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Discussion on Toll-like Receptor 9 Signaling Mediates the Anti-inflammatory Effects of Probiotics in Murine Experimental Colitis

Dear Sir:

We have some concerns with the Rachmilewitz et al.[1] article published in GASTROENTEROLOGY recently, that concluded "live microorganisms are not required to attenuate experimental colitis."

Animal models per se have limitations, particularly when the sample size is limited (here <10 per group). The dextrose sodium sulfate model can be difficult to interpret. Was mild disease created and if so how much inflammation was ameliorated, in terms of clinically relevant colitis? The low MPO levels are inconsistent with the severity of the histological scores. The authors scoring system evaluates depth and extent of ulcers, yet ulcers are not a common feature in this model characterized by several degrees of crypt damage.

DNAse treated probiotics lost their anti-inflammatory properties as shown in Tables 5, 6, 7 so would pancreatic secretion, very rich in DNAses, not degrade the DNA therapy? The DNA extracts activated the NF-κB pathway resulting in higher production of IL-12 and IL-6, 2 potent pro-inflammatory agents whose blockage dramatically reduces inflammatory colitis in animal models of colitis, including in the TNBS model.[2,3] How did stimulation of these cytokines result in an anti-inflammatory effect? Other investigators have observed that bacterial DNA (CpG nucleotides) may actually aggravate inflammation and the severity of the disease when given after the onset of DSS colitis.[4,5] Thus, the use of an unspecified mixture of bacterial DNA from several strains should be avoided. Furthermore, the bacterial composition of VSL#3 is not fully known in terms of which strains are in the highest numbers per batch, and which reach the intestine in numbers sufficient to convey benefits. Were the dominant organisms streptococci, and if so would the DNA from these organisms alone convey the anti-inflammatory effect making the other strains unnecessary? If the effect is caused by a combination of DNA from all 8 or 9 strains in the product, is it a nonspecific interaction that could be achieved by any bacterial DNA? To date, very few studies, and none using dead bacteria, have resulted in significant clinical reduction in symptoms and signs of colitis. To suggest that unreliable, so-called probiotic products sold by certain companies and found to contain mostly nonviable bacteria[6] could, and should, be used to treat colitis, is neither appropriate nor supported by the results of this animal study.

The study has merit in describing a potential mechanism of action of probiotic strain extracts, but the findings cannot rule out other attributes of probiotics, including by-products not found in VSL#3 strains, and immunomodulatory activity induced via other pathways, such as IL-10 synthesis and secretion.[7] The primary author previously failed to show a remediation of immune-mediated dinitrobenzene sulfonic acid-induced colitis,[8] again illustrating that clinical studies are needed before over-interpreting these animal data in relation to a disease in humans.

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host."[9] As such, strains that do not confer a benefit, for example in treating colitis, would not be termed probiotic for that application. This might seem a petty comment, but in practical terms it is critical that healthcare professionals know exactly what it means to be a probiotic. By defining a product or active strain properly, and proving efficacy along with mechanism of action, we will collectively advance this important emerging field, and determine how best to apply the knowledge to retention and restoration of health in our patients.

Numerous publications and trade journals have picked up the press release that followed this paper, including *Nature* online,[10] who refused a rebuttal, and taken literally the concluding message that viable probiotics are "not required." Sadly, this is to the detriment of probiotic research generally and at a time when it is turning the corner from a somewhat murky folklore to a scientific and clinically sound field offering new light in the many dark tunnels of intestinal and other diseases.

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Reply. The aim of our investigations was to explore the mechanism of actions of probiotics in models of experimental colitis. Regarding these mechanisms, our studies provides genetic, immunologic, and biochemical evidence, rather than *opinionated scholarship*. Since in this work we demonstrated that immunostimulatory properties of the bacterial DNA are responsible for the anti-inflammatory effects mediated by viable probiotic bacteria, it was unavoidable to reach other conclusions than those that we had raised. The current definition of probiotics as live bacteria was not being challenged by our study. The mechanisms attributed to probiotics were challenged.

Below are specific comments in response to several of the points raised in the letter:

Because of the drawbacks of every model of experimental colitis, including DSS induced colitis, our work showed that the probiotic DNA is effective also in TNBS–induced colitis and in spontaneous colitis in IL-10 knockout mice.

The fact that oral administration of the probiotic DNA was effective rules out a complete efficacy of pancreatic DNAse. In fact, this issue was tested in Figure 3*A*. The data presented in this Figure indicate the percentage of the oral dose of naked pDNA that is absorbed could be detected at systemic sites. For the delivery of irradiated probiotics, we assumed an additional protective role for the probiotc bacterial cell wall on probiotic DNA from enzymatic digest by pancreatic DNAse. Therefore, the probiotic DNA is available to mediate its effect (see Figure 3*C*). These results are in contrast to an in vitro enzymatic activity of DNAse, which was titrated to provide a complete digest of the reaction's substrate (probiotic DNA).

The activation of NF-κB pathway (Figure 1) was presented in bone marrow–derived macrophages and was used in order to show that probiotic DNA has immunostimulatory properties.

We selected the bacterial preparation VSL-3 only because of the solid published clinical data with this preparation. To demonstrate that our findings do not relate only to this preparation, *E. coli* DNA was also used and shown to mediate similar properties as was shown for VSL-DNA. We agree with Reid that it is worthwhile to elaborate on whether all the strains in the VSL-3 preparation share the same properties. In fact, our results provide a simple method to do so, i.e., evaluation of the immunostimulatory properties of each of the probiotic bacterium in this preparation.

Nowhere in the paper did we claim that unreliable probiotic preparations should be used to treat colitis.

We did not rule out possible involvement of other mechanisms including the secretion of IL-10 (see Discussion). However, Reid's suggestion that induction of IL-10 may be involved in our studies is unlikely as our data indicate anti-inflammatory effects of probiotc DNA in IL-10 KO mice (Table 8).

We agree with Reid et al. that it is now important to demonstrate that viable probiotics, irradiated probiotics or synthetic ISS–ODN are effective in modulation of human disease such as IBD. We do believe that a critical scientific approach leads to new findings, adds knowledge, and provides insight. Only this discipline can turn the probiotic field from *murky folklore* to a solid and sound science and consequently to the expansion of probiotic uses for the benefit of humankind.

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