Analysis of the Fecal Microflora and Its Enzymatic Activity in Individuals Genetically Predisposed to Colon Cancer

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ABSTRACT

The role of the fecal microflora in the induction of colon cancer was investigated in individuals believed to be genetically predisposed to colon cancer. Subjects were members of families with increased occurrence of colon and endometrial carcinomas characteristic of the cancer family syndrome. Group 1 consisted of 5 cancer family syndrome individuals previously diagnosed with colon cancer. Group 2 consisted of 6 cancer family syndrome individuals previously diagnosed with endometrial cancer but free of colon cancer. An environmental control group (Group 3) consisted of 8 spouses of subjects in Groups 1 and 2. Quantitative bacterial cultures and assays of \( \beta \)-glucuronidase and \( \alpha \)-dehydroxylase activity were performed on fecal samples. No differences in bacterial quantities or levels of \( \beta \)-glucuronidase or \( \alpha \)-dehydroxylase activity were found among Groups 1, 2, and 3 or between spouse pairs. The results fail to associate quantities or enzymatic activity of the intestinal flora to colon cancer in individuals believed to be genetically predisposed to colon cancer.

INTRODUCTION

It has been hypothesized that the intestinal microflora is responsible for production of carcinogens and/or cocarcinogens in the gut resulting in colon tumor formation (1, 10, 30). Quantitative bacterial cultures of fecal samples from populations at high and low risk for colon cancer have shown some differences in fecal bacterial profiles (1, 10, 12); however, these differences have not been confirmed (6, 7, 20).

Analyses of related bacterial metabolism in the gut have correlated higher levels of activity of the enzymes \( \beta \)-glucuronidase and \( \alpha \)-dehydroxylase with populations at high risk for colon cancer (20, 21). \( \beta \)-Glucuronidase degrades conjugated products from the liver, releasing toxic substances and possibly carcinogens or precarcinogens within the bowel (13, 14). \( \alpha \)-Dehydroxylase converts primary bile acids to secondary bile acids, which act as colon tumor promoters in animal models (3, 23).

The risk factors in the preceding studies were determined by environmental criteria such as geographical location and/or dietary habits. High risk for colon cancer may also be determined genetically, as suggested by a number of syndromes, including inherited adenomatosis of the colon and rectum, Gardner’s syndrome, Peutz-Jegher’s syndrome, and the CFS (15). Investigators have found an increase in excretion of cholesterol and a decrease in excretion of coprostanol and coprostanone among familial polyposis patients compared with related and unrelated controls (2, 16, 26). Other fecal constituents including the fecal microflora have not been examined in these genetically determined high-risk groups.

Members of families fulfilling the criteria for the CFS are believed to be genetically predisposed to colon cancer. The purpose of this investigation was to study the fecal bacteria and its associated metabolic activity in CFS family members with diagnosed cancer of both the colon and outside the colon. Any differences between the 2 groups might ultimately be associated with the expression of the genetic predeterminant(s) controlling colon cancer.

MATERIALS AND METHODS

Subjects

CFS has been described by Lynch and Krush (18). Characteristics of this syndrome are: (a) high incidence of adenocarcinomas of multiple anatomical sites, most frequently of the colon and endometrium; (b) multiple primary malignant neoplasms; and (c) early age of onset. The syndrome follows an autosomal dominant mode of inheritance.

Names of subjects for this study and their family and medical histories were obtained from the Preventive Medicine Department, Creighton University, Omaha, Nebr. Subjects were selected on the basis of the following criteria: (a) subjects were members of families characterized by CFS or spouses of CFS family members; (b) subjects consumed a high-meat, Western-type diet; (c) subjects had received no antibiotic therapy for at least 4 weeks prior to specimen collection; and (d) colon cancer subjects had been treated by minor resection only.

Fecal Sample Collection and Transport

Complete contents of a single bowel movement were collected from each subject. Feces were collected aseptically in clean, nonsterile Ziploc plastic storage bags (268 x 279 mm; Dow Chemical Co., Indianapolis, Ind.), which were opened and placed in a holder attached to the toilet seat. Immediately following collection, the specimen was homogenized by kneading for 2 to 3 min. Approximately 1 g was added to each of 2 preweighed tubes containing 9.0 ml glycerol broth with glass beads (4). The tubes and the remainder of the specimen were frozen within 15 to 30 min on dry ice for transport to the laboratory, where they were stored at \(-70^\circ\). All specimens were collected and processed at the subjects’ homes.

Bacterial Cultures

Primary Isolation. Quantitative cultures were initiated within 4 weeks of specimen collection and were performed as described by Sutter et al. (25). One tube of the diluted specimen was weighed, placed in an anaerobic chamber (type B; Coy Manufacturing Co., Ann Arbor, Mich.), and allowed to reach room temperature in an atmosphere of 80% oxygen-free nitrogen, 10% carbon dioxide, and 10% hydrogen. After thorough mixing of the sample on a Vortex-type mixer, a series of 10-fold dilutions (\(10^{-2}\) to \(10^{-8}\)) was made in PRAS dilution blanks (11).
Media used, purpose, and dilutions plated on each are described in Table 1.

### Bacterial Identification

Plates were examined for growth after 48 hr of incubation. Each colony type was enumerated and identified. Identification of isolates was performed on single, isolated colonies, which were grown in pure culture when necessary.

Group identification of isolates was based on Gram stain reaction and morphology; colonial morphology; antimicrobial susceptibility; catalase, indole, and lecithinase production; spore formation; growth on bile; esculin hydrolysis; and metabolic end-product analysis.

Metabolic end products were analyzed on a Packard gas chromatograph, 7400 series. Columns were packed with 10% SP-1000-1% H3PO4 on Chromosorb W-AW 100/120 mesh (Supelco, Inc., Bellefonte, Pa.).

### Enzyme Assays

**Controls.** Control specimens were obtained from a laboratorian. After homogenization of the samples, a set of 10⁻¹ dilutions was made by placing 1.0-g aliquots into preweighed PRAS dilution blanks (9.0 ml). The tubes were weighed, thoroughly mixed, and stored at −70°.

**Preparation.** The day of assay, the undiluted subject specimen and control tubes were removed from the freezer and thawed in a 37° water bath. Each assay was performed on triplicate aliquots and on the control specimen.

The thawed patient sample was moved into the anaerobic chamber where 1.0-g aliquots were added to preweighed PRAS dilution blanks (9.0 ml). A 1.0-g aliquot was also placed on a preweighed glass slide. The slide with the sample was weighed and placed in a glass Petri dish containing desiccant. The Petri dish was placed in a drying oven at 100° for 24 hr, after which the dried sample and slide were again weighed and the dry weight of the specimen was calculated.

**β-Glucuronidase Activity.** Samples for β-glucuronidase activity were prepared according to Reddy and Wynder (21). Standards were weighed and the dry weight of the specimen was calculated. Activity is defined as the amount of enzyme that will liberate 1.0 µg of p-nitrophenol per minute.

**7α-Dehydroxylase.** 7α-Dehydroxylase activity was determined according to the methods of Mastromarino et al. (19), using as substrate 14C-cholic acid (specific activity, 40 to 60 µCi/mmol; New England Nuclear, Boston, Mass.). Following extraction from the incubation mixture, bile acids were separated by thin-layer chromatography. The fraction containing the dehydroxylated bile acid was collected, charred at 180° for 30 min. Bile acids appeared as yellow bands which were identified by comparison with the standards. Radioactivity in each band was determined in a Packard Tri-Carb Model 3320 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Activity was calculated as the percentage of cholic acid microbially converted to deoxycholic acid and was expressed as activity/100 mg dry feces.

### Statistical Methods

Data from subject groups were compared using one-way analysis of variance. The matched-pair t test was used in comparing individuals.

### RESULTS

**Subjects.** Subjects were divided into 3 groups for examination. Group 1 consisted of 5 CFS participants who had been diagnosed and treated for colon cancer. Three females, 64, 64, and 77 years old, respectively, and 2 males, 63 and 54 years old, respectively, made up this group. Their respective ages at the time of colon cancer diagnosis were 48, 47, 47, and 47 years. Group 2 consisted of 6 CFS family members with no evidence of colon cancer but in whom either endometrial or breast cancer had occurred. This group consisted entirely of females, 66, 64, 72, 56, 38, and 55 years old, respectively, whose ages at the time of endometrial or breast cancer diagnosis were 49, 46, 39, 45, 29, and 34 years old, respectively. Group 3 consisted of 6 spouses of the individuals in Groups 1 and 2. The individuals in the latter group had no known genetic risk for colon cancer but environmental and dietary exposure similar to that of Groups 1 and 2. Group 3 consisted of 6 males and 2 females. Ages were not available for 3 subjects in this group, the remaining subjects were 50, 59, 65, 69, and 70 years old at the time of specimen collection.

Subjects were requested to complete dietary questionnaires. It was determined that all consumed meats other than poultry and fish at least 4 times/week. Subjects indicated that meals with no meat were generally consumed at breakfast. Four subjects in Group 1 consumed diets unaffected by surgery while 2 subjects reported a limited intake of fresh fruits and dairy products.

### Table 1

**Medium for quantitative cultures**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Dilutions to be plated®</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannitol salt agar</td>
<td>10⁻², 10⁻⁷, 10⁻⁸</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Streptococcus KF agar</td>
<td>10⁻², 10⁻⁷, 10⁻⁸</td>
<td>Enterococci</td>
</tr>
<tr>
<td>Sheep blood agar</td>
<td>10⁻², 10⁻⁴, 10⁻⁷</td>
<td>Predominant aerobic flora</td>
</tr>
<tr>
<td>MacConkey’s agar</td>
<td>10⁻², 10⁻⁴, 10⁻⁷</td>
<td>Aerobic and facultatively anaerobic Gram-negative bacilli</td>
</tr>
<tr>
<td>Sabouraud’s agar</td>
<td>10⁻², 10⁻⁴, 10⁻⁷</td>
<td>Yeast</td>
</tr>
<tr>
<td>Anaerobic incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampin agar</td>
<td>10⁻², 10⁻⁴, 10⁻⁷</td>
<td>Fusobacterium sp. and Clostridium sp.</td>
</tr>
<tr>
<td>Bifidobacterium agar</td>
<td>10⁻², 10⁻⁷, 10⁻⁸</td>
<td>Bifidobacterium sp.</td>
</tr>
<tr>
<td>Kanamycin-vancocycin laked-blood agar</td>
<td>10⁻², 10⁻⁴, 10⁻⁸</td>
<td>Bacteroides sp.</td>
</tr>
<tr>
<td>Neomycin-vancocycin</td>
<td>10⁻², 10⁻⁴, 10⁻⁷</td>
<td>Fusobacterium sp. and Veillonella sp.</td>
</tr>
<tr>
<td>Lactobacillus selective agar</td>
<td>10⁻¹, 10⁻², 10⁻⁸</td>
<td>Lactobacillus sp.</td>
</tr>
<tr>
<td>Sheep blood agar</td>
<td>10⁻¹, 10⁻³, 10⁻⁷</td>
<td>Total counts nd predominant flora</td>
</tr>
<tr>
<td>Rich agar</td>
<td>10⁻², 10⁻⁵, 10⁻⁷</td>
<td>Fastidious organisms</td>
</tr>
<tr>
<td>Phenyl ethyl alcohol agar with vitamin K</td>
<td>10⁻², 10⁻⁵, 10⁻⁷</td>
<td>Gram-positive organisms</td>
</tr>
<tr>
<td>Neomycin agar</td>
<td>10⁻², 10⁻³, 10⁻⁷</td>
<td>Anaerobic cocci and Clostridium sp.</td>
</tr>
<tr>
<td>Cyclerine mannose agar</td>
<td>10⁻⁲, 10⁻⁴, 10⁻⁷</td>
<td>Lecithinase-negative Clostridium sp.</td>
</tr>
<tr>
<td>Egg yolk agar*</td>
<td>10⁻¹, 10⁻³, 10⁻⁷</td>
<td>Clostridium sp.</td>
</tr>
</tbody>
</table>

* 0.1-ml portion of each dilution is plated on each medium used (25).

® Dilutions to be plated on egg yolk agar were heated in an 80° water bath for 10 min prior to inoculation in order to detect spore formers.

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Surgical therapy of the subjects in Group 1 included the following: right colectomy (2 subjects), removal of a 22-cm section of cecum (one subject), removal of a 22-cm section of the descending colon (one subject), and resection of the right colon and distal ileum (one subject).

Quantitative Cultures. Storage of aliquots of a control specimen in glycerol broth at $-70^\circ$ for up to 4 weeks did not decrease quantities of bacteria recovered by culture. Mean values of each group of bacteria found in Groups 1, 2, and 3 are listed in Table 2. No significant differences were noted in quantities of any bacterial group between the subject groups. Comparisons between spouse pairs showed no statistically significant differences between CFS family members and their spouses in quantities of any bacteria.

β-Glucuronidase Activity. The mean values of total β-glucuronidase activity in each group are shown in Table 3. The mean difference in conversion of cholic acid to deoxycholic acid between spouse pairs was 5%, and the standard deviation of the difference was 36%. No significant differences were noted in either comparison. The mean value of the 7α-dehydroxylase control sample was 72.0 ± 3.1, with a coefficient of variation of 4%.

DISCUSSION

Cancer of the large intestine represents 15% of all malignant neoplasms in the United States (5). The incidence is highest in countries of relatively advanced socioeconomic status, and correlations have been found between colon cancer incidence and certain dietary patterns. These patterns include high levels of dietary animal fats and proteins and low levels of dietary fiber and unrefined carbohydrates. Based on these correlations, it has been suggested that cancers of the digestive tract might be caused by the ingestion of carcinogens or by dietary components that are metabolized (possibly by the intestinal microflora) within the digestive tract to carcinogenic compounds.

A unique population of CFS family members was available to us to investigate the proposed relationship of fecal microflora to colon cancer. Three groups of individuals were selected from this population. Subjects in Group 1 had expressed their genetic predisposition for cancer in the form of large bowel carcinoma. Members of Group 2 had expressed their genetic predisposition in the form of endometrial or breast cancer. The purpose of this investigation was to detect any differences in the intestinal microflora between these 2 groups that might be correlated with the development of colon cancer in one group but not in the other. The control group (Group 3) was included to establish the influence of dietary or environmental differences.
Dietary information obtained from subjects in this study was not extensive but did indicate the consumption of a typical Western diet in all cases. Differences that were reported were felt to be minor.

The study showed no significant differences in quantities of bacterial species either among Groups 1, 2, and 3 or between spouse pairs. This failure to demonstrate significant differences suggests that absolute numbers of fecal bacteria may not vary in relation to development of colon cancer in CFS family members, at least not in a manner detectable by current techniques.

Because of the difficulties encountered in collecting specimens from various locations throughout the United States, we elected to have specimens shipped frozen. Crowther (4) has shown that survival of microorganisms during freezing is enhanced by diluting specimens in glycerol broth and that losses of most species are insignificant using this method of transport. Our preliminary studies showed no decrease in total culture counts after storage at —70°. Hedges et al. (9) further examined this method of shipping fecal specimens and found that numbers of colony-forming units on egg yolk lactose sugar medium were reduced 20- to 50-fold after freezing in glycerol broth. Lecithinase-positive clostridia were particularly susceptible to freezing. Bacterial quantities in this study were low in comparison with results of other investigators. Our total anaerobic counts were approximately 1 log lower than counts from nonfrozen specimens reported by Mastromarino et al. (20). These differences in bacterial quantities may have been related to our shipping method.

Levels of carcinogen-related metabolism were also assessed. ß-Glucuronidase activity did not differ among Groups 1, 2, and 3 or between spouse pairs. Fecal 7α-dehydroxylase activity was also measured, with no significant differences noted among Groups 1, 2, and 3, or between spouse pairs.

While it is possible that some enzyme-producing bacteria may be lost during freezing, our assays were designed to measure existent enzymatic activity. ß-Glucuronidase and 7α-dehydroxylase activities are both stable at frozen temperatures (21, 27). Our control specimens maintained enzymatic activity at —70° throughout the time period of the study. Investigators have shown that 7α-dehydroxylase activity can be induced in some species but not in others (24, 28). Maximum induced activity occurs during log-phase growth 2 to 3 hr after exposure to cholic acid. Our incubation period (3 hr) was not long enough to allow bacteria to reach log-phase growth and produce significant amounts of enzymatic activity. Thus, we feel that our results are valid assessments of enzyme activity present in the specimen at the time of collection.

Our failure to demonstrate increased enzymatic activity in CFS colon cancer subjects may be explained in several ways: (a) Colon carcinogenesis may involve enzymatic activities other than or in addition to the 2 studied here. Other metabolic processes that have been theoretically linked with colon cancer include cholesterol degradation and reduction of N-nitroso compounds in feces. (b) Our population size was small due to the difficulty of obtaining good samples from a wide range of locations. Larger numbers of subjects may point out differences in fecal constituents not noted in this study. (c) Difficulties were encountered in maintaining the precision of the ß-glucuronidase assay. Fresh standards were prepared daily, and standards and samples were maintained at 4° until the incubation period began. Nevertheless, control values varied widely from day to day. It is glucuronidase mi...
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