Helicobacter pylori infection is associated with oxidatively damaged DNA in human leukocytes and decreased level of urinary 8-oxo-7,8-dihydroguanine

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Abbreviations: 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxo-7,8-dihydroguanine glycosylase (hOGG1); 8-oxodG, 8-oxo-7,8-dihydroguanosine (8-oxodG) and 8-oxo-7,8-dihydroguanine (8-oxoGua) as well as the level of oxidatively damaged DNA in leukocytes. Using high performance liquid chromatography prepurification/gas chromatography with isotope dilution mass detection methodology, we examined the amount of oxidatively damaged DNA products excreted into urine and the amount of 8-oxodG in the DNA of leukocytes' (with the the HPLC/EC technique in three groups of children: (i) control group, (ii) H. pylori infected children and (iii) children with gastritis where H. pylori infection was excluded. The levels of 8-oxodG in DNA isolated from leukocytes of H. pylori infected patients and in the group with gastritis without H. pylori infection were significantly higher than in DNA isolated from the control group. The mean level of 8-oxoGua in urine samples of children infected with H. pylori was significantly lower than in the urine of the group with gastritis without H. pylori infection. The data suggest that inflammation itself, not just H. pylori infection, is responsible for the observed rise of 8-oxodG level in cellular DNA, and increase in the base damage in DNA isolated from human gastric mucosa was detected during the early stages of H. pylori infection (5). Moreover, a correlation was detected between levels of oxidatively damaged DNA and the intensity of gastritis in H. pylori infected patients (4).

An alternative approach to assessing oxidatively damaged DNA on the level of the whole organism is determination of urinary excretion of oxidatively modified bases/nucleosides.

It is generally accepted that 8-oxo-7,8-dihydroguanine (8-oxoGua) excised from DNA by cellular repair is excreted into the urine without further metabolism (6). There is some evidence that the nucleotide excision repair (NER) system may act upon common, non-bulky, oxidative DNA damage such as 8-oxoGua and thymine glycol (Tg) (7–9), presumably to yield, in the first instance, lesion-containing, oligonucleotide products. Lesion-containing oligomers from NER may be subject to intra- or extra-cellular 5′-3′ exonuclease digestion to ultimately produce oligomers 6–7 nt long. The latter may however be degraded further and such poorly characterized post-excision processing may ultimately yield 8-oxodG. There is some evidence that lesion-containing oligomers may be present in urine and it is feasible that these may in part be derived from NER (10), although the presence of 8-oxodG-containing oligomers in urine is controversial (11). Oxidatively damaged DNA bases are mostly repaired by the base excision repair (BER) pathway, although some components of the NER pathway may also play a role in the repair of some oxidized bases in DNA (12,13). Therefore, the assays which are able to determine the level of 8-oxodG as well as the amount of 8-oxoGua (as well as other base products) in urine may better reflect oxidative damage of cellular DNA. The analysis of 8-oxoGua in urine presents particular difficulties (14,15) and until recently there has been no reliable assay for its detection. Recently a new methodology was...
developed which allowed for simultaneous determination of 8-oxodG and 8-oxoGua as well as 5-(hydroxymethyl)uracil (5-HMUrA) in the same urine sample. This method involved an HPLC prepurification followed by gas chromatography with isotope dilution mass spectrometric detection (16). Using this method we have found that urinary excretion of 8-oxoGua and 8-oxodG does not depend on diet in the case of humans and may reflect involvement of different repair mechanisms (17). Moreover, our data indicated that urinary excretion of 8-oxoGua is higher in cancer patients in comparison with the control group (18).

There are some evidences which suggest that H. pylori infection may lead to development of several extragastroduodenal pathologies (3). Helicobacter pylori infection is usually acquired during childhood and persists throughout the individual’s life span and thus can be responsible for persistent oxidative stress that in turn may be associated with several pathologies.

In order to assess whether the infection may impose oxidatively damaged DNA in other organs than in the one targeted (stomach) we decided, for the first time, to analyse the two kinds of oxidatively damaged DNA biomarkers: urinary excretion of 8-oxodG and 8-oxoGua as well as the level of oxidatively damaged DNA in leukocytes. (Leukocytes are often used as surrogate cells, which are supposed to inform about oxidative stress—measured as a certain level of 8-oxodG— in other tissues) (19). There is a great deal of experimental data which suggests that inflammation leads to the production of ROS, with subsequent oxidatively damaged DNA. To verify a possibility that oxidatively damaged DNA might be the result of an inflammatory condition not necessarily linked with H. pylori infection the above described parameters were analysed in three groups of children: (i) control group, (ii) H. pylori infected children and (iii) children with gastritis where H. pylori infection was excluded.

Materials and methods

Patients

The study was conducted in three groups of children. The control group (i) consisted of 9 boys and 11 girls without gastritis and duodenitis. Median age in this group was 13.5 years (range 10–17 years). None had a history of chronic inflammation disease and cancer and none had acute infection. All of them had also negative C13 urea breath test which is considered to be an accurate diagnostic test for the detection of H. pylori infection (20).

The patient group (ii) comprised 10 boys and 13 girls with recognized gastritis and duodenitis without H. pylori infection. The median patient age was 11.5 years (range 5–18 years). No patient had any other inflammation disease or cancer history. The patient group (iii) comprised 10 boys and 10 girls with recognized gastritis and duodenitis and H. pylori infection. The median patient age was 12 years (range 7–17 years). No patient had any other inflammation disease or cancer history.

All the patients with recognized gastritis and duodenitis were examined with gastroduodenal endoscopy because of permanent abdominal pain. During the endoscopy the mucose picture was estimated and a biopsy was made. The biopsy specimens were examined with histological and urease tests. In the case of gastritis and duodenitis the histological picture showed inflammation of single- and poly-nucleated cells, glandular mucose atrophy or intestinal metaplasia. However, in specimens with the H. pylori infection there was also lymphoid nodule (MALT) hyperthrophy in mucose and, in addition, specific bacterial cells (H. pylori). The patients with positive urease test and specific changes in histological picture were recognized as H. pylori infected.

The control group was chosen in such a way that the following criteria matched the patient group: eating habits, age, body weight and sex. All children were non-smokers.

The study was approved by the medical ethics committee of The Ludwik Rydygier Medical University Bydgoszcz, Poland, No. 405/2003 (in accordance with Good Clinical Practice, Warsaw 1998). All the parents of patients and patients older than 16 years gave informed consent.

Isolation of leukocytes from venous blood

Venous blood samples from the patients were collected. The blood was carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich, St Louis, MO) and leukocytes were isolated by centrifugation according to the procedure laid down by the manufacturer.

DNA isolation and 8-oxodG determination in DNA isolates

DNA from leukocytes was isolated using the method as described earlier (21). Determination of 8-oxodG by the mean of high performance liquid chromatography (HPLC/EC technique was described previously (22).

Urine analysis

Spot urine samples were collected. Aliquots of Aliquots of 0.5 nmol of [15N5] 8-oxoGua, 0.05 nmol of [15N5] 8-oxodG and 10 μl of acetic acid (Sigma, HPLC grade) were added to 2 ml of human urine. Isotopic purity of the applied standards was 97.65 and 98%, respectively. After centrifugation (2000 × g, 10 min), supernatant was filtered through a Millipore GV13 0.22 μm syringe filter and 500 μl of this solution was injected into the HPLC system. In the pilot study isotopically labelled internal standards of unmodified compounds (1 nmol of [13C3] Gua and 1 nmol of [15N5] dG) were added to the urine samples to monitor fractions containing both these compounds and to avoid overlapping of the peaks containing the modified and unmodified base/ nucleoside. Isotopic purity of the applied base and nucleoside standards was 96.4 and 98.0%, respectively.

Urine HPLC purification of 8-oxoGua and 8-oxodG was performed according to the method described by Gackowski et al. (17,18).

Gas chromatography with isotope dilution mass detection (GC/MS) analysis was performed according to the method described by Dizdaroglu (5,23), adapted for additional [15N5] 8-oxoGua analyses (m/z 445 and 460 ions were monitored.)

Statistical analysis

All results are expressed as mean ± SD. The STATISTICA (version 6.0) computer software (StatSoft, Tulsa, OK) was used for the statistical analysis. Student’s t-test (for variables with normal distribution) and Mann–Whitney’s U-test (for variables with abnormal distribution) were carried out. For normal distribution, variables were analysed by the Kolmogorov–Smirnov test with Lilliefors correction. Statistical significance was considered at P < 0.05.

Results

The levels of 8-oxodG in DNA isolated from leukocytes of H. pylori-infected patients and in the group with gastritis without H. pylori infection were significantly higher than in DNA isolated from the control group (5.16 and 5.70 versus 4.00 per 10⁶ dG, respectively, Table I). The mean levels of 8-oxodG are in the range of values reported recently by others (24,25). Interestingly, 8-oxodG level in the DNA of lymphocytes may vary significantly according to country. In Ireland the level is very close to that reported in our study (26). The mean level of 8-oxoGua in urine samples of children infected with H. pylori reached the value of 7.86 ± 2.50 nmol/mmol of creatinine (Table I). This level was significantly lower than in the urine of the group with gastritis without H. pylori infection, where the levels reached the values of 12.34 ± 6.48 nmol/mmol of creatinine (P = 0.0063). In the control group the mean level was 9.37 ± 4.51 nmol/mmol of creatinine. This value was not statistically different from that of the two other groups (Table I). The concentration of the modified nucleoside in urine samples was similar in all groups of subjects (Table I). We did not find correlation between any of these parameters and the age of the studied subjects.

Discussion

It has been demonstrated previously that H. pylori infection may lead to increase of 8-oxodG level in gastric mucosa of the patients (5). Since this lesion has mutagenic properties, the
increased level may be a causative factor involved in development of gastric cancer. The results of the present study show that *H. pylori*-induced rise in the level of the lesion is not restricted to gastric mucosa. We have found that in DNA isolated from leukocytes of patients infected with *H. pylori* the level of 8-oxodG was significantly higher than in leukocytes of control subjects.

Our findings may, at least partially, be explained by the previous reports that described a significant increase in oxidative stress in the blood of infected subjects. As a consequence a significant increase in serum malondialdehyde and decrease in ascorbic acid concentration were observed in *H. pylori*-infected patients with gastritis in comparison with control subjects (27).

However, the level of the modification was also elevated in patients who suffered gastritis without signs of *H. pylori* infection which in turn suggests that the inflammation itself, not just *H. pylori* infection, is responsible for the observed rise of 8-oxodG level in leukocytes.

Since the level of the modified nucleoside/base in urine may be a good indicator of oxidative DNA insult on the level of the whole organism and may be used as indicator of DNA repair (28,29), in the present study we examined whether the amount of 8-oxoGua and 8-oxodG excreted into urine is higher in both groups of children with gastritis when compared with the control group.

To analyse oxidatively damaged DNA, in our work we decided to analyse urinary excretion of oxidatively modified bases/nucleosides using HPLC/GC/MS methodology. In addition to unequivocal identification of the analysed compounds and high sensitivity, isotopically labelled internal standards used in this approach allowed compensation for eventual losses of the analysed products. It is also worth mentioning that in addition to 8-oxodG analyses our methodology enabled measurement of the modified base (8-oxoGua).

For urinary 8-oxodG no differences were found between the study groups. Surprisingly, the level of 8-oxoGua was significantly lower in urine of *H. pylori*-infected children than in the two other groups although only the difference between *H. pylori*-infected children and the other group of children with gastritis was statistically significant. Witherell *et al.* (30) have found that patients with *H. pylori* infection had significantly lower amounts of 8-oxodG in the urine than in the urine of persons without infection. The inconsistency between our results and the aforementioned work may be explained, at least partially, by a different methodological approach. In the Witherell *et al.* study 8-oxodG level was determined with enzyme-linked immunosorbent assay (ELISA). However, it has been shown that ELISA estimates were about two times higher than HPLC or HPLC/GS/MS estimates (31). This in turn suggests that the monoclonal antibody used in the ELISA kit is not sufficiently specific for the detection of 8-oxodG (32). It is possible that the antibody used in the ELISA kit may be cross-reactive towards 8-oxoGua. Therefore, the changes detected in the work of Witherell *et al.* (30) may describe 8-oxodG plus 8-oxoGua excretion. The authors concluded that: (i) DNA repair is deficient in infected subjects, (ii) that inflammation destroys the adduct or (iii) urinary 8-oxodG is not an accurate measure of gastric DNA damage. In the light of our results the second possibility should be ruled out, since in the group of children with the inflammatory condition without *H. pylori* infection a significant increase in urinary excretion of 8-oxoGua was found. The elevated level of both—8-oxodG in leukocytes DNA and urinary excretion of 8-oxoGua—in children with gastritis without *H. pylori* infection—may be a sign of oxidative stress on the level of the whole organism, caused by inflammation. A similar phenomenon was described by us for cancer patients (18). However, in children with *H. pylori* infection a decrease in the level of modified base in urine was observed which seems to be specific for this group of patients. The obvious question is what is responsible for this phenomenon.

A number of literature reports and our data indicate that the BER, namely human 8-oxo-7,8-dihydroguanine glycosylase (hOGG1) is responsible for the presence of 8-oxoGua in urine (for a discussion see Refs 29 and 33). We propose that a possible explanation of the observed decrease of 8-oxoGua urinary excretion in *H. pylori*-infected children is generation of nitric oxide (NO) associated with the infection (34,35). Generation of NO, in turn, results in a reduction of the activity of hOGG1, the main enzyme responsible for the removal of 8-oxoGua from cellular DNA (36).

The infection acquired in childhood is carried for life in the majority of infected individuals and can cause diseases such as peptic ulcers, chronic atrophic gastritis and gastric cancer later in life (37). Moreover, there is some evidence which suggests that the infection may lead to the development of several extragastroduodenal pathologies (3). Our results suggest that premutagenic oxidatively damaged DNA which arises as a consequence of the infection may serve as a source of mutations which may lead to cancer development.

In conclusion, in the light of the presented data it is likely that severe DNA damage imposed by *H. pylori* on the level of the whole body during early childhood may be associated with development of many types of cancer later on. However, at present it is impossible to answer directly the question concerning the involvement of oxidatively damaged DNA associated with the infection in cancer origin since full development of the disease in response to carcinogen exposure takes 20–40 years. Nevertheless, it should be remembered that ROS are one of the most attractive factors to explain the possibility of an association between cancer formation and *H. pylori* infection because besides the fact that ROS are induced by the pathogen, they have well established mutagenic potential and they are also products of endogenous processes. Moreover, inhibition of the removal of 8-oxoGua, which may be a consequence of the infection, can be one of the factors responsible for the accumulation of this potentially mutagenic modification.

### Table I. Analytical parameters of the study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>8-oxodG/10^6dG in leukocytes’ DNA</th>
<th>8-oxoGua excretion (nmol/mmol creatinine)</th>
<th>8-oxodG excretion (nmol/mmol creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (N = 20)</td>
<td>4.00 ± 1.06</td>
<td>9.37 ± 4.51</td>
<td>1.53 ± 0.52</td>
</tr>
<tr>
<td>Gastritis without <em>H. pylori</em></td>
<td>5.70 ± 1.48^a</td>
<td>12.34 ± 6.48^c</td>
<td>1.98 ± 1.12</td>
</tr>
<tr>
<td><em>H. pylori</em> infected (N = 20)</td>
<td>5.16 ± 1.74^d</td>
<td>7.86 ± 2.50</td>
<td>1.79 ± 0.59</td>
</tr>
</tbody>
</table>

^aStatistically significant difference versus control group (Student’s t-test \( P < 0.05 \)).

^bStatistically significant difference versus control group.

^cH. pylori infected group.
Acknowledgements

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References


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