Relevance to Human Cancer of *N*-Nitroso Compounds, Tobacco Smoke and Mycotoxins.
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EXPOSURE TO *N*-NITROSAMINES AND OTHER RISK FACTORS FOR GASTRIC CANCER IN COSTA RICAN CHILDREN

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The hypothesis that endogenous chemical nitrosation in the normal stomach in early life could play a crucial role in inducing chronic atrophic gastritis/intestinal metaplasia in later life was tested by applying the *N*-nitrosoproline (NPRO) test to 12-h urine samples from about 50 children (aged 8–14 years) living in high- and low-risk areas for stomach cancer. The median values of NPRO and the sum of four nitrosamino acids analysed were 0.28–0.84 μg/12 h and 0.75–1.75 μg/12 h, respectively. The NPRO level after proline intake was significantly higher in children from a high-risk area than in those from a low-risk area ($p < 0.04$), and markedly reduced after ingestion of ascorbic acid and proline ($p < 0.05$). Urinary nitrate level was lower than that of adults. NPRO levels on the day of proline intake, however, correlated well with nitrate levels ($p < 0.001$), indicating that children in a high-risk area in Costa Rica have high endogenous nitrosation potential. Blood samples were also collected from about 300 children (aged 7–20 years) and analysed for antibodies against *Campylobacter pylori*, a suspected gastritis-causing bacteria. About 71% of children in both high- and low-risk areas for stomach cancer had antibodies. In addition, raw and cooked beans, which are consumed very frequently in Costa Rica, were collected from families in both areas and analysed for levels of nitrite/nitrate, total *N*-nitroso compounds and genotoxicity in the SOS chromotest. Mean levels of total *N*-nitroso compounds in an aqueous extract (pH 2) of cooked bean samples from high- and low-incidence areas were similar (0.4–0.6 nmol/g of cooked beans). Acid-catalysed nitrosation of the same aqueous extracts produced levels up to 2.4 μmol/g of cooked beans. There was no difference in mean levels of nitrosation-dependent total *N*-nitroso compounds between samples from the two areas. Only two out of 11 extracts from the low-incidence area and two out of 14 from the high-incidence area showed weak direct genotoxicity. After acid-catalysed nitrosation, all samples were genotoxic at similar levels.

Stomach cancer is the most common cause of death from cancer in Costa Rica: in 1984, it caused 33% of deaths from cancer in males and 22% in females. For the period of 1980–83, the age-adjusted incidence rate was 58.8 per 100 000 for men and 25.2 per

100 000 for women. Although these incidence rates are declining, they are the highest in Latin America, and internationally they are second only to those observed in Japan. There is, however, great variation in rates in this small country (51 000 km2) which has a relatively homogeneous population: for men, the rate varies from 84.2 per 100 000 in the highlands of the centre of the country to 25.4 in the coastal areas (Sierra *et al.*, 1989). These data suggest that environmental factors and habits in the different regions could be important risk factors.

It has been postulated that *N*-nitroso compounds, in particular *N*-nitrosamides, are formed intragastrically by bacteria colonized in the stomach, on the basis of studies of patients with chronic atrophic gastritis (a precancerous condition of the stomach; Correa *et al.*, 1975; Correa, 1988). However, it is a matter of controversy whether the level of *N*-nitroso compounds formed in the stomach of such patients is higher than that of normal subjects (Bartsch *et al.*, 1989); patients with chronic atrophic gastritis did not excrete more *N*-nitrosoproline than patients with a normal stomach after ingestion of proline and nitrate (Crespi *et al.*, 1987). On the basis of these results and epidemiological data, in particular that from studies of migrants, it has been hypothesized that *N*-nitroso compounds formed by acid-catalysed chemical nitrosation in the normal stomach in early life could play a crucial role in gastric carcinogenesis (Crespi *et al.*, 1987; Ohshima *et al.*, 1988).

We have postulated that bacterial colonization in the stomach, especially by the bacterium *Campylobacter pylori*, recently identified in human stomach mucosa as a possible etiological agent of gastritis, may contribute to gastric carcinogenesis by inducing chronic irritation or inflammation. We tested these hypotheses in Costa Rican children living in high- and low-risk areas for stomach cancer, using the *N*-nitrosoproline test and serological examination for *C. pylori* infection to assess exposures.

One community in a high-risk area (Turrubares; age-adjusted incidence rate of gastric cancer, 61.3/100 000) and one in a low-risk area (Hojancha; 18.7/100 000) for stomach cancer were selected on the basis of the following criteria: rural community, a large enough population of school children, similar ethnic characteristics and accessibility. A list was produced of all children aged 8–14 years in each community, and 25 subjects were selected at random from each list. Two samples of 12-h overnight urine were collected from children after they had ingested 500 mg proline and 200 mg ascorbate or 500 mg proline alone 1 h after the evening meal. The urine samples were analysed for *N*-nitrosamino acids and nitrate as exposure markers, as described previously (Ohshima *et al.*, 1987).

Blood samples were also collected from about 200 children aged 7–13 years and from about 80 volunteers aged 14–20 in the same communities and analysed for antibodies to *C. pylori*, using a method described by Peña *et al.* (1990).

Levels of N-*nitrosamino acids and nitrate*

The results of analyses for *N*-nitrosoamino acids and nitrate are given in Table 1. The levels of *N*-nitrosamino acids were much lower (1/5–1/10) than those detected in adult urines collected in Japan, China, Poland and other countries (Bartsch *et al.*, 1989). It is not known whether the low levels in Costa Rican children are due to their low body weight or to other factors related to age, or to geographical, environmental and dietary factors, such

Table 1. Median (95% confidence intervals) amounts of *N*-nitrosamino acids (μg/12 h) and nitrate (mmol/12 h) excreted in 12-h urine[a]

Urinary component[b]	Low-risk area		High-risk area	
	Day 1 (proline plus vitamin C)	Day 2 (proline)	Day 1 (proline plus vitamin C)	Day 2 (proline)
Volume (ml)	415.00 (395–542)	445.00 (396–559)	327.00 (293–473)	377.00 (286–460)
N-Nitrosamino acids				
NPRO	0.28 (0.21–0.51)	0.54 (0.43–1.09)	0.66 (0.44–1.29)	0.84 (0.89–2.44)
NTCA	0.48 (0.38–1.27)	0.52 (0.37–1.48)	0.40 (0.33–1.64)	0.77 (0.64–1.80)
NMTCA	0.05*(0.05–0.10)	0.05 (0.04–0.51)	0.05 (0.04–0.40)	0.05 (0.04–0.30)
Sum	0.75 (0.73–1.80)	1.25 (1.08–2.82)	1.62 (1.00–3.13)	1.75 (1.67–4.44)
Nitrate	0.23 (0.22–0.38)	0.20 (0.21–0.39)	0.29 (0.26–0.66)	0.23 (0.17–0.87)

[a] 26 children (19 boys and 7 girls) and 25 children (15 boys and 10 girls) from high- and low-risk areas for stomach cancer, respectively, participated in the study; their mean ages were 11.3 and 10.5 years, and mean body weights were 36 and 34 kg, respectively

[b] NPRO, *N*-nitrosoproline; NTCA, *N*-nitrosothiazolidine 4-carboxylic acid; NMTCA, *N*-nitroso-2-methythiazolidine 4-carboxylic acid

as a high intake of ascorbic acid (dietary data obtained in these areas are currently being analysed; Sierra *et al.*, in preparation). All comparisons of levels of *N*-nitrosoproline were statistically significant. Thus, the level was reduced by ingestion of ascorbic acid with proline compared with that seen with proline alone in both areas (low-risk area, $p < 0.01$; high-risk area, $p < 0.05$; Wilcoxon test); and the level was significantly higher in children in the high-risk area than in those in the low-risk area after ingestion of ascorbic acid plus proline ($p < 0.02$) and after ingestion of proline alone ($p < 0.04$; both Mann-Whitney test). Thus, the level of *N*-nitrosoproline after proline intake was higher in children from the high-risk area than in those from the low-risk area and markedly reduced after ingestion of ascorbic acid and proline. The nitrate level was also lower (1/2–1/3) than that in adult urines; however, the *N*-nitrosoproline levels on the day of proline intake correlated well with the nitrate levels ($p < 0.001$). These results indicate that children living in this high-risk area in Costa Rica have a higher potential for endogenous nitrosation than those living in the low-incidence area, although the absolute values are lower than those of adult subjects in other countries.

Infection with Campylobacter pylori

As shown in Table 2, seropositivity for *C. pylori* in young subjects was 62.6-71.7% for IgG antibody and 38.7–73.0% for IgA antibody. There was no difference between subjects living in high- and low-risk areas; however, seropositivity and average antibody values were greater in some groups of children in the age range 14–20 years than in that of 7–13 years. These results indicate that the majority of the children studied were infected with *C. pylori*

Table 3. Concentrations of total *N*-nitroso compounds (NOC) and genotoxicity in the SOS chromotest (SOS induction potency; SOSIP) of acidic aqueous extracts of cooked beans collected in high- and low-risk areas for stomach cancer in Costa Rica

Area	No. of samples analysed[a]	Mean (range) of total NOC (nmol/g cooked beans)		Mean (range) of SOSIP (per g cooked beans)		SOSIP/μmol total NOC
		Before nitrosation	After nitrosation	Before nitrosation	After nitrosation	(After nitrosation)
High-risk	14/13	0.57 (0–1.7)	1231 (560–1600)	4 (0–28)	96 (38–162)	58 (26–92)
Low-risk	11/11	0.42 (0–1.8)	1322 (798–1810)	8 (0–44)	80 (22–189)	45 (12–80)

[a] Before (first figure) and after nitrosation (second figure). Nitrosation was carried out *in vitro* at pH 2 at 37°C for 60 min with 100 mmol/l $NaNO_2$. The reaction was stopped by adding an excess of sulfamic acid. Total NOC concentration was determined by the method of Pignatelli *et al.* (1987) and genotoxicity by the SOS chromotest as described by Malaveille *et al.* (1989).

Conclusion

We have demonstrated that Costa Rican children living in a high-risk area for stomach cancer have a greater potential for endogenous nitrosation than children living in a low-risk area; the levels of *N*-nitrosamino acids detected in their urine were, however, much lower than those in adults living in other countries. *C. pylori* infection, assessed by serological examination, was found to be very prevalent in children in both areas, indicating that gastritis occurs very early in Costa Rican children and persists for a long time. The genotoxicity and total concentrations of *N*-nitroso compounds in beans consumed very frequently in Costa Rica, before and after nitrosation *in vitro*, were similar for the two areas. Further studies are required to identify other factors that determine the risk for developing gastric cancer in the inhabitants of the high-risk area.

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