

Case-Control Study of Colorectal Cancer and Fecal Mutagenicity¹

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ABSTRACT

Fecal mutagenicity was measured in 68 patients with colorectal cancer and in 114 controls, using *Salmonella* tester strains TA98 and TA100 with and without S9 activation. Samples were also tested for fecapentaenes by high-performance liquid chromatography, to permit the separation of fecapentaene and non-fecapentaene mutagenicity. Overall, no significant case-control differences in fecal mutagenicity were observed. However, when samples containing high concentrations of fecapentaenes were excluded, non-fecapentaene TA98 mutagenicity was observed in eight cases (12%) and only four controls (4%), resulting in an estimated relative risk of 4.4 (95% confidence interval = 1.0-21.1). The association of colorectal cancer risk with non-fecapentaene TA98 mutagenicity could not be explained as an artifact of diagnostic workup or gastrointestinal bleeding among the cases. Smoking could also be excluded as a source of the TA98 mutagenicity seen, but possible dietary origins are still being explored.

INTRODUCTION

Correlational studies have indicated that fecal mutagenicity is elevated in populations known to be at high risk for colorectal cancer (1-5). These observations have suggested the possibility of identifying fecal carcinogens that cause colorectal cancer while in passage through the bowel. To date, three small case-control studies of fecal mutagenicity have failed to show marked differences in *Salmonella* mutagenicity results between colorectal cancer patients and controls (6-8). However, these case-control studies, each with less than 25 cases, were completed before the identification of the fecapentaenes, the most prevalent and highly concentrated fecal mutagens found in North American populations (9-11). The fecapentaenes are bacterial and mammalian mutagens but have not been shown to be carcinogenic (12, 13). Moreover, the excretion of fecapentaenes when measured directly by high-performance liquid chromatography appears to be significantly decreased in colorectal cancer patients compared to controls (14, 15). Thus, to search for other mutagens that may be elevated in colorectal cancer cases, while taking into account the mutagenic effects of the fecapentaenes, we have completed a much larger case-control investigation of fecal mutagenicity than previously reported, and for each sample we have performed both fecapentaene measurements and *Salmonella* mutagenicity assays.

SUBJECTS AND METHODS

Study Population. We recruited patients newly diagnosed with adenocarcinoma of the colon or rectum, seen during the study period (April

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1985-June 1987) by clinical collaborators at three Washington, DC area hospitals, National Naval Medical Center, Walter Reed Army Medical Center, and George Washington University Hospital. Since we planned to measure fecal mutagenicity both prior to and following treatment, cases scheduled for immediate surgery following diagnosis and those residing outside the Washington, DC metropolitan area were not eligible ($n = 26$). Of the 162 presumably eligible cases, 21 (13%) were not reached before surgery and 49 (30%) refused to participate due to the intensive study demands. An additional 14 cases dropped out before collecting a stool sample, leaving 78 participating cases. Nine of these participants were found to have adenomatous polyps or nonneoplastic bowel conditions following surgery or pathology review and were excluded from analysis. One subject's single sample was insufficient to permit mutagenicity testing. As a result, 68 cases comprised the analytic study group.

For controls, we recruited patients awaiting elective surgery for nononcologic, nongastrointestinal conditions at the three study hospitals. Controls at each hospital were frequency matched to cases on age and sex. We approached 315 potential controls, and found 277 to be eligible based on the criteria of Washington, DC area residency and availability for follow-up. Of the 277 eligible subjects, 55 (20%) were not reached before surgery, 95 (34%) refused to participate, and 13 (5%) dropped out before collecting a stool, leaving 114 participants. The distribution of control surgeries was as follows: 62 orthopedic or neurosurgical procedures (54%), 31 hernia repairs (27%), 16 vascular procedures (14%), and 5 other general surgeries, primarily of the thyroid (5%).

Collection Procedures and Laboratory Methods. Cases and controls were asked to collect four 2-day stool samples at home. The first sample was collected before hospitalization and treatment, which usually involved surgery. Three follow-up collections were scheduled at 1, 3, and 6 months following surgery. Participation for these postsurgical collections was somewhat erratic and scattered around the scheduled times. Thus, the presurgical period is highlighted in the results, because this period had the best participation and because the presurgical assay results preceded any possible effects of hospitalization or treatment.

For each 2-day collection, the subjects received a Styrofoam chest filled with dry ice. They collected stool into a plastic container held by a collection bonnet placed on the toilet rim, then placed the container immediately into the dry ice chest. Subjects were shown how to use the collection kits to avoid urine contamination, and this proved not to be a problem. The number of stools making up a pooled sample ranged from 1 to 7 (average, 2-3). After the 2-day collection was completed, stools were transported to the lyophilization laboratory and were freeze dried in the individual containers without ever thawing. The lyophilate was pooled and mixed for the entire 2-day collection, then stored at -40°C or colder in sealed, air-tight containers. Aliquots of the samples were sent in screw-top vials on dry ice to the two testing laboratories.

Fecapentaenes were measured at the Anaerobic Microbiology Laboratory of Virginia Polytechnic Institute (Blacksburg, VA). Samples (1 g) of the freeze-dried materials were extracted and analyzed by HPLC,⁴ as previously described (16). Briefly, freeze-dried samples were extracted in 20 ml acetone supplemented with butylated hydroxytoluene. Each extract was vacuum filtered, evaporated under vacuum at 60°C ,

⁴ The abbreviations used are: HPLC, high-performance liquid chromatography; FP, fecapentaene(s); RR, relative risk; AIA, aminoimidazoazaarene; CI, confidence interval.

stopped under argon, and placed in ice. The evaporated extracts were (a) resuspended in 0.5 ml of prechilled HPLC solvent, (b) filtered through an Acrodisc LC-13 filter (0.5 μ m) into 1-ml septum-capped vials, and (c) sealed under argon.

We determined the concentration of total fecapentaenes in each extract on a Waters liquid chromatograph by using a radial compression module and 8-mm (5- μ m) silica cartridges, with chloroform:isopropanol (95:5) containing 50 μ g/ml butylated hydroxytoluene. The flow rate was 2 ml/min. The HPLC was calibrated by using dilutions of known concentrations of synthetic fecapentaene-12; the peak areas of these standards were then correlated with those of the experimental samples. Absorbance was monitored at 365 nm on a Waters 440 UV detector (AUFS 0.05) and peak areas were integrated on a Hewlett-Packard 3390A recording integrator. The reading corresponded to "total fecapentaenes," combining fecapentaene-12 and fecapentaene-14 (17). The validity of high-fecapentaene readings was routinely confirmed by demonstrating the characteristic fecapentaene UV absorption triplet (300–380 nm) via UV spectroscopy of the HPLC eluates.

Salmonella/mammalian microsome mutagenicity assays were conducted at the Microbial Mutagenesis Screening Laboratory of the National Cancer Institute Frederick Cancer Research Facility (Frederick, MD). To extract each sample, 2.5 g were added to 50 ml of acetone and the suspension was shaken for 30 min at room temperature. The suspensions were vacuum filtered and the filtrate was evaporated with a rotary evaporator with the water bath set at 50°C. For the initial screening tests, 1.5 ml of acetone were added and the flask was hand shaken. The suspended residue was added in 0.15-ml aliquots so that each plate contained 250-mg equivalents of the original lyophilized stool sample.

The plate-incorporation assays were performed as recommended by Ames *et al.* (18) with the modifications of Andrews *et al.* (19). The stock cultures of TA98 and TA100 were maintained as frozen permanents at -80°C, and fresh 15-h cultures were grown in Oxoid No. 2 nutrient broth on a reciprocating shaker at 37°C. For the preparation of S9 mix, male Syrian golden hamsters were induced with 500 mg/kg of Aroclor 1254 (one dose suspended in corn oil injected i.p. 5 days before sacrifice by decapitation). Each plate with S9 mixture contained 3 mg of protein. Each strain was tested with and without the addition of the S9 mix. All experiments were conducted in duplicate under yellow lighting, with appropriate negative controls (cells only and acetone plus cells) and a positive control (2-nitrofluorene). Duplicate plates were incubated at 37°C for 48 h and revertant colonies were counted by using a hand-held tally. The mean number of revertants for duplicate test plates was compared to the mean number of revertants for the negative controls.

When a test sample yielded at least 1.5 times the mean number of spontaneous revertants (the background), a dose response was constructed by using four doses of 50-, 100-, 200-, and 400-mg equivalents of lyophilate. A sample was judged to be mutagenic if two consecutive doses yielded at least twice the background. Samples screening positive (at 1.5 times background) but not judged definitely mutagenic were termed "borderline." Results were recorded, therefore, as "nonmutagenic," "borderline," or "mutagenic." For completeness, both borderline and definitely mutagenic results will be presented, although the focus will be on definitely mutagenic results.

All fecapentaene and mutagenicity assays were performed without knowledge of which sample derived from which individual. As a quality control measure at the end of the study, 1 of every 20 samples ($n = 36$) was resubmitted in a masked fashion for repeat assay by both laboratories. Mutagenic samples were purposely overrepresented among the repeats. The fecapentaene and mutagenicity assays were highly replicable. The Spearman rank correlation coefficient comparing first and replicate fecapentaene measurements was 0.91. Early measurements completed up to 2 years before the quality control batch were as well replicated as recent values, indicating that there was no significant laboratory drift or degradation of samples over time. The percentage of exact agreement for the repeated mutagenicity assays was 78% for TA98, 89% for TA98 plus S9, 89% for TA100, 86% for TA100 plus S9, and 80% for the strongest response obtained by any assay. All but one disagreement between original and replicate mutagenicity results

represented single-category misses, *e.g.*, a sample called borderline on original assay was called negative or positive when repeated.

In additional laboratory measurements, case ($n = 37$) and control ($n = 51$) presurgical specimens collected in the first year of the investigation were also analyzed by HemoQuant for fecal hemoglobin (20) to assess the effect of bleeding, which was common in cases, on fecal mutagenicity.

Statistical Analyses. We originally intended to compare cases to controls with respect to fecal mutagenicity by using each of the four *Salmonella* assay combinations to test samples from the four collection periods (total of 16 case-control comparisons). However, fecal mutagenicity was uncommon in our population, occurring in less than 10% of the acetone extracts for any given assay. When high-FP samples were excluded, the percentage of mutagenic samples was further reduced. Therefore, to generate more meaningful results than the multiple comparisons and small proportions of positivity would permit, we pooled the mutagenicity data in two ways. First, we recorded for each sample the strongest mutagenic response detected with any of the four assays (TA98 or TA100 with or without S9 activation). Also, for each individual we recorded the most mutagenic sample of those collected ($n = 1-4$) during the entire study period.

To control for FP effects, samples with fecapentaene concentrations of 1000+ ng/g dry stool were termed "high-FP samples" and were excluded as indicated in the tables. The choice of 1000 ng/g as the exclusion cutoff was based on earlier methodological work, in which we correlated fecapentaene concentrations and mutagenicity results in 718 stool specimens, including those that comprise the present investigation (11). Those results indicated that samples containing over 1000 ng FP/g were almost three times as likely to be mutagenic as samples with lower FP concentrations, presumably reflecting FP mutagenicity. Moreover, samples containing over 1000 ng FP/g would yield at least 0.25 μ g FP per screening plate in the mutagenicity assays, a dose in the range known to increase the number of induced revertants in testing of synthetic racemic fecapentaene-12 (21).

The association of fecal mutagenicity and colorectal cancer risk was evaluated for each of the four collection periods, using the Mantel χ^2 test for trend (22). In selecting this statistic, we were assuming that borderline mutagenicity might sometimes represent true but weak mutagenic effects. The association of bleeding and mutagenicity was tested by the standard χ^2 statistic.

When we considered the most mutagenic sample donated by each individual, it was necessary to adjust for the number of samples collected ($n = 1-4$), to take into account the possibility that subjects with more samples would be more likely to have at least one mutagenic sample. For these multivariate analyses, we calculated the estimated RR, the ratio of colorectal cancer risk in an exposed group to that in the corresponding unexposed. A relative risk of 2.0 for fecal mutagenicity, for example, would indicate that colorectal cancer occurs twice as often among individuals with mutagenic stools than among those without; conversely, a relative risk of 0.5 would indicate that the occurrence is one-half as great. If the 95% CI computed for the RR estimate excludes 1.0, this corresponds roughly to statistical significance at the level of $P = 0.05$. In our examination of fecal mutagenicity as a risk factor, we took into account the potentially confounding effects of number of stools collected (1, 2, 3, or 4), as well as of age (<56, 56–65, 66+), race (white/nonwhite), sex, admitting hospital, and fecal blood (<7 mg versus 7+ mg heme/g dry stool) by sequential stratified analyses (23). Potential interactions between factors were assessed by computation of the Breslow-Day heterogeneity χ^2 statistic (24).

RESULTS

The cases and controls differed slightly with regard to demographic features. A total of 67% of cases were male and 77% were white, with a median age of 65 years. Fifty-four % of the controls were males and 88% were white, with a median age of 60 years. Parameters of stool sampling were quite similar in the two groups, including the mean number of collection periods in which the subjects participated (2.6 for cases versus 2.8

for controls), the mean number of stools contained in the total collections (7.5 for cases *versus* 7.3 for controls), and the timing of the different collections related to the date of surgery.

For any specific assay and time period, percentages of positivity were very low ($\leq 10\%$) for both cases and controls; thus, our ability to detect case-control differences was limited. Still, a few assay-specific observations made prior to pooling and FP exclusions bear mention. During the presurgical period, TA98 mutagenicity in the absence of S9 activation was observed more frequently in cases (10% of cases *versus* 4% of controls; $P = 0.26$), while TA100 mutagenicity without S9 was observed more frequently in controls (3% of cases *versus* 9% of controls; $P = 0.20$). For each of the follow-up collections, only about one-half of the cases and controls participated, and the scant data were considered unreliable. The excess of TA100 mutagenicity in controls appeared to persist through the 1- and 3-month collections. No other trends were apparent, especially for TA98 or TA100 in the presence of S9, which yielded very few definitely mutagenic samples ($\leq 4\%$) in either cases or controls during any of the collection periods.

To pool the data obtained from the four different assays, we compared cases and controls with regard to the strongest mutagenic response detected in TA98 or TA100 with or without S9 activation, for samples in each of the four collection periods (Table 1). When all samples were considered without consideration of fecapentaene concentration, no significant differences in the pooled assay results were found. The percentages of samples mutagenic in at least one assay were 11% for cases *versus* 11% for controls in the presurgical period, 8% *versus* 12% at 1 month following surgery, 8% *versus* 10% at 3 months, and 6% *versus* 8% at 6 months. When we excluded high-FP samples, an excess of mutagenicity was observed among the cases in the presurgical period ($P = 0.06$; Table 1). This case-control difference derived from increased non-fecapentaene TA98 mutagenicity among cases (9% of cases presurgery *versus* 2% of controls; $P = 0.05$). Cases also had somewhat greater

non-fecapentaene TA98 mutagenicity in the three postsurgical collection periods, but these results were based on very small numbers and were not statistically significant.

In addition to pooling the different assays, we also attempted to increase the statistical power of the case-control comparison by recording each subject's most mutagenic sample, combining the results for the presurgical and three follow-up collections. When all samples were considered, no statistically significant associations were observed. The results excluding high-FP samples are shown in Table 2. TA100 mutagenicity in the absence of high-FP samples was rare, resulting in very broad confidence intervals for the relevant estimates in Table 2. However, non-fecapentaene TA98 mutagenicity was associated with a 4-fold excess in risk that achieved marginal statistical significance (lower limit of the confidence interval was 1.0). This elevation in risk persisted when the RR estimate was adjusted sequentially for age, sex, hospital of admission, and race.

The remainder of the analysis focused on TA98 mutagenicity, in an attempt to clarify the association with risk of colorectal cancer. Considering the original group of samples before the deletion of high-fecapentaene specimens, 10 of 68 cases (14%) and 11 of 114 controls (10%) had at least one sample definitely mutagenic in TA98 (estimated RR adjusted for number of samples = 2.7, 95% CI = 0.8-8.6). Deletion of high-fecapentaene samples eliminated 2 cases and 7 controls from this group, including 1 control with 2 TA98 mutagenic samples, leaving the 8 cases and 4 controls noted in Table 2. All of the excluded subjects exhibited TA100 mutagenicity as well as TA98 mutagenicity, whereas only 2 of the remaining 12 subjects, with TA98 mutagenicity not easily explainable by high fecapentaenes, had any TA100-positive samples. Most subjects had only one mutagenic stool out of several collected; only 2 cases and 2 controls in the group of 12 had more than 1 mutagenic stool and none of the 12 had consistently mutagenic samples.

To determine whether the elevated mutagenicity in cases was an artifact of gastrointestinal bleeding, we examined TA98 mutagenicity in 88 presurgical samples for which HemoQuant analyses had also been performed. Nonphysiological bleeding, defined *a priori* as 7+ mg heme/g dry stool, was found in 20 of 37 cases (54%) compared with 7 of 51 controls (14%). Bleeding

Table 1 Fecal mutagenicity in 68 colorectal cancer patients and 114 controls, before and after surgery

Collection period	Most mutagenic assay result ^a	All samples ^b		High FP samples excluded ^c	
		Cases	Controls	Cases	Controls
Presurgery ^c	Total	63	112	53	82
	Borderline (%)	14	7	15	6
	Mutagenic (%)	11	11	11	6
χ^2 , trend		0.6, $P = 0.44$		3.4, $P = 0.06$	
1 mo Postsurgery	Total	39	69	35	57
	Borderline (%)	10	10	9	11
	Mutagenic (%)	8	12	6	11
χ^2 , trend		0.4, $P = 0.55$		0.8, $P = 0.38$	
3 mo	Total	36	67	24	47
	Borderline (%)	8	6	0	6
	Mutagenic (%)	8	10	8	2
χ^2 , trend		0.0, $P = 0.89$		0.3, $P = 0.59$	
6 mo	Total	36	66	26	46
	Borderline (%)	8	9	4	11
	Mutagenic (%)	6	8	4	4
χ^2 , trend		0.2, $P = 0.68$		0.5, $P = 0.49$	

^a Strongest mutagenic activity detected either by TA98 or TA100 with or without S9 activation.

^b Many subjects did not participate in all collection periods.

^c Samples with 1000+ ng FP/g excluded, along with three samples from subjects who did not have FP measurements in the presurgery period.

Table 2 Estimated RR and 95% CI associating colorectal cancer and fecal mutagenicity, excluding samples with elevated fecapentaenes

Subject's most mutagenic sample ^a	Cases	Controls	RR ^b (95% CI)
TA98			
Nonmutagenic	52	84	1.0 (referent)
Borderline	5	11	0.8 (0.2-2.7)
Mutagenic	8	4	4.4 (1.0-21.1)
TA98 + S9			
Nonmutagenic	55	89	1.0 (referent)
Borderline	8	7	1.8 (0.5-6.1)
Mutagenic	2	3	1.1 (0.1-9.2)
TA100			
Nonmutagenic	60	93	1.0 (referent)
Borderline	3	2	3.2 (0.4-30.6)
Mutagenic	2	4	0.7 (0.1-4.8)
TA100 + S9			
Nonmutagenic	62	97	1.0 (referent)
Borderline	2	1	3.2 (0.2-95.8)
Mutagenic	1	1	1.5 (0.0-57.1)
Most mutagenic assay result ^c			
Nonmutagenic	47	75	1.0 (referent)
Borderline	9	14	1.1 (0.4-3.1)
Mutagenic	9	10	1.5 (0.5-5.6)

^a Most mutagenic response in all samples ($n = 1-4$) collected for each subject.

^b Relative risks adjusted for number of samples provided ($n = 1-4$).

^c Mutagenic activity detected either by TA98 or TA100 with or without S9 activation.

was significantly associated with increased TA98 mutagenicity among the controls ($\chi^2 = 7.8$; d.f. = 2; $P = 0.02$) but not among the cases ($P = 0.95$). Stratification of the RR analysis by bleeding status revealed that the excess risk associated with TA98 mutagenicity was limited to the subgroup of samples without evident bleeding (RR = 7.6; 95% CI = 1.0–56.7). There was no suggestion of risk associated with TA98 mutagenicity among subjects found to be bleeding ($P = 0.09$ for interaction).

DISCUSSION

In an investigation limited by small percentages of mutagenic samples, we have observed a possible association between colorectal cancer risk and non-fecapentaene mutagenicity, detected in acetone-based fecal extracts by *Salmonella* tester strain TA98 without S9 activation. This finding, combined with our earlier demonstration of significantly lowered fecapentaene excretion in colorectal cancer cases (15), illustrates the difficulties in fecal mutagenicity research. There is no universal way to measure fecal mutagenicity; the results are highly dependent on extraction and assay methods (25). Also, not all mutagens are carcinogens, thus mutagenicity results "lump" the effects of multiple compounds of different etiological importance. Fecapentaenes, specifically, are mutagens in bacterial and mammalian assays but may not be carcinogens (13). Fecapentaene excretion is common in North American populations and, thus, mutagenicity due to fecapentaenes must be separated from the effects of other rarer and less concentrated fecal mutagens that may be more likely colorectal carcinogens (11). We sought to achieve this separation by excluding samples from the analysis that contained over 1000 ng fecapentaenes/g dry stool, a concentration in the range known to induce mutagenic revertants in *in vitro* testing of pure compound (21). The limitations of this approach should be noted. We may have eliminated other mutagens that occurred by chance in high-FP samples, and we may have failed to exclude some lower-FP samples that still exhibited FP mutagenicity for whatever reason. In general, however, we feel confident that the exclusions were properly focused, since the deleted samples almost uniformly showed both TA100 and TA98 mutagenicity, like fecapentaenes (21), while the remaining TA98-positive stools were almost all inactive in TA100. Thus, it is possible that we have identified a discrete subset of TA98 mutagenicity that is associated with colorectal cancer.

We can only speculate regarding the origins of this TA98 mutagenicity. Case-control differences in fecal mutagenicity might have been produced by the cancer itself or the clinical procedures used to diagnose it. Thus, in a separate investigation, we considered the possibility that the colorectal diagnostic work-up undergone by cases could have influenced their excretion of mutagens. We took repeated measurements of fecal mutagenicity as 86 subjects underwent sigmoidoscopy, barium enema, and/or colonoscopy for symptoms compatible with colorectal cancer (e.g., rectal bleeding, constipation). We observed no effect of the diagnostic work-up on fecal mutagenicity, although the statistical power of this methodological investigation was again limited by the small numbers of mutagenic samples.⁵

As shown in the results, we also investigated the influence on fecal mutagenicity of gastrointestinal bleeding, which was common in cases before surgery but infrequent in controls. Gastrointestinal bleeding appeared to increase TA98 fecal muta-

genicity and must, therefore, be taken into account in future studies of mutagenicity and colorectal cancer. In our investigation, non-fecapentaene TA98 mutagenicity was associated with colorectal cancer only in the absence of bleeding. We have no ready explanation for this puzzling interaction. Still, the greatly elevated RR estimate seen in the nonbleeding group suggests that bleeding does not entirely explain the increased TA98 mutagenicity observed in cases; thus other explanations must be sought.

It remains possible that some aspect of colorectal cancer apart from bleeding could have resulted in TA98 mutagenicity and given rise to the association seen. This type of concern plagues all case-control comparisons of biological measurements, but is especially relevant in this investigation, where the association of non-fecapentaene TA98 mutagenicity and colorectal cancer was seen most strongly during a single collection period before surgery. Since colorectal surgery does not return the bowel to its prediseased state, to address this concern completely will require predisease measurements of TA98 fecal mutagenicity in subjects who ultimately develop colorectal neoplasia. As a more practical alternative, it might be useful to conduct a large cross-sectional study of subjects with colorectal neoplasia of increasing severity, to see if malignancy is associated with altered assay results for TA98 mutagenicity, as well as for the variety of other stool measurements that are currently under study as potential biomarkers of risk.

If the excess of TA98 mutagenicity that we observed in cases proves not to be a chance finding or an artifact of colorectal cancer, then it will be necessary to look for environmental origins of possible etiological importance. Two well-documented and common environmental sources of TA98 mutagenicity are tobacco smoke (26) and cooked meats, including red meat, fowl, and fish (27–30). We were immediately able to exclude smoking as a possible source of mutagenicity since only 1 of the 12 subjects demonstrating non-fecapentaene TA98 mutagenicity smoked in the days preceding the collection of a mutagenic stool sample. In contrast, the 12 subjects all reported eating broiled, fried, or baked meat immediately prior to the start of the collection of their mutagenic 2-day stool samples, as well as prior to most of their nonmutagenic stools. Since virtually all other study subjects also reported recent intake of cooked meats, we conclude that cooked meat ingestion does not invariably lead to detectable TA98 fecal mutagenicity, even in individuals who sometimes exhibit it. Still, a substantial body of laboratory evidence supports the interesting possibility that the TA98 mutagenicity we found more often in cases might derive at least partly from cooked meats.

Hayatsu *et al.* (29) reported TA98 fecal mutagenicity arising in three volunteers following the ingestion of fried ground beef. The mutagens were shown to resemble closely the heterocyclic amines called aminoimidazoazaarenes produced by cooking beef, lamb, chicken, pork, and fish at high temperatures (26–30). Several AIAs have been proven to be carcinogenic (28). It is known, moreover, that some AIAs can be activated by bacterial flora of the human colon, leading to direct-acting mutagens whose activity is reduced by the addition of S9 (31, 32). Correspondingly, the TA98 mutagenicity associated with risk of colorectal cancer in our data was detected in the absence of S9 activation, perhaps representing a particular metabolic handling of some ingested AIAs.

A major difficulty in testing this hypothesis is the insensitivity of standard fecal extraction and assay methods in detecting mutagens in stool. The overall percentage of mutagenic samples in our population was low (9.5%) but not inconsistent with

⁵ Unpublished data.

other investigations in which unincubated stool samples were tested (5, 7). Methodological insensitivity, combined with very small sample sizes and the confounding effects of fecapentaenes, may explain the negative results found in previous studies attempting to correlate diet, fecal mutagenicity, and colorectal cancer (5-7, 33). Our study was much larger than previous investigations of this complex topic, but still suffered from lack of statistical power. To conduct even larger studies will be difficult, because of the expense and logistical difficulties related to collecting and processing stools from individuals in a community setting. Fortunately, monoclonal antibody and mass spectrometric techniques have recently been developed which should permit more practical and sensitive epidemiological studies of meat-derived TA98 fecal mutagens (34, 35). Ultimately, we hope to use these methods to compare colorectal cancer patients and controls with respect to mutagen excretion, controlling for patterns of meat ingestion.

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