Diet-Induced Changes in the Colonic Environment and Colorectal Cancer Prevention Workshop

Nutrition Science Research Group
Division of Cancer Prevention
National Cancer Institute

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Welcome and Introduction — *Drs. Jon Story and Peter Greenwald*

Dr. Story, Chair of the workshop, welcomed participants and thanked them for attending. A Professor in the Department of Foods and Nutrition at Purdue University on sabbatical with the Nutrition Science Research Group, Dr. Story gave a brief overview of the roles that diet components and colonic bacteria may play in the development of colon cancer. He described the processes by which the normal epithelium progresses to hyperproliferative epithelium, aberrant cryptic foci, small adenomas, large adenomas, and colon cancer. He explained that the workshop was intended to address the following three broad questions:

- How does diet modify colonic bacterial populations?
- What is the impact of colonic bacteria on the nature of metabolites in the colon?
- How does the colonic environment (intestinal cell genomics, colonic microorganisms, and microbial metabolites) alter susceptibility to colorectal cancer?

Dr. Greenwald, Director of the Division of Cancer Prevention at the National Cancer Institute (NCI), also welcomed attendees and thanked them for their participation. Dr. Greenwald then described some of the history of the relationship of dietary fiber and colorectal cancer and the involvement of NCI in that history.

Colonic Bacteria and Colorectal Cancer — *Dr. Michael J. Hill*

Dr. Hill, Chairman of the European Cancer Prevention Organization, participated in the workshop via conference call and provided background by explaining that the following hypotheses were examined during the period of 1968–1990:

- Gut bacteria can synthesize carcinogens/promoters from dietary components.
- The diet determines the composition of the gut flora and its enzymatic activity.
- The diet determines the conditions under which those enzymes act.

- This explains a mechanism for the relationship between diet and cancer risk.

The intestinal microflora produce carcinogens, promoters, and inhibitors from dietary substrates. Dr. Hill listed a number of carcinogens/promoters produced by gut bacteria from dietary substrates such as protein, fat, carbohydrates, and glycosides. Carcinogens include N-nitroso compounds (from basic amino acids and lecithin), ethionine (from methionine), and carcinogenic aglycones (from plant glycosides). Promoters include volatile phenols (from tyrosine) and various metabolites from tryptophan.

In earlier studies, investigators examined the effect of dietary change on fecal bacterial flora. None of the diet changes (high protein, high fat, high fibre, etc.) had any effect on the composition of the bacterial flora of feces. Even a fiber-free diet yielded a large decrease in fecal mass, had no significant effect on the composition of the fecal flora. However, in examining the effect of changes in macronutrient intake on the bacterial flora of ileostomy fluid, researchers discovered that diet has a dramatic effect on fecal flora in the proximal colon. In a stable ileostomy (i.e., one that has been established for a number of years) the distal ileum modifies to become a water absorbing mucosa (like the colon) making the stable ileostomy very similar to the intact caecum. In a series of 3-week studies conducted by Dr. Hill and colleagues, it was found that increasing the dietary intake of fat from 50 g/day to 150 g/day resulted in significant increases in the amount of *Bacteroides* and *Clostridium*. Increasing protein intake from 40 g/day to 120 g/day resulted in significant increases in facultative bacteria. Additionally, increasing bran intake resulted in large increases of all bacterial flora, and a 30 g pectin supplement produced changes in sentinel enzymes.

Dr. Hill and colleagues found further evidence that almost all the metabolism of substrates occurs in the proximal colon. In studying assays from surgery patients, those who underwent a total colectomy demonstrated a total lack of cholesterol reduction and bile acid metabolism in feces. They also had no urinary volatile phenols or cyclic secondary amines produced by the colonic flora—because they had no colonic flora. Patients who underwent a left hemicolectomy had no change in metabolism because they had normal proximal colons. They only lost the storage area of the distal colon; these patients retained the site of metabolism, he explained. The proximal colon is a fluid environment for the bacteria to get at the substrate. Once the lumenal contents have been dehydrated to the consistency of normal feces, the bacteria do not have as good access to their substrates. Therefore, Dr. Hill and colleagues concluded that the main site of metabolism is the proximal colon—that is where an effect of diet on the composition of the flora can be shown.

So in comparing these two ends of the colon, the cecum/ileostomy vs. the rectum/feces, a big effect is seen in the proximal colon but gradually diminishes as you move along the colon, disappearing by the time you reach the last section. The hypothesis is that digestion and absorption in the small bowel is not 100%. The types of nutrients lost to the small bowel depends on the type of food entering the mouth. On a high protein diet, some protein enters the cecum; on a high fat diet, fat enters the cecum, etc. The usable food entering the cecum is fermented by the bacteria in the cecum such that, by the time it gets to the transverse colon, it has all been fermented and the effect of any change in the diet will have disappeared. By the splenic flexure,

any effect on pH due to production of SCFA will be lost due to absorption from the colon. For example, feeding lactulose results in a decrease in the pH of the cecum from the normal 7.5 to about 4.0. But the pH of the transverse and distal colon is almost back to the control value. He suggested that there will be a similar effect on reduction-oxidation potential, with the dramatic effects of diet change seen in the cecum gradually diminishing as you go up the ascending colon to the transverse colon.

So the factors that affect the flora - nutrient supply, and physico-chemical conditions - are no longer related to diet by the time the food components reach the distal colon. This explains the lack of effect of diet change on the fecal flora.

Dr. Hill discussed some of the metabolic activities of gut bacteria, noting that the biochemical activity of the gut flora is similar in range to that of the liver—the range of metabolic activities carried out by the flora and liver are mostly detoxification reactions. He also noted that there is a tremendous range in the activity of any enzyme within a particular species, and that the diet determines both the conditions under which those enzymes act and the overall metabolic activity. Diet determines transit time, the pH of the proximal colon, and changes in redox conditions. Diet, by acidifying the lumen or by binding, also precipitates some of the metabolites within the colon and makes them less available for bacterial metabolism and for the action of carcinogens and tumor promoters.

Dr. Hill cited the results of three studies as evidence for the presence of a mechanism for the relationship between diet and cancer risk: (1) germ-free animals have significantly fewer tumors than conventional animal models after exposure to some carcinogens; (2) rats treated with antibiotics had a significant decrease in the number of tumors in a breast cancer study; and (3) lactulose has a protective effect against colon cancer in rats. Dr. Hill was asked if the shift in the location of colon cancers in the United States from the left side to right side could be related to dietary changes. He replied that diet could play a role, although there is no direct evidence. Unlike distal tumors, proximal tumors tend to be related to the microsatellite instability pathway. It may be that in the United States, there has been a dietary change that is favoring the microsatellite instability pathway.

Molecular Analysis of the Gastrointestinal Microbiota ***Dr. H. Rex Gaskins***

Dr. Gaskins, Associate Professor in the Departments of Animal Sciences and Veterinary Pathobiology at the University of Illinois, Urbana-Champaign, explained that the intestinal ecosystem is comprised of two complex cellular communities. One of these communities is comprised predominantly of anaerobic bacteria; the other is comprised of a variety of host cells that provide a defense against the microbiota. Dr. Gaskins explained that this structure and multitiered nature of defense evolved in response to the persistent challenges associated with harboring a dense and complex microbiota, not in response to periodic exposure to pathogens.

Dr. Gaskins presented a brief history of the understanding of intestinal microecology from the early 1960s to the present. He stated that cultivation-dependent microbiology is a major limitation because the nutritional and growth requirements must be known—it is only possible to grow those organisms whose mode of metabolism is known and can be isolated in culture. In

addition, given the complexity of the normal microbiota and the nature of cultivation-based techniques, using this approach becomes intractable because of diversity. In the early 1990s, it was recognized that information from the 16S rRNA genes can be used to study complex ecosystems in the natural environment. Dr. Gaskins explained the justification for the 16S rRNA approach, which is a cultivation-independent approach for studying microbial ecosystems, as follows: (1) the genes are present in all bacterial cells; (2) the sequence is highly conserved, which facilitates detection; (3) the presence of highly variable regions, which enables discrimination at (sub)species to higher phylogenetic levels; and (4) the presence of a large 16S rRNA database (approximately 20,000 sequences in the Ribosomal Database Project; http://rdp.cme.msu.edu/html/).

Dr. Gaskins described the primary uses and limitations of the following techniques: cultivation, 16S rDNA sequencing, denaturing gradient gel electrophoresis (DGGE)/thermal gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single-strand conformation polymorphism, fluorescence *in situ* hybridization (FISH), and dot blot hybridization. He placed each of these techniques in the context of three parameters: (1) community structure, (2) bacterial identity, and (3) relative abundance of individual organisms within that community. To demonstrate the varying efficacy of these techniques for certain parameters, Dr. Gaskins presented the combined results of three studies, comparing the cultivation, FISH, and dot blot hybridization techniques for detecting counts of four target groups of fecal bacteria from different individuals. In terms of total coverage, cultivation yielded a 10–50 percent coverage rate, FISH yielded a 64 percent rate, and dot blot hybridization yielded a 70 percent rate. Dr. Gaskins cited studies that presented the following key findings from molecular ecological analyses:

- There is marked variation in bacterial population profiles among individuals. These findings have been corroborated in mice, pig, and human studies.
- Among individuals, the dominant bacterial community remains stable over time.
- Host genotype affects the gastrointestinal (GI) bacterial community.
- There is segmental variation in bacterial population profiles along the GI tract.
- Fecal bacterial populations reflect distal colon profiles only.
- A comparative analysis of 16S rRNA sequences amplified from human feces indicated that:
  - Less than 25 percent of the molecular species (operational taxonomical units [OTUs]) identified corresponded to previously cultivated organisms
  - Three phylogenetic groups contained 95 percent of the OTUs identified.

Dr. Gaskins noted that host genotype is one of the primary determinants of bacteria. He presented data from two studies that demonstrate the utility of these techniques for comparing the effect of diet on intestinal microbiota. The first was a study of a total parenteral nutrition piglet model in which 16S rDNA polymerase chain reaction (PCR) DGGE analyses of the ileal

microbiota were compared in terms of enteral versus parenteral nutrition. The second study involved an approach to analyze the metabolic molecular ecology of the intestinal sulfate-reducing bacteria in mice.

Dr. Gaskins summarized his presentation by stating that the 16S rRNA gene is an ideal phylogenetic marker that consists of variable and conserved regions. 16S rRNA-based approaches allow for the quantitative and qualitative elucidation of the composition and abundance of bacterial species, and how their presence relates to diet and health. The choice of a particular molecular-based approach will depend on the question at hand and consideration of the limitations of each technique. He concluded his talk by suggesting the following approaches: (1) clone libraries for species identification and composition of the bacterial community, (2) fingerprinting techniques for community structure analysis, and (3) dot blot hybridization or FISH to measure abundance.

ISSUE I: HOW DOES DIET MODIFY COLONIC BACTERIAL POPULATIONS?

Effects of Inulin and Oligofructose on Colonic Microflora *Dr. Venket Rao*

Dr. Rao, a Professor in the Department of Nutrition Science at the University of Toronto, began by stating that probiotics and prebiotics selectively modify the microflora to bacteria that benefit the host. Probiotics are the bacteria themselves; prebiotics are those compounds that selectively promote the growth of the beneficial bacteria. Prebiotics are selected based on two characteristics: (1) reaching the colon undigested and unabsorbed in the upper GI tract, and (2) being selectively utilized by the resident *Bifidobacteria*. Based on these criteria, inulin-type fructans represent an effective prebiotic substrate, according to Dr. Rao. Naturally occurring sources of fructans include asparagus, banana, chicory, garlic, artichoke, onion, leek, and wheat. Processed foods containing fructans include beverages, breakfast cereals, butter, candy, chocolate, ice cream, and yogurt.

Dr. Rao described the *in vitro* fermentation of inulin and oligofructose, presenting data from studies on the effect of fructans on the growth of *Bifidobacteria,* the effect of different sugars on the *in vitro* growth of human fecal bacteria, and the *in vitro* effects of fructans on the composition of human fecal microflora. There is *in vitro* evidence that inulin and fructans selectively activate the growth of *Bifidobacteria.* A study examining the minimum oligofructose dose affording a prebiotic effect *in vitro* found a prebiotic effect even at doses as low as 1g/day. Dr. Rao reported that the bifidogenic effect was optimized at a dosage of 4g/day.

He also reviewed studies on the *in vivo* fermentation of inulin and oligofructose. Data were presented which demonstrated the effect of oligofructose on predominant intestinal microflora, *in vivo* effects of oligofructose on fecal microflora, *in vivo* effects of inulin on fecal microflora, and the effect of inulin on *Bifidobacterium*-fermented milk induced fecal *Bifidobacteria*. These studies show the sustainability of the enhanced prebiotic activity of inulin on *Bifidobacteria.*

Dr. Rao described a study he and his colleagues conducted in which fructan doses of 0, 225, 300, and 400 mg/day were administered to eight human subjects. They measured fecal bacteria,

breath hydrogen, fecal wet and dry weights, and pH, and found a dose-response relationship between fructan intake and physiological effects among subjects who did not have high baseline *Bifidobacteria* levels. A study on the effect of inulin intake on fecal microflora found that inulin increased the proportion of *Bifidobacteria* from 20 percent to 71 percent.

Dr. Rao described two directions for future research: (1) studies on the long-term intake of fructans by healthy human subjects to establish a dose-response relationship between the intake of fructans and their bifidogenic effects; and (2) studies on the effects of fructans on the fecal *Bifidobacteria* levels in subjects at risk for acute and chronic GI disorders, including colon cancer. He concluded his presentation by reiterating the following points:

- There is convincing evidence that chicory inulin and its hydrolysis product (oligofructose) stimulate the growth of *Bifidobacteria.*
- There is no correlation between the dose of fructans ingested and the increase in fecal *Bifidobacteria.*
- The lower the starting level of fecal *Bifidobacteria,* the greater the increase at any given dose of fructans.
- *In vitro* studies indicate that a fructan dose of 4 g/day increases the *Bifidobacteria* level, while decreasing the number of *Bacteroides* and *Coliform.*
- There are no published human studies demonstrating the minimum effective dose of fructans.

Dietary Fiber ***Dr. Judith A. Marlett***

Dr. Marlett, a Professor in the Department of Nutrition Science at the University of Wisconsin, Madison, discussed some of the observations stemming from the development of an *in vitro* system to study the rate and extent of dietary fiber fermentation. The system was designed to ask two basic questions:

- Is microbial adaptation to the substrate important? Microbial composition may depend on the substrate to which it has been exposed. When subsequently exposed to a different substrate, this adaptation may alter the ability of the bacteria to metabolize the new substrate.
- What should be the source of the inoculum? For this study, Dr. Marlett and colleagues chose cecal and fecal contents. The collection site and source of the inoculum was varied and came from rats fed nonpurified diet or purified diet to which psyllium seed husk (PSH) or lyophilized canned green peas were added.

The investigators measured the substrate that left the ileum and was exposed to the microflora. Dr. Marlett noted that the carbohydrate composition of the PSH and the pea fiber were similar. She shared data on the disappearance of the total expressed carbohydrates, whether the inoculum

came from cecal contents of the rats or from feces. The investigators found that the cecal inocula were able to ferment carbohydrates more extensively than fecal inocula. Inocula from the animals that had been exposed to PSH were not able to ferment cellulose in the first 24 hours. She explained how the data from this study can be used to obtain initial rates of fermentation as well as minimum and maximum rates of fermentation.

In all instances, the cecal source of inoculum produced more short-chain fatty acids (SCFAs) than did the fecal source. She explained that nonpurified diet was very complex and difficult to ferment and thus the bacteria present in response to this diet are more capable of producing SCFAs. The cecal inoculum initial production rate of SCFAs in animals fed the nonpurified diet was up to twofold greater than the rate in response to fecal inoculum.

Dr. Marlett also described a study of 75-day old male pigs on controlled diets that were administered 10 percent D-tagatose or no D-tagatose. D-tagatose is a steroisomer of fructose that often is used as a low-calorie bulk sweetener but is not highly prevalent in the food supply. Contents of the cecum and from the middle-third of the colons of the pigs were collected. Investigators found a fourfold increase in response to the cecal source of inoculum and a ninefold increase in response to the colonic inoculum from pigs given D-tagatose. She noted however, that the results of studies seeking to measure variables in an *in vitro* situation that reflect what occurs *in vivo* vary greatly depending on the design of the system used.

Dr. Marlett concluded her presentation by listing some of the important aspects of fermentation studies, including: (1) source of inoculum, (2) substrate, (3) conditions, (4) purpose of fermentation, (5) anaerobic processes, and (6) transport of SCFAs. She also noted that fermentation is not carried out for the benefit of the host; it is the primary route by which the microflora get their energy.

ISSUE 2: WHAT IS THE IMPACT OF COLONIC BACTERIA ON THE NATURE OF METABOLITES IN THE COLON?

Short-Chain Fatty Acids and Colon Cancer Prevention — *Dr. Joanne Lupton*

Dr. Lupton, Regents Professor and William W. Allen Chair in Nutrition at Texas A&M University described the relationship between SCFAs and colon cancer, focusing on the role of butyrate. Dr. Lupton argued that with respect to colon cancer, the single most important attribute of dietary fiber is its fermentability. As dietary fiber reaches the colon, the colonic microflora ferment it to a variety of different substances, including SCFAs. She described three clinical intervention trials on dietary fiber and colon cancer that were designed to determine whether or not dietary fiber is protective against colon cancer using polyp recurrence as an intermediate marker None of these trials showed a protective effect of dietary fiber on polyp recurrence. It remains unclear why these studies did not show an effect; one possibility is that polyp recurrence is not a prognostic indicator of later tumor development..

One school of thought is that the most protective type of fibers are the nonfermented or poorly fermented fibers such as wheat bran and cellulose. Poorly fermented fibers can dilute luminal

constituents such as carcinogens, procarcinogens, tumor promoters, bile acids, and other lumenal constituents such as ammonia . Poorly fermented fibers also may accelerate colonic transit, thus providing less access of these carcinogens, procarcinogens, and tumor promoters to the colonic mucosa. Of the poorly fermented fibers, wheat bran appears to be the most effective *in vivo* diluter.

However, there is another school of thought that the highly fermentable fibers such as pectin, guar, and oat bran are more protective against colon cancer. Fibers are fermented to produce SCFAs, and some of the highly fermentable fibers produce large amounts of SCFAs. One of these, butyrate, has been shown, in *in vitro* studies, to promote differentiation and apoptosis and to downregulate proliferation in human colon cancer cell lines, resulting in a protective effect. She described efforts to reproduce these *in vitro* studies *in vivo.*

Studies have found that wheat bran diets fed to mice leads to less aberrant crypt formation and higher fecal butyrate levels than fiber-free diets. Dr. Lupton and colleagues compared wheat bran and oat bran in rats and found, as expected, that oat bran was a more fermentable fiber than wheat bran. Rats given oat bran had significantly higher levels of butyrate in the proximal and distal colon compared with rats given wheat bran. However, they found that rats given wheat bran had fewer tumors than animals fed oat bran and concluded that increasing the amount of butyrate in the lumen, per se, is not protective against colon cancer.

Dr. Lupton described a study of sodium butyrate in drinking water administered at 0, 1, or 2 percent concentration to rats that were treated with the carcinogen dimethylhydrazine. Rats administered butyrate had more tumors than control animals possibly due to the presence of sodium. A study using tributyrin was conducted to see if the promotive effect of sodium butyrate was due to high sodium levels. Tributyrin feeding resulted in a tenfold increase in concentrations of fecal butyrate compared with controls but no difference in dysplasia or tumor incidence. A study of slow-release pellets of sodium butyrate found increased levels of butyrate in the colon and the apoptotic index increased with butyrate, but there was no effect of butyrate on cell proliferation, aberrant crypt formation, or colon tumor incidence.

She concluded her presentation with the following points:

- The statement that fiber may protect against colon cancer by its fermentation products, particularly butyrate, is not supported by *in vivo* data.

- This does not mean that butyrate is not protective, only that a protective effect has not been documented.

- Future consideration should be given to:
  - Timing of butyrate administration
  - Amount of butyrate
  - Effects of the rest of the diet, particularly fat.

Gut Microbial Metabolism of Soy Isoflavones ***Dr. Suzanne Hendrich***

Dr. Hendrich, a Professor in the Department of Food Science and Human Nutrition at Iowa State University and colleagues have been studying the bioavailability of soybean isoflavones for 10 years. They started conducting single-meal feeding studies of soy and noticed that some subjects repeatedly had significantly higher levels of isoflavones excreted in their feces and urine as well as higher plasma levels compared with other study subjects. It was hypothesized that increased gut microbial degradation resulted in reduced bioavailability.

Dr. Hendrich and colleagues conducted a number of studies examining the *in vitro* fecal degradation and disappearance of isoflavones. In a study of 15 subjects followed for 1 year, subjects sorted into two or three distinct phenotypes in terms of degradation characteristics. For example, in some individuals there was no effect on certain isoflavones after 24 hours, while in other individuals, the same isoflavones disappeared within 1 hour. The investigators sorted a set of young men in another study according to fecal isoflavone degradation rate constant and found that peak isoflavone plasma concentrations correlated inversely with the degradation rate constant.

She and her colleagues also conducted a study of Caucasian and Asian subjects and found that a significantly higher number of Asian subjects had a high isoflavone degradation rate constant phenotype as compared with Caucasian subjects. The mechanisms behind these results are unclear; however, habitual exposures to certain diets may play a role, Dr. Hendrich speculated. There are many dietary differences between Asians and Caucasians. In this study however, there were no significant dietary differences between the two groups. The only difference that sorted by phenotype was gut transit time (GTT), which was overall significantly shorter in Asians than Caucasians, although it is not clear why. The GTT for Asian low degraders was significantly more rapid, than in Asian high degraders and there was a difference between the phenotypes in their ability to absorb and excrete the isoflavone genistein (low degraders excreted threefold more genistein over 24 h after a single isoflavone meal than did high degraders). In Caucasian subjects, low and high degraders differed in fecal disappearance of these compounds *in vitro,* but the phenotype was not expressed in vivo (as isoflavone absorption/excretion differences) presumably because of the long GTT in all Caucasian subjects.

The investigators recently completed a study that sorted 11 high isoflavone degraders (*in vitro* degradation rate constant greater than 0.3) and 11 low isoflavone degraders (*in vitro* degradation rate constant lower than 0.2). Both groups were fed soy supplements for 7 days and no difference in isoflavone bioavailability was observed, perhaps because a less stringent criterion for the difference in isoflavone degradation in vitro was used than in previous studies, as well as smaller numbers of subjects.

The gut microflora's ability to degrade or dramatically alter isoflavones may define some human subpopulations who absorb isoflavones to differing degrees, but the functional significance of this is not known. Sorting by phenotypes may be helpful in determining the health effects of differences in isoflavone degradation. Thus far, only GTT has been identified as a factor that appears to make a difference in the ability of the gut microorganisms to degrade isoflavones. Dr. Hendrich hypothesized that up to a point, the longer the gut is exposed to isoflavones, the more chance it has to absorb them. At some point however, the ability to absorb will be lost, so determining the optimal GTT may be helpful.

Dr. Hendrich and her group have started to conduct *in vitro* fecal incubation studies with saponins. The most common saponin in soy food products is soy saponin 1. There appear to be two distinct populations: people who are more rapidly able to deglycosylate or remove the sugars in soy saponin 1, and those who remove the sugars more slowly. Four out of the five subjects whose feces removed the sugars more rapidly were Asian subjects (the other subjects were Caucasians, both rapid or slow deglycosylators). She concluded her presentation by noting that at present, it is not known whether it is more beneficial to be a high metabolizer of isoflavones or a low metabolizer of isoflavones.

Catechins/Lycopene ***Drs. Beverly Clevidence and Volker Mai***

Dr. Clevidence, a Research Nutritionist at Beltsville Human Nutrition Research Center, U.S. Department of Agriculture, described a series of carotenoid feeding studies in which data were collected on the accumulation of carotenoids in plasma and colon cells. Dr. Clevidence noted that while there currently are no quantitative measures, the levels of carotenoids in colon cells increase strikingly in individuals fed five servings per day of carotenoid-rich foods. The possibility was raised that carotenoids may directly protect colonocytes or may protect indirectly by altering the interaction between colonocytes and gut microflora.

Dr. Clevidence and colleagues studied the bioavailability of catechins in black tea. Tea was selected because of its high catechin concentrations and because it is less complex than fruits and vegetables. In addition, the composition of tea is known, the major phytochemicals are water soluble, and it is thought that catechins are the bioactive components of tea. Catechins in black tea are present as free catechins and as condensation products that are generated during the oxidation of tea leaf to form black tea. Microbal enzymes are thought to act on catechins and their condensation products to produce small molecular weight phenolic acids, and these compounds may be physiologically important. When black tea was administered to subjects, only small amounts of each of the four major catechins were detected in plasma, although she noted that their potentially important, but poorly delineated breakdown products were not analyzed. She concluded her remarks by introducing Dr. Volker Mai, a Cancer Prevention Fellow in the Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, NCI.

Drs. Mai, Clevidence, and colleagues conducted a double-blind randomized human feeding study to determine whether the polyphenols in black tea could change the bacterial flora in the gut, resulting in increased excretion of fecal bile acids and a reduction of serum lipid levels. They collected fecal samples from 15 subjects on a controlled diet at baseline, week 2, and week 3. The study included both men and women who drank five servings of black tea or placebo beverage per day. Fecal samples were analyzed using FISH. DNA was extracted from the frozen samples and the V6 to V8 region of the bacterial 16S rDNA was amplified by PCR for TGGE.

Tea drinking did not consistently affect the amounts of the seven bacterial groups that were analyzed with specific probes, yet it did decrease the amounts of bacteria that were detected by the universal bacterial probe. Preliminary TGGE analyses showed that the profiles for each

subject vary less than expected from the FISH studies. Dr. Mai and colleagues are planning to examine changes in the composition of the bile acids and relate them to changes in the composition in the gut flora.

The researchers also will be conducting a study on the effect of oolong tea on the intestinal microflora. Oolong tea has more free catechins than black tea because it is not oxidized to the degree of black tea.

ISSUE 3: HOW DOES THE COLONIC ENVIRONMENT (INTESTINAL CELL GENOMICS, COLONIC MICROORGANISMS, AND MICROBIAL METABOLITES) ALTER SUSCEPTIBILITY TO COLORECTAL CANCER?

Muc2/SCFA *Drs. Leonard Augenlicht and Anna Velcich*

Dr. Augenlicht, Professor of Medicine and Cell Biology and Director of the Molecular Oncology Program at Albert Einstein College of Medicine and Cancer Center, explained how colonic epithelial cells have evolved and adapted to their environment. These cells will respond to agents they have seen in a programmed way, but not to agents they have not seen, because they do not know how to respond. Colonic carcinoma cells *in situ* respond to butyrate and other SCFAs in a programmed way—over a time course, they undergo a series of discrete events. Dr. Augenlicht noted that the mitochondrial membrane potential is involved in triggering both apoptosis and plays a role in early regulation of cell cycle. He described one study that found that all of the events triggered by butyrate appear to be linked to the cell's ability to metabolize SCFAs and utilize them as an energy source through β-oxidation within the mitochondria. The relative and different abilities of mitochondrial populations within an individual to metabolize SCFAs efficiently have not been well studied.

He and his colleagues conducted a number of studies with SCAD mice, which have a homozygous deletion in a nuclear-encoded enzyme that is incorporated in the mitochondria and downregulates SCFA metabolization. Wild-type and mutant SCAD animals were fed a controlled diet and were found to gain roughly the same amount of weight over a certain period of time. When the diets of these mice were supplemented with wheat bran, weight gain was similar to controls in wild-type mice but the SCAD mice did not gain as much weight. It is assumed that this difference is the result of utilization of wheat bran as an energy source due to fermentation by one population of bacteria in the colon and the uptake and utilization of SCFAs through the colon. Animals that were mutant for this enzyme cannot metabolize SCFAs efficiently and, as a consequence, did not gain weight as quickly as the wild-type mice. The researchers asked the following questions: Is this pathway of utilizing SCFAs important in the normal regulation of colonic epithelial mucosa maturation pathways? What would be the effect of eliminating the ability of a mouse to utilize that pathway on these processes?

In examining apoptosis rates in the intestinal mucosa of SCAD wild-type and mutant mice, the investigators found that apoptosis is significantly decreased over the proximal distal colon in mutant mice, indicating that they used this pathway to regulate apoptosis. No decrease was found in the duodenum. Dr. Augenlicht explained that SCFAs are produced principally by microbial fermentation in the lower part of the intestine, not in the duodenum. In conducting

studies to find genetic evidence of the time course response to butyrate over 48 hours, Dr. Augenlicht and colleagues studied the ratio of expression in treated versus untreated cells as a log ratio and a function of time. They found a monotonic increase in the number of frequencies that are altered in expression and control. He compared upregulated and downregulated pathways in response to butyrate and sulindac, noting that, in spite of similar effects on the cell cycle, these two compounds had very different patterns of gene expression.

Dr. Augenlicht noted that changes in β-catenin signaling are important for a number of cell functions. He described a series of studies examining changes in β-catenin activity and formation in response to butyrate and how they affect initiation and transcription of the c-myc gene using a variety of methods. He described further analyses of the response of butyrate and sulindac on the number of c-myc transcription sites. Butyrate downregulates c-myc transcription while sulindac upregulates the message, which explains sulindac's toxic side effects.

Dr. Velcich, Associate Professor of Medicine in the Department of Oncology at Albert Einstein Cancer Center and Montefiore Medical Center, described a new mouse model that has been developed in which the Muc2 gene has been knocked out. She described a number of studies designed to explore the interface between colonic and epithelial cells of mice with a knocked out Muc2 gene. Dr. Velcich explained the process for knocking out the Muc2 gene. When fed a regular diet, these mice were found to have a decreased number of goblet cells, decreased mucus production, an increased number of proliferating cells in the small and large intestine, and a decrease in apoptosis. Her research group found no mechanisms to compensate for the loss of Muc2. Most important, the Muc2-/- mice develop invasive adenocarcinoma throughout the small and large intestine, and the rectum.

Bacterial Infection as a Risk Factor for Colon Cancer ***Dr. David B. Schauer***

Dr. Schauer, Associate Professor in the Division of Bioengineering and Environmental Health at Massachusetts Institute of Technology said that there may be pathogens that cause pathologic alterations in the colon that may predispose to cancer. *Helicobacter pylori* may be one such pathogen. He explained that *H. pylori* is a persistent infection that causes chronic gastritis, increases the risk for gastric adenocarcinoma, and is considered to be a Group 1 carcinogen by the World Health Organization. There are other *Helicobacter*s involved—those that affect the liver, for example. One of these, *H. hepaticus,* causes chronic hepatitis that progresses to hepatocellular carcinoma in male mice of susceptible strains. Although *H. hepaticus* elicits chronic inflammatory changes that result in the development of liver cancer, it initially infects the large intestine, not the liver. Its primary niche is the mucus gel layer in the cecum and colon, and it is not known how it gets from the large intestine to the liver.

Mice infected with *H. hepaticus* that have a disregulated immune response develop very severe mucosal inflammation and proliferation that Dr. Schauer likened to the human equivalent of irritable bowel disease (IBD). He and his colleagues are working to develop rodent models infected with *Helicobacter* as a model for human IBD. He noted that all of the genes that are thought to be critical in *H. pylori's* ability to cause chronic gastritis and increased risk of gastric cancer are absent in *H. hepaticus.* There are some important similarities between *H. hepaticus*

and *H. pylori* in terms of chemokine increases and hypoproliferation of epithelial cells, but the mechanisms of action are different.

Dr. Schauer described another organism, *Citrobacter rodentium,* which causes severe transmissible murine colonic hyperplasia, which involves severe but self-limiting hyperproliferation of epithelial cells that lasts 4–6 weeks after experimental infection. Adult mice that are infected appear to be clinically healthy; they eat normally and do not have diarrhea, despite the hyperproliferation occurring in the colon. This hyperproliferative state, however, can promote chemically initiated tumors in the colon. Dr. Schauer said that *Citrobacter* infection in mice is a model for human *Escherichia coli* infection that causes the same type of histopathological changes.

Dr. Schauer and colleagues infected MIN mice with *C. rodentium* at 1 month of age and determined the tumor burden at 6 months of age. They found a fourfold increase in colonic tumors in the group which had been infected, corresponding to the increased proliferation. There was no significant change in the number of tumors in the cecum or ileum of these mice, because there is no hyperproliferation there, Dr. Schauer explained. Wild-type mice infected with *C. rodentium* experience the same hyperproliferation seen in the MIN mice, but it disappears and they do not develop tumors.

Dr. Schauer described a study in which biopsies taken from the duodenum of children with enteropathogenic *E. coli* infection were analyzed. The researchers found changes in cytokinetics that were very similar to those seen with *C. rodentium* in the colon of mice. In this study, the cells proliferated more towards the surface, a risk factor for cancer. Importantly, Dr. Schauer said, animals and humans do not have to appear to be clinically sick to have a pathogen present. It is important to consider pathogen interactions with resident microbiota as well. His group is approaching this by sequencing the 16S rRNA of the eight species of anaerobic bacteria in flora mice (mice that have their entire microbiota made up of 8 species of anaerobic bacteria). They are developing quantitative PCR methods for real-time determination of what each one of these species does, and they hope to apply the technique to animals that are infected with different pathogens.

Sulfotransferases ***Dr. Hansruedi Glatt***

Dr. Glatt, a Professor in the Department of Nutrition Toxicology at the German Institute of Human Nutrition, introduced the human xenobiotic-metabolizing system of the gut. It differs substantially from that in the liver and is targeted to xenobiotic metabolites formed by intestinal bacteria, e.g. via deglycosidation or reduction. Human intestinal mucosa expresses high levels of conjugating enzymes. Of particular interest are sulfotransferases (SULTs). Sulfo conjugates are water-soluble and require transmembrane transporters for penetrating cell membranes. Therefore, they can be easily excreted, at least if they are stable. However, some types of sulfoconjugates are chemically reactive, which is explained by the fact that sulfate is a good leaving group in certain chemical linkages. These reactive sulfo conjugates can induce mutations and lead to the formation of tumors. Usually, it is possible to predict whether a sulfoconjugate will be stable or reactive based on its chemical structure.

Dr. Glatt noted that the first electrophilic metabolite of a carcinogen to be discovered was a sulfoconjugate, *N*-sulfooxy-2-acetylaminofluorene. He described the chemical characteristics of this compound. This sulfoconjugate has not been found to be mutagenic in classical *in vitro* studies, possibly because it does not penetrate the cell membrane. Dr. Glatt and colleagues hypothesized that *N*-sulfooxy-2-acetylaminofluorene would be mutagenic if it was generated within the cell. They constructed new *Salmonella* strains and demonstrated the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in strains expressing human SULT enzymes. They also have demonstrated expression of this toxifying enzyme form in the human colon.

Dr. Glatt also presented data on SULT1B1 enzyme activity in different species with regard to the activation of promutagens and expression in colon mucosa. He showed results for human, rat, mouse, and dog SULT1B1 enzymes. The human enzyme was unique in that it readily activated 4-hydroxycyclopenta[*def*]chrysene (a metabolite formed by intestinal bacteria from the environmental polycyclic hydrocarbon 4- oxycyclopenta[*def*]chrysene) and 6-hydroxymethylbenzo[*a*]pyrene to mutagens and was found to be highly expressed in colon mucosa. The SULT1B1 enzymes from the other species investigated showed only weak activation of these compounds and/or only weak expression in colon mucosa.

Dr. Glatt described the metabolism of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). This heterocyclic amine is formed when muscle meat is heated and belongs to the small number of chemicals that have been demonstrated to induce colon tumors in laboratory animals. The activation of this carcinogen requires two steps, the *N*-hydroxylation (which primarily occurs in the liver by a cytochrome P450 expressed specifically in that tissue) and sulfoconjugation or *O*-acetylation (which may occur in the target tissue). He described the hypothesis that *N*-OH-PhIP is formed and glucuronidated in the liver, that the glucuronide is excreted into the gut where it is deconjugated by bacteria, and that the resulting free *N*-OH-PhIP is reabsorbed and activated in the colon mucosa. To test this hypothesis and in particular to determine the role of intestinal bacteria, the formation of DNA adducts by PhIP was studied in normal and germ-free rats. Levels of DNA adducts in liver were similar in both groups. Colonic mucosa showed higher levels of DNA adducts than liver. Although DNA adducts were found in colon mucosa of both groups, they were higher in normal rats than in germ-free rats. Thus, intestinal bacteria enhanced the activation in colon mucosa (possibly via hydrolysis of glucuronidated metabolites of PhIP), but additional mechanisms of transfer of *N*-OH-PhIP to colonic mucosa appear to occur.

Dr. Glatt concluded his talk by stating that there are special xenobiotic-metabolizing enzyme systems in the colon that are different from those in other tissues. These enzymes that interact with the intestinal bacteria are more species-dependent in terms of expression level and activity than enzymes in other tissues. A model for detecting the relevance to human colon carcinogens is needed.

Gut-Associated Immune System, Diet, and Colon Cancer ***Dr. Catherine J. Field***

Dr. Field, an Associate Professor in the Department of Agricultural, Food, and Nutritional Science at the University of Alberta described the immune system as the host's defense against destructive forces from outside and within the body. She discussed the two components of the human immune system, the innate immune system and the acquired immune system. The innate immune system does not require previous exposure to an antigenic challenge. It is comprised of

cellular components (phagocytes, natural killer cells); physical barriers (mucus membranes, skin); and the reticuloendothelial system. The acquired immune system develops as humans interact with the environment and has two main responses: cell-mediated (CD4-T cells, CD8-T cells) and humoral (B-cells).

Dr. Field briefly described the structure and function of the GI immune system. She noted that the immune system comprises an organ about the size of the liver and 25 percent of that immune system is found in the gut. The immune system constantly is being challenged by the diet. Food, bacterial antigens, and carcinogens all come in contact with the gut environment. She added that it is believed that on a daily basis, 80 percent of immunoglobulin A is secreted into the lumen. The lymphocytes that line the small and large intestine are specialized cells that may play a major role in preventing an abnormal response to the environment, and they form a special relationship with the cells of the gut.

She also described the interaction between diet and gut-associated lymphoid tissue (GALT). Small changes in diet can provoke significant, although not necessarily negative, changes in the gut. She noted that the results of any animal study on intestinal flora will vary greatly depending on the animals' diets. There has been a great deal of interest in the mechanisms of prebiotics and probiotics, and how they might be implemented in the prevention of colon cancer in the healthy intestinal tract. Dr. Field discussed some of the proposed mechanisms for the effect of gut microflora on immune functions, including: (1) direct contact of bacteria with GALT, (2) bacterial-secreted substances, (3) production of SCFA (butyrate anticancer effects), (4) modulation of mucin production/composition, and (5) structure/function changes in epithelial/colonic cells.

Dr. Field discussed immunosurveillance, the concept that there are parts of the immune system that try to eliminate transformed cells. Evidence supporting this concept includes chronic inflammation risk, lymphoid infiltrates found in many tumors, spontaneous regression of some tumors, and immunomodulatory agents used in treatment. She noted that this is a controversial area, and concluded by asking the question: Could diet influence mutations in tumor suppressor genes and protooncogenes?

Goals for Future Research ***Moderator: Dr. Story***

Dr. Story asked workshop participants for ideas and guiding questions that need to be asked to progress this field. Discussion led to comments/questions in the following 4 areas:

1) Bacterial populations and their assay:

    a) Recent technological advances have made it possible to fully characterize bacterial populations. The scientific community is beginning to come to terms with the fact that the standard culture techniques are not giving a complete, comprehensive picture.
    b) However, developing the necessary databases to perform the appropriate genomic studies will be labor intensive. There is the need to develop initial databases and fully characterize bacterial populations to obtain the genetic sequence data needed to develop the appropriate probes.

c) Communities of bacteria, e.g., of aerobes versus anaerobes, may reveal relationships that are useful and less demanding to assay.

2) Colonic bacteria and colorectal cancer:

a) Are microbes involved in colon carcinogenesis? Is it important to study them? Clearly more preliminary data are needed to allow more complete evaluation of the relationship between colonic bacterial species and colon cancer risk (as discussed in 1), but other questions persist:
b) Is there a bacterial target that researchers should be looking for?
c) Are the techniques developed enough so that researchers can screen larger numbers of people and try to build a relationship between cancer incidence and a group of bacteria?
d) Many of the bacterial forms present in the intestinal tract may not be culturable with currently available technology. There are additional classes of microorganisms that are not being detected.
e) Where along the colon should samples be taken to examine this relationship? Is there a mechanism to measure the bacteria in the proximal colon? Could one be developed?
f) Are there some microorganisms that researchers are missing? Are there specific pathogens we are currently not recognizing? Comprehensive analyses of bacterial communities are needed to answer these questions, as discussed above. There is no technologic barrier, but a good deal of work is required to get to the point where bacterial populations can be fully characterized.

3) Colonic environment:

a) At the molecular level, APC mutation or inactivation is a fundamental component of many colon cancers. In APC animal models, the cancers are located in the small intestine, not the large intestine. It may be important to understand this phenomenon, and investigate how and why the site-specificity of cancers is driven by environmental factors. In this and other currently used animal model systems, there are not enough data on mechanisms of action to allow effective use.
b) Researchers need to remember that the microflora have a preferred substrate. If the microflora are supplied with adequate amounts of this substrate (mostly carbohydrates), the microflora will prefer to metabolize it rather than other molecules.
c) What does butyrate do to the transformed epithelial cell? Is dose important? What intracellular concentration is important?
d) Do antimicrobial compounds such as polyphenols have an impact on intestinal microflora? What is the extent of their impact?
e) The constituents of diets fed to animals for study vary dramatically and affect the outcome.
f) How do diet and the microflora impact the immune system, particularly its involvement in cancer defense?

4) General comments/questions:
a) Is there an interdisciplinary unit that would make it feasible to encourage more human studies? Who would sponsor the interdisciplinary collaboration? The program project

and grant supplementation approaches may help foster these collaborations and combine crossdisciplinary expertise.

Dr. Story adjourned the workshop by thanking attendees for their comments/questions and for their participation.