Fecal Mutagens in Two Japanese Populations with Different Colon Cancer Risks


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ABSTRACT

Human feces from 223 Japanese in Hawaii at high risk for colon cancer and feces from 166 Japanese of northern rural Japan at low risk for colon cancer were shown to contain mutagenic activity under five different test conditions.

The first assay, using the Ames TA98 and TA100 Salmonella test, detected ether-soluble mutagens in the presence and absence of rat liver microsomes. Of these, the TA98 direct-acting mutagens are present more frequently in the feces of the high-risk population than the low-risk population at a high level of statistical significance (p < 0.01). TA98 mutagens activated by rat liver microsomes also occur significantly more frequently in the feces of the Japanese from Hawaii (p < 0.05).

Mutagens detected by TA100 in the presence and absence of rat liver microsomes are not commonly found in either Japanese population.

The second bacterial test system used to detect fecal mutagens uses Escherichia coli rec-. This system detects water-soluble fecal mutagens which are also present more frequently in the high-risk population than in the low-risk population (p < 0.05).

MATERIALS AND METHODS

Participants of the Japan-Hawaii Cancer Study in Honolulu, Hawaii, and Akita Prefecture, Japan, formed the population base of this investigation (19). Japanese men and women were randomly selected to submit a stool specimen for fecal mutagen analysis. If a person had a previous resection for a gastrointestinal lesion or was currently receiving antibiotic medication, he or she was disqualified from the study.

Over 95% of the qualified subjects agreed to participate. In all, there were 223 participants from Hawaii and 166 participants from Akita. Approximately 10% of the subjects from Hawaii were born in Japan; the rest were born in Hawaii.

Comparisons of age, colon cancer incidence, height, weight, blood pressure, and hematocrit were done. The blood pressure was recorded from the left arm with the person in a sitting position. Diastolic blood pressure was based on the disappearance of the sound. The hematocrit was based on the microtechnique.

A diet recall questionnaire was used to estimate each subject's weekly consumption of different food items found in a Western or Japanese diet. The details of the dietary method were published previously (11). Briefly, it was designed to quantitate the recent intake of specific food items with the assistance of photographs of weighed small, medium, and large portions of each item. The questionnaire containing the dietary and clinical variables was available on 221 subjects from Hawaii and on 95 subjects from Japan.

The method of stool collection has been described elsewhere (10). The donor took home a dry ice container, a polyethylene bag, and a metal toilet bowl frame. After the stool specimen was expelled into the bag, it was frozen at −20°C until the time of analysis.

The processing of feces for mutagen testing was as follows (4). The feces were dried without thawing by lyophilization and ground to a fine powder in a mortar. Fecal powder (1 g) was transferred to a 100-ml
beaker and suspended in 15 ml of diethylether by stirring on a magnetic stirrer for 15 min. The mixture was then filtered through fluted filter paper. The residue was washed with 15 ml of ether and then reextracted as before. The filtrates were combined and evaporated to dryness in a rotary evaporator under water aspirator pressure. The residue was dissolved in 5 ml of dimethyl sulfoxide and autoclaved for 10 min at 121°C. Aliquots of 0.1 ml, corresponding to 0.02 g of dried feces, were tested with TA98 and TA100 in the presence and absence of rat liver microsomes according to the plate incorporation procedure of Ames et al. (1). The rat liver microsomal preparation was obtained from Aroclor-treated rats.

Each sample was run in triplicate, and all experiments were incubated for 48 hr. Spontaneous reversion rates for TA100 were routinely 140 to 180 revertants, for TA98 30 to 40, and were determined in sextuplicate in the presence and absence of S9 preparation for each set of experiments. These numbers of spontaneous revertant colonies agree closely with those reported by McCann et al. (16). Each culture of the Salmonella strains was tested for ampicillin resistance and crystal violet sensitivity (1). The ethyl ether used for extraction was routinely tested for peroxides, and a solvent blank was routinely included with each series of tests. The number of revertant colonies was counted on each experimental plate and divided by the average number of spontaneous colonies on control plates run with each set of experiments. Colony counts of each plate for each experiment were within 30% of the mean of the 3 replicate plates, or the experiment was repeated. Experiments in which the mutation ratio was less than 0.7 were repeated at lower amounts of feces extract until a mutation ratio of at least 0.9 was obtained.

The results are presented as mutation ratio rather than the number of net revertants, because the mutation ratio has the same significance whether the tester strain TA100 or TA98 was used. This is especially important when low levels of mutagens are being measured, and 17 to 20 net revertants are at the level of minimum significance for TA98, but 60 to 80 net revertants are necessary when using TA100. The approximate number of net revertants may be obtained from the mutation ratio by multiplying the mutation ratio value by 35 for TA98 and subtracting 35 from the product for TA98 and 140 for TA100 and subtracting 35 from the product for TA98 and 140 from the product for TA100.

Extracts were prepared for measurement of water-soluble mutagens by ether extraction of 0.5 g lyophilized powdered feces and suspension of the residue in 2.5 ml water. The resulting mixture was stirred for 15 min at room temperature and centrifuged at 5000 × g for 15 min, and the supernatant was autoclaved for 10 min.

In the E. coli recA assay, 0.1 ml of extract was added to a well cut with a 5-mm heated glass tube in a hard agar nutrient broth plate seeded with E. coli JC5519 (recBC) and to a well cut in a control plate seeded with a lawn of the parent wild-type strain E. coli AB1157 (13). If zones of inhibition much greater than 6.0 mm, including the 5 mm of the center well, were obtained with AB1157, the experiment was repeated with lower amounts of extract added to the center well. All experiments were carried out in triplicate, and the results were recorded as the diameter of the net zone of inhibition after subtracting the diameter of the zone of inhibition observed on the E. coli AB1157 plates. Minimal net zones of inhibition of 2.0 mm were considered necessary to establish the presence of mutagens in a sample.

In examining a subset of 25 representative fecal samples of all types in which the extraction and testing were carried out 2 to 4 times, it was found that the mutation ratio could be reproduced within 0.2 of the first observed mutation ratio. Fecal mutagens appear to be stable, and fecal samples stored over 1 year lose little of their mutagenic activity. Another subset of active fecal samples did not lose activity upon autoclaving when compared to unsterilized aliquots. A third subset of inactive samples was first tested with autoclaving. When restested without autoclaving, none showed any activity. In other experiments, many of the fecal mutagens were little affected by exposure to 100° for 15 min and acidic (pH 1.0) conditions (data not shown). Methylene chloride is equally effective as ethyl ether in extraction of mutagens from lyophilized powdered fecal samples.

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**Table 1**

<table>
<thead>
<tr>
<th>Food item</th>
<th>Hawaii (n = 221)</th>
<th>Akita (n = 95)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>196.4</td>
<td>12.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Wieners</td>
<td>33.0</td>
<td>30.4</td>
<td>0.727</td>
</tr>
<tr>
<td>Bacon</td>
<td>8.6</td>
<td>0.2</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Sliced tomatoes</td>
<td>108.4</td>
<td>166.0</td>
<td>0.100</td>
</tr>
<tr>
<td>Lettuce</td>
<td>222.2</td>
<td>94.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Celery</td>
<td>35.1</td>
<td>10.9</td>
<td>0.032</td>
</tr>
<tr>
<td>Coffee</td>
<td>2512.2</td>
<td>46.8</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Milk</td>
<td>524.9</td>
<td>406.5</td>
<td>0.251</td>
</tr>
<tr>
<td>Japanese</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sashimi (raw fish)</td>
<td>30.5</td>
<td>27.5</td>
<td>0.079</td>
</tr>
<tr>
<td>Dried fish</td>
<td>8.0</td>
<td>690.6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Dried cuttlefish</td>
<td>1.3</td>
<td>4.3</td>
<td>0.130</td>
</tr>
<tr>
<td>Kamaboko (fish cake)</td>
<td>14.8</td>
<td>15.1</td>
<td>0.920</td>
</tr>
<tr>
<td>Tofu (soybean curd)</td>
<td>141.4</td>
<td>210.0</td>
<td>0.025</td>
</tr>
<tr>
<td>Rice</td>
<td>1186.2</td>
<td>1915.0</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Hakusaizuke (picked Chinese cabbage)</td>
<td>28.5</td>
<td>178.9</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Takawan (picked turnip)</td>
<td>12.2</td>
<td>63.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Ume (pickled plum)</td>
<td>4.9</td>
<td>13.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Tsukudani (seaweed paste)</td>
<td>2.3</td>
<td>3.1</td>
<td>0.323</td>
</tr>
<tr>
<td>Green tea</td>
<td>766.8</td>
<td>662.1</td>
<td>0.610</td>
</tr>
<tr>
<td>Gen mai cha tea</td>
<td>314.8</td>
<td>1164.4</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Fecal extracts apparently contain toxic substances, which in some cases produce mutation ratios less than 1.0 but do not affect the density of the background lawn in the Salmonella testing systems. Retests of these samples at lower amounts of extracts produce mutation ratios near 1.0 but have never resulted in reclassifying the fecal sample as containing mutagens.

There is no evidence of a decrease in background lawn or of changes in revertant colony size on plates that have higher mutation ratios. A test of revertant colonies for ability to synthesize histidine is positive for 90% of the colonies. Dose response experiments for representative fecal samples show a linear response with increasing volume of extract.

**Statistical Methods.** Age-adjusted and age-sex-adjusted comparisons of demographic, clinical, and dietary variables were performed using the direct method of adjustment (14). Mean values of these variables were adjusted using the entire sample of study subjects as the reference population.

Univariate comparisons of the proportions of subjects having mutation ratios >2.0 were made by origin (Japan versus Hawaii), age group, and sex, using (uncorrected) χ² tests of significance. Where sample sizes were too small to justify this, Fisher's exact test (9) was used instead. Multivariate (origin, continuous age, sex) analyses were performed using the multiple logistic risk function model. The logistic coefficients were estimated by the method of maximum likelihood (7), using Newton-Raphson iterative solutions of the maximum likelihood equations. Separate logistic models of mutagenicity risk were constructed for each of the 5 different mutagen assays. The computer program used for these logistic analyses was the LOGIST procedure (12) in the statistical analysis system, Release 79.3B, as implemented on the International Business Machines Model 370/158 at the University of Hawaii Computing Center.

**RESULTS**

A comparison of Japanese men and women in Honolulu and rural Akita by weight, height, and other parameters was done.
The food items in the questionnaire were separated into Western or Japanese foods on an a priori basis in Table 1. Of the Western foods, the Japanese in Hawaii consumed more beef, bacon, lettuce, celery, and coffee than did their Japanese peers. With regard to Japanese foods, Akita residents ate (data not shown). The men from Hawaii were taller and heavier than their counterparts in Akita, while the women from Hawaii were taller but not notably heavier. There were no significant differences in blood pressure or hematocrit between the 2 geographic groups.
much more dried fish, tofu (soybean curd), rice, hakusaizuke (pickled Chinese cabbage), takuwan (pickled turnip), ume (pickled plum), and gen mai tea than did Japanese participants in Hawaii. A correlation of dietary habits and mutagen assay results will be presented in a future publication.

Charts 1 to 3 show the frequency of values of mutation ratios or net zones of inhibition found in the fecal samples tested. Univariate comparisons of the proportion of subjects having fecal samples with a mutation ratio >2.0 by origin (Japan versus Hawaii), age group, and sex are shown in Table 2. With respect to origin, these proportions are significantly different for 4 of the 5 mutagen assays, with p <0.05 in each case. For TA100 with S-9, the proportions are almost identical. Of the 4 assays showing significant differences, 3 demonstrate significantly higher proportions of mutagenic samples in the Japanese subjects from Hawaii. However, with TA100 (direct acting), it is the Japanese subjects from Japan who have the significantly higher rate of mutagenicity (as it is defined here).

Ignoring origin and comparing these "mutagenicity rates" by sex over the entire study population showed that only for TA98 (direct acting) there was a significant difference (higher mutagenicity rate in females). Also, when similar comparisons were made by age group (regardless of origin and sex), only for

**Table 2**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>E. coli S-9</th>
<th>TA98</th>
<th>TA100</th>
<th>TA100 + S-9</th>
<th>TA98 + S-9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japan</td>
<td>M</td>
<td>&lt;35</td>
<td>0.267</td>
<td>0.348</td>
<td>0.245</td>
<td>0.348</td>
<td>0.348</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>≥35</td>
<td>0.267</td>
<td>0.348</td>
<td>0.245</td>
<td>0.348</td>
<td>0.348</td>
</tr>
<tr>
<td>Hawaii</td>
<td>M</td>
<td>&lt;35</td>
<td>0.267</td>
<td>0.348</td>
<td>0.245</td>
<td>0.348</td>
<td>0.348</td>
</tr>
<tr>
<td></td>
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<td>0.267</td>
<td>0.348</td>
<td>0.245</td>
<td>0.348</td>
<td>0.348</td>
</tr>
</tbody>
</table>

* All p values from uncorrected X2 tests unless otherwise noted.
* Correlation of dietary habits and mutagen assay results will be presented in a future publication.
TA98 and TA98 with S-9 were there significant differences (higher mutagenicity rates in older subjects for both assays). In general then, mutagenicity rates were definitely related to origin and sometimes to sex and age group also, depending on the mutagen assay used. Comparison of mutagenicity rates by origin was also done within sex categories separately. This showed that the significant differences by origin mentioned earlier were mostly due to the female subjects, with direction of differences just as before. However, for the E. coli rec test, the higher mutagenity rate among female Japanese subjects from Hawaii was not quite significant at the (approximate) 5% level for E. coli rec, TA98, and TA98 with S-9 even after adjustment for age and sex. Also, the negative association (note the sign of the logistic coefficient for origin and the coding conventions used) of lower mutagenicity rates in Japanese subjects from Hawaii is significant ($p = 0.060$) for TA100 (direct acting). Note the significant association with sex for TA98 over and above that due to origin. First-order interaction effects among these 3 variables were also investigated and found to be not significant for E. coli rec and TA98 with S-9. For both assays, each of the 3 possible first-order interaction effects had $p > 0.12$. For the remaining 3 assays, the interaction effects (whether significant or not) could not be reported here due to computer program convergence problems of the estimation procedure.

**DISCUSSION**

The characteristics of the mutagen tester bacteria used in these experiments have allowed us to detect 5 separate types of fecal mutagens. Each type may contain a range of mutagens of different chemical structures. Furthermore, some fecal samples probably contain more than one mutagen type. Despite these difficulties, it is useful to separate the fecal mutagens into groups in order to guide future efforts in isolating compounds with the highest probability of being important in the causation of large-bowel cancer. Mutagens that are consistently found in high incidence in fecal samples from populations at high risk for colon cancer are the most likely candidates.

That mutagen differences do exist in the feces of different populations is seen by the low levels of direct-acting TA100 mutagens that we have found in our high-risk Hawaiian Japanese population, compared to the much higher level that Bruce and Dion (3) found in the feces of their high-risk Canadian population or that Reddy et al. (20) have found in the feces of a group of New Yorkers and that Ehrich et al. (8) have found in the high-risk white South Africans. The incidence of direct-acting TA98 mutagens in these latter high-risk populations, however, agrees closely to the level of this mutagen class found in our high-risk population. The study by Bruce and Dion (3) on 17 colon cancer patients and 17 age-matched control patients shows the incidence of TA100 direct-acting mutagens to be the same, suggesting that the TA100 direct-acting mutagen may not be involved in the etiology of colon cancer.

The choice of the minimum mutation ratio which establishes the presence or absence of mutagens in a fecal sample is very important, as this determines the levels of each type of mutagen in the population. The minimum mutation ratio suggested for this purpose is 1.5. This has been proposed by McCann et al. (16), and we have found that the experimental variation in our results would support this as a minimum mutation ratio. Comnoner et al. (5) have discussed this matter in some detail and have concluded that a minimum mutation ratio of 3.0 is necessary to separate mutagenic from nonmutagenic compounds. Ehrich et al. (8), however, have chosen 2.0. Although the choice of the minimum mutation ratio greatly affects the incidence of the various mutagen classes, it does not alter the basic conclusion of this study that the high-risk Japanese from...
Hawaii have more fecal mutagens than do the low-risk Japanese of Japan. As the incidence falls with higher mutation ratios, the relative incidence of mutagens between the 2 populations increases and becomes large at a mutation ratio near 2.0, since the low-risk population contains only a few fecal samples with high mutagen content (Charts 1 and 2). The level of the significance of the differences between the 2 populations is not greatly affected whether 1.5 or 2.0 is chosen as the minimum mutation ratio value.

Recent work by Commoner et al. (6), Sugimura et al. (24), and Spingarn and Weisburger (22) has shown that a variety of S-9-activated mutagens can be formed in cooked meat or by pyrolysis of amino acids and proteins. In addition, various polynuclear hydrocarbons have also been detected in cooked fish and meat. These mutagens may be the source of the TA98, S-9 mutagens we have detected in our study of human fecal mutagens, but further work will be necessary to clarify this point.

Our work is part of a prospective study of Japanese in Hawaii. Individuals contributing feces from this study will be followed in order to determine whether the presence or absence of any of these fecal mutagens is an indication of risk for large-bowel cancer (23).

REFERENCES

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