Old Herborn University Seminar Monograph 19:
Defence mechanisms of the innate system: influence of microbes.
Editors: Peter J. Heidt, John Bienenstock, Tore Midtvedt, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 89-92 (2006).

DIVERSE EFFECTS OF TLR SIGNALLING ON INTESTINAL INFLAMMATION

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SUMMARY

Probiotics are live commensal microorganisms of the intestinal tract that confer multiple gastro-intestinal health benefits to the host. Yet, the exact mechanisms by which probiotics ameliorate experimental colitis in animals and human inflammatory bowel disease are largely unknown. We tested whether the attenuation of experimental colitis by live probiotic bacteria is mediated by Toll-like receptor signalling and whether non-viable probiotics are similarly effective. Administration of probiotic DNA ameliorated the severity of experimental colitis whereas methylated probiotic DNA, calf thymus DNA and DNAse treated probiotics had no effect. The colitis severity was attenuated to the same extent by delivery of non-viable, γ-irradiated or viable probiotics, but not by heat-killed probiotics, in wild type mice, in mice deficient in Toll-like receptor 2 or in mice deficient in Toll like receptor 4. In contrast, we did not observe any inhibition of experimental colitis by probiotics, in mice deficient in MyD88 or Toll-like receptor 9.

In subsequent studies, we identified that Toll-like receptor 9-induced type-1 IFN mediates the anti-inflammatory effects in experimental colitis. The addition of neutralisation antibodies to type-1 IFN abolished the anti-inflammatory effects whereas the administration of recombinant IFN-β mimicked the anti-inflammatory effects induced by Toll-like receptor 9 agonists.

Taken together, these results indicate that the protective effects of probiotics are mainly mediated by their own DNA rather than by their metabolites or their ability to colonise the colon. These finding underscore the diverse effects of indigenous microbial TLR ligands in intestinal homeostasis and intestinal inflammation and suggest that strategies, which modulate type-1 IFN may be of therapeutic value for intestinal inflammatory conditions.

INTRODUCTION

Inflammatory bowel disease (IBD) includes Crohn's disease and ulcerative colitis, both of which are characterised by flare up periods with possible life-long relapses. Clinical and experimental evidence suggest that the aetiology of IBD is multifactorial involving susceptibility genes and environmental factors, such as intestinal microflora or their products, and it is the interaction of these factors with the immune system that leads to dysregulated mucosal immunity

and chronic intestinal inflammation (*Shanahan*, 2001).

Probiotics are live commensal microorganisms of the intestinal tract that confer health benefits to the host by one or more of the following means: Production of various anti-microbial metabolites, competitive exclusion of enteric pathogens, neutralisation of dietary carcinogens, and modulation of mucosal immune responses. Current probiotic therapy is mainly advocated for its immunomodulatory properties and anti-inflammatory activities at mucosal sites (*Hooper* et al., 2001, *Shanahan*, 2001).

The rationale for using probiotics in IBD is based on evidence implicating enteric bacteria in the pathogenesis of various models of murine colitis and IBD in humans (*Podolsky*, 2002). Indeed, probiotic therapy has been effective for the attenuation of experimental colitis (*Madson* et al., 2001), prevention of pouchitis, and maintenance of remission of pouchitis, Crohn's disease and ulcerative colitis (*Hart* et al., 2003). Despite these beneficial effects, the exact mechanisms and the molecular pathways by which probiotics ameliorate experimental colitis and IBD are largely unknown.

Toll-like receptors function as sentinels of innate immunity. By recognising signature microbial compounds they trigger the transcriptional activation of pro-inflammatory cytokines (e.g., IL-12, TNF-α) and chemokines (e.g., RANTES), as well as co-stimulatory molecules (e.g., CD40). The activation of this pro-inflammatory program initiates defence mechanisms that are vital for host survival (*Takeda* et al., 2003). Indeed, various $TLR^{-/-}$ mice as well as mice deficient in TLR-related adaptor proteins (e.g., MyD88) fail to mount protective responses and succumb to various microbial infections (*Kopp* et al., 2003). Bacterial DNA and its synthetically derived immunostimulatory DNA sequence oligonucleotides (ISS-ODN, also known as CpG-ODN) contain unmethylated CpG dinucleotide motifs within consensus sequences and are ligands of TLR9. Like other TLR ligands such as LPS, ISS-ODN has a broad range of activities on the mammalian innate immune system. In particular, ISS-ODN induces the secretion of Th1-type cytokines and up-regulates the expression of co-stimulatory molecules on antigen presenting cells (*Krieg* 2002). These immunostimulatory characteristics of ISS-ODN have been utilised to elicit Th1-dependent immune responses (*Roman* et al., 1997) and mucosal immunity (*Horner* et al., 1988), leading to an enhanced host defence against invading pathogens.

Since ISS-ODN mimics the immunomodulatory activities of bacterial DNA, we hypothesised a role for probiotic DNA in the inhibition of colonic inflammation and therefore conducted studies which explored the mechanisms by which probiotics ameliorates experimental colitis

DISCUSSION

Persuasive evidence indicates that intestinal microflora play an important role in the initiation and the perpetuation of murine experimental colitis and human IBD (*Podolsky*, 2002). However, the molecular mechanisms by which probiotics exert their therapeutic effects have not been identified. The impact of probiotics on intestinal barrier function, their diverse metabolic activities, their competitive exclusion of intestinal indigenous microflora, and their interaction with the mucosal immune system have all been implicated in mediating their therapeutic effects (*Hooper* et al., 2001, *Shanahan*, 2001).

In a recent study, we provided biochemical, immunologic and genetic evidence that implicated TLR signalling, especially TLR9, in mediating the protective effect of probiotics (VSL-3) on experimental colitis (*Rachmilewitz* et al., 2004). The administration of γ-irradiated probiotics effectively ameliorated experimental colitis, as did the administration of viable probiotics. Since the irradiated probiotics were unable to grow in culture, it is unlikely that either their metabolites or their competitive inhibition with indigenous microflora were responsible for the protective effects on the colonic mucosa. Therefore, we reasoned that the anti-inflammatory activities could be the product of the activation of innate immunity (e.g., via TLR) by structural microbial probiotic components (*Rachmilewitz* et al., 2004).

To further verify the role of TLR signalling in the probiotic-induced amelioration of experimental colitis, mice deficient in TLR2, TLR4, TLR9 and MyD88 were treated with dextran sodium sulphate (DSS) and irradiated probiotics. The administration of γ-irradiated probiotics ameliorated the clinical, biochemical, and histological parameters of colitis in TLR2 and in TLR4 deficient mice but not in TLR9 and MyD88 deficient mice indicating the involvement of the TLR9 signalling pathway in the observed amelioration of colonic inflammation (*Rachmilewitz* et al., 2004). The inhibition of colonic inflammation by probiotic DNA or by ISS-ODN was reproduced in DSS- and TNBS-induced colitis as well as in spontaneous colitis in IL-10 deficient mice (*Rachmilewitz* et al., 2002, 2004). Thus, in contrast to the current paradigm related to the pro-inflammatory role of TLR-activated innate immunity, our data indicated that TLR9 signalling results in the activation of an anti-inflammatory program that attenuates inflammation in different models of experimental colitis.

Subsequent studies addressed the molecular basis for the anti-inflammatory effects induced by TLR9 signalling in models of experimental colitis. We found that two genetically distinct, but phenotypically similar animals responded differently to ISS-ODN administration (*Katakura* et al., 2005). While DSS-induced colitis in $RAG^{-/-}$ mice was inhibited by ISS-ODN, colitis in SCID mice was not. We utilized these ISS-responsive and ISS-resistant phenotypes to dissect the anti-inflammatory role of TLR9 signalling in colonic inflammation (*Katakura* et al., 2005). Analysis of the response to ISS-ODN of these two mouse strains revealed defective TLR9-induced type-1 IFN production in SCID mice. Furthermore, we observed that IFN-a/$bR^{-/-}$ mice are extremely sensitive to colitis inflicted by DSS and that the administration of ISS-ODN to these mice increased their mortality. In addition, the lack of inhibition of DSS-induced colitis in ISS-treated wild type mice adoptively transferred with bone marrow derived macrophages (BMDM) from IFN-α/$\beta R^{-/-}$ but not with BMDM from wild type mice also suggests that TLR9-induced type 1 IFN inhibits the inflammatory response of activated macrophages. Finally, the administration of recombinant IFN-β to DSS-treated mice mimicked the anti-inflammatory effects on colonic inflammation induced by ISS-ODN (*Katakura* et al., 2005). Collectively, these set of data indicate that type-1 IFN has a physiological and protective role on colonic injury and that it also cross-regulates the other pro-inflammatory activities induced by TLR9 triggering (*Katakura* et al., 2005). Indeed, in subsequent preliminary studies we observed that basolateral administration of IFN-α to polarised monolayers of intestinal epithelial cells protected the cells against apoptosis and disruption of the epithelial tight junctions. Thus, type-I IFN may protect against colonic inflammation by preventing epithelial barrier dysfunction.

Recent study documented the protective effect of other TLR ligands on colonic injury (*Rakoff-Nahoum* et al., 2004). Our previous studies identified the anti-inflammatory effects of TLR9 agonists on experimental colitis and identified that TLR-induced type-1 IFN mediates these protective effects on colonic inflammation. These findings and the hypersensitivity to DSS observed in IFN-$\alpha/\beta R^{-/-}$ mice expand the already known activities of type-1 IFN and indicate an important role for type-1 IFN in intestinal homeostasis. Taken together, these results suggest that strategies designed to trigger a type-1 IFN response in the intestinal tract, by the administration of certain TLR ligands (e.g., ISS-ODN, probiotic DNA) or probiotics are of therapeutic value for intestinal inflammatory conditions.

ACKNOWLEDGEMENTS

This work was supported by NIH grants AI40682 and DK35108.

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