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IRRITABLE BOWEL SYNDROME: ROLE OF GUT BACTERIA AND BACTERIAL TOXINS

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SUMMARY

Irritable bowel syndrome (IBS) is a common condition affecting 10-15% of the population. While the precise aetiology remains unknown, our group has focused on the contributions of altered gut flora to IBS, including links between acute gastroenteritis and post-infectious IBS (PI-IBS) and the role of small intestinal bacterial overgrowth (SIBO). Breath testing and culture of small intestinal aspirates indicate that SIBO is present in over one-third of IBS subjects and almost two-thirds of diarrhoea-predominant IBS (D-IBS) subjects, but only one-tenth of non-IBS subjects. The success of antibiotic therapies in impacting IBS phenotypes also supports a bacterial role in IBS. Four large-scale multi-centre trials indicate that rifaximin significantly reduces SIBO and non-constipation IBS with little bacterial resistance or reduction of benefit in successive retreatments. To elucidate the mechanisms underlying PI-IBS, we developed a rat model using *Campylobacter jejuni*, the most common identified cause of acute gastroenteritis. This model mirrors many findings in human IBS subjects, including altered stool form more than three months after clearing initial infection, SIBO, increased intraepithelial lymphocytes (IELs), reductions in deep muscular plexus interstitial cells of Cajal (DMP-ICCs), required for normal intestinal motility, and increases in specific mucosal defence mediators. Common to all pathogens causing gastroenteritis is cytolethal distending toxin (Cdt). We developed a rat model using a mutant *C. jejuni* lacking Cdt, which demonstrated significantly ameliorated bowel phenotypes including SIBO and IELs and no reduction in DMP-ICCs, strongly implicating Cdt in the development of IBS phenotypes. Further, early exposure to *C. jejuni* appears to mitigate the acute effects of second infections, which suggested immunity might play a role in IBS development. Our recent data indicating molecular mimicry between Cdt and neural elements of the gut support this hypothesis and may form the basis for future biomarkers and potential therapies directed at the underlying causes of IBS.

INTRODUCTION

Irritable bowel syndrome (IBS) is the most common chronic medical condition in the U.S. accounting for nearly 30% of all gastroenterology related health care costs (*The Lewin Group*, 2001; *Thompson*, 1994) and affecting 10-15% of the population (*Drossman* et al., 1982; *Thompson* and *Heaton*,

1980). This condition does not discriminate by age and the prevalence of this condition is equally common among young adults. These facts make IBS a very important disease state. However, the aetiology of IBS has remained unknown.

Historically, IBS was treated as largely a psychological disorder and anti-depressants were used as treatment for IBS. Our group has validated microbe-mediated theories in the pathophysiology of post-infectious IBS (PI-IBS) and small intestinal bacterial overgrowth (SIBO) and spent the last 10 years characterizing alterations in gut flora that may contribute to IBS.

SIBO AND IBS

Over the last decade, we have developed a new hypothesis in IBS that focused on bloating as a universal complaint in IBS subjects. Through a series of studies, we demonstrated that SIBO, which results from reduced gut motility, is more common in IBS. This was initially controversial as we used breath testing as a surrogate of SIBO. While we recognized the limitations of this technique, it was apparent that the breath test was frequently abnormal in IBS compared to healthy controls. Despite continued controversy, a key meta-analysis by our group has secured that breath testing is important in IBS and that the odds of having a positive breath test in IBS compared to healthy controls is far greater (*Shah* et al., 2010). Since then, two studies have conclusively demonstrated through culture of the small bowel that IBS subjects have higher coliform (colon bacteria) levels than healthy persons (*Posserud* et al., 2007; *Pyleris* et al., 2012).

SMALL BOWEL MICROBIOME IS ALTERED IN IBS

In another aspect of our on-going work to identify the mechanisms responsible for IBS, we have begun to explore how changes in gut microbial populations contribute to IBS. Since September 2009, subjects undergoing upper endoscopy have been recruited to a repository developed by our collaborator Dr. Evangelos Giamarellos-Bourboulis in Athens, Greece. Samples and data obtained include serum, genetic material, subject phenotypes, and aspirates from the small intestine (*Pyleris* et al., 2012). Conventional cultures of these small intestinal samples demonstrated that 39.3% of IBS subjects had SIBO (defined as $>10^3$ cfu/ml), compared to 11.3% of non-IBS subjects (subjects with GI complaints that did not have IBS). Moreover, 60% of D-IBS subjects were found to have SIBO (*Pyleris* et al., 2012). More recently, we have begun to characterize the specific microbial populations present in the small intestine in IBS vs. control subjects. Preliminary deep sequencing analyses suggest that the microbial profile (microbiome) in the small intestine of IBS subjects is markedly different from that in healthy controls, with significantly reduced microbial diversity and overrepresentation of a few specific genera (*Chang* et al., 2013). The overgrowth of these specific microbial populations, possibly in concert with the loss of other microbes with important protective effects, may contribute to the gut symptoms of IBS subjects.

TREATING SIBO IN IBS

While the uncovered connection between IBS and SIBO was important, it was equally important to identify treatment options for those who suffer from IBS. Based on our growing evidence for the connection to SIBO, we initiated a series of studies to test the use of antibiotics in IBS. The first was the use of neomycin in the first double blind antibiotic study (*Pimentel* et al., 2003). Although neomycin successfully improved IBS in the study, neomycin was a poor antibiotic for SIBO. Thus we began looking for an alternate non-absorbed antibiotic. Rifaximin had all the properties that made it ideal for IBS. It was almost completely non-absorbed, had no issues with bacterial resistance, was highly effective for SIBO, was not related to conventional antibiotics (thus would not create cross-resistance) and did not change stool flora. Since identifying this antibiotic, we have conducted four large-scale multicentre trials to examine the efficacy of rifaximin (*Pimentel* et al., 2011a, 2006). The most recent of these were the completion of the Target 1 and 2 trials published singularly in the New England Journal of Medicine. This final phase III study conclusively showed that rifaximin is effective in IBS without constipation and that a 2-week treatment had benefits lasting a minimum of 12 weeks. No previous IBS therapy had this kind of effect, suggesting that we impacted a potential mechanism of IBS. We also examined the effects of retreatment with rifaximin in subjects with non-constipated IBS, and found that more than 75% of subjects who initially responded to rifaximin also responded to subsequent treatments, with no significant reduction in benefit for up to 5 successive retreatments (*Pimentel* et al., 2011b). Furthermore, there was no change in the duration of benefit (median time between treatments) for successive retreatments (*Pimentel* et al., 2011b).

POST-INFECTIOUS IBS

Over the last decade, it has been established that intestinal pathogens play a significant role in the development of IBS. Numerous studies have shown that IBS can be precipitated by an episode of acute gastroenteritis, and that up to 57% of subjects who otherwise had normal bowel function may continue to have altered bowel function for at least 6 years after recovering from the initial acute illness (*Neal* et al., 2002). Based on two recent meta-analyses of this research, approximately 10% of subjects who have documented acute gastroenteritis develop IBS, with a summary odds ratio of 6 to 7 for PI-IBS (*Halvorson* et al., 2006; *Thabane* et al., 2007). As gastroenteritis is extremely common, so-called PI-IBS may in fact constitute a large proportion of IBS cases. Thus, reducing risk factors for IBS development after acute gastroenteritis may have an impact on the incidence of IBS. Although the mechanisms of PI-IBS remain unclear, investigators have identified certain risk factors for the development of IBS after gastroenteritis. The two most significant of these are duration/severity of gastroenteritis and female sex (*Gwee* et al., 1999; *Neal* et al., 1997). Stress, manifest as recent traumatic life events, and a neurotic personality trait were also predictors of PI-IBS (*Gwee* et al., 1999). Evidence of low-grade inflammation is evident in PI-IBS patients.

Rectal biopsies demonstrate mildly elevated intraepithelial lymphocytes and entero-endocrine cells that persisted 12 months after infection with *Campylobacter jejuni* (*Spiller* et al., 2000), which is the most common cause of acute gastroenteritis in the US (*Tauxe*, 1992). Increased rectal lymphocytes also occur in general IBS patients, but to a lesser degree (*Dunlop* et al., 2003). Elevated expression of pro-inflammatory cytokine IL-1β was detected in *C. jejuni* PI-IBS rectal biopsies (*Gwee* et al., 2003) and in *Shigella* PI-IBS recto-sigmoid and terminal ileum biopsies (*Wang* et al., 2004). Thus, acute gastroenteritis may increase the risk of developing IBS in a susceptible individual through persistent low-grade activation of the gut immune system, or possibly through establishment of an intestinal dysbiosis, defined as an alteration of the composition of the gut flora. Animal infection models of PI-IBS will play a key role in characterizing the mechanistic pathways and underlying alterations in this process.

RAT MODEL OF PI-IBS

To study the underlying mechanisms of PI-IBS, we developed and validated a rat model (*Pimentel* et al., 2008) using the human pathogen *C. jejuni* as the infective agent. Over the past 8 years, we have characterized and validated this model, which exhibits phenotypes that closely mimic those seen in human patients with PI-IBS. These include: altered stool consistency that persists three months after the clearance of the acute infection and the development of SIBO (*Pimentel* et al., 2008), as well as increased rectal intraepithelial lymphocytes and reduced numbers of deep muscular plexus interstitial cells of Cajal (DMP-ICCs) (*Jee* et al., 2010). The latter is particularly significant as ICCs and myenteric nerves are known to be required for normal intestinal motility, including phase III of interdigestive motor activity (*Nieuwenhuijs* et al., 1998). Further, evidence suggests that a deficiency of phase III motor activity, which is known to induce SIBO (*Nieuwenhuijs* et al., 1998; *Vantrappen* et al., 1977), is associated with the development of SIBO in IBS (*Nieuwenhuijs* et al., 1998). We have also confirmed alterations in mucosal defence mediators such as TNF-α in post-infectious rats (*Sung* et al., 2013).

ANTIBIOTIC PROPHYLAXIS PREVENTS THE DEVELOPMENT OF IBS-LIKE CHARACTERISTICS IN A RAT MODEL OF *C. JEJUNI* INFECTION

Antibiotic therapy (*Lembo*, 2008; *Pimentel* et al., 2003, 2006; *Sharara* et al., 2006) and the *C. jejuni* vaccine (*Monteiro* et al., 2009) mitigate the effects of gastroenteritis in humans (*Lembo*, 2008; *Pimentel* et al., 2003, 2006; *Sharara* et al., 2006). Since duration of gastroenteritis is a risk factor for IBS (*Neal* et al., 1997), these approaches may be effective in preventing IBS. To determine the effect of prophylactic antibiotic therapy on the development of post-infectious IBS-like symptoms in rats, we gavaged rats with either rifaximin (200mg rifaximin daily for 3 days) and *C. jejuni* 81-176 (5×10^8 cfu, given on day 2) (C+/R+), or with *C. jejuni* alone (C+/R-). The

two groups were then compared for both acute *C. jejuni* colonization, and for the development of post-infectious IBS-like symptoms three months later. We found that rats that received rifaximin prophylaxis (C+/R+) cleared the initial *C. jejuni* infection significantly faster than the C+/R- group (10.3±7.1 days vs. 12.6±5.9 days, $p<0.01$) (*Pimentel* et al., 2011c). Further, we found that after 3 months, the C+/R- rats had a greater persistent variability in stool % wet weight than C+/R+ rats ($p<0.01$), and that the average stool consistency was closer to normal in C+/R+ rats than in the C+/R- rats (*Pimentel* et al., 2011c).

CYTOLETHAL DISTENDING TOXIN (CDT) IS REQUIRED FOR EXPRESSION OF AN IBS-LIKE PHENOTYPE IN THE RAT MODEL

The Cdt toxin is common to all bacterial organisms that cause post-infectious IBS, and thus a potential candidate toxin mediator of IBS. To evaluate the effect of Cdt on the post-infectious IBS-like symptoms in rats, rats exposed to a Cdt knockout (Cdt-) mutant of *C. jejuni* were evaluated for stool form three months after clearing the initial *C. jejuni* infection, and compared to results obtained in rats infected with wild type *C. jejuni* (Cdt+). Stool samples were collected and evaluated over a 3-day period to obtain an average stool form. Stool form at 3 months post-infection was significantly better in the Cdt- group. Furthermore, 42% of the Cdt+ rats had altered stool form 2 out of 3 days, as compared to 18% of the Cdt- rats ($p=0.028$, Fisher's exact test). In addition, the stool wet weight in the Cdt+ rats demonstrated an alternating pattern of wet and dry, such that the variance was 8.4±6.4, compared to 4.2±2.4 for the Cdt- rats ($p<0.001$) (*Morales* et al., 2011; *Pokkunuri* et al., 2012).

To evaluate the effect of Cdt on gut histology, DMP-ICC were also examined in Cdt- rats. Three months after clearance of the initial *C. jejuni* infection from their stool, the ileum was resected and CD117 immunostaining (anti c-kit) was performed on cross-sections of mucosa. The results revealed normal DMP-ICC staining in the ilea of these rats, which is in marked contrast to the reduced numbers and altered appearance of DMP-ICC seen in sections from rats exposed to wild type *C. jejuni* (*Morales* et al., 2011; *Pokkunuri* et al., 2012). Taken together, these data strongly implicate Cdt as directly contributing to the development and severity of PI-IBS phenotypes.

POTENTIAL ROLE OF IMMUNITY IN THE DEVELOPMENT OF PI-IBS

Epidemiologic studies from developing countries indicate that the prevalence of PI-IBS in developing countries is lower than or similar to that in developed countries, despite much higher incidence of *Campylobacter* enteritis, which suggested to us that acquired immunity might play a role in PI-IBS. To begin to determine whether immunity was involved in the effects of *C. jejuni* and Cdt in the gut, we infected 50 rats with *C. jejuni* 81-176 as juveniles, and then re-infected them with the same strain two months later, and

compared the results to those from another 50 rats exposed for the first time as adults. Fewer rats infected for a second time (juvenile then adult (J+/A+)) had detectable stool *C. jejuni* compared to their first infection ($p<0.05$) and compared to the 50 rats that only received *C. jejuni* as an adult (J-/A+) ($p<0.05$). In addition, the number of days of loose stool was less for a second infection than the first (1.58±0.16 vs. 2.50±0.29 days, $p<0.05$) and the overall duration of the second infection was shorter than the first ($p=0.001$). These data suggest that exposure to *C. jejuni* early in life mitigated the acute effects of a second infection (*Sung* et al., 2013).

We next compared the development of SIBO and stool phenotypes during the post-infectious period. Interestingly, 47% (23/49) of J+/A+ rats developed SIBO, as compared to 26% (13/50) of J-/A+ rats ($p=0.019$), but J-/A+ rats that developed SIBO had greater alterations in stool consistency than J+/A+ rats that developed SIBO ($p<0.01$) (*Sung* et al., 2013). These data suggest that while rats exposed to *C. jejuni* early in life were not protected against the development of SIBO, the second infection was better tolerated and resulted in less severe stool phenotypes, perhaps suggesting the development of tolerance to the SIBO. Moreover, for rats who were first exposed as infants and then reinfected, we found that those who had diarrhoea during the first exposure were more likely to develop SIBO than those who did not ($p=0.02$) (*Sung* et al., 2013). This result mirrors findings in humans that suggest a relationship between the severity of acute illness and the likelihood of subsequent development of IBS (*Halvorson* et al., 2006; *Thabane* et al., 2007), and led to our hypothesis that immunity might play a significant role in the development and severity of PI-IBS phenotypes. Recent data generated in our laboratory support this hypothesis, and further suggest that the effects of immunity are the result of molecular mimicry between Cdt and endogenous neural elements of the gut (*Pimentel* et al., submitted). These results may form the basis for future biomarkers and potential therapies that could be directed at the cause of IBS, rather than just treating SIBO.

PERSPECTIVES AND FUTURE DIRECTIONS

In summary, our work to date indicates that alterations in gut microbial populations contribute to IBS and the development of IBS phenotypes. Specifically, we have shown that SIBO, which is caused by reduced gut motility, is present in human IBS subjects, particularly D-IBS subjects, and can be ameliorated using antibiotics such as rifaximin. Further, acute gastroenteritis caused by *C. jejuni* and similar pathogens can precipitate PI-IBS. Using a rat model, we have shown that acute *C. jejuni* infection exhibits altered gut histology including reductions in the DMP-ICC which are required for gut motility as well as increased IELs and immune response mediators, and results in altered stool phenotypes and the development of SIBO in the post-infectious phenotypes. Further, we have shown that these are likely potentiated by the bacterial toxin Cdt. Lastly, recent work by our group indicates that the small intestinal microbiome is markedly different in IBS subjects, with significantly reduced microbial diversity and overrepresentation of a few specific genera, and that immunity may play a significant role in the devel-

opment of IBS, due to molecular mimicry between Cdt and neural gut moieties. Future work will include characterization of the small intestinal microbial signatures in IBS vs. healthy controls, and the development of biomarkers and targeted therapies for IBS based on immune targets. The results will allow us to significantly impact the diagnosis and treatment of IBS.

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