by arrowheads. (B) Immunohistochemical staining of *S. typhimurium* in cecum from the same bird as in A. Bacteria are identified within a vacuole by brown staining (arrow). (C) Bursa contains mild infiltrates of heterophils (arrow) in the connective tissue between follicles, but not within the bursal follicles. (D) Immunohistochemical staining of *S. typhimurium* (arrow) within a vacuole in bursal follicles from the same bird as in C.](http://iai.asm.org/)

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**TABLE 3. Percent survival of salmonellae in MQ-NCSU cells after phagocytosis**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>% Survival after:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td></td>
</tr>
<tr>
<td>χ3181</td>
<td>18.82 ± 3.16</td>
</tr>
<tr>
<td><em>S. pullorum</em></td>
<td></td>
</tr>
<tr>
<td>χ3423</td>
<td>5.68 ± 1.06</td>
</tr>
</tbody>
</table>

* Percent survival was calculated as the percent of inoculum cultured after phagocytosis and gentamicin killing of extracellular bacteria. An increase in percent indicates bacterial replication; a decrease indicates killing of the bacteria.
lication had occurred by both isolates. No decrease in phagocyte viability was detected by trypan blue exclusion (data not shown). In some experiments, an average of 30 bacteria per macrophage were cultured after 5 h of incubation. Visual confirmation of replication was obtained by Wright’s staining of MQ-NCSU macrophages experimentally infected with *S. typhimurium* and *S. pullorum*. At 1 h postphagocytosis, small numbers of both *S. typhimurium* and *S. pullorum* were seen within the vacuoles. By 7 h post-gentamicin treatment, most vacuoles contained large clusters of bacteria (data not shown).

**Live animal challenge studies.** *Salmonella* was not cultured from control birds at any time during the course of the experiment. Immunohistochemical staining was negative for *Salmonella* in the control birds throughout the study, and tissues were observed to be histologically normal. Organized lymphoid tissue was not observed in the intestines of the control birds.

The isolates demonstrated different intestinal colonization dynamics in this study. Both *S. typhimurium* and *S. pullorum* were isolated from the cecum of challenged birds 1 day after inoculation at approximately 10⁶ CFU. While this level was maintained in the *S. typhimurium*-challenged birds throughout the experiment, the number of *S. pullorum* cells decreased to levels only detected by enrichment on the 2nd day postchallenge. Both isolates were cultured from small and large intestinal tissues 1 day postchallenge at approximately 10⁶ CFU. However, while *S. typhimurium* maintained this level throughout the experiment, *S. pullorum* decreased by 4 log units on the 2nd day postchallenge.

Immunohistochemical staining identified numerous *S. typhimurium* cells associated with the superficial surface of the mucosal epithelium, the lamina propria, and the lumenal contents of the cecum, 1 day after challenge. Figure 1 shows the microscopic events 2 days after *S. typhimurium* challenge. The presence of *S. typhimurium* was accompanied by marked heterophilic and mononuclear infiltration into the lamina propria, and heterophils were migrating between mucosal epithelial cells into the intestinal lumen (Fig. 1A). Multiple crypt abscesses were also present. On day 3 postchallenge, the luminal contents of the cecum and the ileum were filled with heterophils in the *S. typhimurium*-challenged bird; however, few heterophils were seen in the lamina propria or epithelium. The mucosal villi of the cecum were markedly flattened. On both days, large numbers of *S. typhimurium* bacteria were confirmed by immunohistochemical staining in areas of inflammation in...
the cecum (Fig. 1B), with moderate numbers in the small intestine. On day 4, the inflammatory infiltrate was primarily mononuclear and present in the cecum and small intestine in the S. typhimurium-infected bird. The cecal mucosal epithelium was attenuated, abscesses were present in the cecal crypts, and individual cell necrosis was distributed throughout the lamina propria. Intestinal luminal contents contained numerous necrotic heterophils and cellular debris. Large numbers of S. typhimurium bacteria were seen within areas of inflammation of the cecum as well as within the intestinal lumen. Clusters of bacteria were seen within vacuoles in intestinal epithelial cells and within mononuclear cells.

In contrast, no significant intestinal lesions were observed in the S. pullorum-challenged bird at 1 day postchallenge. Immunohistochemistry revealed that S. pullorum was present only within the lumen of the cecum and was rarely associated with the mucosal epithelium. On day 2 postchallenge, S. pullorum cells were rarely seen within vacuoles of the cecal epithelium, and only mild heterophilic infiltration was apparent (Fig. 2). By day 3, crypt abscesses and a marked heterophilic and mononuclear infiltration in the lamina propria were present in the cecum of the S. pullorum-challenged bird. Heterophils were seen between mucosal epithelial cells and in the intestinal lumen. The epithelium and lamina propria of the ileum were diffusely infiltrated by heterophils. Immunohistochemistry revealed S. pullorum within the contents of the cecal lumen and within vacuoles in the epithelial mucosa. The major inflammatory component of the cecum in the S. pullorum-challenged bird on day 4 was mononuclear, with very few heterophils except those present in the multifocal areas of crypt abscesses. The cecal lumen was filled with heterophils and cellular debris; immunohistochemistry showed bacteria primarily in the lumen of the cecum.

The isolates were similar in their invasion of the liver; the number of each isolate peaked in the liver by 3 days postchallenge (Table 4). Bacteria were also demonstrated within areas of inflammation in the liver by using immunohistochemistry. In contrast, the isolates displayed different dynamics in invasion of the bursa. While both isolates were cultured from the bursa on all days, numbers of S. pullorum bacteria were decreasing by day 4 postchallenge, while numbers of S. pullorum bacteria were increasing. The culture data were corroborated by microscopic pathology and immunohistochemistry. Surprisingly, the pathology associated with S. pullorum invasion of the bursa occurred earlier and was more severe than in the S. typhimurium lesions. On day 2 postchallenge, S. typhimurium elicited a mild heterophilic infiltration around the follicles of the bursa (Fig. 1C); immunohistochemistry confirmed the presence of bacteria in these areas (Fig. 1D). S. pullorum, in contrast, induced marked bursal inflammation (Fig. 2C). Bursal follicles were depleted of lymphocytes and infiltrated with heterophils and macrophages. Immunohistochemistry revealed that S. pullorum cells were present within the areas of bursal inflammation and within vacuoles of mononuclear cells in this organ (Fig. 2D); however, bacteria were not found in the bursal lumen. On day 4, the bursal architecture was effaced by a marked infiltration of heterophils and mononuclear cells with accompanying necrosis and marked lymphoid depletion. Large numbers of both organisms were present within areas of inflammation in the bursa. The pathology of the S. pullorum lesions was more severe, with bursal follicles exhibiting lymphoid lysis and necrosis.

**DISCUSSION**

In this study, the broad-host-range pathogen S. typhimurium and the host-specific avian pathogen S. pullorum were shown to interact similarly with avian leukocytes. Both mammalian and avian PMNs were competent killers, although macrophages were not as efficient as PMNs at killing Salmonella. Furthermore, both isolates were also capable of replicating within macrophages in vitro in comparable numbers. The interactions of PMNs or macrophages with Salmonella have been studied in great detail by using mammalian models (1–4, 10, 39, 47), but only a few studies have evaluated the ability of avian leukocytes to kill Salmonella (42). Histological reports on experimental broad-host-range Salmonella infection in mammalian and avian models confirm that intestinal colonization by Salmonella initiates an inflammatory response characterized by infiltration of the infected site by PMNs (7, 18, 32, 37, 44). Studies establishing the importance of PMNs in host resistance have demonstrated that experimentally infected neutropenic animals do not exhibit gastroenteritis; however, these animals die from septic disease following systemic spread of the organism (18, 24). In addition, transepithelial signaling to PMNs occurs for those serotypes known to produce gastroenteritis in humans (30); however, non-human pathogens did not exhibit this signaling in human-derived cells, which suggests that the intensity and outcome of disease depend largely on the pathogen-host interactions involved in colonization (31). Thus, PMNs apparently play a crucial role in eradicating luminal pathogens, thereby preventing their dissemination.

PMN migration is typically followed by an infiltration of macrophages (21). Although macrophages are important in clearing bacteria, there is evidence that these phagocytes, capable of surviving intracellularly, may play a key role in pathogenesis by serving as hosts for those organisms. The capacity for Salmonella species to intracellularly replicate within murine-derived macrophages has been previously demonstrated by several techniques (2, 10). For serotypes such as S. typhimurium, known to produceenteric fever in mice, survival and replication in macrophages are essential for virulence, and macrophages serve as a vehicle of dissemination (12, 16). Several studies suggest that the site of intestinal invasion may contribute to the host specificity of Salmonella serotypes (25, 36, 43). Barrow et al. have reported that, for Salmonella serotypes that exhibit host specificity for chickens and mice, specificity is the result of the ability to survive within organs of the

### TABLE 4. Tracking viable salmonellae in vivo after oral challenge of 1-day-old chicks over a 4-day period

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Intestine (days postchallenge)</th>
<th>Cecum (days postchallenge)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>S. typhimurium y3181</td>
<td>6.51 ± 0.36</td>
<td>6.77 ± 0.23</td>
</tr>
<tr>
<td>S. pullorum y3423</td>
<td>6.38 ± 0.84</td>
<td>2.51 ± 1.52</td>
</tr>
</tbody>
</table>

* Two chicks were sacrificed daily, and the numbers of salmonellae were determined in selected organs. Results reflect the means (log_{10}) of CFU detected per organ.
reticuloendothelial system rather than the ability to penetrate intestinal epithelium (5). However, the gut-associated lymphoid tissue of birds is poorly organized; cecal tonsils and Peyer’s patches do not develop in young birds until 2 weeks of age (23, 44). In our study, even though S. pullorum was rarely found associated with epithelial cells, it was found within macrophages in the intestinal mucosa, bursal follicles, and liver on the 2nd day postchallenge, suggesting that these organisms were taken up and disseminated by macrophages. While S. typhimurium was primarily localized to intestinal tissues, the route of pathogenesis for S. pullorum involved rapid dissemination to the bursa, a lymphoid organ unique to birds.

Invasion assays performed in vitro demonstrated that S. typhimurium was capable of invading both human and avian cells, while S. pullorum was poorly invasive on these and other avian species-derived cells. Histopathology and immunohistochemistry confirmed that S. typhimurium was located within vacuoles in the intestinal epithelium and accompanied by an infiltrate of heterophils on the 1st day postchallenge. In contrast, while S. pullorum was seen in the luminal contents of the cecum and large intestine, close association with the mucosa was not observed until the 2nd day postchallenge, and inflammation was not observed until the 3rd day postchallenge. Nonetheless, bursal follicles were depleted and contained S. pullorum by this time.

The results from our study indicate that infection with S. pullorum is not dependent upon rapid penetration of the intestinal epithelial cells. Rather, dissemination to the bursa occurs prior to the manifestation of enteritis. Hassan and Curtiss also observed transient inflammation of the bursa 2 days after 1-day-old chicks were orally challenged with S. typhimurium (22). Our histopathology of S. pullorum infection illustrated a targeting of lymphoid tissue, similar to the progression of disease caused by other host-specific salmonellae (12, 13, 28). Although the presence of organized lymphoid tissue was not observed in the cecum and intestine of these chicks, the bursa was a major site of infection, as confirmed by culture and immunohistochemical staining. Therefore, by preferentially invading lymphoid tissues instead of targeting intestinal epithelium, it is possible that S. pullorum exhibits tissue tropism for organized lymphoid tissue in chickens similar to the way S. typhimurium targets gut-associated lymphoid tissue in mice.

REFERENCES


