

Yersinia enterocolitica Invasin-Dependent and Invasin-Independent Mechanisms of Systemic Dissemination

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We report here invasin-dependent and invasin-independent mechanisms in which the enteropathogen *Yersinia enterocolitica* is able to disseminate from the lumen of the small intestine to the spleen. The invasin-dependent route is clearly discernible in mice devoid of intestinal Peyer's patches and mesenteric lymph nodes.

The food-borne pathogen *Yersinia enterocolitica* can efficiently colonize and induce disease in the small intestine (4, 5). Following ingestion, the bacteria colonize the lumen and invade the epithelial lining of the small intestine, resulting in the colonization of the underlying lymphoid tissues known as Peyer's patches. A direct lymphatic link between the Peyer's patches and mesenteric lymph nodes may result in dissemination of the bacteria to these sites, resulting in mesenteric lymphadenitis. Dissemination to extraintestinal sites, such as the spleen, has also been reported (4, 5, 10, 24).

Colonization of the intestinal lymphoid tissues requires transmigration of the bacteria from the intestinal lumen across an epithelial tissue barrier. Specifically, antigen-sampling intestinal epithelial cells known as M cells are thought to be critical for this trans migratory process (1, 9, 22, 23). The epithelium overlying the Peyer's patches has a high concentration of M cells; however, these cells have recently been identified throughout the non-Peyer's patch areas of the small intestine (3, 13). Furthermore, *Y. enterocolitica* and the related pathogen *Yersinia pseudotuberculosis* produce at least three invasion proteins, invasin, Ail, and YadA, which could potentially promote adherence to and invasion of M cells (2, 11, 12, 21, 25, 31). Invasin, the principle invasion factor of *Y. enterocolitica* and *Y. pseudotuberculosis*, binds to β_1 -chain integrin receptors with high affinity, which promotes internalization (12, 32). These receptors are found at high levels on the luminal side of M cells but not on the luminal side of enterocytes (6). Therefore, the frequent high-level colonization of Peyer's patches by *Y. enterocolitica* is probably due to the adjacent localization of a highly invadable cell population.

In contrast, a conceptual model of dissemination of *Y. enterocolitica* from the lumen of the small intestine to the spleen has yet to be clearly defined. Although this event is similar to colonization of Peyer's patches in that the epithelial barrier of the small intestine would need to be breached, little is known about the order of events occurring beyond this stage. Previous reports have suggested the possibility of at least two distinct routes the bacteria may take to reach extraintestinal sites (18,

24). The first route relies on the colonization of the Peyer's patches, which can then be used as a staging ground for spread into the blood and/or lymph, ultimately resulting in the appearance of bacteria in other tissues. A secondary route would bypass the Peyer's patches and lead to systemic colonization. Furthermore, the possibilities of additional avenues for dissemination have yet to be excluded.

In order to assess the anatomical role Peyer's patches play in disseminated *Y. enterocolitica* infection, we took advantage of a genetically engineered strain of mouse which lacks the ability to develop any organized intestinal lymphoid tissues, including Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes. These mice lack the gene which encodes the lymphotoxin- α (LT α) protein. Signaling of heterotrimers of LT α and LT β (LT $\alpha_{1\beta 2}$) through the lymphotoxin- β receptor (LT β R) is required for the development of Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes in mice (7, 17, 26, 29). However, LT $\alpha^{-/-}$ mice still develop M cells, which are found in the villi throughout the small intestine (13). Following oral infection with (5 to 8) $\times 10^8$ CFU of wild-type *Y. enterocolitica* (JB580v [14]), bacterial dissemination to the spleen was evident in the LT $\alpha^{-/-}$ mice (Fig. 1). By day 3 and continuing to day 7, spleen colonization was similar in the LT $\alpha^{-/-}$ and C57BL/6J mouse strains. Bacteria were also apparent in the spleens of mice lacking the gene for LT β R, which are also devoid of intestinal lymphoid tissue (8) (data not shown). This supports the idea that Peyer's patch and subsequent mesenteric lymph node colonization is not required for *Y. enterocolitica* dissemination from the intestinal lumen to the spleen.

Deletion of either the LT α gene or the LT β R gene in mice results in many alterations of the immune system beyond the lack of peripheral lymph nodes, such as the Peyer's patches and mesenteric lymph nodes (7, 19, 20). Mice lacking these genes also have a disrupted splenic architecture, lack a follicular dendritic cell network, and lack the ability to form germinal centers. However, they do not appear to be lacking any specific immune cell populations, they develop a thymus, and they appear to have a normal lymphatic vasculature. It is possible that a defect in the immune system other than the anatomical absence of the Peyer's patches and mesenteric lymph nodes is resulting in the spleen colonization observed in the LT $\alpha^{-/-}$ and LT β R $^{-/-}$ mice. In order to determine if LT α or LT β R has

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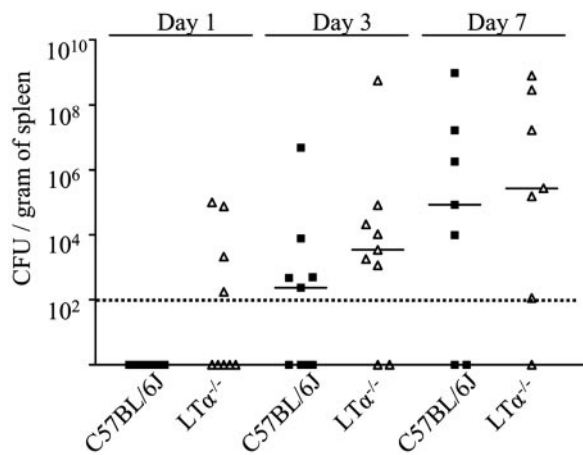


FIG. 1. Colonization of C57BL/6J (■) and $LT\alpha^{-/-}$ (Δ) mouse spleens following oral inoculation with $(5 \text{ to } 8) \times 10^8$ CFU wild-type *Y. enterocolitica* (JB580v). Infections were performed as described previously (10). At days 1, 3, and 7 postinfection, spleens were dissected and macerated into suspensions. Colonies were enumerated by plating serial dilutions of the suspensions onto *Yersinia* selective agar plates (Becton-Dickinson, Sparks, MD). The graph displays the combination of two independent experiments which used four to five mice per group per time point. The dashed line indicates the limit of detection for the assay.

an inherent immunological role in controlling *Y. enterocolitica* infection, we compared bacterial colonization in the spleens of $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$ mice to that for control C57BL/6J mice following intraperitoneal infection and performed 50%-lethal-dose (LD_{50}) analysis. The numbers of *Y. enterocolitica* in the spleens of mice following intraperitoneal inoculation were essentially identical in $LT\alpha^{-/-}$, $LT\beta R^{-/-}$, and C57BL/6J mice at day 3 postinfection (data not shown). This is suggestive that the immunological defects conferred due to the lack of either the $LT\alpha$ gene or the $LT\beta R$ gene do not contribute to the early stages of controlling the infection. The average LD_{50} s from two independent analyses for all three strains of mice were as follows: for C57BL/6J, 6.6×10^7 ; for $LT\beta R^{-/-}$, 1.2×10^9 (18-fold higher than the value for C57BL/6J mice); and for $LT\alpha^{-/-}$, 2.2×10^7 (threefold lower than the value for C57BL/6J mice). At this time, we do not know why the $LT\beta R^{-/-}$ mice are more resistant to oral *Y. enterocolitica* infection than the control mice. However, these mice and the $LT\alpha^{-/-}$ mice do not appear to have a general immunodeficiency which might account for the spleen colonization observed in Fig. 1.

Recently non-Peyer's patch M cells were described (13). These M cells are dispersed among the villi of the small intestine and are found in C57BL/6J mice and in Peyer's patch-deficient mice, including $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$ mice (13). Intestinal villous M cells have been shown to take up green fluorescent protein-expressing *Salmonella enterica* serovar Typhimurium, *Y. pseudotuberculosis*, and interestingly, *Escherichia coli* expressing *Y. enterocolitica* invasin but not *E. coli* without invasin protein (13). It has yet to be shown whether or not invasion of intestinal villous M cells results in bacterial dissemination to extraintestinal tissues. However, these data allude to a Peyer's patch-independent dissemination route initiating by translocation through intestinal villous M cells. Fur-

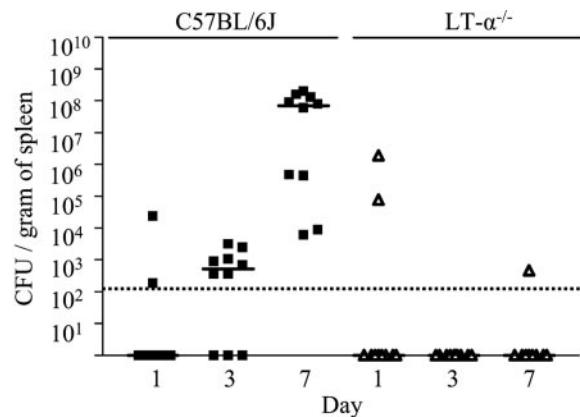


FIG. 2. Colonization of C57BL/6J (■) and $LT\alpha^{-/-}$ (Δ) mouse spleens following oral inoculation with $(5 \text{ to } 8) \times 10^8$ CFU of *inv* mutant *Y. enterocolitica* (JP273v). Infections were performed as described previously (10). Spleen colonization was assessed as described in the legend to Fig. 1. The graph displays the combination of two independent experiments which used five mice per time point. The dashed line indicates the limit of detection for the assay.

thermore, it suggests that access to this route by *Y. enterocolitica* could be dependent on invasin.

In order to test if dissemination to the spleen is invasin dependent, we orally infected C57BL/6J and $LT\alpha^{-/-}$ mice with an *inv* mutant strain of *Y. enterocolitica* (JP273v). This mutant has previously been reported to have a delay in Peyer's patch colonization compared to wild-type bacteria (24). Nevertheless, it eventually colonizes the Peyer's patches, mesenteric lymph nodes, and spleen and has an LD_{50} equivalent to that for wild-type bacteria, suggesting at least one splenic dissemination route is not invasin dependent. The *inv* mutant was highly attenuated in its ability to disseminate from the intestine to the spleen in $LT\alpha^{-/-}$ mice compared to that in C57BL/6J mice (Fig. 2). However, as previously reported, the *inv* mutant was still able to disseminate to the spleens of mice with Peyer's patches (Fig. 2). This suggests that the intestinal villous M cells together with invasin mediate efficient dissemination of *Y. enterocolitica* in the absence of Peyer's patches.

Infrequent colonization of the spleen was seen on day 1 after infection by the *inv* mutant. This is similar to what was seen when $LT\alpha^{-/-}$ mice were infected with wild-type *Y. enterocolitica* strain JB580v (Fig. 1). Interestingly, this was not true when the $LT\beta R^{-/-}$ mice were compared to C57BL/6J mice (data not shown). At this time we are uncertain as to why this may be occurring. Further experimentation is necessary in order to determine the mechanism contributing to this differential phenotype.

A third dissemination route has been suggested from research concerning *Salmonella enterica* serovar Typhimurium and how these bacteria are sampled by intestinal CD18-expressing phagocytes (27, 30). These phagocytes reside directly underneath the intestinal epithelium and extend antigen-sampling dendrites into the lumen of the small intestine. Strains of *S. enterica* serovar Typhimurium incapable of targeting the Peyer's patches were associated with CD18⁺ cells and circulating in the blood 1 h postinfection. Furthermore, bacteria were impaired in their ability to disseminate to the spleen in

CD18-deficient mice. We tested for this but were unable to find any blood-borne *Y. enterocolitica* during the first hour of infection (data not shown). However, the assay samples only 100 μ l of blood from each individual mouse, and the bacterial load may very well be below the limit of detection. Furthermore, wild-type *Y. enterocolitica* colonizes the spleens of CD18-deficient mice after oral infection (data not shown). The difference in phenotypes might be explained by the differences in lifestyles between *S. enterica* serovar Typhimurium and *Y. enterocolitica*. While *S. enterica* serovar Typhimurium survives and replicates within CD18⁺ macrophages in vivo, *Y. enterocolitica* is currently believed to primarily reside extracellularly and to resist uptake by phagocytic cells (15, 16, 28).

Taken together, these data suggest at least two routes in which *Y. enterocolitica* can disseminate from the small intestine to the spleen: one dependent and one independent of invasins. Wild-type mice infected with invasins-producing *Y. enterocolitica* should have both routes available. This information is critical when assessing virulence defects of both bacterial and mouse mutants.

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