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Invasion and Dissemination of *Yersinia enterocolitica* in the Mouse Infection Model

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Abstract. *Yersinia enterocolitica* is one of the most common causes of food borne gastrointestinal disease. After oral uptake yersiniae replicate in the small intestine, invade Peyer´s patches of the distal ileum and disseminate to spleen and liver. In these tissues and organs yersiniae replicate extracellularly and form exclusively monoclonal microabscesses. Only very few yersiniae invade Peyer´s patches and establish just a very few monoclonal microabscesses. This is due to both *Yersinia* and host specific factors.

25.1 Introduction

Yersinia enterocolitica and *Yersinia pseudotuberculosis* cause food-borne gastrointestinal disease in humans. While *Y. pseudotuberculosis* is primarily an animal pathogen that only rarely causes disease in humans, *Y. enterocolitica* is one of the most common causes of gastrointestinal disease in the moderate and subtropical climates of the world. It may present as enteritis, terminal ileitis, or mesenteric lymphadenitis (pseudoappendicitis) with watery or sometimes bloody diarrhoea. In patients with iron overload states (e.g. haemochromatosis, haemolytic anemia) a systemic infection can ensue leading to focal abscess formation in liver and spleen (Bottone 1997). A similar disease results after oral infection of mice with yersiniae replicating in the small intestine, invading Peyer´s patches (PPs) of the distal ileum, and disseminating to liver and spleen. Extracellular replication of yersiniae in these tissues and organs leads to the formation of microabscesses. This is made possible by the injection of *Yersinia* outer proteins (Yops) by a type three secretion system (T3SS) which paralyses phagocytes of the innate immune system (reviewed in Heesemann et al. 2006). The precise mechanisms that lead to the formation of microabscesses *in vivo* are not clear. Invasion of PPs is mediated by several non-fimbrial adhesins such as invasin and the *Yersinia* adhesion A (YadA). Both of these surface proteins interact with β1 integrins of eukaryotic cells and are believed to mediate adherence and invasion of M cells (reviewed in Isberg and Barnes 2001 and Heesemann et al. 2006). Invasin is able to directly bind β1 integrins of host cells (Isberg and Leong 1990; Leong et al. 1990), whereas YadA interacts with extracellular matrix (ECM) proteins such as collagen and fibronectin and with host cell β1 integrins by ECM bridging (Heise and Dersch 2006; Eitel and Dersch 2002). β1 integrins are expressed on the apical surface of M cells but not by enterocytes (Clark et al. 1998). PP invasion is therefore believed to be mediated by M cells of the

follicle-associated epithelium. Invasin is the most important invasion factor of *Yersinia* and is known to be essential for early invasion of PPs in the mouse oral infection model (Marra and Isberg 1996; Pepe and Miller 1993). The surface exposed C-terminal region of invasin consists of five globular domains (D1-5) that protrude 18 nm from the bacterial surface in *Y. pseudotuberculosis* (Hamburger et al. 1999). *Y. enterocolitica* invasin lacks the D2 self association domain. The adhesion unit that is responsible for the high affinity interaction with β1 integrins is formed by the D4-D5 domains. Preferential invasion of M cells by *Y. pseudotuberculosis* has been demonstrated by microscopy of mouse ligated gut loops (Clark and Jepson 2003). M cells were found to carry multiple adherent/invading yersiniae suggesting translocation of multiple bacteria to submucosal tissue (Clark et al. 1998). After translocation across the mucosal barrier by M cells, yersiniae disseminate from PPs to mesenteric lymph nodes (Grutzkau et al. 1990; Hanski et al. 1989; Simonet et al. 1990). Further dissemination to spleen and liver probably does not occur via PPs and lymph nodes. Recently it was shown that organized intestinal lymphoid tissue was not required for dissemination of yersiniae to spleen and liver (Handley et al. 2005). It was furthermore demonstrated that yersiniae colonizing spleens and livers were derived from the gut lumen but not mesenteric lymph nodes (Barnes et al. 2006). The precise mechanism of dissemination from the gut lumen to the liver and spleen are not well understood at this time. Early after infection, microabscesses formed by yersiniae in liver and spleen of mice consist primarily of neutrophils (Autenrieth et al. 1993; Carter 1975). During the course of infection, lesions are populated by mononuclear cells and exhibit a granulomatous character (Autenrieth et al. 1993). We have recently studied abscess formation in the oral mouse infection model using RFP- (red fluorescent protein) and GFP- (green fluorescent protein) expressing yersiniae. We were able to show that oral *Y. enterocolitica* infection of mice leads to monoclonal microabscess formation in PPs, spleen and liver. Furthermore experiments with red and green fluorescing yersiniae revealed that only very few yersiniae were able to invade PPs from the gut lumen and that both *Yersinia* and the host contribute to this phenomenon.

25.2 Monoclonal Abscess Formation by *Yersinia*

In the mouse oral infection model yersiniae disseminate from the gut lumen to PPs of the distal ileum, lymph nodes, liver and spleen (Trülzsch et al. 2004). In these tissues and organs, yersiniae replicate predominately extracellularly and form microabscesses. Recently we asked the question whether many bacteria were required for microabscess formation or if a single bacterium was sufficient to initiate abscess formation in PPs, liver, and spleen. To answer this question we infected mice orally with an equal mixture of red and green fluorescing yersiniae (expressing RFP or GFP) (Oellerich et al. 2007). At different time points after infection, organs were removed, sectioned, and abscesses were analysed by fluorescence microscopy. These experiments revealed that *Yersinia* microabscesses in PPs, liver, and spleen were

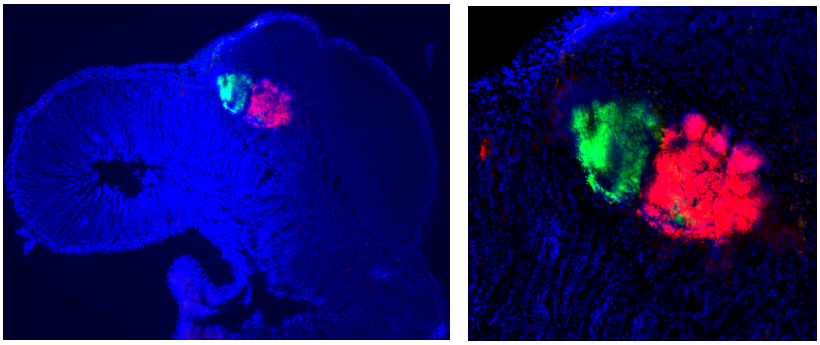

Fig. 1. Typical cryosection (10 μm) of a DAPI stained PP from a Balb/c mouse infected orally with an equal mixture of 10^9 red and green fluorescing *Y. enterocolitica*, 5 days post infection. One red and one green monoclonal microabscess can be seen within the PP. (See color plate.)

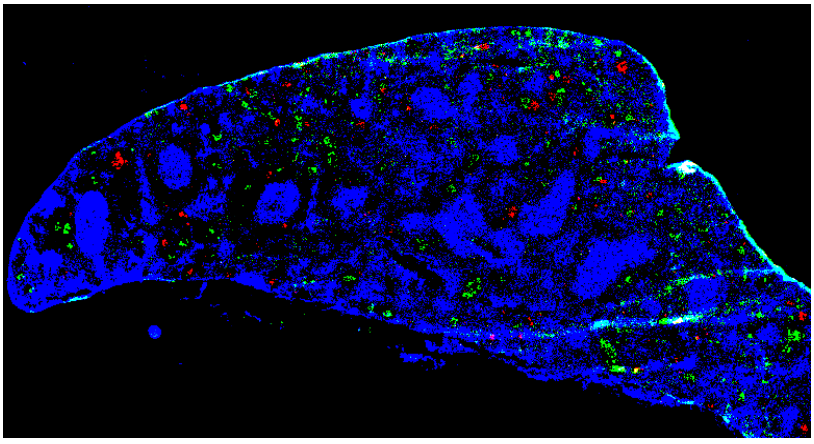

Fig. 2. Typical cryosection (10 μm) of a DAPI stained spleen from a Balb/c mouse infected orally with an equal mixture of 10^9 red and green fluorescing yersiniae, 5 days post infection. Hundreds of red and green fluorescing monoclonal microabscesses can be seen throughout the organ. (See color plate.)

monoclonal as evidenced by the fact that microabscesses were exclusively single colored after infection with a mixture of red and green fluorescing yersiniae (Fig. 1, Fig. 2) (Oellerich et al. 2007). Two scenarios are conceivable when multiple yersiniae invade PPs or disseminate to liver and spleen: either multiple bacteria need to associate prior to forming an abscess or a single bacterium could be sufficient to

initiate abscess formation. Recently we demonstrated monoclonal abscess formation according to the latter scenario. Only very few monoclonal microabscesses were observed in any given PP, whereas hundreds of monoclonal microabscesses were seen in cross sections of liver and spleen tissue 5 days after infection (Fig. 2). These results showed that bacterial dissemination from the gut lumen to liver and spleen was much more efficient than dissemination of bacteria from the gut lumen to PPs. This is in line with a recent publication demonstrating that yersiniae colonizing spleen and liver were derived from a replicating pool of bacteria in the intestine rather than disseminating via PPs and lymph nodes (Barnes et al. 2006). Monoclonal abscess formation in liver and spleen is presumably due to single yersiniae disseminating from the gut lumen to these organs. Single yersiniae may be trapped in the capillary vessels of liver and spleen which could subsequently be plugged by the proliferating yersiniae leading to monoclonal abscess formation.

25.3 Clonal Invasion of Peyer´s Patches

The fact that only very few (1-4) monoclonal microabscesses form in each PP, even at a high oral infection dose, suggests clonal invasion of PPs by *Yersinia* (Oellerich et al. 2007). This observation could be the result of many yersiniae initially invading a given PP with only very few bacteria surviving the initial encounter with the host immune response. This latter possibility has been suggested by the electron microscopic observation of multiple yersiniae invading M cells (Clark et al. 1998; Clark and Jepson 2003). However these experiments were performed using the murine ligated gut loop model which cannot be compared to *in vivo* oral infections. To support the finding that it is limited invasion of PPs which is responsible for our observation, we performed infection experiments with neutropenic and oxidative burst deficient mice. Neutrophils are known to be critical in early host defense against *Yersinia*. It has been shown that the initial inactivation of yersiniae that implant in liver and spleen during the first few hours of infection is primarily the feat of neutrophils (Conlan 1997). Besides accumulating in liver and spleen early after infection, neutrophils have been demonstrated in PPs within 24 h post infection (Carter 1975). Therefore mixed infections with red and green fluorescing yersiniae were performed using neutropenic mice (Oellerich et al. 2007). If many yersiniae were to invade PPs and only a few to survive the influx of neutrophils, an increase in the number of microabscesses per PP for neutropenic vs immunocompetent mice would be expected. The same should hold for mice impaired in the oxidative burst ($p47^{phox-/-}$ mice). Infection of these mice with *Yersinia* resulted in essentially the same picture seen with immunocompetent mice (Oellerich et al. 2007). These findings support the clonal invasion hypothesis. If the observation of just a few clones of *Yersinia* establishing microabscesses in a certain PP was solely the result of many yersiniae invading PPs with just a few surviving the initial encounter with the immune response, then a 10 fold higher infection dose would be expected to lead to a 10 fold higher number of monoclonal microabscesses per PP. Recently we were able to show that this was not the case lending support to the clonal invasion hypothesis (Oellerich

et al. 2007). Clonal invasion would imply that signature-tagged mutagenesis would not be a suitable tool to identify attenuated yersiniae in mouse PPs. In fact a study on the dissemination of signature-tagged *Y. pseudotuberculosis* mutants in the mouse model previously noted that barriers must exist that limit the number of bacteria that are able to reach mesenteric lymph nodes and spleen. Such barriers were responsible for the failure of signature-tagged mutagenesis to identify attenuated mutants of *Y. pseudotuberculosis* in mesenteric lymph nodes and spleen (Mecsas et al. 2001). However, dissemination of these mutants to PPs was not studied.

25.4 Sequential Invasion of Peyer´s Patches is Inhibited by the Host

The simplest explanation for clonal invasion would be limited contact between *Yersinia* and M cells in the mouse model. Clonal invasion of PPs could furthermore be a *Yersinia* specific characteristic, the result of the host response to *Yersinia* infection, or a combination of both. Invasin is the most important invasion factor for PPs (Pepe and Miller 1993) and is known to bind β-integrins of M cells (Clark et al. 1998). From *in vitro* studies it is known that invasin expression is high at ambient temperature and down-regulated at the host temperature of 37°C (Pepe et al. 1994). It was therefore of interest to determine whether yersiniae also down-regulate invasin expression in the gut lumen after oral infection. Recently we demonstrated, by Western blotting of the small intestinal content, that this is the case (Oellerich et al. 2007). The lack of invasin expression in the small intestine obviously restricts early bacterial invasion to a short time period after oral uptake during which invasin is still present on the bacterial surface. Another possible explanation for clonal invasion could be that *Yersinia* actively prevents invasion of PPs by injecting Yops into M cells thereby paralyzing these cells and preventing *Yersinia* uptake. To look into this possibility we recently performed co-infection experiments with YopH, -O, -P, -E, -M, -T, and -Q mutants expressing RFP and GFP (Trülzsch et al. 2004). These experiments however revealed a similar number of monoclonal microabscesses per PP as seen for wild type yersiniae (unpublished results). Besides several conceivable *Yersinia* specific factors, the host response to infection could be limiting *Yersinia* invasion of PPs. To look into this possibility and to determine if *Yersinia* was able to invade and form microabscesses in previously abscessed PPs, we performed sequential infection experiments, orally inoculating mice with green fluorescing yersiniae followed by red fluorescing yersiniae two days later (Oellerich et al. 2007). These experiments revealed that yersiniae orally inoculated two days after a primary *Yersinia* infection, preferentially invaded those PPs that were not initially abscessed. The freshly inoculated yersiniae of the successive infection were invasion competent since they invaded and replicated in “naïve” PPs but showed severely reduced ability to establish microabscesses in previously abscessed PPs. This indicates that the host severely limits sequential infection of PPs and is obviously one important reason why only very few monoclonal microabscesses are seen in a certain PP after infection with a high bacterial dose. Presumably PPs are only permissive for invasion of multiple yersiniae if they are invaded concomitantly. This hypothesis is supported by

the fact that with logarithmically increasing infection doses, the number of microabscesses per PP increases only in a linear fashion. Possibly a signal generated locally in a certain PP shuts off antigen sampling and *Yersinia* uptake by M cells of that PP only. Alternatively it is possible that further yersiniae invade but are rapidly eliminated by the "activated" PP. Very rarely were previously abscessed PPs invaded and abscessed by a subsequent *Yersinia* infection. Possibly only the invasin/β-integrin mediated invasion process is inhibited by the host with residual invasion taking place by alternate mechanisms such as YadA, Ail or inter-epithelial dendritic cells transporting yersiniae to the subepithelium, which has been demonstrated for *Salmonella* (Vazquez-Torres et al. 1999). Finally we have shown that some yersiniae replicating in the gut lumen continue to invade PPs between days 2 and 5 of infection. However the number of microabscesses per PP remains constant during this time period supporting the finding that the host limits invasion of previously abscessed PPs.

25.5 Summary

In summary we have recently demonstrated monoclonal abscess formation in PPs, spleen, and liver by *Yersinia* in the mouse oral infection model indicating that single yersiniae are able to initiate abscess formation. Only very few *Yersinia* cells establish microabscesses in PPs of the small intestine presumably due to clonal invasion of PPs. This is probably due to both the host severely limiting sequential infection of PPs and *Yersinia* down-regulating invasin expression in the small intestinal lumen.

25.6 References

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