

## Discussion

Leader: Cathryn Nagler, PhD

Dr Nagler: I want to start the discussion with the concept of dysbiosis. This table is taken from a recent commentary by Balfour Sartor [*Proc Natl Acad Sci U S A* 2008;105:16413-16414].

<b>A disturbed balance of beneficial and detrimental bacteria (dysbiosis) could promote intestinal inflammation.</b>	
Balance of beneficial vs detrimental commensal enteric bacterial species helps determine intestinal homeostasis vs inflammation	
<b>Potentially injurious species in susceptible hosts</b>	<b>Protective species</b>
<i>Bacteroides vulgatus</i> , <i>B thetaiotaomicron</i>	<i>Lactobacillus</i> species
<i>Escherichia coli</i> (adherent/invasive)	<i>Bifidobacterium</i> species
<i>Enterococcus faecalis</i> (nonpathogenic)	<i>Escherichia coli</i>
<i>Klebsiella pneumoniae</i>	<i>Bacteroides thetaiotaomicron</i>
<i>Fusobacterium varium</i>	<i>Faecalibacterium prausnitzii</i>
<i>Helicobacter hepaticus</i> and other intestinal species	
<i>Bifidobacterium animalis</i>	
	Sartor B: <i>Proc Natl Acad Sci U S A</i> 2008;105:16413-16414.

Sartor suggests that the balance of beneficial vs detrimental commensal bacteria species regulates intestinal homeostasis vs inflammation. He targeted inflammatory bowel disease (IBD), but I suggest that this concept could be applied more broadly and could include diabetes and food allergy. I highlighted *Helicobacter hepaticus*, which is my favorite inflammation-inciting agent, and *Lactobacillus* and *Bifidobacterium* species. So we do not have to restrict the dysbiosis concept to a particular bacterial or viral infection that can influence the composition of the microbiota. Perhaps *Helicobacter* infection does not necessarily act directly as a pathogen—most of us are populated by *Helicobacter* species—but perhaps it alters the composition of the

microbiome in a way that allows for either a decrease in protective bacterial species or an increase in pathogenic species.

Does that seem reasonable? The diabetes data I showed by Wen and colleagues were interesting [*Nature* 2008;455:1109-1113], but the investigators do not know which bacterial species regulates the susceptibility to diabetes or protection against diabetes. Apparently they are different populations, but both are MyD88 independent.

Dr McSorley: What do you think of the inflammatory bowel disease (IBD) literature in regard to that? Most people are using these IBD models. If this dysbiosis concept is true, it would be critical, and we could test it in TLR5 knock-out mice, which were reported to develop rectal prolapse [Vijay-Kumar M et al: *J Clin Invest* 2007;117:3909-3921]. Mice in our colony do not develop rectal prolapse, but no one defines these strains when they publish data on them.

Dr Nagler: Another problem with *Helicobacter* infection is that in academic mouse colonies, it is endemic, but it varies from cage to cage. Mice in one cage will be infected, and those in another cage will not. That variation complicates the results of experiments unless researchers do not use large numbers of mice and track the infection mouse by mouse. This is done fairly easily by using fecal DNA.

Dr McSorley: Why *Helicobacter*? Do you look back at what is driving this response?

Dr Nagler: No. However, it has been implicated in colitis in many mouse strains. It is found in human patients; it has been implicated in colon cancer and is known to cause gastric ulcer. The enterohepatic *Helicobacter* species in humans is not well characterized, but it is a focus in many groups in the ongoing human microbiome project. The association of *Helicobacter* with food allergy is completely unexplored. We are interested in this possible association because it could explain the big increase in food allergies over the last 10 years. As Dr Brandtzaeg mentioned, the

microbial deprivation hypothesis, or the hygiene hypothesis as originally presented, was too simplistic. The increase in food allergies must have something to do with various populations of microbes and their influence on each other, which we are only beginning to explore.

Dr Bienenstock: What do you know about *Helicobacter*-negative humans that you would like to leave with us that we can extrapolate to food allergies?

Dr Nagler: This has not been studied, but it could be fairly easily. We could compare people with food allergies with nonallergic family members and see whether *Helicobacter* has anything to do with the allergies.

Dr Bienenstock: Researchers are studying the question of balance or imbalance in the microbiome. Agnes Wold and colleagues, for instance, has data on *Staphylococcus aureus*, which has become a major player in the first couple of years of life [Lundell AC et al: *Clin Exp Allergy* 2007;37:62-71]. They have developed a whole theory about the role of *Staph aureus* in development of food allergy. Presumably, we can extrapolate that theory to other microbes that also could displace or change the balance of the microbiome.

Dr Nagler: That is why I showed Sartor's table. It shows that many microbes probably can do this. Understanding what they are and the nature of their interaction is another level of complexity in the influence of the microbiome on the mucous layer. Evidence is emerging that shows how the composition of the mucosa-associated bacteria influences the mucous layer, and how that affects the ability of other bacteria to approach the epithelium and influence immune responsiveness [Young VB, Schmidt TM: *Adv Exp Med Biol* 2008;635:29-40]. When we understand the microbiome better, we can manipulate it.

Dr Versalovic: It is important to consider the relative balance of these classes of organisms. We are limited now by our lack of knowledge about microbial deficiencies, as well as excesses (see

Table, p XX, left column). We have published data about *Faecalibacterium prausnitzii* (see Table, p XX, right column). It is intriguing, and we have learned from it. As we expand our concept of probiotics and beneficial microbes, we will discover new organisms that have potent immunomodulatory features. Certainly emerging data support this notion of dysbiosis.

Dr Lee: There is balance between the beneficial and detrimental groups of bacteria. One group induces inflammation; the other suppresses inflammation. But in your antibiotic study, Dr Nagler, it is the absence of microbes that leads to inflammatory responses. Can you comment on that?

Dr Nagler: Although I and other people call this “antibiotic decontamination,” all it does is knock down the number of bacteria several logs. We still have not identified most of the bacteria; so I could not say what we have eliminated, but plenty of bacteria are still in there. The antibiotic treatment does remove an important population of bacteria—maybe the mucosal-associated population—or it somehow disturbs the balance in a way that removes the protective population or its access to the immune system. My first step in microbiome analysis in this model would be to find out what we removed in the antibiotic-treated mice to determine the effects on TLR4 signaling, and the exact interaction between the bacteria and TLR4 in this model.

Dr Lee: You use a combination of cholera toxin and antibiotics. Have you changed the antibiotic profile? Did you observe any differences?

Dr Nagler: No. We just used this mixed cocktail and looked for maximum effect. This is similar to the cocktail that Medzhitov’s group used in their studies [Rakoff-Nahoum S et al: *Cell* 2004;118:229-241].

Dr Michael Montalto (Abbott Faculty): Are we to assume that the protective species are all similar within the *Lactobacillus* strain or that some are more potent than others?

Dr Nagler: I think some other people here would be better qualified to address that than I am, but I think probably not. I do not think that all species are welcome. *E coli* is Gram-negative, for sure, and Dr Bienenstock mentioned *Staph aureus*. They are not all what we have considered to be in the probiotic family. I certainly would put *B fragilis* on the protective side, based on Kasper's work that I alluded to earlier—that polysaccharide can modulate inflammation [Mazmanian SK et al: Nature 2008;453:620-625].

Dr Versalovic: Saying "*Lactobacillus*" is a bit like saying "T cells." We know that, within a specific species—*Lactobacillus reuteri*, for example—that individual strains can potentially stimulate the immune system. Others can downregulate and have very potent anti-inflammatory features. The issue is one of resolution. Clearly, there are good examples within the *Lactobacillus* genome of potent anti-inflammatory organisms, but we could flip it the other way when we talk about different strains. The phrase "antibiotic decontamination" itself has a bias that is being reconsidered.

Dr Brandtzaeg: Bringing this back to real life, you said that in less than 10 years, peanut allergies in the United States have at least doubled. If this is true, do you really think that the bacterial composition has changed that much in 10 years, even in the face of increased use of antibiotics? What about looking for some toxic factor? Cholera toxin breaks all mucosal tolerance; you have to use that toxin to induce efficient sensitization through the gut. There is discussion in Europe about a possible toxic sequence of the Cry proteins in the construct of genetically modified food, transferred from the *Bacillus thuringiensis* (*Bt*) bacteria that were used to make the construct. Perhaps there is some cholera toxin-like expression in a genetically modified food such as maize,

consumption of which has increased enormously during these 10 years. That could be a parallel development—increased intake of genetically modified maize and then increasing peanut allergy—reflecting break of mucosal tolerance by Cry toxin from *Bt*.

Dr Nagler: I do not think that these numbers about the increase are necessarily reliable. I think the increase is real, but it has not happened over the last 10 years. Certainly, within a generation. No one would argue with at least a generational change in peanut allergy in this country. That cannot be explained by genetics, so it has to be environmental, whether it is a microbial component, a mix of microbial components, or toxins.

Dr Brandtzaeg: Is there no discussion in this country about genetically modified food? As I said, that could introduce a toxic component.

Dr Nagler: I have not heard any such discussion.

Dr Kuitunen: Why have food allergies increased? In Europe, a lot of elimination diets are prescribed—too many, in my view—because of various symptoms in infants. In a recent study on mice, researchers assessed the effect of ovalbumin transfer via breast milk. The development of tolerance in offspring was induced by the transfer of ovalbumin in the milk [Verhasselt V et al: *Nat Med* 2008;14:170-175]. These avoidance diets might be one reason for the increase in food allergies. Dr Brandtzaeg, what is your view of the effect of these breast-milk antigens on the development of the IgA system?

Dr Brandtzaeg: In that study, lactating mice were fed albumin. The breastfed mice babies were protected from asthma if the mother was fed oral albumin after delivery. The study also showed that a combination of antigen and TGF- $\beta$  was needed in the breast milk to induce this tolerance.

That paper has created a lot of discussion around the world. Perhaps we should not be as strict about food avoidance during pregnancy and breastfeeding as some people have advocated.

Now people at meetings I have attended are saying, "do not worry about allergen avoidance during breastfeeding and pregnancy; women cannot avoid them entirely anyway." With regard to gluten intolerance, there is a large European Union study on introduction of gluten very early in genetically prone infants to see whether that can delay or inhibit the presentation of celiac disease. Perhaps an early allergen exposure is a natural thing, and especially mixed feeding with allergen and secretory IgA from breast milk.

Dr Rueda: What do you think about the role of hydrolyzed protein in stimulating the immune system, or not stimulating the immune system enough, and consequently increasing the development of allergy, or not, during the first months of life?

Dr Brandtzaeg: Partially hydrolyzed formula, in the opinion of some researchers, could induce tolerance, since it has some intact antigen. Tolerance is an active process; you need antigens to activate the immune system. Clearly, we have to distinguish between prevention and treatment. If we want to treat a patient with severe food allergy, we need to have completely degraded antigens or amino acid-based diets. But in prevention, this discussion is not included. Studies are being conducted to see whether partially hydrolyzed formulas can prevent allergies, but researchers find it difficult to select a study population because most patients who become allergic lack a family history. They are scattered in the general population, so the researchers would have to treat everyone.

Dr Montalto (Abbott Faculty): What happens to the microbiome when a premature infant is born and they hit him or her with a cocktail of vancomycin, gentamycin, and neomycin? Is it wiped out, or do all the bad bugs go away and the good ones stay?

Dr Nagler: I do not think there is information on that yet. However, it could have a detrimental effect by removing populations of bacteria that might be beneficial. This needs to be studied.

Dr Montalto (Abbott Faculty): Can you envision a designer protective species bug that is resistant to the antibiotics that normally would wipe it out?

Dr Nagler: Yes, but microbial constituents might be even more attractive. In our model, for instance, we are looking for a TLR4 agonist that is antibiotic resistant.

Dr Rueda: Dr Brandtzaeg, one of your slides showed how the levels of IgA in saliva increased during the first months of life and went on increasing during the first years of life. How do these levels relate to the IgA in the intestine and the rest of the mucosa? Might they be a good indicator of the IgA levels in the rest of the mucosa?

Dr Brandtzaeg: Salivary IgA, particularly parotid secretory IgA, probably reflects the immune response of the upper respiratory airways better than the gut. However, we probably cannot use it as a reliable indicator of the development of the mucosal immune system in general. There was a question about selective IgA deficiency, which generally shows a mild phenotype in humans.

This deficiency may increase some diseases such as inflammatory bowel disease and celiac disease, but the gut in most people with selective IgA deficiency is okay. They do, however, have increased allergies and upper airway problems. One explanation for why most people with the deficiency do well is that there is compensation with secretory IgM antibodies in the gut, which is not so predictable in the airways. The polymeric IgA receptor, or SC, will pump out IgM as compensation for lack of IgA.

Confusion has been introduced into this field by studies of the IgA knock-out mouse, which also has secretory IgM compensation, especially in the gut, as well as a reduced antigen presentation capacity. So this mouse has a defect in proinflammatory potential. The confusion is due to the need to distinguish between that model—the IgA knockout—and our model, which is

a knockout of the polymeric Ig receptor, or SC. In our model, all the secretory immune system is blocked and there is no antibody compensation at all.

Dr Robert Miller (Abbott Faculty): Does research suggest that the microbiome develops differently in infants delivered vaginally than it does in those delivered by caesarian section? If so, are those differences associated with any type of disease?

Dr Nagler: In our mouse model, we remove the *Helicobacter* infection by caesarian rederivation of the mice. The disease that is induced in the caesarian rederived mice is not the same as the disease in the naturally infected mice, which suggests not only an influence beyond just removing *Helicobacter* of the caesarian rederivation, but also a difference between perinatal infection and adult infection. Extrapolation from studies of animals after caesarian or vaginal birth suggests there is a big difference in the microbiome. This has not been studied to any great extent.

Dr McSorley: A comment about these protective species. I do not closely follow the literature on *Lactobacillus*. Are its anti-inflammatory effects an active process, or do the *Lactobacilli* fill a niche so that the species that would normally take hold are not there? Dr Nagler: As Dr Versalovic pointed out, there are many different subspecies of *Lactobacillus*, all with different characteristics. What model do we use to study them? If you study them in vivo, you know whether it is the interaction of the bacteria with the epithelium that influences the education of dendritic cells, but that is difficult to model in vitro.

Dr McSorley: That is an important question with regard to probiotics. The stage of life at which you give probiotics—whether the patient has a fully intact flora at that point—determines whether the probiotic fails or succeeds. It should not matter whether it is actively anti-inflammatory.

Dr Nagler: We started the antibiotic decontamination protocol at 2 weeks because it is difficult to modulate the flora in adult mice. We have done some limited experiments with *Lactobacilli*. We have found that we need to start introducing the bacteria before weaning because the composition of both the flora and the gut-associated tissue changes dramatically at weaning. That is why administration of probiotics for treatment of allergy in adults is not efficacious.

Dr Kuitunen: Some reports indicated that children born by C-section have more allergic diseases. We are interested in examining this in the cohort we are following for 5 years. We want to supplement the children born by C-section with probiotics because this target group could benefit most. We are analyzing data now that suggest the frequency of caesarian deliveries in northern Europe is about 17%, and according to some reports, the frequency in the United States is as much as 30%. So this target group is quite large.

Dr Brandtzaeg: We have a large cohort of children in Oslo that is followed carefully. In that cohort, egg allergy is eight-times higher among those born by C-section than among those born vaginally, but only when there is a positive family history for allergy [Eggesbø M et al: *J Allergy Clin Immunol* 2003;112:420-426]. Also, a recently published meta-analysis shows that asthma is 20% higher among children born by C-section, so that must have something to do with colonization [Thavagnanam S et al: *Clin Exp Allergy* 2008;38:629-633]. Studies of certain strains of probiotic *Lactobacilli* show stimulation of increased production of IgA; that is one aspect of anti-inflammatory action [Rautava S et al: *Pediatr Res* 2006;60:221-224].

Dr Buck: Following up on the comments of Dr Kuitunen and Dr Brandtzaeg, we know there was delayed bacterial colonization in preterm infants, as well as in full-term infants born by C-section. What are the consequences of delayed colonization besides allergies?

Dr Versalovic: We need to rethink how babies are being cared for. I hesitate to say "treated," although I suppose we could call it treatment because we may be dealing with a microbial deficiency syndrome. Certainly there is a population of children that is relatively deficient in specific classes of microbes, and those are the children who may need to be targeted with specific prebiotics, probiotics, or symbiotic regimens. The data emerging from some of the meta-genomic studies support the fact that colonization is delayed in children born by C-section.

Dr Buck: Dr Brandtzaeg, would you speculate on the mechanisms involved in the increase of ileal secretory IgA by fructooligosaccharides (FOS)?

Dr Brandtzaeg: You are referring to the Japanese study with the mice model [Nakamura et al. *Clin Exp Immunol* 2004;137:52-58]. I asked the senior author at a meeting about the mechanism, and he said he did not know. One possibility is the fermentation to butyrate. Butyrate has quite a positive effect on the IgA pump, but it could be other mechanisms or a combination of mechanisms changing the composition of the flora and so on.

Dr Buck: You talked about the increase in the polymeric immunoglobulin receptor.

Dr Brandtzaeg: Yes, I alluded to that. Butyrate is an active component in that respect. The senior author was happy when I suggested that fermentation of FOS to butyrate might be the mechanism that increases the ileal secretory IgA level.

Dr Buck: Any thought of just increasing microbial loads, thus acting directly rather than indirectly?

Dr Brandtzaeg: Yes, that is one possibility, but we really do not know.