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NOTES

Secreted Effector Proteins of *Salmonella enterica* Serotype Typhimurium Elicit Host-Specific Chemokine Profiles in Animal Models of Typhoid Fever and Enterocolitis

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Infection of bovine ligated loops with the *Salmonella enterica* serotype Typhimurium wild type but not a *sipA sopABDE2* mutant resulted in fluid accumulation, polymorphonuclear cell infiltration, and expression of CXC chemokines, particularly GROα. None of these *sipA sopABDE2*-dependent responses was observed in murine-ligated loops. The majority of GROα transcripts localized to bovine intestinal epithelium. Thus, different disease outcomes between mice (i.e., no diarrhea) and calves (i.e., diarrhea) may be due to differences in *sipA sopABDE2*-dependent CXC chemokine gene expression in epithelial cells.

Salmonella enterica serotype Typhimurium causes enterocolitis in humans, a localized infection characterized by diarrhea and by pathological changes that are most severe in the intestine and mesenteric lymph nodes (24, 25). In contrast, *S. enterica* serotype Typhi causes typhoid fever, a systemic infection characterized by fever, while diarrhea is considered to be an insignificant symptom developing in only one-third of patients (28). A striking difference between the host responses elicited during infections with serotype Typhimurium and serotype Typhi in humans is the type of inflammation observed in the intestine. Analysis of biopsy samples reveals that inflammation in the intestines of patients infected with serotype Typhimurium is characterized by an infiltrate that is composed primarily of polymorphonuclear (PMN) cells (6, 27), while inflammation in typhoid fever patients is caused predominantly by infiltration with monocytes (21, 39). Similarly, the predominant cell type (representing 95% of fecal leucocytes) in stools from typhoid fever patients is mononuclear, whereas PMN cells predominate (representing 75% of fecal leucocytes) in stool samples from enterocolitis patients (13).

Serotype Typhimurium induces fluid accumulation and PMN cell influx in bovine, but not murine ligated ileal loops. Experimental infections of calves or mice with serotype Typhimurium are commonly used as animal models to study the pathogenesis of typhoid fever or enterocolitis, respectively. Serotype Typhimurium causes a typhoid fever-like disease without diarrhea in mice, while infection of calves results in a localized infection characterized by diarrhea (49). We compared the host responses to infection with serotype Typhimurium in mice and calves using the ligated-ileal-loop model. Bovine ligated-ileal-loop surgeries were carried out as described previously (34, 50). In mice, anesthesia was induced and maintained with Propofol (Abbot Laboratories, Chicago, Ill.), an approximately 2-cm incision was made in the abdomen, an ileal loop of 5 to 8 cm was ligated and injected with 0.15 ml of sterile Luria-Bertani (LB) broth or 1×10^8 CFU. To prevent dehydration, mice were given two doses of 0.5 ml of sterile saline subcutaneously. Fluid accumulation and inflammatory changes elicited by the serotype Typhimurium wild type (IR715) (41) were compared to those elicited by a *sipA sopABDE2* mutant (ZA21) (50) and sterile LB broth. The *sipA* (*sspA*), *sopA*, *sopB* (*sigD*), *sopD*, and *sopE2* genes encode effector proteins of the invasion-associated type III secretion system (TTSS-1) that are required for eliciting PMN cell influx and fluid accumulation in bovine ligated ileal loops (11, 18, 34, 47, 50). Fluid accumulation in bovine ligated ileal loops inoculated with the serotype Typhimurium wild type (IR715) was significantly higher ($P > 0.05$) than that elicited by inoculation with LB broth or the *sipA sopABDE2* mutant (ZA21) at 4 and 8 h postinfection (Fig. 1A). In contrast, inoculation of murine-ligated ileal loops with either the serotype Typhimurium wild type (IR715), the *sipA sopABDE2* mutant (ZA21), or sterile LB broth did not result in significant differences in fluid accumulation at any of the time points collected (Fig. 1B). The serotype Typhimurium wild type was recovered at approximately 10-fold-higher numbers ($P > 0.05$) from bovine and murine Peyer's patches than the *sipA sopABDE2* mutant at early times (1 h) but not at later times (8 h) postinfection (data not shown). Reduced recovery of the *sipA sopABDE2* mutant

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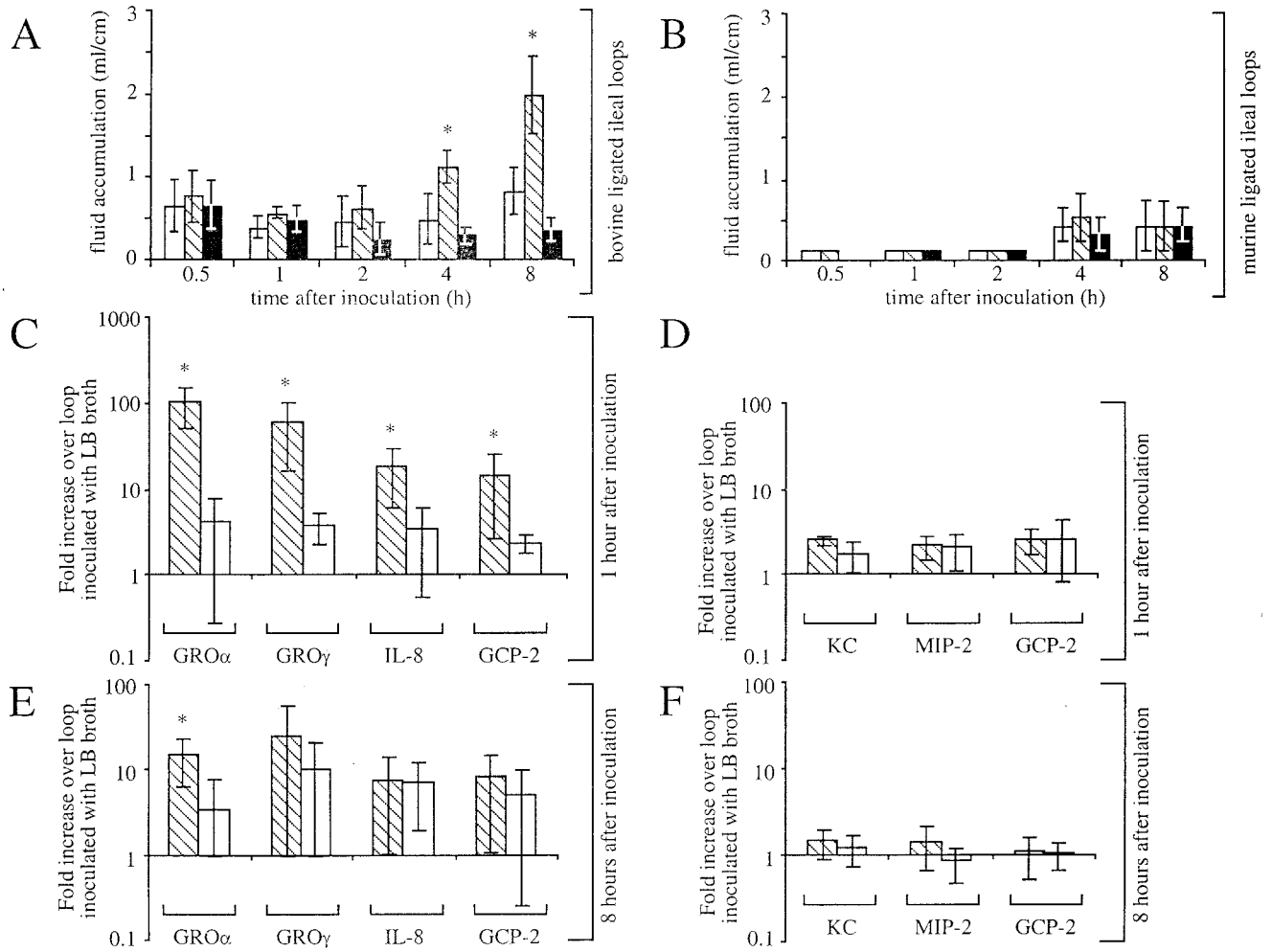

FIG. 1. Kinetics of fluid accumulation and CXC chemokine expression in bovine and murine ligated ileal loops. (A and B) Fluid accumulation elicited in bovine (A) and murine ligated (B) ileal loops is shown. Each data point represents the average (±standard deviations) from three independent experiments. Asterisks indicate that the amount of fluid elicited by serotype Typhimurium wild type (IR715; hatched bars) was significantly higher ($P < 0.05$) than that elicited by the *sipA sopABDE2* mutant (ZA21; open bars) and sterile LB broth (solid bars). (C to F) The change in induction (*n*-fold) of CXC chemokine gene expression in bovine (C and E) or murine (D and F) ileal Peyer's patches infected with wild-type (hatched bars) or *sipA sopABDE2* mutant (open bars) strains compared to expression in Peyer's patches inoculated with LB broth at 1 h (C and D) or 8 h (E and F) after inoculation is shown below. Asterisks indicate a significant increase in chemokine mRNA level in loops infected with the wild type (IR715) compared to those inoculated with the *sipA sopABDE2* mutant (ZA21).

from tissue collected at early times after infection may be explained by the requirement of SopB, SopE2, and SipA for invasion of human intestinal epithelial cells by serotype Typhimurium in vitro (17, 29, 51, 52).

Hematoxylin-and-eosin-stained sections of bovine and murine ileal Peyer's patches were examined by light microscopy. The degree of inflammatory cell infiltration and the severity of intestinal epithelial detachment were scored by two pathologists as described previously (34). A higher degree of monocyte infiltration was observed in sections collected from both bovine and murine ileal Peyer's patches infected with the serotype Typhimurium wild type (IR715) than in those collected from loops infected with the *sipA sopABDE2* mutant (ZA21) or sterile LB broth (Table 1). The host response observed in ligated ileal loops of mice differed from that in calves mainly by the degree of PMN cell infiltration. Infection with the serotype Typhimurium wild type (IR715) resulted in perivascular PMN cell infiltration in the bovine lamina propria by 1 h postinfection, and this inflammatory response progressed to severe diffuse PMN cell infiltration in the bovine ileal mucosa and submucosa by 8 h postinfection (Table 1). The *sipA sopABDE2* mutant (ZA21) caused only a mild PMN cell infiltration in the bovine ileal mucosa with minimal lesions. In contrast, the serotype Typhimurium wild type and the *sipA sopABDE2* mutant (ZA21) elicited only a mild PMN cell infiltration in the murine ileal mucosa.

The exposure of the ileal mucosa to a large bacterial inoculum for the duration of the experiment represents a physiological limitation of the ligated-ileal-loop assay. This limitation may be responsible for the severe detachment of surface epithelia observed within only 8 h after inoculation of murine ligated ileal loops with the serotype Typhimurium wild type (Table 1), while little or no inflammatory changes are observed in the intestinal mucosa at 1 day after oral infection of mice

TABLE 1. Development of inflammatory infiltrates and tissue injury in the bovine and murine ileal mucosa following inoculation with the serotype Typhimurium wild type, the *sipA sopABDE2* mutant, or sterile LB broth

Loop source	Pathological change	Loop inoculated with	Avg[a] qualitative score of pathological changes[b] in tissue collected at the following times (h) after inoculation of loops				
			0.5	1	2	4	8
Calves	Tissue-associated PMN infiltration	Wild type	1.7	2.7	3.3	2.7	4.0
		sipA sopABDE2 mutant	1.7	1.3	2.7	1.7	2.3
		LB broth	2.0	2.0	2.0	1.3	1.0
	Intravascular PMN infiltration	Wild type	0.7	1.7	2.3	1.3	2.3
		sipA sopABDE2 mutant	1.0	0.7	2.3	2.0	1.7
		LB broth	1.0	0.5	1.3	1.3	1.0
	Monocyte infiltration	Wild type	2.0	1.7	1.7	2.3	3.0
		sipA sopABDE2 mutant	1.9	1.3	2.3	1.7	2.0
		LB broth	1.7	2.0	1.7	1.7	1.3
	Detachment of surface epithelia	Wild type	0.0	1.0	1.4	1.7	3.3
		sipA sopABDE2 mutant	0.7	0.3	2.0	1.0	1.3
		LB broth	0.0	1.4	1.0	0.3	0.7
Mice	Tissue-associated PMN infiltration	Wild type	0.0	0.0	0.3	0.0	0.3
		sipA sopABDE2 mutant	0.0	0.3	0.7	0.7	1.3
		LB broth	1.0	0.0	0.0	0.0	0.3
	Intravascular PMN infiltration	Wild type	0.5	0.7	1.0	1.0	1.0
		sipA sopABDE2 mutant	0.3	1.0	2.3	3.0	2.7
		LB broth	1.0	1.0	0.7	0.0	1.3
	Monocyte infiltration	Wild type	1.5	3.0	3.3	3.0	3.5
		sipA sopABDE2 mutant	2.0	2.3	1.3	2.3	2.3
		LB broth	1.7	1.0	1.7	0.7	2.3
	Detachment of surface epithelia	Wild type	0.0	0.7	2.0	2.0	4.8
		sipA sopABDE2 mutant	0.3	0.3	0.0	0.0	1.0
		LB broth	0.0	0.0	0.0	0.0	0.7

[a] Each data point represents the average score from three independent experiments.
[b] The histopathological changes of murine and bovine Peyer's patches were assessed in a blinded manner by two veterinary pathologists and independently scored on a scale of 0 to 5, with 5 being the highest level of histologic changes.

(20, 36). In calves, on the other hand, lesions developed with kinetics similar to those observed during an oral infection (34, 43). Intravascular PMN cells were seen in the microvasculature of the murine ileal serosa in all treatment groups (data not shown), suggesting that the response in the mouse was due to surgical manipulations rather than to infection.

TTSS-1 effector genes are required for induction of CXC chemokine gene expression in bovine—but not murine—Peyer's patches. The major difference detected between the inflammatory infiltrates in calves and mice was the presence or absence of a large amount of PMN cells in the ileal mucosa (Table 1). Since trafficking of leukocytes is largely controlled by chemokines (23), we investigated whether the different compositions of inflammatory infiltrates observed in mice and calves in response to serovar Typhimurium infection may be reflected by the expression in infected tissue of different subsets of these chemoattractants. According to the number and arrangement of N-terminal cysteine residues, chemokines are divided into four subfamilies, including CX3C (three amino acid residues between the first two cysteine residues), CXC (one amino acid between the first two cysteine residues), CC (the first two cysteine residues being adjacent), and XC (lacking the first cysteine residue) (53). Different subsets of chemokines direct the migration of specific subsets of leukocytes (23). For instance, monocyte chemotactic protein 1 (MCP-1), MCP-2, macrophage inflammatory protein 1α (MIP-1α), and RANTES (i.e., regulated upon activation, normal T-cell expressed and secreted) act mainly on monocytes and belong to a subgroup of human CC chemokines, which are encoded by genes clustered on human chromosome 17q11.2 (53). In contrast, interleukin 8 (IL-8), growth-related oncogene α (GROα), GROγ, and granulocyte chemotactic protein 2 (GCP-2) are encoded by genes clustered on human chromosome 4q12-q13 and belong to a subgroup of human CXC chemokines controlling migration of PMN cells (53). While counterparts of the human genes encoding MCP-1, MCP-2, MIP-1α, RANTES, IL-8, GCP-2, GROα, and GROγ are also present in the bovine host (1, 30–32, 44, 45), mice exhibit a number of genetic differences. That is, mice do not possess the CC chemokine MCP-1 but instead express a functional analogue, the monocyte chemoattractant JE (3). Furthermore, mice do not possess IL-8, GROα, or GROγ, but instead produce the CXC chemokines keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) (4, 33, 42, 46). Murine KC and MIP-2 share sequence homology with human and bovine GRO proteins (31) and are involved in controlling PMN cell trafficking (38). Unlike other cytokines, CXC and CC chemokines are generally not stored within cells; rather, their production is induced at the transcriptional level upon appropriate stimulation (2, 10). We therefore investigated the expression of chemokines in bovine and murine tissues by detecting transcripts at 0.5, 1, 2, 4, and 8 h postinfection by using semiquantitative reverse transcriptase PCR (RT-PCR) as described previously (35) and by using primers specific to bovine MCP-1, MCP-2, MIP-1α, RANTES, IL-8, GCP-2, GROα, and GROγ as well as primers specific to murine JE, MCP-2, MIP-1α, RANTES, KC, GCP-2, and MIP-2 (Table 2).

Infection with the serotype Typhimurium wild type (IR715)

TABLE 2. Primers used in this study for RT-PCR and real-time PCR

PCR type	Target	Primer sequence[a]	Annealing temp (°C)	No. of cycles	Product or amplicon size (bp)
RT					
	b-IL-8	TGCCTAAACCCCAAGGAAAAGTG AACCCTACACCAGACCCACACAGAAC	53.5	25	205
	b-GROα	GATTCACCTCAAGAACATCCAGAGC AGAACTGCCAAACACATTCACACC	55.0	25	396
	b-GROγ	CAAAGAGGGAAAAGAGGAATCACC AAGGGCTGGCATAATGTGGG	52.0	25	335
	b-GCP-2	TTCGCCACTATGAGACTGCTATCC TCCAGACAGACTTCCCTTCCATTC	60.0	25	284
	b-MCP-1	AAACCAAACTCCAAAGCCTTGAG TTCTTGCGAGGACACTTCCACC	52.5	25	335
	b-MCP-2	ATTCTGTGTCTGCTGCTCGTGG TTCAAGGCTTCGGTGTTC	55.3	25	283
	b-MIP-1α	TCTGCCCTTGCCTGTTGTTC TCGGTGATGTATTCCTGGACCC	55.2	25	252
	b-RANTES	CCAGGAGTATTTCTACACCAGC AGCACTGAGGGTCTTTCACAGC	56.5	25	298
	m-KC	TGGGATTCACCTCAAGAAC AGTGTTGTCAGAAGCCAGCG	53.9	25	355
	m-MIP-2	CCCAGACAGAAGTCATAGCCAC AATAAGTGAACTCTCAGACAGCG	55.8	25	366
	m-GCP-2	GGCATTTCTGTTGCTGTTCACG CTTTCTTCTCTTCACTGGGGTCAG	56.3	25	343
	m-JE	GGAAAAATGGATCCACACCTTGC TCTCTTCCTCCACCACCATGCAG	58.3	25	581
	m-MCP-2	TGCTTCTTTGCCTGCTGCTC TGCTTGTAACATCTCTCTGCCTGG	56.8	25	358
	m-MIP-1α	TGACCTGGAACTGAATGC TGTGACCAACTGGAGGGATG	54.4	25	242
	m-RANTES	CATCCTCACTGCAGCCGCC CCAAGCTGGCTAGGACTAGAG	56.2	25	319
Real time					
	b-IL-8	AAGTGGGTGCAGAAGGTTGTG GGAGCATGGGTTTTTCCTTTC			79
	b-GCP-2	CCAGTGTCCCCAGGAAGCT GTCCAGGAGCCTTATGGAAGTCT			103
	b-GROα	TTACTTTTTGTAGAGAAGATTGTCAGTTGTT CCAAGGGATATTTAGATCATTGTCATT			121
	b-GROγ	TTGGATGGCTGTTCCAGAAGTA GCCTTAGGAGGTGGTGATTCCT			78
	b-GAPDH	TTCTGGCAAAGTGGACATCGT GCCTTGACTGTGCCGTTGA			92
	m-KC	ACCCAAACCGAAGTCATAGCC TTCAGGGTCAAGGCAAGCC			60
	m-MIP-2	TGAGTGTGACGCCCCCA TTTTTGACCGCCCTTGAGAG			71
	m-GCP-2	ACGCTGCGCAGCATCA GCTCCGTTGCGGCTATG			59

[a] Sequences for bovine and murine chemokine genes were obtained from GenBank. Top row, primer 1; bottom row, primer 2.

TABLE 3. Expression profile of chemokine genes in bovine and murine ileal Peyer's patches determined by RT-PCR

Chemokine	Loop inoculated with	Avg.[a] intensity of RT-PCR products[b] from tissue collected at the following times (h) after inoculation of loops				
		0.5	1	2	4	8
Bovine GROα	Wild type	21,659	43,862	39,525	41,398	44,374
	sipA sopABDE2 mutant	1,681	7,308	8,595	22,765	10,401
	LB broth	2,366	3,256	924	3,019	3,658
Bovine GROγ	Wild type	12,736	43,331	44,574	35,691	43,014
	sipA sopABDE2 mutant	6,159	12,780	30,285	37,517	23,707
	LB broth	3,202	5,622	3,213	4,187	9,227
Bovine IL-8	Wild type	25,839	38,272	37,428	37,213	36,497
	sipA sopABDE2 mutant	19,988	28,175	25,751	38,657	31,395
	LB broth	6,430	12,314	11,371	17,239	11,525
Bovine GCP-2	Wild type	20,906	30,238	28,735	38,998	34,338
	sipA sopABDE2 mutant	22,666	23,162	32,724	31,325	36,786
	LB broth	15,025	14,587	11,331	10,291	18,894
Bovine MCP-1	Wild type	19,865	34,807	29,242	23,868	27,201
	sipA sopABDE2 mutant	5,566	7,482	16,178	16,824	11,345
	LB broth	3,616	3,846	2,506	1,153	772
Bovine MCP-2	Wild type	38,738	49,545	44,096	44,129	52,014
	sipA sopABDE2 mutant	21,107	30,192	38,427	49,680	43,826
	LB broth	20,511	22,795	22,415	20,622	19,531
Bovine MIP-1α	Wild type	3,150	4,711	6,019	5,824	5,353
	sipA sopABDE2 mutant	7,216	4,817	3,699	6,524	5,791
	LB broth	2,067	4,096	4,977	2,330	2,483
Bovine RANTES	Wild type	41,907	42,042	42,018	34,219	39,325
	sipA sopABDE2 mutant	46,135	43,184	43,064	39,054	38,471
	LB broth	32,058	41,615	39,265	30,620	29,846
Murine KC	Wild type	47,636	52,148	50,812	52,744	53,246
	sipA sopABDE2 mutant	40,688	50,866	50,594	51,208	49,622
	LB broth	18,581	42,701	39,421	50,579	40,688
Murine GCP-2	Wild type	8,616	16,655	47,264	54,468	56,123
	sipA sopABDE2 mutant	6,878	6,359	41,984	52,237	48,917
	LB broth	4,738	5,689	15,315	45,895	39,984
Murine MIP-2	Wild type	37,232	52,471	51,698	51,403	56,766
	sipA sopABDE2 mutant	26,215	52,049	55,777	56,673	44,451
	LB broth	15,546	33,140	40,279	56,931	44,342
Murine JE	Wild type	28,370	27,814	28,592	30,706	33,599
	sipA sopABDE2 mutant	7,686	14,969	13,464	6,401	6,417
	LB broth	12,960	15,756	12,344	15,927	17,240
Murine MCP-2	Wild type	12,243	38,362	45,164	43,510	49,735
	sipA sopABDE2 mutant	1,528	25,614	43,678	48,953	35,019
	LB broth	1,732	17,727	27,713	30,071	26,441
Murine MIP-1α	Wild type	14,196	26,057	35,651	39,379	38,554
	sipA sopABDE2 mutant	18,240	13,436	27,968	29,018	18,001
	LB broth	8,580	19,396	10,920	16,760	16,433
Murine RANTES	Wild type	29,994	40,021	35,289	31,557	40,429
	sipA sopABDE2 mutant	35,293	26,349	26,708	32,241	42,878
	LB broth	32,054	19,464	32,946	40,776	38,599

[a] Each data point represents the average of three independent experiments.
[b] Intensity of bands was determined using the NIH image software.

caused a significant ($P < 0.05$) elevation in the expression of two bovine CC chemokines (MCP-1 and MCP-2) and two bovine CXC chemokines (GROα and GROγ) in tissue from bovine Peyer's patches compared to infection with the *sipA sopABDE2* mutant (ZA21) (Table 3). Induction of GROα gene expression was most pronounced, being significantly ($P < 0.05$) elevated at all time points and reaching a peak at 1 h postinfection of bovine loops. In contrast, only the expression of two murine CC chemokines (i.e., JE and RANTES) was significantly ($P < 0.05$) higher in murine Peyer's patches in-

fected with the serotype Typhimurium wild type (IR715) than in those infected with the *sipA sopABDE2* mutant (ZA21) (Table 3). These data showed that the TTSS-1 effector genes *sipA*, *sopA*, *sopB*, *sopD*, and *sopE2* were only required in the calf for eliciting elevated expression of PMN cell chemoattractants (i.e., GROα and GROγ).

To quantify the differences in CXC chemokine gene expression observed between the treatment groups, real-time PCR analyses were performed with RNA samples collected at 1 and 8 h postinoculation. Real-time PCR was performed by using the SYBR Green method according to instructions provided by the manufacturer of the PCR kit (Applied Biosystems, Foster City, Calif.). Primers for murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased (Biosource International, Camarillo, Calif.), and the remaining primers are listed in Table 2. Reverse transcription of total RNA (2 μg) in a mixture containing 100 μl of 5.5 mM $MgCl_2$, 500 μM dNTP, 2.5 μM random hexamers, and 1.25 U of MultiScribe reverse transcriptase per μl was performed at 48°C for 30 min. Real-time PCR was performed for each cDNA sample (4 μl/reaction) in duplicate by using gene-specific primers (300 nM) and an ABI Prism 7700 thermocycler (95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). This experiment was performed twice for each total RNA sample. The threshold cycle (C_T) value was determined for each sample, and the mRNA concentration for each target gene was quantified by using a comparative C_T method (Applied Biosystems). Real-time PCR amplification of GAPDH transcripts was used to normalize the cDNA concentrations of different samples (which was carried out with the assumption that expression of GAPDH does not change during infection). The normalized amount of transcripts relative to the amount of transcripts present in samples from an uninfected control loop was given as $2^{-\Delta C_T \pm S}$, where S is the standard deviation.

Both the serotype Typhimurium wild type (IR715) and the *sipA sopABDE2* mutant (ZA21) elicited similar increases (4.8-fold or less) of murine CXC chemokine (KC, MIP-2, and GCP-2) gene expression compared to transcript levels in loops inoculated with sterile LB broth at both 1 and 8 h postinfection (Fig. 1D and F). In sharp contrast, the serotype Typhimurium wild type (IR715) elicited substantially higher CXC chemokine gene activation in the calf than the *sipA sopABDE2* mutant (ZA21). These differences were most pronounced for expression of the bovine GROα and GROγ genes at 1 h after infection (Fig. 1C). In addition, expression of IL-8 and GCP-2 was consistently elevated at 1 h postinfection of bovine ligated ileal loops with the serotype Typhimurium wild type (IR715) compared to that due to infection with the *sipA sopABDE2* mutant (ZA21). The fact that differences between the wild type and the *sipA sopABDE2* mutant in their ability to induce expression of IL-8 and GCP-2 at 1 h postinfection were detected by real-time PCR but not by RT-PCR analysis is likely due to the higher sensitivity and accuracy of the former method. Collectively, these data further supported the idea that the TTSS-1 effector genes *sipA*, *sopA*, *sopB*, *sopD*, and *sopE2* were required for elevated expression of CXC chemokine genes in bovine but not murine intestinal tissue.

Localization of GROα transcripts in bovine ileal Peyer's patches by in situ hybridization. The most dramatic changes in the host response observed during this study were a TTSS-1-dependent induction of CXC chemokine gene expression, particularly GROα, in the bovine ileal mucosa (Fig. 1). Previous in vitro studies have shown that infection of human epithelial cell lines with *Salmonella* serotypes can elicit the production of various CXC chemokines (8, 19, 26, 48), thereby suggesting that epithelial cells may be a source of these PMN cell chemoattractants in vivo. However, expression of CXC chemokines can be induced in vitro upon appropriate stimulation in nearly every type of cell that has been examined (2, 10), thereby illustrating the need to experimentally test the assumption that epithelial cells are a significant source of GROα production in vivo. To this end, we localized bovine GROα transcripts in bovine tissue by using in situ hybridization in tissue collected 1 h after inoculation. The bovine GROα gene was PCR amplified from a bovine cDNA library by using the primers listed in Table 2 (b-GROα) and labeled with psoralen-biotin according to instructions provided by the manufacturer (Ambion). This probe was used for in situ hybridization by using 5-μm sections of formalin-fixed and paraffin-embedded tissue samples. Deparaffinized sections were treated with a target retrieval solution at 95°C for 40 min (Dako) and with proteinase K (DAKO) at room temperature for 5 min. Endogenous peroxidase activity was quenched by incubating the sections in a solution of 0.3% hydrogen peroxide in methanol for 20 min. After denaturation of RNA for 5 min at 65°C, hybridization was carried out at 37°C for 1 h in a humidified chamber. Specificity of the labeling was confirmed in two control experiments in which the GROα probe was either omitted or replaced by biotinylated bacterial plasmid DNA. Hybridization of probes was detected by subsequent incubation with a primary streptavidin-peroxidase concentrate followed by one cycle of signal amplification with biotinyl tyramide solution and a secondary streptavidin-peroxidase concentrate. Sections were incubated with a diaminobenzidine solution, counterstained with Meyer's hematoxylin, dehydrated, and mounted.

In samples from loops infected with the serotype Typhimurium wild type (IR715), bovine GROα transcripts were detected primarily in enterocytes lining the intestinal crypts, in the base of absorptive villi, and in the follicle-associated epithelium of lymphoid nodules in Peyer's patches (Fig. 2A). Positive signals were observed less frequently in epithelial cells located at the tips of absorptive villi and occasionally in mononuclear leukocytes in the lamina propria. However, PMN cells were negative for the production of GROα transcripts. The amount of GROα transcripts detected in sections collected from loops inoculated with the *sipA sopABDE2* mutant (ZA21) or LB broth was strongly reduced (Fig. 2B) compared to that in sections from wild-type-infected loops. These data demonstrate that intestinal epithelial cells represent the principal cell type producing GROα in the initial (1 h postinoculation) response to serotype Typhimurium infection.

The precise mechanism for the *sipA sopABDE*-dependent induction of GROα gene expression in bovine epithelial cells is presently unclear. Serotype Typhimurium induces the expression of IL-1β mRNA in bovine intestinal tissue (35), but it is not known whether this cytokine is proteolytically activated and subsequently contributes to the induction of CXC chemokine gene expression in epithelial cells. The production of GROα by human intestinal epithelial cell lines in response to *S. enterica* serotype Dublin infection suggests a direct interac-

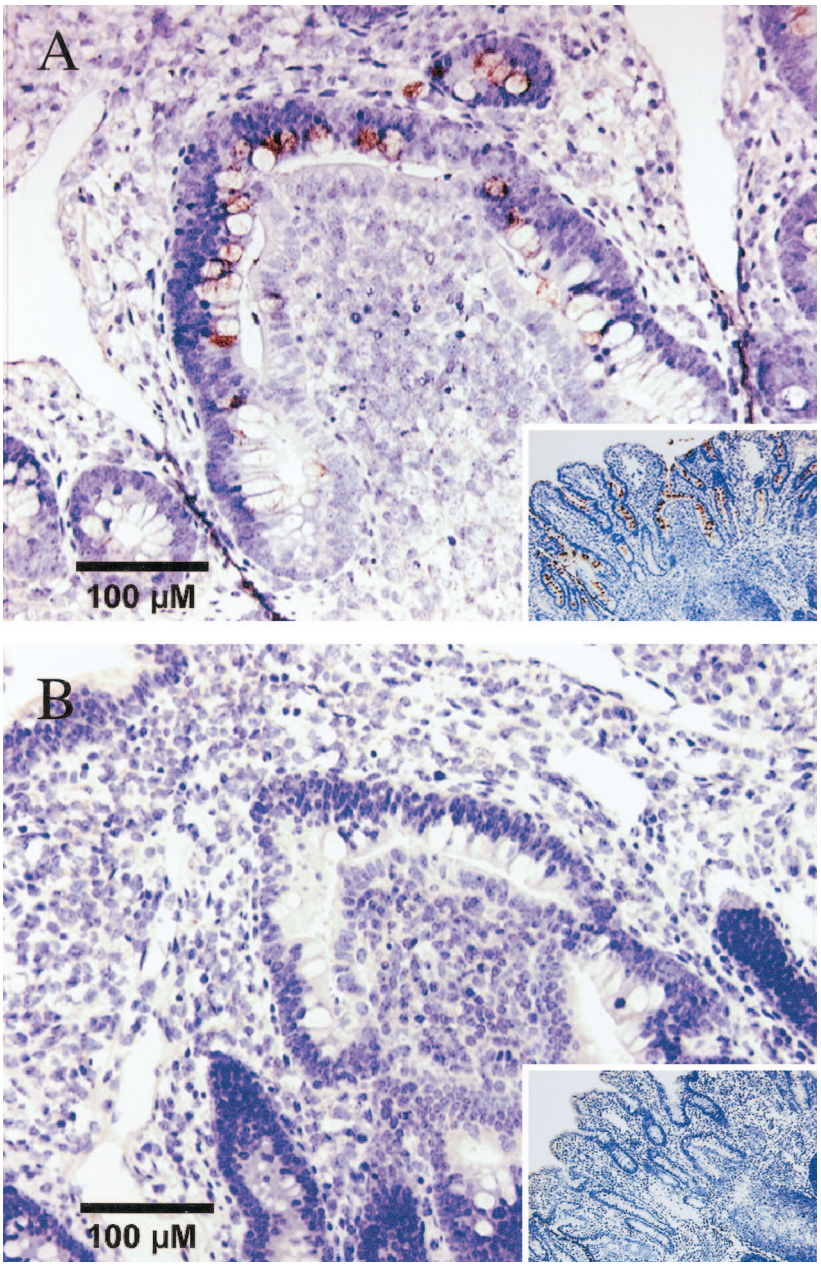

FIG. 2. Localization of GROα transcripts in bovine ileal Peyer's patches by in situ hybridization. The brown signal is produced by hybridization with GROα-specific mRNA, and slides were counterstained with hematoxylin to visualize cells (blue signal). (A) Section of bovine ileal Peyer's patches 1 h after infection with the serotype Typhimurium wild type (IR715). The insert shows a section at a lower magnification. (B) Section of bovine ileal Peyer's patches 1 h after inoculation with sterile LB broth. The insert shows a section at a lower magnification.

tion between bacteria and enterocytes as an alternate mechanism for the *sipA sopABDE*-dependent production of this CXC chemokine (48). Whether the TTSS-1 is required for inducing GROα production in human epithelial cells has not previously been studied. However, the serotype Typhimurium TTSS-1 is required for eliciting the production of human IL-8 in cultured epithelial cells (14) and was essential for inducing bovine IL-8 production in vivo (Fig. 1). It is presently a matter of debate whether TTSS-1-mediated invasion is required for IL-8 production (7, 12). TTSS-1-mediated invasion of epithelial cells may facilitate recognition by Nods or Toll-like receptors (16, 37), thereby triggering proinflammatory signaling events leading to CXC chemokine gene expression (15). An alternative mechanism by which TTSS-1 effector proteins may elicit CXC chemokine gene expression in epithelial cells is by directly engaging components of proinflammatory intracellular signaling cascades (5, 9, 22, 40). Regardless of whether CXC chemokine expression is induced through Toll-like receptors or by direct interaction of TTSS-1 effector proteins with intracellular targets, a correlate of the hypothesis that CXC chemokine expression results from a direct interaction between bacteria and enterocytes is that the relevant proinflammatory signaling

cascades must differ between murine and bovine intestinal epithelium, since the wild type and the *sipA sopABDE* mutant elicited similar CXC chemokine profiles in the mouse (Fig. 1D and F).

In summary, our data provide convincing evidence that induction of CXC chemokine expression accompanied by PMN cell infiltration is a key event that distinguishes the host response in calves from that elicited in mice during serotype Typhimurium infection. Furthermore, our study suggests that future studies should focus on GROα as the main CXC chemokine, the expression of which is induced by the TTSS-1 of serotype Typhimurium during enterocolitis in calves.

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