

Bacterial Translocation in Alymphoplasia (*aly/aly*) Mice

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Bacterial translocation (BTL) is defined as the passage of viable bacteria from the gastrointestinal tract to the organs. This study was to elucidate the roles of Peyer's patches (PPs) and/or mesenteric lymph nodes (MLNs) in BTL. Alymphoplastic mutant mice and phenotypically normal heterozygous mice were dominantly colonized with streptomycin-resistant *Escherichia coli* and BTL was examined. In PP- and MLN-competent mice, BTL to MLNs was detected in 100% of mice, but BTL to organs was rare (25%). On the other hand, in PP- and MLN-deficient mice, BTL to organs was detected in 91% of mice. The results clearly indicate that PPs are not the only site for bacterial entry.

Key words: Alymphoplasia mouse, bacterial translocation, indigenous bacteria, mesenteric lymph node, Peyer's patch.

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Bacterial translocation (BTL) is defined as the passage of viable bacteria from the gastrointestinal tract to the mesenteric lymph nodes (MLN) and other visceral organs (BERG & GARLINGTON 1979). This phenomenon is promoted by mucosal barrier disruption, intestinal bacterial overgrowth, and host immunosuppression (BERG & ITOH 1986). Although host immunosuppression is one of the enhancing factors for BTL, the role of gut-associated lymphoid tissues (GALT), especially Peyer's patches and MLN, in BTL is not clear.

Alymphoplasia (*aly/aly*) mice are a spontaneously occurring strain with mutant nuclear factor- κ B-inducing kinase (SHINKURA *et al.* 1999). These mice are characterized by the systemic absence of lymph nodes and Peyer's patches with immunodeficiency (MIYAWAKI *et al.* 1994), although mature lymphocytes are present (MIYAWAKI *et al.* 1994; SUZUKI *et al.* 2001). These mutant mice and/or the lymphotoxin-deficient mice, both of which show a similar phenotype of systemic alymphoplasia, were used for studying the roles of GALT in intestinal infections (BARTHEL *et al.* 2003; KWA *et al.* 2006; MORIMOTO *et al.* 2006; RAY & KRIEG 2003)

The purpose of this study was to elucidate the roles of Peyer's patches and/or MLN in the translocation of indigenous bacteria using Peyer's patch- and MLN-deficient mice.

Material and Methods

Animals: Specific pathogen-free male *aly/aly* and female *aly/+* mice, purchased from CLEA Japan, Inc. (Tokyo, Japan), were used for breeding in a bioBubble™ clean room (The Colorado Clean Room Company, Ft. Collins, CO, U.S.A.). Male homozygous (*aly/aly*) mice and female heterozygous (*aly/+*) mice were mated because female homozygous (*aly/aly*) mice display a lactation defect and fail to nurture their pups. The offspring were transferred to a biosafety level (BSL) 2 animal facility, acclimated for a few days, and then used for the experiment at 10 weeks of age. The mice were housed in plastic cages with wood chip bedding and were fed commercial pellets sterilized by γ -irradiation at 30 kGy (CL-2; CLEA Japan, Inc.) and autoclaved water *ad libitum*. All animal experiments were conducted with the approval of the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Bacteria: Streptomycin-resistant *Escherichia coli* C25 (FRETER 1956) were kindly provided by Prof. Berg (Louisiana State University Health Sciences Center—Shreveport, retired). The organisms were suspended in brain-heart infusion broth (Becton Dickinson and Co., Sparks, MD, U.S.A.) and incubated by shaking at 37°C for 16 hrs.

Decontamination and *E. coli* C25 colonization: Experimental BTL was examined using the method described by BERG (1981), with minor modifications. Briefly, mice were inoculated orally with 0.5 ml antibiotic-containing water (2 mg/ml streptomycin sulfate and 1500 units/ml penicillin G sodium salt (Sigma Chemical Co., St. Louis, MO, U.S.A.)) and continued to be given the antibiotic-contained water as drinking water ad libitum for 3 days. The antibiotic-decontaminated mice were then inoculated orally with 0.5 ml *E. coli* C25 suspension (7.3×10^9 cells/ml) and received drinking water containing 2 mg/ml streptomycin sulfate and 10% overnight culture of *E. coli* C25 (7.3×10^9 cells/ml) for 4 days.

Testing for *E. coli* C25 translocation: Mice with *E. coli* C25 were euthanized with CO₂ gas and the body surfaces were disinfected with 70% ethanol. Blood was taken from the heart without opening the thoracic cavity. The MLN were excised aseptically from *aly*⁺ mice, placed in a preweighed 15 ml centrifuge tube containing 0.5 ml sterile phosphate-buffered saline (PBS), weighed, and homogenized with a grinder (Cosmo Bio Co., Ltd., Tokyo, Japan). Equal portions (0.2 ml) of the homogenate were plated onto 2 MacConkey agar plates (EIKEN CHEMICAL CO., Ltd., Tokyo, Japan). In the case of *aly/aly* mice, MLN were not examined due to the absence of MLN. The spleen, liver, and left kidney were then removed, placed in 15 ml preweighed centrifuge tubes containing 1.0 ml sterile PBS, weighed, and homogenized, and 0.1 ml of each was directly plated onto MacConkey agar plates to determine the number of translocating cells. The plates were incubated aerobically at 37°C for 24 hrs. The limits of quantification were dependent on the weight of organs and about 33, 130, 45, and 60 colony forming units (CFU)/g in MLN, spleen, liver and kidney, respectively. For qualitative detection of BTL, 2 ml Trypticase™ Soy Broth (Becton Dickinson and Co.) was added to each tube of the remaining ho-

mogenate of the organs. Blood samples were also cultured with Trypticase™ Soy Broth. The tubes were incubated aerobically at 37°C for 24 hrs, then the bacterial growth was confirmed on MacConkey agar plates. The cecum was also removed, weighed, and homogenized, and serial dilutions were placed onto MacConkey agar plates. The identity of *E. coli* C25 was confirmed by an API® Rapid 20E system (bioMérieux Japan, Ltd., Tokyo, Japan).

Statistical analysis: The incidences of BTL were compared by Fisher's exact test. The mean cecal populations of bacteria were compared by the Student *t* test. Probabilities of less than 0.05 were considered significant.

Results

Nineteen offspring, both male and female, were used for the experiment. Out of 19 offspring, 8 mice were *aly*⁺ and 11 were *aly/aly* phenotypes, distinguished based on the presence/absence of MLN and Peyer's patches.

BTL to MLN was detected in all (8/8) of the *aly*⁺ mice (Table 1). The number of translocating cells (average ± S.D.) was 108.0 ± 51.1 CFU/MLN (Table 2). The incidence of BTL to the spleen, liver, kidney, and blood was 1/8, 2/8, 0/8, and 0/8 in *aly*⁺ mice and 5/11, 9/11, 0/11, and 0/11 in *aly/aly* mice (Table 1), respectively, although the number of translocating cells was below the quantitation limit in most of the mice (Table 2). BTL to MLN and/or other visceral organs (spleen, liver, and kidney) was detected in most individuals, both *aly*⁺ (8/8) and *aly/aly* (10/11) mice. However, in *aly*⁺ mice, BTL was mostly limited to the MLN, and BTL to other visceral organs was rare (2/8). Conversely, in *aly/aly* mice, BTL was often detected in the visceral organs (10/11), although *aly/aly* mice lack MLN as an inherited trait. There was no significant difference in the incidence of total BTL (to MLN and/or other visceral organs),

Table 1

Incidence of bacterial translocation to MLN and other visceral organs and cecal population

	MLN	spleen	liver	kidney	blood	total	visceral organs ^a	cecal population (CFU/g)
<i>aly</i> ⁺	8/8	1/8	2/8	0/8	0/8	8/8	2/8	$1.15 \pm 0.59 \times 10^{10}$
<i>aly/aly</i>	NA	5/11	9/11	0/11	0/11	10/11	10/11	$1.31 \pm 0.48 \times 10^{10}$
P	NA	NS	*	NS	NS	NS	**	NS

Data are shown as the number of mice positive for bacterial translocation/number of total mice examined.

^a – Incidence of bacterial translocation to spleen, liver or kidney * P<0.05, ** P<0.01.

NA – not applied

NS – not significant

Table 2
Number of translocating bacteria in MLN and other visceral organs of male (m) and female (f) mice

aly/+ mice												
	1 (f)	2(m)	3(m)	4(m)	5(m)	6 (f)	7 (f)	8 (f)				incidence
MLN (CFU/MLN)	86.7	96.9	129.7	201.4	58.3	104.5	40.5	146.3				8/8
spleen (CFU/g)	–	–	–	–	–	–	–	+	*			1/8
liver (CFU/g)	+	–	–	–	–	–	–	409.2				2/8
kidney (CFU/g)	–	–	–	–	–	–	–	–				0/8
blood (CFU/g)	–	–	–	–	–	–	–	–				0/8
aly/aly mice												
	1(m)	2(m)	3(m)	4(m)	5(m)	6(m)	7 (f)	8 (f)	9 (f)	10(m)	11 (f)	incidence
spleen (CFU/g)	–	–	+	+	+	–	–	+	–	139.9	–	5/11
liver (CFU/g)	+	+	–	+	+	+	–	+	+	+	+	9/11
kidney (CFU/g)	–	–	–	–	–	–	–	–	–	–	–	0/11
blood (CFU/g)	–	–	–	–	–	–	–	–	–	–	–	0/11

* positive but under the quantitation limit.

(The bacterial colonies were not detected by direct plating, but bacterial growth was detected after enrichment.)

except for BTL to visceral organs ($P < 0.01$) between *aly/+* and *aly/aly* mice. No BTL was detected in the kidney or blood of *aly/+* or *aly/aly* mice (Table 1). The cecal populations were not significantly different between *aly/+* and *aly/aly* mice (Table 1). No differences between sexes were observed in this study, although both female and male mice were used.

Representative results of a single experiment are presented in this manuscript, however, similar results were obtained in preliminary experiments.

Discussion

Microfold (M) cells, which are located in the follicle-associated epithelium of the Peyer's patches, are known to be preferred sites of attachment and entry of various pathogens, such as *Shigella* spp., *Yersinia* spp., *Listeria* spp., and *Salmonella* spp. (JENSEN *et al.* 1998; MILLER *et al.* 2007). Unlike these overt pathogens, the role of Peyer's patches in BTL of non-pathogenic indigenous bacteria is not clear and is controversial. There have been several reports that some immunosuppressants and chemotherapeutics induce BTL of indigenous bacteria in laboratory animals or human patients, because of host immunosuppression (BERG 1983; BALZAN *et al.* 2007; CERC I *et al.* 2007; KOH *et al.* 2005; PENN *et al.* 1991). However, SUZUKI *et al.* (1996) and NAKAYAMA *et al.* (1997) report that

treatment with cyclophosphamide, one of the immunosuppressants and chemotherapeutics, reduced BTL of *E. coli* in mice because of the decrease and/or damage of M cells and macrophages of Peyer's patches. Conversely, WELLS & ERLANDSEN (1991) report that indigenous bacteria might translocate via the cecum and colon rather than the small intestine and stomach. SILVA *et al.* (1996) also report that Peyer's patch-tied animals did not show significantly decreased BTL to MLN compared with Peyer's patch-untied animals.

Our results, using Peyer's patch- and MLN-deficient mice, clearly indicate that Peyer's patches are not the only site for bacterial entry, because BTL was found in Peyer's patch-deficient *aly/aly* mice. As for MLN, there are 2 possibilities to be considered. One possible interpretation is that MLN play an important role in arresting translocating bacteria to visceral organs, because BTL was almost entirely limited to MLN in MLN-competent *aly/+* mice but often spread to other visceral organs in MLN-deficient *aly/aly* mice. TOKYAY *et al.* (1992) report that BTL may occur mainly via mesenteric lymphatics to MLN and thence into other systemic organs. The other possible interpretation is that BTL to MLN and BTL to visceral organs occur by different mechanisms or routes. MAINOUS *et al.* (1991) report that portal blood, rather than mesenteric lymph, may be the major route of BTL to systemic organs. STENBÄCK *et al.* (2002) report that mesenteric lymphadenec-

tomy did not result in increased BTL. In this case, immunodeficiency, rather than absence of Peyer's patches and MLN, may account for a high incidence of BTL to visceral organs in *aly/aly* mice. In addition, genetically immunodeficient mice, such as nude, SCID, and beige mice, are reported to show a high incidence of BTL to MLN and visceral organs (OHSUGI *et al.* 1996; OWEN & BERG 1980).

In conclusion, it is clear that Peyer's patches are unnecessary for BTL of non-pathogenic indigenous bacteria, although the role of MLN in BTL is still not clear.

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