Detection of Mutagens in Cervical Mucus in Smokers and Nonsmokers

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Abstract

The Salmonella mutagenicity test was used to analyze cervical mucus specimens from 364 smokers and 333 nonsmokers to determine whether the association between smoking and mutagenic cervical mucus that we reported previously among women diagnosed with dysplasia would apply to a larger group of healthy women (E. A. Holly et al., J. Natl. Cancer Inst., 76: 983-986, 1986). Women smokers and nonsmokers between the ages of 18 and 49 who attended eleven clinics and physicians' offices in the San Francisco Bay area for a routine Pap smear were examined to determine whether smokers were more likely to have mutagenic substances in their cervical mucus. About 4% of smokers and 8% of nonsmokers had positive mutagenicity test results (P = 0.02). Cervical mucus with a large number of microorganisms was more likely to have a positive mutagenicity test result than that with fewer microorganisms (test for trend, P = 0.01). Mutagenicity results varied by race and clinic location but were not associated with smoking behavior, sexual behavior, gynecological diagnosis, or diet. Further work is needed to develop methods to detect mutagens in specific body fluids.

Introduction

Mutagens are substances that have a genotoxic effect on human tissues, and the presence of mutagens in body fluids is an indicator of the ingestion, excretion, and possible metabolism of these chemicals (1). In vitro detection of mutagens in body fluids was made possible in the 1970s by the development of the Salmonella/mammalian-microsome mutagenicity test (hereafter called the Salmonella mutagenicity test) (2). Mutagenic activity was demonstrated in cigarette smoke condensates (3) and in concentrated urine from smokers by Yamasaki and Ames (4). The association of mutagenic urine with smoking and passive smoking has been confirmed by several recently reviewed studies (5) and has provided a possible explanation for the higher risk of bladder cancer among smokers. Mutagenicity testing of other body fluids including gastric juice (6), amniotic fluid (7), and sputum (8) has been attempted less frequently and has met with methodologic problems and yielded inconsistent results.

Women smokers have been noted to have an elevated risk of cervical cancer compared to nonsmokers after controlling for other confounding factors, but the biological rationale for the association has not been established (9, 10). Cotinine and nicotine have been detected in the cervical mucus of smokers (11) and in cervical lavage from women exposed to passive smoke (12), supplying evidence that constituents of smoke reach the uterine cervix and providing a valuable indicator of smoking status (13). Cotinine and nicotine are not among the mutagenic components of tobacco smoke (14), although they may be a marker for the presence of an undetected mutagen (13). In 1986, we reported an association between cigarette smoking and mutagenic cervical mucus in women with cervical dysplasia (15). The purpose of the current study was to determine whether the association between smoking and mutagenic cervical mucus that we found in women who had been examined at the UCSF Dysplasia Clinic and who were at high risk of cervical cancer (15) would apply to a larger group of healthy women smokers and nonsmokers.

Materials and Methods

The current study was conducted in women who attended 11 San Francisco Bay area physicians offices and clinics, including seven Planned Parenthood clinics, between May 1987 and November 1990. Women smokers and nonsmokers between the ages of 18 and 49 were asked to participate in the study when they visited their physicians for a routine Pap smear. In addition to the 631 normal, healthy women recruited from the physicians offices, 66 women from the UCSF Dysplasia Clinic were asked to participate to determine whether smokers with dysplasia had different results on the test for mutagenicity of cervical fluids than did healthy women smokers and women who did not smoke.

Women who consented to participate were interviewed by professional interviewers about smoking habits, passive smoking environment, sexual and reproducr-
tive history, contraceptive use, history of sexually transmitted diseases, and recent diet. The diet questions were limited to foods and drinks thought to be related to mutagenicity of body fluids and included questions about consumption of alcohol; soft drinks; coffee; cruciferous vegetables; and grilled, broiled, and barbecued meats.

Smokers and nonsmokers were recruited from each clinic in approximately equal numbers. Former smokers (n = 32) had not smoked for a mean of 8 years (SD = 6.4) and were included with nonsmokers. Current smokers were asked about number of years smoked, usual number of cigarettes per day, and time of day they last smoked a cigarette.

Cervical mucus specimens were collected by physicians after the routine Pap smear was obtained. The woman’s cervix was flushed and reflushed with 1.5 ml of sterile nonpyrogenic electrolyte solution, and the fluid containing cervical mucus was retrieved, placed in a test tube, and frozen. All specimens were kept frozen until they were transferred to LLNL for testing. Upon arrival at LLNL, the cervical mucus specimens were immediately cultured by laboratory personnel for yeast, lactobacillus, and other microorganisms. Microorganisms other than lactobacilli were cultured on TSA agar, and yeast and lactobacilli were grown using selective agars, Sabouraud dextrose and Rogosa SL agars, respectively. Cervical mucus specimens were kept frozen (–20° C) at LLNL for no longer than 14 days, and Salmonella mutagenicity tests were completed in batches after adequate specimens were collected from all medical clinics. Salmonella mutagenicity tests were conducted according to the plate incorporation method described by Ames and Maron (16).

The Salmonella test for mutagenicity uses a strain of Salmonella typhimurium bacteria that contains a selected mutation to prevent the bacteria from synthesizing the amino acid histidine. Certain mutagens will cause a DNA frameshift change that will reverse this mutation and allow the bacteria to grow and divide, and an increased number of revertant bacteria over background provides an indicator of the presence of a mutagen. There are several strains of the specially developed Salmonella bacteria, and each contains a different mutation for the gene that controls histidine metabolism, allowing response to different types of mutational events.

In the current study, cervical mucus that had been dissolved in the organic solvent DMSO was combined with molten soft agar; TA98 Salmonella bacteria, the strain most sensitive to mutagenic dietary factors and to tobacco products such as polycyclic aromatic hydrocarbons (PAHs) and aromatic amines; and S9, a metabolic agent prepared from livers of Aroclor-treated rats that permits detection of substances that must be metabolized to be mutagenic (17). The cervical mucus of 56 subjects was tested with TA100, another Salmonella strain sensitive to cigarette smoke constituents. The test results for subjects tested using TA100 did not differ from those tested with TA98, and the results were combined for analysis. The specimens were tested in duplicate at two doses: 200 and 400 μl of cervical secretion, the highest doses compatible with the test and the availability of sample. Routine examination of the background lawn of bacterial growth revealed no toxicity at either the 200- or the 400-μl dose. After the molten agar cooled and solidified, the plates were inverted and incubated at 37°C for 48 h. After incubation, the number of revertant colonies was counted and reported as number of multiples times the number of spontaneous revertant colonies. The test was considered positive if the number of revertant colonies was 2.0 times the background count. A positive control that contained 2-aminoanthracene and a negative control that contained DMSO and S9 but no test substance were used with each batch. Laboratory personnel had no knowledge of the smoking status of the subjects, of responses to questions asked in the interview, or of Pap smear results. The same technician conducted all tests for mutagenicity.

The above methodology is standard (16) and was used in both this and our earlier study (15), although different laboratories conducted the tests. However, a modification of the sample preparation was made in the current study by LLNL personnel to reduce the possibility of contamination by bacteria normally found in cervical mucus. The cervical samples were thawed, and an aliquot was plated for the culture of microorganisms. Prior to mutagenicity testing, the cervical mucus was dissolved in DMSO at a ratio of 1:1 DMSO to cervical sample and allowed to incubate overnight (18 h) at 25°C to sterilize the samples before testing. In the earlier study (15), the specimens were incubated with the DMSO for 30 to 60 min prior to plating, which was standard sample preparation at the time the tests were conducted.

The samples obtained from the women attending the UCSF Dysplasia Clinic were split to evaluate the effect of the modified sample preparation on test results. One-half of each sample was tested using the above method, which included overnight incubation with DMSO. The other half of the sample was tested using the sample preparation used in the earlier study in which cervical mucus specimens were incubated with DMSO for 30 to 60 min prior to combining with the other ingredients.

Data analysis was conducted using BMDP statistical software programs (18). Univariate and multivariate odds ratios were computed for factors of interest among smokers and nonsmokers using logistic regression analysis, with 95% confidence intervals, P values, and tests for trend computed when appropriate (19).

**Results**

Eighty-five % of women who were approached for this study agreed to participate, with 364 smokers and 333 nonsmokers interviewed and examined. Sixty-eight % of the women who smoked reported that they smoked less than half a pack of cigarettes a day and were classified in some analyses as light smokers. Women who smoked more than half a pack a day were classified in these same analyses as heavy smokers.

Table 1 shows the test results for women by smoking status, race, and clinic. About 4% of smokers and 8% of nonsmokers had a positive result on the Salmonella test for mutagenicity (P = 0.02). A somewhat higher proportion of nonsmokers than smokers had a positive test in all race groups except Hispanics, although the results could have been due to chance. Asian and Hispanic women were somewhat more likely to have a positive test than white non-Hispanic and black women, but the results could have been due to chance. A higher proportion of nonsmokers than smokers had a positive test.
result in all clinics except Clinic FR, where none of the women had positive test results. However, the difference between smokers and nonsmokers was statistically significant only for women who attended Clinic PP ($P = 0.05$), where the number of positive test results was highest.

Table 2 displays the results of the Salmonella mutagenicity test for selected dietary factors. Women who had eaten meat or cruciferous vegetables in the 24 h prior to the test had similar results on the test for mutagenicity to those who had not eaten these foods. Women who had consumed broiled meat were slightly more likely to have mutagenic cervical mucus, and this result approached statistical significance ($OR = 2.3; Cl = 0.89-5.8; P = 0.09$).

Table 3 shows the results of the Salmonella test for mutagenicity by number of cervical microorganisms and sample preparation method. Five % of samples tested with the original sample preparation and 6% of samples tested with the modified sample preparation had a positive test result. When the modified sample preparation method was used, cervical mucus with a large number of cervical microorganisms was more likely to have a positive Salmonella mutagenicity test result than that with fewer microorganisms. Although the OR was elevated to about 1.5 for the number of microorganisms greater than 200 microorganisms/ml using the original sample preparation method, the number of positive tests was very small.

Women who were currently using oral contraceptives and those who were not were equally likely to have a positive test for mutagenicity ($OR = 0.87; Cl = 0.41-1.8; P = 0.84$). When compared with women who last had sexual intercourse in the 2 days before the examination, women who last had intercourse more than 5 days before the examination were somewhat more likely to have mutagenic mucus ($OR = 2.7; Cl = 0.88-8.2; P = 0.09$).

Table 1: Number and percentage positive on the Salmonella test for mutagenicity among women smokers and nonsmokers by race and clinic

<table>
<thead>
<tr>
<th>Foods consumed</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>% positive</td>
</tr>
<tr>
<td>Cruckiferous vegetables</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>Meat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Pork</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Poultry</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Meat preparation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Broiled</td>
<td>7</td>
<td>11%</td>
</tr>
<tr>
<td>Fried</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Grilled</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Baked</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>Boiled</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Discussion

In contrast to our previous study in women with cervical dysplasia (15), the test for mutagenicity of cervical mucus in the current study detected no difference between healthy women smokers and nonsmokers. Differences were noted between smokers and nonsmokers in the current study on several other factors, and these have been published previously (20). Smokers were slightly older than nonsmokers (mean age, 29.7 years versus 28.3 years) (20), and subsequent analyses were adjusted for age. Smokers were less educated, less likely to be Hispanic, and somewhat more likely to be black when compared to white non-Hispanic women. They were more likely to be separated, divorced, or living as if married in a relationship of at least 6 months when compared to married and single women, and consumed larger quantities of coffee, beer, liquor, soft drinks, and beef than did nonsmokers (20). Smokers also were more sexually active with earlier age at first intercourse and a higher number of sexual partners, and smokers had a higher number of cervical microorganisms (20).

Interpretation of the Salmonella mutagenicity test results in the current study is hampered by the low number of positive results. The different results from the
two studies may be attributable to different study populations or to a change in the test methodology. The population in our earlier study (15) comprised 82 women from the UCSF Dysplasia Clinic, while the population in the current study included 631 women from other Bay area clinics and 66 women from the UCSF Dysplasia Clinic. The current study also used different methods to prepare the mucus specimens for testing by incubating the specimens with DMSO overnight to prevent contamination by normal cervical microorganisms. However, neither sample preparation method nor dysplasia status affected the proportion of positive test results.

In our earlier study, women who had smoked a cigarette within 7 h of the sample collection were more likely to have a positive test result for mutagenicity than smokers who had not consumed a cigarette in that time period (15), but neither time since last cigarette nor number of cigarettes was associated with mutagenicity in the current study. Both number of cigarettes and time since last cigarette have been shown to be associated with mutagenic urine (4, 21).

With the possible exception of broiled meat, a positive result on the test also was not associated with the ingestion of foods that have produced detectable mutagens in urine in other studies (22, 23). Cooked meats contain heterocyclic amine carcinogens (24), and this study noted a higher consumption of some meats among smokers (20). Dietary mutagens may not be excreted in cervical mucus or the test may have been unable to detect the levels of mutagens that were present.

Results from our earlier study (15) noted a higher proportion of mutagenic mucus among women smokers who attended the UCSF Dysplasia Clinic, raising the question of whether these results were related to the diagnosis of dysplasia. A study in India of 98 women, most of whom were nonsmokers, noted that 23.4% of women with cervical intraepithelial neoplasia had mutagenic cervical mucus compared to 3.9% of healthy controls (25, 26). In our current study healthy women and women with dysplasia had similar results on the test for mutagenicity, but the number of women with dysplasia was too small to detect a difference between the smokers and the nonsmokers.

The sample preparation methodology used in this study is the same as that used in a small study of 55 healthy women in Washington, DC (13, 27). That study noted that 9% of smokers and 13% of nonsmokers had mutagenic cervical mucus when the mucus was incubated overnight with DMSO before testing. This result is comparable to the 4% of smokers and 8% of nonsmokers who had a positive test in the current study using the same sample preparation methods.

### Table 1

<table>
<thead>
<tr>
<th>Colony counts/ml cervical wash</th>
<th>Positive test results (N = 36)</th>
<th>Negative test results (N = 568)</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>Positive test results (N = 3)</th>
<th>Negative test results (N = 61)</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microorganisms&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Positive test results (N = 36)</td>
<td>Negative test results (N = 568)</td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
<td>Positive test results (N = 3)</td>
<td>Negative test results (N = 61)</td>
<td>OR</td>
<td>95% CI</td>
<td>P</td>
</tr>
<tr>
<td>≤200</td>
<td>5</td>
<td>169</td>
<td>1.0</td>
<td></td>
<td>0.60</td>
<td>1</td>
<td>26</td>
<td>1.0</td>
<td></td>
<td>0.60</td>
</tr>
<tr>
<td>201-8500</td>
<td>9</td>
<td>194</td>
<td>1.6</td>
<td>0.47-5.5</td>
<td>0.02</td>
<td>21</td>
<td>35</td>
<td>1.5</td>
<td>0.11-17.1</td>
<td>0.76</td>
</tr>
<tr>
<td>&gt;8500</td>
<td>22</td>
<td>225</td>
<td>3.3</td>
<td>1.2-10.2</td>
<td>0.02</td>
<td>1</td>
<td>26</td>
<td>1.0</td>
<td></td>
<td>0.60</td>
</tr>
</tbody>
</table>

*See text for definition.

<sup>a</sup> Includes yeast, other microorganisms, not lactobacillus.

<sup>b</sup> 201 to >8500.
clohexane (8). However, control subjects who smoked but were not exposed to factory air did not have mutagenic expectorate (8). The detection of mutagens in gastric juice has been shown to be affected by the presence of histidine, which increased the number of revertant colonies, and by the presence of substances that inhibit mutagenesis (6). Detection of mutagens in concentrated (freeze-dried) amniotic fluid of smokers was hampered by methodological problems and yielded inconsistent results, possibly because of the presence of histidine in amniotic fluid (7). Further work is needed to develop methods to isolate mutagens from cervical mucus. Mucus may contain substances similar to those in saliva that inhibit mutagenic activity. Mutagens in cervical mucus may need further extraction, such as that used on expectorate, to remove them from extraneous constituents.

Researchers using the Salmonella mutagenicity test have noted that minor changes in the procedure can cause different results. DeRaat et al. (31) documented that a change as minor as differing quantities of bottom agar on the incubation plates changed the numbers of spontaneous revertants in the Salmonella mutagenicity test using TA98 and other strains of Salmonella. Increasing the number of TA98 bacteria added to the test has been noted to increase the sensitivity of the test to benzo(a)pyrene in the urine of smokers (30). The effect of incubating cervical mucus overnight with DMSO has not been published. A recent analysis of the stability of mutagens in the frozen urine of smokers reported that mutant response decreased slightly with increased storage time (up to 175 days), although the results were not statistically significant (32). Storage time in the current study was limited to 14 days. While DMSO is generally the solvent of choice for mutagenicity assays because of its ability to dissolve a variety of chemical substances, it is not always the best solvent. It is known to react with certain chemicals and to inhibit the mutagenic effect of some chemicals (33). Although it is unlikely that DMSO was able to inactivate the mutagens in the cervical mucus, it is not currently known how DMSO reacts with the various components of cervical mucus, especially in the prolonged period of time used in the current study. However, positive control specimens did remain positive and were not significantly affected by the overnight incubation with DMSO.

Men and women are exposed to a variety of mutagenic chemicals in the environment and workplace. Since its development nearly 20 years ago, the Salmonella test for mutagenicity has demonstrated its value to detecting these mutagens and their metabolites in human body fluids, especially urine. While the methods to isolate mutagens from urine have been improved during this period, the methods used to isolate mutagens from other body fluids are less well understood, and further work is needed.

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References


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