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■ Brief Communication ■

Analysis of Inflammatory Cytokines from the Cecum and Proximal Colon of Mice Infected with Enterotoxigenic *Bacteroides fragilis*

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Enterotoxigenic *Bacteroides fragilis* (ETBF) causes inflammatory diarrhea in humans and animals and is also implicated in colorectal cancer. ETBF-infected mice exhibit a prominent large intestinal inflammation characterized by neutrophil infiltration and induction of the Th17 response. In this study, we examined differences in the secreted cytokine profile of the cecum and proximal colon of ETBF-infected mice using an antibody array. Of the cytokines examined, we found that the cecal tissues from ETBF-infected mice secreted elevated levels of G-CSF, IL-6, IL-17 and LIX compared to non-toxigenic *Bacteroides fragilis* (NTBF) and Mock infected mice. The proximal colon tissues from ETBF-infected mice secreted higher levels of G-CSF, IL-6, KC, LIX, MIP-1g and MCP-1. This study demonstrates that the cecum and colon should be considered separately when assays are used to determine immune responsiveness to enteric infections.

Key Words: Enterotoxigenic *Bacteroides fragilis*, Protein array, Cytokine, Colitis, Toxin

Enterotoxigenic *Bacteroides fragilis* (ETBF) is an intestinal commensal bacteria which causes inflammatory diarrhea in laboratory animals (mice, gerbils), livestock and humans (Rhee et al., 2009; Sears et al., 2008; Yim et al., 2013). In addition, ETBF infections have been implicated in colorectal cancer in both humans and mice (Goodwin et al., 2011; Toprak et al., 2006; Wu et al., 2009). The only known virulence factor for ETBF is the secretion of a 20-kDa secreted zinc-dependent metalloprotease called *Bacteroides fragilis* toxin (BFT) or fragilysin (Franco et al., 1997; Moncrief et al., 1995). Treatment of the human colonic epithelial cell line HT29/c1 with purified BFT or bacterial supernatants of ETBF strains induce E-cadherin cleavage, secretion of IL-8 and cellular proliferation (Wu et al., 1998; Wu et al., 2003; Wu et al., 2004). Mice orally infected with ETBF exhibited acute diarrhea within 2~3 days accompanied by cecal contraction, inflammation of the large intestine and neutrophil infiltration of intestinal tissues (Rabizadeh et al., 2007; Rhee et al., 2009).

The inflammatory response in ETBF-infected mice is skewed toward a Th17 profile and is accompanied by STAT3 activation and production of the Th17 related cytokines (IL-17, IL-23, IL-6, TGF-b) (Wu et al., 2009).

The intestinal inflammation observed in ETBF infected mice is specific to the large intestine as inflammation is

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not observed in the small intestine and is only restricted to the cecum and colon (Rhee et al., 2009). It is unclear why ETBF induces only inflammation of the large intestine but not the small intestine. ETBF can be found in the luminal contents of the small intestine of ETBF infected mice and the small intestinal tissues ex vivo explants cultured in the presence of purified BFT undergo E-cadherin cleavage similarly to cecal and colonic tissues (Rhee et al., 2009). We postulate that either secretion of BFT is regulated differently in the small intestine versus the large intestine. Alternatively, anatomical and histologic disparities between the small and large intestine may contribute to ETBF responsiveness. The cecum and colon are anatomically, histologically and functionally different in mice although both are considered to be part of the large intestine. Previously, we found that C57BL/6 mice infected with ETBF exhibited regional differences in large intestinal inflammation (Rabizadeh et al., 2007). This difference in inflammation may be explained by the types of cytokines secreted in these different regions of the large intestine. Therefore, in this study, we used an antibody array which can detect the presence of multiple inflammatory cytokines and examined the cytokine profile in two regions of the large intestine, the cecum and proximal colon, from ETBF-infected mice during acute inflammation. To determine the spectrum of inflammatory cytokines produced by the infected tissues of ETBF infected mice, we used the RayBio Mouse Inflammation Antibody Array I (RayBiotech Inc, Norcross, GA, USA) which is a membrane based protein detection system that allowed simultaneous of 40 inflammatory cytokines and inflammation related proteins.

Specific pathogen-free 4-week-old male C57BL/6 mice were purchased from Central Lab Animals (Seoul, Korea). Mice infection was conducted as described previously (Rhee et al., 2009). In brief, mice were given water with clindamycin (100 mg/L) and gentamicin (300 mg/L) to promote *B. fragilis* colonization and for consistency of infection. Antibiotic water was initiated 7 days prior to bacterial inoculations and discontinued one day prior to bacterial inoculation. Mice (3 per group) were inoculated with 1×10^9 colony forming units (CFU) of ETBF (strain 86-5443-2-2), non-toxigenic *B. fragilis* (NTBF) (strain NCTC 9343 transformed with pFD340) or PBS (Mock). One week post-inoculation, ETBF and NTBF colonization in the stool was approximately $1\sim5\times10^9$ CFU/gram stool showing that both bacterial species colonized the mice equally. Mock infected mice had no growth of bacteria in the stool. Only the ETBF-infected mice exhibited signs of intestinal inflammation and diarrhea as observed previously (Rhee et al., 2009). Seven days post-infection, mice were euthanized and the large intestine excised and washed extensively with cold PBS to remove bacteria and debris. From each mouse, five 3-mm full-length punch biopsies were collected from the cecum of each mouse and placed into one well of a 24-well plate. The tissues were cultured with 1 ml of DMEM/F12 media containing 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml)and gentamicin (100 ug/ml). Supernatant was harvested after 24 hours of culture. The proximal colon (adjacent to the cecum) from each mouse was also processed in an identical manner. The supernatants from each group three mice were pooled and stored at -20℃ until used.

The antibody array experiment was conducted as suggested by the manufacturer. In brief, antibody dotted membranes were blocked for 30 min and then incubated with 500 ul of pooled tissue supernatant for 2 hr. The unbound proteins were washed and then an antibody cocktail containing biotinylated antibodies specific for each cytokine were added to the membrane for 1.5 hr. The membranes washed again and then cultured with HRP-conjugated strepavidin for 2 hr. The membranes were washed extensively and overlayed with the detection buffer. The chemiluminescence signal emanating from the membranes were exposed to X-ray film. The developed film was scanned, the dot intensity quantitated by densitometry (FusionCapt Advance software, Vilber Lourmat, France) and the relative expression levels were compared. Internal positive controls were used to normalize the results from different membranes being compared. The position and list of the target proteins

Table 1. List and position of antibodies in the membrane array.

Raybio Mouse Inflammation Antibody Array 1

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	POS	POS	Blank	BLC	CD30L	Eotaxin	Eotaxin-2	Fas ligand	Fractalkine	G-CSF
2	NEG	NEG	NEG	NEG	Blank	BLC	CD30L	Eotaxin	Eotaxin-2	Fas ligand	Fractalkine	G-CSF
3	GM-CSF	IFNγ	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
4	GM-CSF	IFNγ	IL-1α	IL-1β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
5	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
6	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
7	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNFα	sTNF R Ⅰ	sTNF R Ⅱ	Blank	Blank	Blank
8	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNFα	sTNF R Ⅰ	sTNF R Ⅱ	Blank	POS	POS

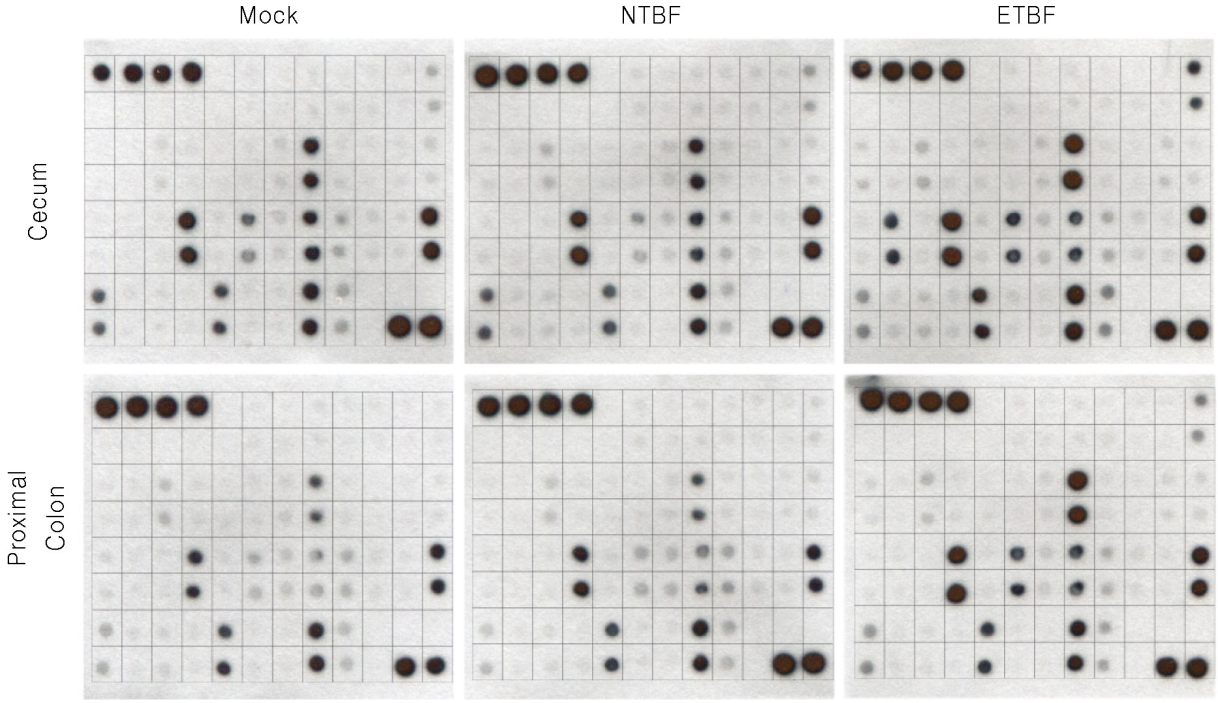

Fig. 1. Spectrum of inflammatory proteins secreted ex vivo from cecal tissues of infected mice. Membranes were cultured with the supernatants from ex vivo explants of the large intestines from Mock-, NTBF- and ETBF-infected mice. Bound proteins were visualized using ECL and development on X-ray film. Scanned images are shown. Each membrane is composed of 12 columns and 8 rows.

assessed in this array are shown in Table 1.

In the cecal supernatants, several proteins (KC, MIP-1g, MCP-1, sTNFR1, TIMP-1) were secreted at equally high levels among the Mock-, NTBF- and ETBF-infected mice (Fig. 1 and 2A). Only four cytokines (G-CSF, IL-6, IL-17 and LIX) were secreted at higher levels in the ETBF-infected tissues compared to the NTBF- and Mock-infected tissues. In the proximal colonic supernatants, several cytokines (G-CSF, IL-6, KC, LIX, MIP-1g, MCP-1) were elevated in the ETBF-infected tissues compared to the NTBF and Mock groups (Fig. 1 and 2B). This result indicates that the

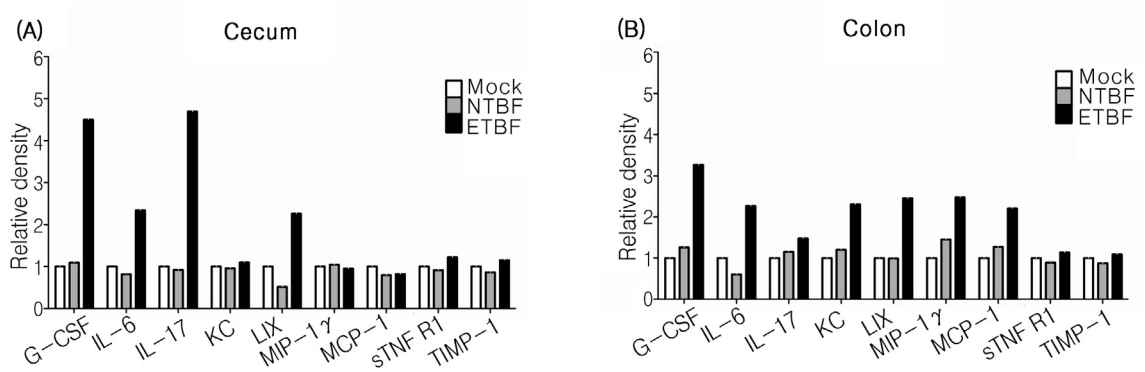

Fig. 2. Densitometry analysis of antibody array. The signal intensities were quantified by densitometry and the relative expression levels of inflammatory proteins shown. Internal positive controls were used to normalize the results from different membranes being compared. (A) cecum, (B) proximal colon.

inflamed proximal colon secretes a much larger repertoire of inflammatory proteins compared to the inflamed cecum. Curiously, IL-17 was only slightly elevated in the proximal colon tissues of ETBF-infected mice but was greatly elevated in the inflamed cecal tissues. It is known that ETBF infection in mice induces a robust Th17 response in the large intestine (Wu et al., 2009). The results from the current study raise the possibility that regional difference exists for induction of IL-17 secretion. The proximal colon secreted several inflammatory cytokines and chemokines. IL-6 is a key inflammatory cytokine involved inflammation. The chemokines IL-17, KC, LIX, MIP-1g and MCP-1 are all involved in chemotaxis of immune cells especially neutrophils. G-CSF is a multifunctional cytokine that stimulates the survival, proliferation and differentiation of neutrophils (Roberts, 2005). Taken together, the presence of these inflammatory proteins indicates that the large intestines of ETBF-infected mice are in a state of active acute colitis. This result is in agreement with prior studies indicating that neutrophil infiltration is a hallmark of ETBF-infected tissues (Rhee et al., 2009).

The cecum and proximal colon are adjacent regions of the large intestine but are anatomically and functionally different. In many cases, these two distinct tissues are often not distinguished in mouse experimental systems. In this study, we show that the cytokine profile of the cecum and colon in response to ETBF infection has similarities and differences. The results from this study highlight the need for examining the cecum and colon separately during experimental analysis.

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