Loxoribine pretreatment reduces *Salmonella* Enteritidis organ invasion in 1-day-old chickens

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ABSTRACT Young poultry exhibit a transient colonization by some food-borne pathogens, including *Salmonella*, during the first week of life that stems from immature innate and acquired defense mechanisms. Consequently, modulation of the hosts' natural immune response is emerging as an important area of interest for food animal producers, including the poultry industry. Toll-like receptor (TLR) agonists have been shown to boost the innate immune response in young chickens and increase their resistance to colonization by *Salmonella enterica* serovar Enteritidis. The objective of the present study was to determine if pretreatment with loxoribine, a TLR7 agonist and immune modulator, protects young chicks from *Salmonella* Enteritidis organ invasion. Loxoribine (0–100 μg) was administered intra-abdominally to 1-d-old broiler chicks, and 4 h later, the birds were challenged orally with *Salmonella* Enteritidis. Twenty-four hours postchallenge, birds were euthanized and the liver and spleen aseptically removed and cultured for *Salmonella* Enteritidis. This was carried out on 3 separate occasions using 26 to 50 chicks per dose per experiment. Pretreatment of chicks with loxoribine (6.25–25 μg) significantly ($P \leq 0.05$) reduced liver and spleen organ invasion by *Salmonella* Enteritidis. Higher doses (50–100 μg) of loxoribine had no effect. The results obtained in this study indicate that there is a potential application for using loxoribine to increase protection of young chicks when they are most susceptible to infections with *Salmonella*.

Key words: chicken, immunomodulation, loxoribine, *Salmonella* Enteritidis, toll-like receptor

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INTRODUCTION

Young poultry exhibit a transient colonization by some food-borne pathogens, including *Salmonella*, during the first week of life that stems from immature innate and acquired host defense mechanisms (Kogut et al., 1994; Kogut et al., 1995; Genovese et al., 1998; Genovese et al., 2000). Traditional management in poultry depends on the use of vaccines, husbandry practices, bio-security, and when necessary, use of broad-spectrum antibiotics. Public outcry for removal of growth-promoting antibiotics in animal feed will ultimately limit this latter practice. In 1986, Sweden became the first country to ban the use of antibiotic growth promoters in food animal production (Cogliani et al., 2011), and the European Union withdrew approval for antibiotic growth promoters in 2006 (Castanon, 2007). Further, the use of other prophylactic drugs, including anticoccidiostats and antihelminthics, is also being limited. Consequently, ways to modulate the hosts' natural immune response is emerging as an important area of interest for all food animal producers, including the poultry industry.

Toll-like receptors (**TLR**) are a group of pattern recognition receptors that are differentially expressed on leukocytes and in nonimmune cells. The pattern recognition receptors detect and initiate the first response against invading bacteria and viruses by recognizing specific pathogen-associated molecular patterns (Janeway and Medzhitov, 2002; Akira et al., 2006). The use of TLR agonists, including flagellin and CpG oligodeoxynucleotides (CpG-**ODN**), have been shown to boost the innate immune response in young chickens and increase resistance against *Salmonella enterica* serovar Enteritidis (Genovese et al., 2007; He et al., 2007). Loxoribine is a guanosine analog that is a potent stimulator of the innate immune system and acts through TLR7 (Heil et al., 2003; Lee et al., 2003). Chicken TLR7 (chTLR7) has been identified and shares 62% identity to human TLR7 (huTLR7) (Philbin et al., 2005). Functional similarities also exist between mammalian TLR7 and chTLR7, in that stimulation with loxoribine promotes

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splenocyte proliferation and cytokine production (Philbin et al., 2005; Jenkins et al., 2009). Numerous in vitro studies using chicken cells (peripheral blood heterophils, splenocytes, or the HD11 chicken macrophage cell line) show that stimulation with loxoribine promotes increased levels of degranulation and oxidative burst responses, elevated interleukin (**IL**)-1β and interferon (**IFN**)-α expression (Kogut et al., 2005; Philbin et al., 2005; Kogut et al., 2006; Jenkins et al., 2009). Collectively, these studies provide data indicating the in vitro immunomodulatory properties of loxoribine, yet, to our knowledge, its role as an in vivo immune modulator in poultry has not been evaluated. Therefore, the objective of the present study was to determine if loxoribine pretreatment reduced organ invasion by *Salmonella* Enteritidis administered orally to 1-d-old-chicks.

MATERIALS AND METHODS

Experimental Chickens

Fertilized eggs from broiler chickens were incubated and hatched under standard conditions (Stromberg, 1975). On the day of hatch, straight-run chickens were placed into heated floor pens (3 × 3 m) containing wood shavings and were provided water and a balanced, unmedicated corn and soybean meal-based chick starter diet ad libitum. The feed contained 23% protein and 3,200 kcal of ME/kg of diet, and all other nutrient levels met or exceeded the requirements established by the NRC (1994). At the conclusion of each experiment, birds were euthanized via CO_2 asphyxiation. The birds were not vaccinated at any time during the experiment nor did they receive any medications. The experiments were conducted in accordance with the recommended code of practice for the care and handling of poultry and followed the ethical principles within the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, and NRC, 1996). The Institutional Animal Care and Use Committee approved all experimental procedures.

Bacteria

A poultry isolate of *Salmonella* Enteritidis (#97–11771) was obtained from the National Veterinary Services Laboratory (Ames, IA). The bacteria were selected for resistance to carbenicillin and novobiocin and were maintained in tryptic soy broth (Difco Laboratories, Sparks, MD) containing antibiotics [100 μg/mL of carbenicillin and 25 μg/mL of novobiocin (**TSB+CN**); Sigma Chemical Co., St. Louis, MO]. The *Salmonella* Enteritidis was cultured in TSB+CN overnight at 41°C and prepared fresh for each experiment; the stock culture was prepared in sterile PBS and adjusted to 1×10^9 cfu/mL as described (Swaggerty et al., 2005).

Loxoribine Preparation and Administration

A sterile stock of loxoribine (10 m*M*) was prepared according to the manufacturer's instructions (InvivoGen, San Diego, CA), and additional dilutions were prepared using endotoxin-free water (Cambrex Corporation, East Rutherford, NJ). Loxoribine was administered (0.1 mL using a 26G 3/8″ intradermal beveled needle) intra-abdominally (**IA**) to 1-d-old-chicks.

Organ Invasion

Chickens were randomly placed into either control or challenged groups and maintained in floor pens housed in separate isolation rooms. One-day-old chicks were challenged orally with *Salmonella* Enteritidis (0.5 mL) 4 h after administration of loxoribine. At 24 h post-challenge, chicks were euthanized and necropsied as described by Ferro et al. (2004). The challenge dose for experiments 1, 2, and 3 was 5.7×10^6 (n = 26–30 chicks per dose of loxoribine), 4.3×10^4 (n = 49 chicks per dose of loxoribine), and 7.7×10^4 cfu/chick (n = 47–50 chicks per dose of loxoribine), respectively. Positive control chicks received an IA injection of sterile PBS (0.1 mL) followed by challenge with *Salmonella* Enteritidis 4 h later. Noninjected and nonchallenged chickens (n = 5) were included for each experiment to confirm the flocks were free of *Salmonella* Enteritidis.

Statistical Analysis

Three separate experiments were conducted using chickens from a different hatch and flock. The experiments were analyzed separately to support the consistency of the results. All analyses were performed with SAS 9.2 (SAS Institute, Cary, NC) using logistic regression that modeled the number of positive infections by *Salmonella* Enteritidis-affected birds relative to the total number of birds challenged for each dose of loxoribine. Odds ratios relative to the control groups of the no loxoribine treatment were reported as significant if $P \leq 0.05$.

RESULTS

Experiment 1

In total, 73.3% of the liver and spleen samples obtained from the control birds were positive for *Salmonella* Enteritidis (Table 1). Pretreatment of birds with loxoribine (25 μg) significantly ($P = 0.039$) reduced the percentage of *Salmonella* Enteritidis-positive birds to 46.4%, and it was determined they were 3 times less likely to be infected than controls not given loxoribine. Administration of higher doses of loxoribine (50 and 100 μg) did not significantly affect *Salmonella* Enteritidis organ invasion compared with controls. For this and all subsequent experiments, the noninjected, nonchal-

Table 1. Organ invasion by *Salmonella* Enteritidis in 1-d-old chicks pretreated with loxoribine (InvivoGen, San Diego, CA)

Experiment	Loxoribine[1] (μg)	No. of *Salmonella* Enteritidis-positive chicks/no. challenged	*Salmonella* Enteritidis-positive[2] (%)	Likelihood of being positive compared with controls	*P*-value
1	0	22/30	73.3		
	25	13/28	46.4	3× lower	0.039
	50	18/29	62.1	Same	0.30
	100	23/26	88.5	2.8× higher	0.16
2	0	19/49	38.8		
	6.25	9/49	18.4	2.8× lower	0.02
	12.5	12/49	24.5	Same	0.13
	25	24/49	49.0	1.5× higher	0.30
3	0	25/50	50.0		
	6.25	10/50	20.0	4× lower	0.002
	12.5	3/47	6.4	14.7× lower	≤0.0001
	25	4/48	8.3	11× lower	≤0.0001

[1]One-day-old chicks received specified amounts of loxoribine administered intra-abdominally.

[2]Chicks were given an oral challenge of *Salmonella* Enteritidis 4 h after administration of loxoribine. The challenge dose for experiments 1, 2, and 3 was 5.7×10^6, 4.3×10^4, and 7.7×10^4 cfu/chick, respectively.

lenged chickens were negative for *Salmonella* Enteritidis (data not shown).

Experiment 2

The optimal dose of loxoribine was 25 μg in experiment 1; therefore, the higher doses were omitted and replaced with lower doses (0, 6.25, 12.5, or 25 μg) for experiments 2 and 3 (Table 1). There were fewer control birds that were infected for this experiment (38.8%). Pretreatment of birds with loxoribine (6.25 μg) significantly ($P = 0.02$) reduced the percentage of *Salmonella* Enteritidis-positive birds to 18.4%, and it was determined they were 2.8 times less likely to be infected compared with birds not treated with loxoribine. Administration of 12.5 and 25 μg loxoribine did not significantly affect *Salmonella* Enteritidis organ invasion compared with controls.

Experiment 3

Fifty percent of the liver and spleen samples obtained from the control birds were positive for *Salmonella* Enteritidis (Table 1). Each dose of loxoribine (6.25, 12.5, or 25 μg) significantly ($P \leq 0.002$) reduced organ invasion compared with the control group that was administered PBS. Organ invasion in the group given 6.25 μg of loxoribine was reduced to 20% ($P = 0.002$), indicating those birds were 4 times less likely to be infected by *Salmonella* Enteritidis. The reduction was greater for the 12.5-μg dose of loxoribine (6.4% positive and 14.7 times less likely to have *Salmonella* Enteritidis; $P \leq 0.0001$) and the 25-μg dose of loxoribine (8.3% positive and 11 times less likely to have *Salmonella* Enteritidis; $P \leq 0.0001$).

DISCUSSION

Loxoribine is a guanosine analog that is a potent stimulator of the innate immune system and acts through TLR7 (Heil et al., 2003; Lee et al., 2003). In the present study, loxoribine was evaluated for its efficacy to protect young chicks against *Salmonella* Enteritidis organ invasion. There was some variation in the percentage of control birds that were positive for *Salmonella* Enteritidis (38.8–73.3%) between the 3 experiments. The highest (73.3%) was observed in experiment 1 and was likely due to a higher challenge dose (10^6 cfu/chick) compared with experiments 2 and 3 (10^4 cfu/chick), although those differences (38.8 and 50%, respectively) were probably normal flock-to-flock and experimental variations. There were also some differences in the number of *Salmonella* Enteritidis-infected chicks that were administered the same dose of loxoribine. Twenty-five micrograms of loxoribine was the only dose used in all 3 experiments. In the first 2 experiments, the percentage of *Salmonella* Enteritidis-positive birds was similar (46.4 and 49%), whereas in the third experiment, 8.3% of the birds given 25 μg of loxoribine were positive. Also, when comparing the percentage of positive birds in experiments 2 and 3, where the same doses of loxoribine were administered, the data indicate the chicks used in the third experiment were much more sensitive to the protective effects offered by loxoribine. Most importantly, the overall trend of increased resistance against *Salmonella* Enteritidis organ invasion following loxoribine treatment was evident in all 3 experiments. To our knowledge, we were provided the same line of birds for all 3 studies, so the differences are likely a result of flock-to-flock variation.

Because *Salmonella* Enteritidis is a gram-negative flagellated bacterium, activation of signaling pathways would function primarily via TLR4 and 5 and not TLR7; however, we sought to determine if the immunomodulatory effects of loxoribine were broad spectrum in nature and therefore protective against *Salmonella* Enteritidis. In vitro studies conducted by our laboratory and others show stimulation with loxoribine induces degranulation and oxidative burst responses by heterophils (Kogut et al., 2005; Kogut et al., 2006). Additionally, there are reports of increased cell proliferation accompanied by increases in IL-6, IL-8, and IFN-α cytokine expres-

sion levels in chickens (Philbin et al., 2005; Kogut et al., 2006; Jenkins et al., 2009) and mammals (Jin et al., 1990; Heil et al., 2003; Lee et al., 2003) following exposure to loxoribine. This is important as there are numerous studies that indicate increases in heterophil numbers and enhanced functional efficiency and upregulated cytokine production are involved in protecting chickens from *Salmonella* (Kogut, 2002; Swaggerty et al., 2003; Ferro et al., 2004; Swaggerty et al., 2004; Swaggerty et al., 2005; Redmond et al., 2009).

To our knowledge, there are no in vivo studies using loxoribine as an immune modulator in chickens; however, the earlier in vitro studies suggest that pretreatment with loxoribine had the potential to protect young chicks. In fact, as shown herein, loxoribine administered via the IA route to 1-d-old chicks offered a significant level of protection against organ invasion following an oral challenge with *Salmonella* Enteritidis. The protection offered by loxoribine is similar to that observed with treating 1-d-old chicks with CpG-ODN, a mammalian TLR9 agonist, which has also been shown to prime heterophil-mediated killing mechanisms and is associated with a significant reduction in *Salmonella* Enteritidis organ invasion (He et al., 2007). The mechanism of protection was not evaluated in the present study; however, the data suggest that the innate immune response may have been modulated or primed, thus responding to and eliminating the bacterium before it had a chance to colonize the young chicks. Flagellin, a TLR5 agonist, is another potent stimulator of heterophil-mediated innate immunity in young chickens. A study by Genovese et al. (2007) showed 1-d-old chicks administered flagellin IA had increased numbers of circulating heterophils, elevated numbers of heterophils in the abdominal cavity following IA challenge with *Salmonella* Enteritidis, and significantly less mortality following *Salmonella* Enteritidis challenge in the flagellin-treated birds compared with the controls. Collectively, these data further support a potential role for TLR agonists as immunomodulators for use by the poultry industry.

Numerous studies show that heterophils are a critical component in controlling and eliminating *Salmonella* infections in chickens (Kogut et al., 1994; Stabler et al., 1994; Harmon, 1998; Kogut, 2002; Swaggerty et al., 2003, 2005; Ferro et al., 2004; He et al., 2007; Redmond et al., 2009). Taken together with the in vitro studies using loxoribine and the in vivo CpG-ODN and flagellin studies, one could speculate that in the present study the heterophils were activated and either underwent degranulation or oxidative burst, or that additional heterophils were recruited, or a combination of all to eliminate the *Salmonella* Enteritidis before getting established. Another possibility is that cytokines and chemokines released by heterophils could have contributed to the killing of the bacteria. The expression of cytokines and chemokines as a result of loxoribine stimulation has been reported in chickens (Kogut et al., 2005; Philbin et al., 2005; Kogut et al., 2006; Jenkins et al., 2009) and mammals (Heil et al., 2003; Lee et al., 2003). It is also plausible that *Salmonella* Enteritidis (TLR4 and 5 agonist), in conjunction with loxoribine (TLR7 agonist), synergistically enhanced the host response that, in turn, offered increased protection from organ invasion by *Salmonella* Enteritidis. Reports of such TLR-TLR cross talk show there is enhanced cytokine production by human peripheral blood mononuclear cells following simultaneous TLR4 and TLR7 stimulation in vitro (Ghosh et al., 2007). Further studies to define the mechanism(s) of loxoribine in protecting chicks from *Salmonella* Enteritidis as well as other key poultry and food-borne pathogens should be examined.

In conclusion, the results described in this report demonstrate how administration of loxoribine to young chicks can increase their resistance against orally acquired *Salmonella* Enteritidis. This study advances earlier in vitro experiments and shows the potential application for a novel, nonantibiotic means to prevent infection of young chicks with *Salmonella* Enteritidis, one of the most important food-borne pathogens.

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