Helicobacter pylori Infection and Urinary Excretion of 8-Hydroxy-2-deoxyguanosine, an Oxidative DNA Adduct

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Abstract
To assess whether Helicobacter pylori-related infection increases oxidative DNA damage, we evaluated the association between H. pylori infection and urinary excretion of an adduct of oxidative DNA damage, 8-hydroxy-2-deoxyguanosine (8ohdG). Subjects included 555 healthy persons, ages 20–39, within the Kaiser Permanente Medical Care Program in Northern California. We tested sera for antibodies to H. pylori by ELISA; collected demographic, dietary, smoking, and alcohol data by questionnaire; and assayed 24-h urine samples for 8ohdG with a newly developed ELISA kit. Two hundred eighty-one subjects provided adequate 24-h urine samples for 8ohdG and creatinine assays and had detectable levels of 8ohdG. After adjusting for 24-h urinary creatinine (Ucr) and demographic factors, persons without H. pylori infection had significantly higher amounts of 24-h urinary 8ohdG than infected persons (geometric mean, 18.04 μg 8ohdG/Ucr g versus 14.36 μg 8ohdG/Ucr g, respectively; P = 0.008). Excretion of 8ohdG was higher in whites and Hispanics (17.44 and 18.09 μg/Ucr g) than in blacks (13.21 μg/Ucr g; P < 0.001). Gender was not significantly associated with 8ohdG excretion (16.18 μg/Ucr g for males versus 16.01 μg/Ucr g for females; P = 0.883). Of the dietary factors evaluated, vitamin C negatively correlated (P < 0.001) and carbohydrate intake positively correlated with 8ohdG excretion (P = 0.003). Infection with H. pylori was strongly associated with decreased 8ohdG excretion in the urine. This unexpected finding suggests either that DNA repair is deficient in infected subjects, that inflammation destroys the adduct, or that urinary 8ohdG is not an accurate measure of gastric damage.

Introduction
Helicobacter pylori causes gastric inflammation and gastric cancer (1–5). ROS released by phagocytic cells subsequent to infection have been implicated in this process (6–8). In support of this, gastric mucosal biopsy specimens from H. pylori-infected individuals contain significantly greater amounts of ROS than biopsy specimens from noninfected persons (9). Moreover, the density of H. pylori observed in the gastric mucosa correlates with levels of ROS in gastric tissue (10). If ROS, released consequent to H. pylori related gastritis, are involved in gastric carcinogenesis, then individuals with active H. pylori infection should have greater indices of oxidative DNA damage in the stomach than uninfected persons. This was recently supported by a small study (n = 74), demonstrating significantly higher levels of one marker of oxidative DNA damage, 8ohdG, in mucosal tissue of H. pylori-infected children than in uninfected control tissue (11).

8ohdG is one of the most abundant lesions in DNA induced by ROS (12, 13). It results from attack of a singlet hydroxyl or oxygen radical on guanine. 8ohdG lesions can be excised by DNA repair enzymes before mutation occurs, can undergo cell death, or can lead to permanent changes in DNA structure (14–16). During DNA replication, unrepaired 8ohdG lesions induce G-to-T transversions, which may contribute to carcinogenesis (17). Ostensibly, 8ohdG lesions excised from tissues by DNA repair enzymes enter the bloodstream and are filtered and excreted in urine (18). Shigenaga and Ames (19) demonstrated that when 8ohdG is infused i.v., it does not become degraded during its transport from plasma to urine. This suggests that adducts, once they have reached the bloodstream, should be detected in the urine in a quantity reflective of serum levels. Although little is known about individual differences with respect to DNA adduct formation, tolerance for such adduction, and subsequent DNA repair mechanisms, age, metabolic rate, smoking, and dietary caloric and antioxidant intake may influence adduct formation and/or urinary detection (20). However, dietary sources of 8ohdG did not seem to influence the levels of the adduct detected in urine in an animal model (19).

Most studies of 8ohdG to date have focused on tissue levels of the adduct. Because urinary assays are noninvasive, their use is far simpler than those involving tissue samples. Moreover, urinary levels should reflect total body oxidative stress, a biological measure of great interest. Unfortunately, urinary assays require HPLC with electrochemical detection. This is technically cumbersome and has been inadequately validated as a marker of oxidative damage (19, 21, 22). In the few population-based studies that have been conducted, how-
ever, results conform to the hypothesis that urinary 8ohdG levels correlate with oxidative stress. For example, Lagorio et al. (23) showed a positive linear relationship between exposure to benzene and levels of 8ohdG in human urine, whereas Loft et al. (24) showed greater excretion of 8ohdG in smokers. As yet, however, there has been little consistency in these types of studies.

Recently, an ELISA kit was developed to quantify levels of 8ohdG in human urine (Genox Corp., Baltimore, MD). The availability of this simple commercial assay provides the opportunity to evaluate 8ohdG excretion in large population studies. In our study, we hypothesized that, as a result of the active inflammatory process, urinary levels of 8ohdG would be higher in *H. pylori*-infected individuals than in uninfected persons. To test this hypothesis, we evaluated the association between *H. pylori* infection and 24-h urinary 8ohdG in healthy young adults, adjusting for diet and a measure of lean body mass.

**Materials and Methods**

**Study Population.** Subjects were drawn from a large pool of PHA participants within the Northern California Kaiser Permanente Medical Care Program. Kaiser Permanente Medical Care Program offers an optional lifestyle and health assessment for both new members and established members who have not seen a doctor in 5 or more years. For our study, we recruited a race/ethnicity stratified sample of healthy young adults (ages 20–39) in the PHA program who were not known to have any underlying inflammatory conditions. After providing informed consent, subjects were asked to complete a questionnaire regarding dietary intake, alcohol and caffeine consumption, exercise, and smoking and then to provide 10 ml of sera and a 24-h urine sample. The Health Habits and History Questionnaire was used to assess dietary intake of macro- and micronutrients (25). This semiquantitative food frequency questionnaire, which has been validated in several different populations (26–29), elicits information about the type and quantity of foods eaten over the past month and the daily or weekly consumption of vitamin supplements.

Of 3340 members who enrolled in the PHA between June 1, 1992 and October 31, 1993, 567 were eligible to participate in this study based on age, gender, race, and complete dietary and interview information. Twelve of these subjects reported improbable dietary intake and were excluded from further study. Of the remaining 555 subjects, 281 (50.8%) provided adequate 24-h urine samples and were included in the final analysis.

**Serum Assays.** Sera were frozen at −70°C before testing. They were subsequently assayed by ELISA for the presence of IgG antibodies to *H. pylori* as described previously (30). One negative control, four low-titer *H. pylori*-positive controls, and one high-titer *H. pylori*-positive control were assayed at the same time as the study samples. The cutoff value for seropositivity was set at the mean level of the four low-titer *H. pylori* controls. This assay has been shown to be greater than 90% sensitive and specific for active *H. pylori* infection, as confirmed by bacteriological culturing of pathologicial specimens from 58 infected and uninfected subjects from a separate study. A random selection of 100 sera was also tested for creatinine in a commercial laboratory using a modified Jaffe reaction (Nichols Laboratory, San Luis Obispo, CA).

**Urine Collection, Storage, and Assays.** Urine was collected in plastic containers over a 24-h period by all study participants. Participants were told to void their first morning urine on the first day of collection and to collect every subsequent urine after that time, ending with the following day’s first urine. They were asked to return the urine sample within 24 h of the final collection or to freeze the sample if retained for greater than 24 h. Participants were asked whether the sample they returned represented the complete 24-h period. If the samples were deemed incomplete, the subject and the sample were not included in the analysis. Urine was stored at −70°C at the Kaiser Permanente Division of Research, where the total urine volumes were recorded. Individual aliquots were transported to our laboratory on dry ice and assayed for 8ohdG using a double-antibody ELISA kit (Genox Corp.). This assay uses a murine monoclonal primary 8ohdG antibody that is specific to the 8ohdG adduct in urine (N45.1: Ref. 31).

Urine samples were thawed and centrifuged to remove particulate matter. Six standards of known concentrations of 8ohdG (0.64, 3.2, 16, 80, 400, and 2000 ng/ml) and six blank wells were run concomitantly with triplicate urine samples. Fifty µl of sample urine and 50 µl of primary monoclonal antibody reconstituted in 1% BSA/phosphoric acid buffer were added to a microtiter plate that was precoated with 8ohdG-BSA conjugate. Plates were sealed and incubated for 1 h at 37°C, followed by a wash with 200 µl of 0.05% Tween 20/phosphoric acid buffer. One hundred µl of horseradish peroxidase-conjugated anti-mouse polyclonal secondary antibody solution was then added to each well, incubated, and washed. One hundred µl of hydrogen peroxide/phosphoric acid-reconstituted substrate was then added to each well, followed by reaction termination with 100 µl of 2 N sulfuric acid. Absorbance readings were taken 3 min later with a spectrophotometer at 492 nm. The average of the absorbance readings from the triplicate runs of each individual sample was determined, and the value of 8ohdG for each subject was obtained via comparison to a standard reference curve.

Creatinine values in urine were determined by a commercial laboratory using a colorimetric modified Jaffe reaction (Corning-Nichols Institute, San Luis Obispo, CA).

**Validation of the Urinary Assay.** Studies using the 8ohdG ELISA assay report excellent specificity of the N45.1 monoclonal antibody (31–33). An ELISA for the detection of 8ohdG used; the addition of known concentrations of deoxyribonucleosides including 2′-deoxy-adenosine, 2′-deoxycytidine, 2′-deoxyuridine, and 2′-deoxyguanosine to urine samples had little effect on the amount of 8ohdG detected (31). Similarly, an immunohistochemical study of 8ohdG in renal carcinogenesis showed minimal cross-reactivity between the N45.1 antibody and a wide array of purine- and pyrimidine-containing compounds or their breakdown products that are ubiquitous in urine. The ELISA assay achieved excellent specificity, a lower limit of detection, and a lower risk of production of 8ohdG during the pretreatment steps associated with HPLC (32).

When HPLC with electrochemical detection and the N45.1 ELISA assay were used to measure 8ohdG content in identical urine samples, the two methods had a high degree of correlation. The sensitivity was substantially higher, however, with the ELISA assay (limit of detection, 1 ng/ml). The amount of

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4 J. Parsonnet, unpublished data.

8ohdG detected in urine has not been shown to vary significantly within the range of physiological pH.6

Statistical Analysis. Statistical analysis was performed with SAS software (SAS, Cary, NC). Information from the dietary questionnaire was analyzed by the DIETSYS version 3.0 Dietary Analysis System (25). DIETSYS calculated supplemental and dietary intakes of vitamins separately. Univariate procedures were run for both the outcome variable (8ohdG) and the demographic and dietary variables to assure normal distributions before inclusion in parametric models. Variables that were not normally distributed were either log-transformed or square root-normalized for both univariate and multivariate analyses. For dietary variables in which the log- or root-transformed versions were not normally distributed, tertiles were created to represent individuals with low, medium, or high intake of the given nutrient or vitamin.

Because urinary excretion of 8ohdG should vary with lean body mass, we evaluated the correlation between 8ohdG and three measures of lean body mass: urinary creatinine, serum creatinine, and body weight. Because Ucr correlated most closely with 8ohdG excretion ($r = 0.4$), our outcome variable was expressed as micrograms of 8ohdG per gram of Ucr.

Univariate analyses were conducted using linear regression, ANOVA, or $t$ tests. Variables significant at $P < 0.15$ in univariate analysis were included in multivariate models. A regression matrix was run between all of the continuous dietary variables to test for colinearity before inclusion in a multiple linear regression model. Dietary variables with the strongest associations with 8ohdG excretion were retained, whereas those that were colinear with the retained variables were dropped. The SAS general linear models program (SAS, Cary NC) was used to test for statistical significance of the retained variables while controlling for potentially confounding factors in the model. Variable significance is reflected by $P$ values corresponding to the null hypothesis, whereas geometric means of each variable represent the type three sums of squares in the multiple regression model.

Results

The 281 subjects included 110 white, 82 African American, and 89 Hispanic men and women between the ages of 20 and 39 years. Table 1 lists characteristics of our sample by gender, age, and H. pylori infection. In a previous report for this study population, we found that antibodies to H. pylori infection were more common in males than in females, in Hispanics and African Americans than in whites, in persons from lower educational and income groups, and in persons born in developing countries (34).

Univariate Analysis. For all analyses, 8ohdG per gram of Ucr was log transformed. Values of total 24-h 8ohdG ranged from 1.76 to 143.40 $\mu$g/Ucr g, with a geometric mean value of 17.14 $\mu$g/Ucr g. Seventy-five % of values fell within the range of 7.22 $\mu$g/Ucr g to 26.72 $\mu$g/Ucr g. African-American race was significantly associated with lower levels of 8ohdG excretion compared with whites and Hispanics individually ($P < 0.05$ for both comparisons; Table 2). H. pylori infection was associated with lower levels of 8ohdG excretion in univariate analysis ($P = 0.044$; Table 2). There was a weak but significant correlation between 8ohdG and dietary carbohydrate intake ($P = 0.031$), whereas univariate correlations between dietary kilocalorie, protein, fat, and total vitamins A and C intake were insignificant (Table 3). Of the dietary variables with $P < 0.15$ in univariate regression with 8ohdG, dietary carbohydrates and total vitamin C intake were independent of one another (i.e., not colinear) while showing the strongest associations with 8ohdG excretion. These were, therefore, retained in the multivariate model. Dietary kilocalories, fat, protein, and sodium intake were colinear with carbohydrate intake and were dropped from the model; carbohydrate intake was retained as a marker for these dietary nutrients.

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6 Japan Institute for the Control of Aging, Fukuroi, Shizuoka, unpublished data.
Table 4  Multiple variable linear regression model of 8 ohdG excretion

<table>
<thead>
<tr>
<th></th>
<th>Mean 24-h 8ohdG (µg/g creatinine)</th>
<th>P</th>
</tr>
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<tr>
<td><strong>H. pylori infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>18.04</td>
<td>0.008</td>
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<tr>
<td>Positive</td>
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<td></td>
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<tr>
<td><strong>Gender</strong></td>
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<td></td>
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<tr>
<td>Female</td>
<td>16.01</td>
<td>0.883</td>
</tr>
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<td>Male</td>
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<td></td>
</tr>
<tr>
<td><strong>Race</strong></td>
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<td></td>
</tr>
<tr>
<td>White</td>
<td>17.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>African American</td>
<td>13.21</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>18.09</td>
<td></td>
</tr>
<tr>
<td><strong>Total carbohydrate intake</strong></td>
<td>NA*</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>Total vitamin C intake</strong></td>
<td>NA</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*NA, not applicable.

Multivariate Analysis. *H. pylori* infection was independently associated with lower levels of urinary 8ohdG (*P* = 0.008; Table 4); levels were 26% higher in uninfected persons than in infected individuals. As in the univariate analysis, African-American race continued to be significantly associated with lower levels of 8ohdG excretion (*P* < 0.001). Gender was not significantly associated with 24-h 8ohdG after adjusting for infection status, race/ethnicity, and dietary factors (*P* = 0.88). Daily dietary carbohydrate intake was positively correlated (*P* = 0.003), whereas daily total (dietary and supplemental) vitamin C intake was negatively correlated with 8ohdG excretion (*P* < 0.001). Factors not linked to 8ohdG excretion included: vitamin A intake, β-carotene and α-carotene intake, total antioxidant intake, smoking, education, and income. There were no significant interactions among these variables. Vitamin E intake could not be log- or root-normalized and was not included in the analysis.

Discussion

In this study, we hypothesized that 8ohdG excretion, a putative marker for oxidative stress, would be more pronounced in persons with chronic gastric inflammation, such as that produced by *H. pylori*, than in persons without inflammation. We therefore expected that urinary 8ohdG levels would either be higher in persons with infection or that no difference in 8ohdG levels would be observed because the amount of damage induced by *H. pylori* would be inconsequential in comparison to total body stress. We did not anticipate our actual findings; 8ohdG levels were considerably lower in the urine of *H. pylori*-infected individuals than in uninfected persons. This observation was unlikely to be a spurious finding; the same results were observed in a smaller pilot study and in a subset analysis of 100 randomly selected persons from among our total population. Furthermore, the results did not appear to be due to confounding by dietary variables, smoking, or exposures related to socioeconomic status. This result is particularly enigmatic when one considers that tissue levels of 8ohdG have been found to be elevated with *H. pylori* infection (11). Assuming that the assay is accurately detecting the adduct, this discordance of tissue and urinary levels is best explained in one of two ways: either *H. pylori* infection decreases DNA repair mechanisms; or the inflammatory process is destroying the adduct before it can be excreted in urine.

Hydroxylated guanine lesions are repaired in mammalian DNA via both base and nucleotide excision repair pathways (14, 16). In humans, 8-hydroxyguanine DNA glycosylase promotes base excision repair, whereas a specific 8-hydroxyguanine endonuclease catalyzes nucleotide excision repair of lesions (14). Levels of 8ohdG in tissue may increase either because there is a strong DNA-damaging stimulus or because one of the specific DNA repair mechanisms is deficient. In the former case, one would expect urinary adducts to rise in concert with increasing tissue levels; in the latter case, urinary adducts would not be correlated with quantities in tissues. DNA repair deficiencies have been reported in other inflammatory conditions, including urinary schistosomiasis and inflammatory arthritis. Badawi et al. (35) showed that a repair enzyme involved in removing methylated DNA lesions was significantly lower in bladder tissue from patients with schistosomiasis-related bladder cancer than in tissue from persons with normal bladder histology. This was not the case when tissue from patients with nonschistosomiasis bladder cancer were compared to normal tissue, suggesting that the infection was independently related to DNA repair activity. Harris et al. (36) reported decreased capacity to repair 8-OH-dG in lesions in patients with Bechet's disease, although it was unclear from their findings whether this was related to the disease or to some other factor (36). The inhibition of repair enzymes may involve induction of nitric oxide via inflammatory cell processes (37, 38). Nitric oxide was shown recently to inhibit the major enzyme involved in base excision repair of imidazole ring-opened 8ohdG lesions in bacteria, formamido-pyrimidine DNA glycosylase (39). This enzyme was found to be biochemically indistinguishable from the human nucleotide excision repair enzyme, 8-hydroxyguanine endonuclease, the end-product of which was measured in this study (15, 40). If *H. pylori* inhibits DNA repair (either via its induction of nitric oxide or some other mechanism), increased tissue levels of 8ohdG would be seen in conjunction with decreased urine levels. Alternatively, chronic damaging stimulus accompanying inflammation could deplete DNA repair enzymes in affected tissue and lead to the same divergence of tissue and urine levels.

Another explanation for our finding is that 8ohdG is being destroyed or metabolized in persons with inflammation. If this were the case, then urinary levels of 8ohdG in *H. pylori*-positive patients would not be reflective of either DNA damage or repair. Because excised adducts must be shuttled through the gastric epithelium, the interstitial fluid, the capillary system, the bloodstream, and the liver before being filtered in the kidneys and excreted in urine, numerous steps may impede urinary detection. 8ohdG is not an end metabolite but can be metabolized to hypoxanthine, xanthine, and other purine derivatives (41). The inflammatory response itself may, therefore, mask any increases in oxidative damage that could result from inflammatory ROS. Early animal studies on the stability of 8ohdG when injected i.v. into rats, however, showed that 8ohdG degraded little during transport from plasma to urine (19). This suggests that, if the adduct is being metabolized, the site of degradation is found before reaching the bloodstream (i.e., either the gastric mucosa where *H. pylori* resides or the lamina propria).

Several other explanations for our finding can be hypothesized. Recently, investigators have shown that neutrophils from patients with *H. pylori* infection are insensitive to stimulation by their own *H. pylori* strains but activate normally in response to stimulation by different *H. pylori* strains (42). Thus, it is possible that, after a period of infection, neutrophils in the stomach and in the periphery no longer respond to *H. pylori* and do not produce ROS. This hypothesis, however, is not com-

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pletely tenable because previous research indicates both increased ROS and increased 8OhdG in the mucosa of infected persons. *H. pylori* has also been shown to increase epithelial cell apoptosis. Thus, cells with oxidative damage may be shed in the gastric lumen and the DNA adducts excreted in stool, not in urine. Should this be the case, however, we would have expected 8OhdG levels in the urine to be the same, not lower, in persons with infection as those without. Finally, it is possible that *H. pylori* is merely a marker for an unidentified confounder that protects persons from oxidative DNA damage.

Urinary 8OhdG levels have been touted as a marker for oxidative stress. Indeed in our study, 8OhdG levels did correlate with some measures we would have expected. Daily total intake of vitamin C was significantly correlated with lower levels of 8OhdG. This supports the putative role of ascorbic acid as a potent antioxidant in living systems. Moreover, our findings of a significant inverse correlation between antioxidant intake and oxidative DNA damage corroborates findings of a recent study of antioxidants and oxidative DNA damage in human lymphocytes (43). Our data also support recent results of in vitro experiments demonstrating oxygen radical scavenger activity of ascorbic acid (44, 45). Finally, because increased caloric intake would generally be accompanied by increased excretion of nitrogenous wastes as well as increased metabolic activity of caloric protein, our finding that carbohydrate intake was positively associated with 8OhdG excretion conformed to our expectations. Despite these correlations, however, we remain unconvinced that urinary 8OhdG is a useful tool in measuring oxidative stress in the gastric environment. Although explanations for the negative association with *H. pylori* can be offered, there is little to favor one theory over another. It is quite possible that metabolism of the adduct within the mucosa renders any urinary findings meaningless or that the assay used in this study is cross-reacting with guanine or some other purine metabolite in urine. In light of our findings, we believe further population studies using urinary markers of this adduct should be deferred until the complexities of 8OhdG excretion are unraveled.

References


H. pylori Infection and Urinary Excretion of 80hdG


Helicobacter pylori infection and urinary excretion of 8-hydroxy-2-deoxyguanosine, an oxidative DNA adduct.

H L Witherell, R A Hiatt, M Replogle, et al.


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