In Vivo Nitrosoproline Formation and Other Risk Factors in Costa Rican Children from High- and Low-Risk Areas for Gastric Cancer[1]

Rafaela Sierra, Anne Chinnock, Hiroshi Ohshima, Brigitte Pignatelli, Christian Malaveille, C. Gamboa, Sibylle Teuchmann, Nubia Muñoz, and Helmut Bartsch[2]

University of Costa Rica, San José, Costa Rica [R. S., A. C., C. G.], and International Agency for Research on Cancer, Lyon, France [H. O., B. P., C. M., S. T., N. M., H. B.]

Abstract

The hypothesis that intragastric synthesis of N-nitroso compounds (NOC) in early life could play a role in gastric carcinogenesis was tested by applying the N-nitrosoproline (NPRO) test to about 50 children living in high- and low-risk areas for stomach cancer in Costa Rica. The median values of excretion of NPRO and the sum of three nitrosamino acids (μg/12 h urine) were 10–20% of those in adults from other geographical high-risk areas for stomach cancer. The urinary NPRO level after proline intake was higher in children from the high-risk area ($P < 0.04$) and markedly reduced after ingestion of ascorbic acid together with proline ($P < 0.05$). NPRO levels on the day of proline intake were highly correlated with levels of nitrate excretion ($P < 0.001$).

Mean levels of total NOC in an aqueous (pH 2) extract of cooked beans from the high- and low-risk areas were similar. Acid-catalyzed nitrosation of the extract increased the total NOC concentration up to 1000-fold, but there was no difference between samples from the two areas. About 10% of bean extracts from both areas showed weak direct-acting genotoxicity in Escherichia coli; after acid-catalyzed nitrosation, all samples were genotoxic at similar levels.

The diet of children in the low-risk area satisfied recommended levels of intake of energy and most nutrients except riboflavin and retinol equivalents. Diets from the high-risk area were deficient in energy intake and all nutrients except protein and vitamin C.

Blood samples were collected from 276 children and young adults from the same areas and analyzed for serum antibodies against Helicobacter pylori. Although very high, no significant difference was found in the prevalence of IgG or IgA antibodies between the two regions (R. Sierra, et al., Cancer Epidemiol., Biomarkers & Prev., 1: 449–454, 1992). The results implicate intragastrically formed NOC or other nitrite-derived carcinogens in stomach cancer etiology.

Introduction

Stomach cancer, the most common cause of death from cancer in Costa Rica, in 1984 caused 31% of deaths in males and 19% in females (1). Although the incidence rates are declining, they are the highest in Latin America, and internationally they are second only to those observed in Japan (2). For the period 1984–1988, the age-adjusted incidence rates were 46.4/100,000 men and 21.6/100,000 women. There is, however, great variation in rates in this small country (51,000 km²), which has a fairly homogenous population; for men, the rate varies from 66.1/100,000 in the center of the country to 26.8/100,000 in the coastal area. These data suggest that environmental factors and habits in the different regions are responsible for the differences in risk.

On the basis of studies of patients with chronic atrophic gastritis, a precancerous condition of the stomach, it has been postulated that NOC[3], in particular N-nitrosamides, are formed intragastrically in the colonized stomach via bacterial reduction of ingested NO_3^- to NO_2^-, and subsequent nitrosation of precursors by bacterial enzymes could play a role in the cancer etiology (3, 4). However, it is still unclear whether the level of NOC formed in the stomach of patients with precancerous conditions is higher than that of normal subjects. In most studies (reviewed in Ref. 5), patients with chronic atrophic gastritis did not excrete more NPRO than subjects with a histologically normal stomach after ingestion of proline and nitrate (6). Similarly, gastric juice from patients with precancerous gastric conditions did not contain higher levels of total NOC than that of asymptomatic subjects (7, 8). On the basis of these and other data (in particular from epidemiological studies of migrants), it has been hypothesized that carcinogenic NOC formed in the normal stomach by acid-catalyzed (chemical) nitrosation in early life could play a crucial role in gastric carcinogenesis (6, 9). To collect support for these hypotheses, we have applied the NPRO-test to Costa Rican children living in high- and low-risk areas for stomach cancer.

Chronic infection by the bacterium Helicobacter pylori in human stomach mucosa has been identified as a possible etiological agent causing gastritis that may contribute to gastric carcinogenesis by inducing chronic irritation or inflammation. Therefore, in addition, serum levels of antibodies to H. pylori and of pepsinogens were measured in young

[3] The abbreviations used are: NOC, N-nitroso compounds; NPRO, N-nitrosoproline; NAA, N-nitrosamino acids.

people (8–20 years of age) from the same two areas. Detailed results have been reported elsewhere (10). Food consumption data on children from the high- and low-risk areas were compared. As nitrosation of fava beans produced a powerful mutagen (11, 12), we measured the mutagenicity and levels of total NOC before and after nitrosation of aqueous extracts of beans that are commonly consumed by inhabitants of the two study areas.

Methods

Study Subjects

One community in a high-risk area for stomach cancer (Turrubares; age-adjusted incidence rate, 66.1/100,000 in males) and one in a low-risk area (Hojancha; 26.8/100,000) (2) were selected in Costa Rica on the basis of the following criteria: rural community; a sufficiently large population of school children; and similar ethnic characteristics and accessibility.

NPRO Test. We randomly selected 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, to participate in the study. Their mean ages ± SD (range) were 11.3 ± 2.2 (8–14) and 10.5 ± 1.7 (8–14) years, and mean body weights ± SD (range) were 37 ± 10.4 (19–65) and 34 ± 7.1 (20–52) kg, respectively. Two samples of 12-h overnight urine were collected from the children after they had ingested 500 mg of proline together with 200 mg of vitamin C 1 h after the evening meal on day 1 or 500 mg of proline alone on day 2. The urine samples were collected and analyzed for N-nitrosamino acids and nitrate as exposure markers, as described previously (13).

Analysis of Raw and Cooked Beans

Sixty-two samples of beans (*Phaseolus vulgaris* L.) that had been cooked by the families and stored at room temperature for up to 9 days were collected from the two study areas and kept frozen at −30°C before analyses. Both the raw and cooked beans were analyzed for nitrate according to Green *et al.* (14).

Aqueous extracts of boiled beans were prepared as follows: 4 g of cooked beans were mixed with 4 ml of 0.4 M KCl-HCl buffer of pH 2. After vigorous shaking, the pH was adjusted to pH 2 with 0.2 N HCl. The mixture was incubated with gentle shaking in the dark for 10 min at 37°C and then centrifuged for 10 min at 1900 *g*. Levels of NOC and genotoxic activity before and after *in vitro* nitrosation were measured in the supernatant. Nitrosation was carried out *in vitro* at pH 2 at 37°C for 60 min with 100 mmol/liter $NaNO_2$.

Determination of Total NOC

Total NOC in acidic aqueous extracts of boiled bean samples before and after *in vitro* nitrosation ($NaNO_2$ 100 mmol/liter; pH 2; 1 h at 37°C) were determined by a group-selective method as previously described (15). The limit of detection was 0.01 µmol/liter.

Genotoxic Activity

Acidic aqueous extracts of cooked bean samples, before and after *in vitro* nitrosation, were assayed for genotoxicity by the SOS chromotest as previously described (16).

Food Consumption

Fifty school-aged children, including all of those participating in the NPRO test (7 to 14 years of age), were randomly

selected from each area from the total populations of school-aged children in Hojancha and Turrubares. Data on the food consumption were recorded over a 2-day period for each subject. On day 1 each child was given instructions on how to record foods and drinks consumed during that and the following day. On day 2 the child and mother were interviewed by a final-year nutrition student in order to note the recalled types and amounts of food consumed on day 1. The amounts of food were estimated by weighing similar portions of foods available in the houses or local shops and in a few cases from household measures and life-sized drawings. The same procedure was repeated on day 3. The nutritive value of the foods consumed was calculated by use of various food composition tables (17–19) and the dietetic analysis program for microcomputers "Nutri-Calc" (20). The nutritional adequacy of the diet in each community was determined by comparison with recommended intakes (21–23).

A comparison of food consumed between the two communities was performed for foods or food groups with at least 10 measurements in each community. For each of the foods or food groups and nutrients, the distribution of consumption values was tested for normality with the Kolmogorov-Smirnov one-sample test. For foods with distributions found to be normal, a comparison of the means was performed by using the *z* test, and for the distributions differing significantly from normal, the Mann-Whitney test was used. In the case of nutrients without normal distributions, the distribution of the log-transformed values was tested for normality. The means (real or log-transformed values) of nutrient intake were compared between the two communities by means of the *z* test.

The following nutrients were found to be normally distributed in both communities ($P > 0.05$): energy; protein; carbohydrates; phosphorus; iron; thiamine; and dietary fiber. When the remaining nutrients were converted to their natural logarithms, the log-transformed values of the following nutrients were found to be normal ($P > 0.05$): fat; carotenes; calcium; riboflavin; niacin; and vitamin C. The log-transformed values of vitamin E, retinol, and retinol equivalents were nonnormally distributed ($P < 0.05$) and were, therefore, excluded from further analysis. In the case of vitamin C, some of the records included zero values and these were converted to one-tenth of the minimum value found in order to compare the means of the log-transformed data.

Results

Demographic Comparison of Study Areas. Data taken from the family of children who participated in the dietary study showed that the mean monthly family income (means ± SD in colons, 6532 ± 4945 *versus* 6179 ± 3376) was slightly higher in the low-risk area than in the high-risk area. The proportion of household heads who cultivate their own land was greater in the low-risk area (37.5% *versus* 0) and a higher proportion of household heads was agricultural laborers in the high- (44%) than in the low-risk area (18.8%). Also, significantly more household heads in the low-risk area (82.8%) worked on a permanent basis than in the high-risk area (46.2%). The percentage of families who owned refrigerators was similar in the two communities (40.6% in the low-risk area *versus* 46.2% in the high-risk area).

Levels of NAA and Nitrate in Urine. The results of analyses for NAA and nitrate in the 12-h urine and *P* values for com-

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

Urinary component	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)	415.0 (370–520)	445.0 (395–570)	327.5 (230–515)	377.5 (215–535)
NPRO	0.28 (0.20–0.36)	0.54 (0.36–0.86)	0.66 (0.31–0.98)	0.84 (0.51–1.67)
NTCA	0.48 (0.17–0.80)	0.52 (0.13–0.80)	0.40 (0.15–1.07)	0.77 (0.40–1.30)
NMTCA	0.05[a] (0.05–0.05)	0.05[a] (0.05–0.05)	0.05[a] (0.05–0.19)	0.05[a] (0.05–0.08)
Sum of 3:	0.75 (0.53–1.31)	1.25 (0.57–1.85)	1.62 (0.74–2.06)	1.75 (1.03–3.37)
Nitrate	0.23 (0.18–0.41)	0.20 (0.16–0.41)	0.29 (0.20–0.53)	0.23 (0.15–0.44)

P values of comparison	NPRO	NMTCA[b]	NTCA[b]	Sum	Nitrate
Day 1: high- vs. low-risk area	0.02	0.97	0.41	0.17	0.61[c]
Day 2: high- vs. low-risk area	0.04	0.21	0.61	0.24	0.75[c]
High-risk area: day 1 vs. day 2	0.05	0.73	0.38	0.27	0.21[d]
Low-risk area: day 1 vs. day 2	0.01	0.74	0.08	0.06	0.82[d]

[a] Limit of detection.
[b] NTCA, N-nitrosothiazolidine-4-carboxylic acid; NMTCA, N-nitroso-2-methylthiazolidine 4-carboxylic acid.
[c] Mann-Whitney test.
[d] Wilcoxon test.

parisons are given in Table 1. Comparisons of (median) levels of NPRO in 12-h urine between areas or between day 1 and day 2 dose regimens (*i.e.*, proline with or without vitamin C) were statistically significant. The NPRO 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 ($P < 0.04$). NPRO excretion was reduced in both areas after ingestion of ascorbic acid together with proline, as compared with that seen with proline alone (low-risk area, $P < 0.01$; high-risk area, $P < 0.05$; Wilcoxon test). Levels of N-nitrosothiazolidine 4-carboxylic acid, N-nitroso-2-methylthiazolidine 4-carboxylic acid and nitrate in urine were not different in the two areas, suggesting that these NAA were not useful markers in this study, and none of the NAA, except NPRO, were markedly reduced after intake of ascorbic acid. The NPRO levels on the day of proline intake correlated well with the urinary nitrate levels ($P < 0.001$) but not on the day of proline plus vitamin C intake. These results indicate that children living in the high-risk area in Costa Rica have a higher rate of endogenous nitrosation than those living in the low-incidence area. However, levels of NAA (including NPRO) were only 0.10–0.20 of the values found in urines from adults, and nitrate levels were only one-half to one-third of those found in urines from adults, previously collected from inhabitants of other high-risk areas for gastric cancer (5).

Genotoxicity and Levels of Total NOC and Nitrate in Samples of Beans Consumed Frequently in Costa Rica. On a daily basis, 96% of the families from the study areas consumed beans. Raw and cooked beans were collected from families in the two areas and analyzed for nitrite, nitrate, total NOC, and genotoxicity in the SOS chromotest in *E. coli* (Table 2). The mean levels of total NOC in aqueous extracts (pH 2) of boiled bean samples (stored at room temperature for up to 9 days) from the high- and low-incidence areas were similar. Acid-catalyzed nitrosation of the extracts produced a maximal level of total NOC of 1.8 µmol/g wet weight of cooked beans, but there was no difference in the mean level of (nitrosation-dependent) total NOC between the 2 areas. Storage at room temperature up to 9 days before freezing of cooked beans from the 2 areas had no effect on

their nitrosation-dependent genotoxicity (data not shown). Only 2 of 11 extracts prepared from cooked beans from the low-incidence area and 2 of 14 from the high-incidence area showed weak, direct genotoxicity; after acid-catalyzed nitrosation, all bean samples were genotoxic, with the mean genotoxicity being similar in samples from the two areas. The mean nitrate concentration ± SD of raw beans from the high-risk area (0.76 ± 0.54 µmol/g dry weight; $n = 9$) was about twice as high as that of beans from the low-risk area (0.36 ± 0.12 µmol/g, $n = 6$), while no significant difference was seen in the nitrate levels in boiled beans from the two areas (0.97 ± 0.49 µmol/g, $n = 32$, *versus* 0.89 ± 0.53 µmol/g, $n = 32$, respectively). Furthermore, there was no clear increase or decrease in nitrite or nitrate concentrations in cooked beans stored in a refrigerator or at room temperature for up to 9 days.

Dietary Comparisons. The consumption of milk products, vegetables, maize, sugar, jams, honey, and vegetable fat was significantly greater in the low-risk area and the consumption of processed meat products, bread, and wheat flour was significantly higher in the high-risk area ($P < 0.05$).

Table 3 shows the average intakes of energy and nutrients of the subjects in the two areas. The following nutrients were consumed in significantly greater amounts in the low-risk area: fat; calcium; phosphorus; thiamine; and dietary fiber ($P < 0.001$); energy, protein, carotenes and riboflavin ($P < 0.01$); and carbohydrates and iron ($P < 0.05$). The average amounts of niacin and vitamin C did not differ significantly between the two communities. The values for vitamin E, retinol, and retinol equivalents were not compared because the log-transformed values were not distributed normally.

Table 3 also shows the mean intakes, expressed as percentages of the recommended daily intake, for each area. The diet in the low-risk area satisfies recommended levels of intake of energy and most nutrients except riboflavin and retinol equivalents. In the high-risk area it was deficient in energy and all the nutrients except protein and vitamin C. The study in the high-risk area took place during the green mango season, a major source of vitamin C for these children, and the high vitamin C intake may not apply to other periods of the year. Although several differences in nutrient

Table 2   Concentrations of total NOC and genotoxicity in the SOS chromotest (SOS induction potency) of acidic aqueous extracts of cooked beans collected at high- and low-risk areas for stomach cancer in Costa Rica

Study area	No. of samples analyzed	Mean (range) of total NOC nmol/g cooked beans		Mean (range) of SOSIP[a]/ g cooked beans		Mean (range) for SOSIP/μmol total NOC after nitrosation
		Before nitrosation	After nitrosation	Before 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] SOSIP, SOS induction potency.

Table 3   Comparison of (a) the mean intake of energy and nutrients and (b) of nutritional adequacy of mean food intake in a low- and high-risk area of gastric cancer in Costa Rica

Energy and nutrients	Mean intake ± SD		Comparison of the two areas	Nutritional adequacy[a] (%)	
	Low-risk area	High-risk area		Low-risk area	High-risk area
Energy (kcal)	2103 ± 714.0	1774 ± 665	$0.01 > P > 0.001$	98	82
Protein (g)	54.7 ± 19.4	46.3 ± 19.8	$0.01 > P > 0.001$	149	118
Fat (g)	65.6 ± 33.9	49.0 ± 31.6	$P < 0.001$		
Retinol (μg)	319 ± 1476	265 ± 1488	NA[b]		
Carotenes (μg)	838 ± 938	470 ± 700	$0.01 > P > 0.001$		
Vitamin E (mg)	10.6 ± 8.5	7.1 ± 6.6	NA	145	93
Carbohydrates (g)	334.4 ± 118.2	296.2 ± 118.0	$0.05 > P > 0.01$		
Calcium (mg)	679 ± 695	432 ± 378	$P < 0.001$	115	71
Phosphorus (mg)	878 ± 326	697 ± 307	$P < 0.001$	96	67
Iron (mg)	14.7 ± 5.0	12.8 ± 5.6	$0.05 > P > 0.01$	114	82
Retinol equivalents (μg)	454 ± 1521	340 ± 1547	NA	80	56
Niacin[c] (mg)	11.5 ± 5.5	10.7 ± 5.8	NS[d]		
Vitamin C (mg)	90 ± 93	151.0 ± 193	NS	391	604
Thiamin (mg)	1.13 ± 0.53	0.86 ± 0.52	$P < 0.001$	119	91
Riboflavin (mg)	0.96 ± 0.67	0.73 ± 0.61	$0.01 > P > 0.001$	74	56
Dietary fiber (g)	25.8 ± 12.4	18.8 ± 11.0	$P < 0.001$		

[a] Nutritional adequacy of mean food intake in high- and low-risk areas is expressed as a percentage of recommended daily intake.
[b] NA, not analyzed.
[c] Does not include niacin derived from tryptophan.
[d] NS, not significant.

intake have been noted between the two areas, they may be due to the reported difference in total energy intake (Table 3). Questionnaires on food intake were used mainly to help to interpret results from NPRO-test.

No significant differences in dietary habits were detected between the two areas in terms of the following: use of beans in terms of frequency of consumption, type, preferences, method of preparation and storage of beans; usage of condiments (mostly pickled hot peppers and sauce made from fermented soya); and use of pickled vegetables and fermented drinks and reported use of salt. A significantly ($P < 0.01$) higher proportion of families in the low-risk area added condiments to their food at the table and consumed beverages made from fermented maize.

Discussion

The hypothesis that intragastric formation of nitrite-derived carcinogens in the acidic stomach in early life could play a crucial role in inducing atrophic gastritis and intestinal metaplasia in later life was tested in about 50 Costa Rican children (aged 8–14 years) living in high- and low-risk areas for stomach cancer. By applying the NPRO test, we have demonstrated that the levels of NPRO were significantly higher in urine samples collected in the high-risk area than those for a low-risk area. On the day of proline intake, NPRO was

positively correlated with urinary NO_3^- level ($P < 0.001$). Urinary concentrations and absolute amounts of NAA excreted in 12-h urine were only 10–20% of those detected previously in urine samples from adults in other high-risk countries for gastric cancer; the nitrate level was 30–50% lower than that in the urine of adults (5). It is not known whether these low levels in Costa Rican children are due to a lower body weight or other factors related to age, or diet, such as high intake of ascorbic acid (Table 3). When expressed by body weight (mean body weight of children, 35 kg), NPRO excretion was 20–40% as for adults while nitrate excretion was 0–15% lower in children.

Although the excreted values for NPRO were lower in children than in adults, the results in Table 1 demonstrate that the in vivo NPRO formation after proline intake in children from the high-risk area was elevated and this suggests that increased intragastric formation of nitrite-derived carcinogens could occur, if nitrosatable precursors were present.

As the urinary nitrate level did not markedly differ between the areas (Table 1), the higher nitrosation potential in children of the high-risk area could be due to a diet more deficient in inhibitors of endogenous nitrosation than that in the low-risk area. Alternatively, besides exposure to nitrate from the environment, one has to consider endogenous sources of nitrate. Chronic inflammatory processes, such as

infection with *H. pylori*, have been linked with gastric cancer (although causality has not been established) and this could well be due to the generation of nitric oxide, nitrite, and nitrate by macrophages and other cell types in inflamed tissues (24–27). In cases of persistent bacterial infection and colonization of the stomach, this source of nitrite/nitrate might be of considerable importance for nitrosation reactions *in vivo* also leading to increased nitrate excretion in urine.

Beans have been associated with increased gastric cancer risk (28) and have been found to be mutagenic after nitrosation (11–12); salt increased the mutagenicity of nitrosated black beans collected in Costa Rica (29). In the present study, no differences were found in the frequency of bean consumption, of total NOC content and genotoxicity of cooked beans collected from families living in the high- and low-risk areas. However, the more than 1000-fold increase in total NOC and about 20-fold increase in genotoxicity after nitrosation of bean extract confirm the formation of NOC and mutagens, possibly including nitrosation of the precursor 4-chloro-6-methoxy-indole (12).

The diet of the high-risk area was lower in quantity and quality than that of the low-risk area (Table 3). The latest national nutrition survey in Costa Rica found that the population in general has a high and adequate intake of vitamin C but that of vitamin A is inadequate (30). Our results confirm this observation and showed that the intake of carotenes and other nutrients was significantly lower in the high-risk population.

Results from a related study showed a high prevalence of IgG and IgA antibodies against *H. pylori* in children and young adults in Costa Rica, but the excess prevalence did not differ significantly between the high- and low-risk areas (10). Although early infection and high prevalence of *H. pylori* infection are not always associated with high rates of gastric cancer (31), several studies have found a moderate but significant association between *H. pylori* infection and risk of gastric cancer (32–35). Therefore, we must assume that in Costa Rica other factors in addition to *H. pylori* play an important etiological role. Although endoscopy and gastric biopsies were not performed in the present study, the high pepsinogen C levels and their correlation with the *H. pylori* antibodies titers suggest that a high proportion of these children and young adults had gastritis while it is predominantly antral (10), but no difference was found in the prevalence rate of gastritis in children between the two areas. Salas (36) reported that intestinal metaplasia, considered to be a precancerous lesion, was three times more frequent in young people from the high-risk area of Costa Rica and also found at earlier ages in these areas. Our results on the increased NPRO formation in Costa Rican children of the high-risk area suggest that other mutagenic NOC formed *in vivo* might be one of the factors responsible for progression of *H. pylori*-related gastritis to metaplasia and gastric cancer. This process may be accelerated by two factors: (*a*) the low dietary carotene and retinol intake by inhabitants of the high-risk area as revealed by our dietary survey (Table 3); and (*b*) a lowered antioxidant defense state and reduced nitrite-scavenging capacity of the stomach in patients with gastric precancerous conditions. The gastric juice of patients with chronic superficial and, in particular, atrophic gastritis has been reported to contain significantly lower ascorbic acid and vitamin C levels (sum of ascorbic acid and dehydroascorbic acid) (8, 37). However, plasma levels of vitamin C

were similar in patients with and without gastric precancerous conditions.

References

1. Ministerio de Salud. Anuario 1988. Informaciones Estadisticas San José, 1989.

2. Sierra, R., Parkin, D. M., Muñoz, N., and Barrantes, R. *In:* C. A. Bieber and G. Munoz, Cancer in Costa Rica (Technical Report 1). Lyon, France: International Agency for Research on Cancer, 1988.

3. Correa, P., Haenszel, W., Cuello, C., Tannenbaum, S., and Archer, M. A model for gastric cancer epidemiology. Lancet, *ii:* 58–60, 1975.

4. Correa, P. A human model of gastric carcinogenesis. Cancer Res., *48:* 3345–3360, 1988.

5. Bartsch, H., Ohshima, H., Pignatelli, B., and Calmels, S. Human exposure to endogenous *N*-nitroso compounds: quantitative estimates at high risk for cancer of the oral cavity, oesophagus, stomach and urinary bladder. Cancer Surv., *8:* 335–362, 1989.

6. Crespi, M., Ohshima, H., Ramazzotti, V., Muñoz, N., Grassi, A., Casale, V., Leclerc, H., Calmels, S., Carroen, S., Kaldor, J., and Bartsch, H. Intragastric nitrosation and precancerous lesions of the gastrointestinal tract: testing of an etiological hypothesis. *In:* H. Bartsch, I. O'Neill, and R. Schulte-Hermann (eds.), The Relevance of *N*-Nitroso Compounds to Human Cancer Exposures and Mechanisms, IARC Scientific Pub. No. *84*, pp. 511–517. Lyon, France: International Agency for Research on Cancer, 1987.

7. Pignatelli, B., Malaveille, C., Rogatko, A., Hautefeuille, A., Thuillier, P., Muñoz, N., Moulinier, B., Berger, F., De Montclos, H., Lambert, R., Correa, P., Ruiz, B., Sobala, G. M., Schorach, C. J., Axon, A. T. R., and Bartsch, H. Mutagens, *N*-Nitroso Compounds and their precursors in gastric juice from patients with and without precancerous lesions of the stomach. Eur. J. Cancer, in press, 1993.

8. Sobala, G. M., Pignatelli, B., Schorah, C. J., Bartsch, H., Sanderson, M., Dixon, M. F., Shires, S., King, R. F. G., and Axon, A. T. R. Levels of nitrite, nitrate, *N*-nitroso compounds, ascorbic acid and total bile acids in gastric juice of patients with and without precancerous conditions of the stomach. Carcinogenesis (Lond.), *12:* 193–198, 1991.

9. Ohshima, H., Pignatelli, B., Malaveille, C., Friesen, M., Calmels, S., Shuker, D., Muñoz, N., and Bartsch, H. Markers for intragastric nitrosamine formation and resulting DNA damage. *In:* P. I. Reed and M. J. Hill (eds.), Gastric Carcinogenesis, pp. 175–185. Amsterdam: Elsevier, 1988.

10. Sierra, R., Muñoz, N., Peña, A. S., Biemond, I., van Duijn, W., Lamers, C. B. H. W., Teuchmann, S., Hernandez, S., and Correa, P. Antibodies to *H. pylori* and pepsinogen levels in children from Costa Rica: a comparison of two areas with different risk for stomach cancer. Cancer Epidemiol., Biomarkers & Prev., *1:* 449–454, 1992.

11. Marquardt, H., Rufino, F., and Weisburger, J. H. On the aetiology of gastric cancer: mutagenicity of food extracts after incubation with nitrite. Food Cosmet. Toxicol., *15:* 97–100, 1977.

12. Yang, D., Tannenbaum, S. R., Büchi, G. and Lee, G. C. M. 4-chloro-6-methoxyindole is the precursor of a potent mutagen (4-chloro-6-methoxy-2-hydroxy-1-nitroso-indolin-3-one oxime) that forms during nitrosation of fava bean (*Vicia faba*). Carcinogenesis (Lond.), *5:* 1219–1224, 1984.

13. Ohshima, H., O'Neill, I. K., Friesen, M., Béréziat, J-C., and Bartsch, H. Occurrence in human urine of new sulphur-containing *N*-nitrosaminoacids *N*-nitrosothiazolidine 4-carboxylic acid and its 2-methyl derivatives and their formation. J. Cancer Res. Clin. Oncol., *108:* 121–128, 1984.

14. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P., Wishnok, J. S., and Tannenbaum, S. R. Analysis of Nitrate, Nitrite and [^{15}N] nitrate in biological fluids. Anal. Biochem., *126:* 131–138, 1982.

15. Pignatelli, B., Richard, I., Bourgade, M. C. and Bartsch, H. Improved group determination of total *N*-nitroso compounds (NOC) in human gastric juice by chemical denitrosation and thermal energy analysis. Analyst, *112:* 945–949, 1987.

16. Ohshima, H., Friesen, M., Malaveille, C., Brouet, I., Hautefeuille, A., and Bartsch, H. Formation of direct-acting genotoxic substances in nitrosated smoked fish and meat products: identification of simple phenolic precursors and phenyldiazonium ions as reactive products. Food Chem. Toxicol. *27:* 193–203, 1989.

17. Paul, A. A., and Saethagete, D. A. T. *In:* McCance and Widdowson (eds.), The Composition of Food, Ed. 4. London, England: Elsevier/North Holland Inc., 1978.

18. Murillo, S., and Ulate, E. Table de Composicion de Alimentos y Pesos para Costa Rica. Instituto de Investigaciones en Salud, Universidad de Costa Rica, 1985.

19. INCAP (Nutrition Institute for Central America and Panama). Tabla de Composicion de Alimentos. Guatemala, 1977.

20. Gardena, CA: PCD Systems Nutri-Calc., Inc., and Oakwood Publishing, 1984.

21. INCAP (Nutrition Institute for Central America and Panama). Recommendaciones Dietéticas Diarias para Centro América y Panama. Guatemala, 1973.

22. National Academy of Sciences Recommended Dietary Allowances, Ed. 9. p. 185. Washington: National Academy Press, 1980.

23. FAO/WHO/UNO Energy and Protein Requirements. Geneva, Switzerland, 1985.

24. Liu, R. H., Baldwin, B., Tennant, B. C., and Hotchkiss, J. Elevated formation of nitrate and N-nitrosodimethylamine in woodchucks (*Marmota monax*) associated with chronic woodchuck hepatitis virus infection. Cancer Res., *51:* 3925–3929, 1991.

25. Leaf, C. D., Wishnok, J. S., Hurley, J. P., Rosenblad, M. D., Fox, J. G., and Tannenbaum, S. R. Nitrate biosynthesis in rats, ferrets and humans. Precursor studies with L-arginine. Carcinogenesis (Lond.), *11:* 855–858, 1990.

26. Leaf, C. D., Wishnok, J. S., and Tannenbaum, S. R. Endogenous incorporation of nitric oxide from L-arginine into N-nitrosomorpholine stimulated by *Escherichia coli* lipopolysaccharide in the rats. Carcinogenesis (Lond.), *12:* 537–539, 1991.

27. Wu, Y., Brouet, I., Calmels, S., Bartsch, H., and Ohshima, H. Increased endogenous N-nitrosamine and nitrate formation by induction of nitric oxide synthase in rats with acute hepatic injury caused by *Propionibacterium Acnes* and lipopolysaccharide administration. Carcinogenesis (Lond.), *14:* 7–10, 1993.

28. Correa, P., Cuello, C., Fajardo, L. F., Haenzel, W., Bolaño, O., and de Ramirez, B. Diet and gastric cancer: nutrition survey in a high-risk area. J. Natl. Cancer Inst., *70:* 673–678, 1983.

29. Rojas, N., Sigaran, M., Correa, P., Bravo, A., and Jiménez, F. Salt enhances the mutagenicity of nitrosated black beans. Nutr. Cancer, *14:* 1–3, 1990.

30. Nutrition Department, Ministry of Public Health Encuesta Nacional de Nutrición; Evaluación Dietética 1982. San José, Costa Rica, September, 1986.

31. Parkin, D. M. (ed.). Cancer Occurrence in Developing Countries (IARC Scientific Pub. No. 75). Lyon, France: International Agency for Research on Cancer, 1986.

32. Forman, D., Newell, D. G., Fullerton, F., Yarnell, J. W. G., Stacey, A. R., Walds, N., and Sitas, F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. Br. Med. J., *302:* 1302–1305, 1991.

33. Nomura, A., Stemmermann, G., Chyou, P. H., Kato, I., Pérez-Pérez, G., and Blaser, M. J. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med., *325:* 1132–1136, 1991.

34. Parsonnet, J., Friedman, G. D., Vandersteen, D. P., Chang, Y., Vogelman, J., Orentreich, N., and Sibley, R. *Helicobacter pylori* infection and the risk of gastric carcinoma. N. Engl. J. Med., *325:* 1127–1131, 1991.

35. Simpponen, P., Kosunem, T. U., Valle, J., Riihelä, M., and Seppälä, K. *Helicobacter pylori*: infection and chronic gastritis in gastric cancer. J. Clin. Pathol., *45:* 319–325, 1992.

36. Salas, J. Lesiones precancerosas en el estómago en Costa Rica. Patologia (Mex. City), *15:* 63–79, 1977.

37. Sobala, G. M., Schorah, C. J., Sanderson, M., Dixon, M. F., Tompkins, D. S., Godwin, P., and Axon, A. T. R. Ascorbic acid in the human stomach. Gastroenterology, *97:* 357–363, 1989.

Ornithine Decarboxylase Activity in the Mucosa of Gastric Remnant following Gastric Surgery

Itsuo Nakanishi,[1] Michio Sowa, and Shuzo Otani

First Department of Surgery [I. N., M. S.] and Second Department of Biochemistry [S. O.], Osaka City University Medical School, 1-5-7, Asahimachi, Abeno-ku, Osaka 545, Japan

Abstract

Ornithine decarboxylase (ODC) activity and polyamine levels in mucosal specimens obtained by endoscopic biopsy from the stomal portion and greater curvature of the gastric remnant mucosa taken from 181 patients were determined and compared with the histopathological findings. The results obtained can be summarized as follows. (*a*) The ODC activity was significantly higher in the stomal portion [455 ± 340 (mean ± SD)] of the gastric remnant than in the greater curvature (148 ± 107). The ODC activity of the stomal portion was significantly higher following a Billroth's II method (599 ± 417) than following any other operative method that consists of a Billroth's I method (327 ± 172) and a Roux-en Y (341 ± 191). (*b*) The levels of total polyamine, putrescine, spermidine, and spermine were also significantly higher in the stomal portion of the gastric remnant than in the greater curvature. There were no significant differences in the stomal tissue level of any of these substances among operative procedures used. (*c*) Histopathological changes consisting of glandular dilatation and an irregular glandular structure were detected more frequently in the stomal mucosal specimens, especially following a Billroth's II method.

In summary, the present findings suggest that the measurement of ODC activity may thus be considered as one method of estimating the risk of carcinogenesis.

Introduction

Many studies have been reported on the likely facilitatory effects of the duodenogastric reflux of bile acids (which is a tumor promoter) on stomach carcinogenesis. The activity of ornithine decarboxylase, which is the rate-limiting enzyme of polyamine biosynthesis, is generally accepted as the index of cell proliferation and as a useful index of tumor promotion in many experimental studies. We measured the levels of ODC[2] activity and those of putrescine, spermidine, and spermine in tissue samples obtained by endoscopic biopsy from the stomal portion and the greater curvature of the gastric remnant, and we examined the relation of those lev-

els to the histopathological findings in order to clarify whether any tumor-promoting stimuli continuously existed in the anastomosed region and if the ODC activity and polyamine levels in the stomal mucosa might provide useful information on the risk of carcinogenesis of the gastric remnant.

Materials and Methods

Tissues. Mucosal specimens were obtained via endoscopic biopsy from the stomal portions and greater curvatures at least 5 cm from the stomal portions of the gastric remnants of 181 patients who were followed up at the First Department of Surgery, Osaka City University Medical School. Almost all of them underwent curative operation for gastric cancer. The patients comprised 64 females and 117 males, ranging in age from 27 to 87 years (average, 60.7 years). The time after gastrectomy ranged from 1 to 37 years (average, 4.3 years). The types of operations performed were B-I for 87 patients, B-II for 85 patients, and Roux-en-Y for 9 patients. There were no significant differences between patients treated by B-I and those treated by B-II with regard to the range in age, sex, and the period after operation. The specimens were immediately frozen in liquid nitrogen, preserved at −85°C, and analyzed within 2 weeks.

Enzyme Assay. The ODC activity was measured as described previously (1). Briefly, the specimens were minced and homogenized in 500 μl of 50 mM Tris (pH 7.5) containing 0.25 M of sucrose, 2.5 mM of dithiothreitol, 0.1 mM of EDTA, and 0.2 mM of pyridoxal phosphate with an ultrasonic homogenizer. After centrifugation at $100,000 \times g$ for 60 min at 4°C, 200 μl of the supernatant and 42 nmol of L-[1-^{14}C]-ornithine (0.125 μCi; 10 μl) were put into a test tube. The test tube was tightly stoppered with a rubber stopper equipped with the syringe needle and a filter paper disk pierced with the needle and impregnated with 70 μl of Soluene-350 (Pakard Co, Ltd.). The mixture was incubated at 37°C for 60 min. The reaction was stopped by the addition of 1 ml of 2 M citrate using a syringe through the rubber stopper and the syringe needle. After another incubation at 37°C for 60 min, the paper disk was transferred to a vial containing 5 ml of toluene scintillation fluid (Omnifluor, Daiichi Chemicals, Tokyo, Japan), and the radioactivity was measured in a Beckmann liquid scintillation counter.

Statistics. Tests of statistical significance were performed using nonparametric methods. The generalized Wilcoxon T test was used to evaluate the significance of the differences in the mean ODC activity and polyamine levels in the mucosal specimen from the stomal portion *versus* those from the greater curvature, and the generalized Kruskal-Wallis' H test was used to compare the diffe∙ ∙nt operative methods.
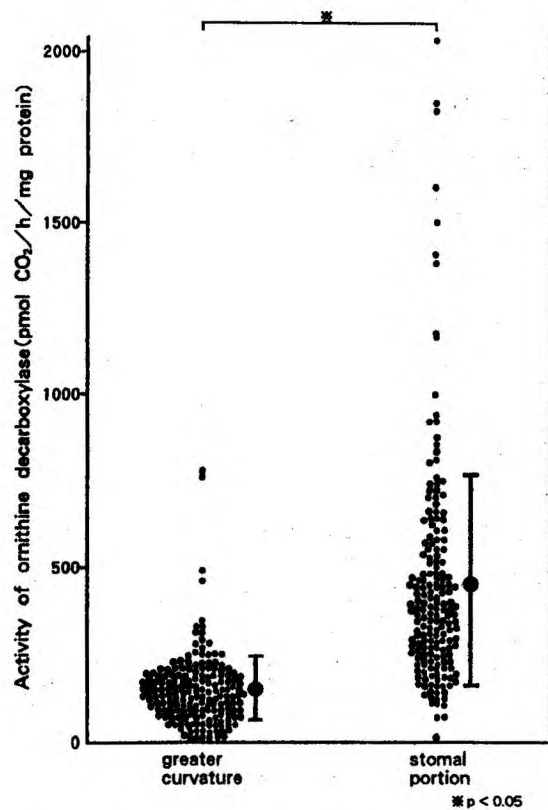
Fig. 1. Changes in ornithine decarboxylase activity of the gastric remnant mucosa at different biopsy sites. Results are expressed as means ± SD (*bars*) of 181 cases.

Fig. 2. Changes in ornithine decarboxylase activity of the gastric remnant mucosa following varying operative procedures [B-I, B-II, and Roux en Y (R-Y)].

Results

Fig. 1 shows ODC activity in the mucosa at different biopsy sites. The ODC activity in the mucosa of the stomal portion was 455 ± 340 pmol CO_2/h/mg protein (mean ± SD) and was significantly higher than that of the greater curvature (148 ± 107 pmol CO_2/h/mg protein). Fig. 2 shows the ODC activity in the mucosa for the different operative methods. The ODC activity was significantly elevated at the stomal portion compared with that at the greater curvature, irrespective of operative methods. As for the stomal portion, the ODC activity following a B-II was 599 ± 417 pmol CO_2/h/mg protein, and that following a B-I was 327 ± 172 pmol CO_2/h/mg protein, and that following a Roux en Y method was 341 ± 191 pmol CO_2/h/mg protein. The ODC activity following a B-II was more significantly elevated than it was with any other operative method.

Table 1 shows the polyamine levels of the stomal mucosa and of the greater curvature after gastric resection by the various operative methods. As for each operative method and/or to summarize them, the putrescine, spermidine, spermine, and the total polyamine levels were significantly higher in the mucosa of stomal portion than in the greater curvature. No significant differences were recognized among the different operative methods. Table 2 shows the histopathological findings consisting of intestinal metaplasia, glandular dilatation, irregular glandular structure, and round cell infiltration. The glandular dilatation and the irregular glandular structure were significantly more frequent in the stomal mucosa than in the greater curvature (65.8
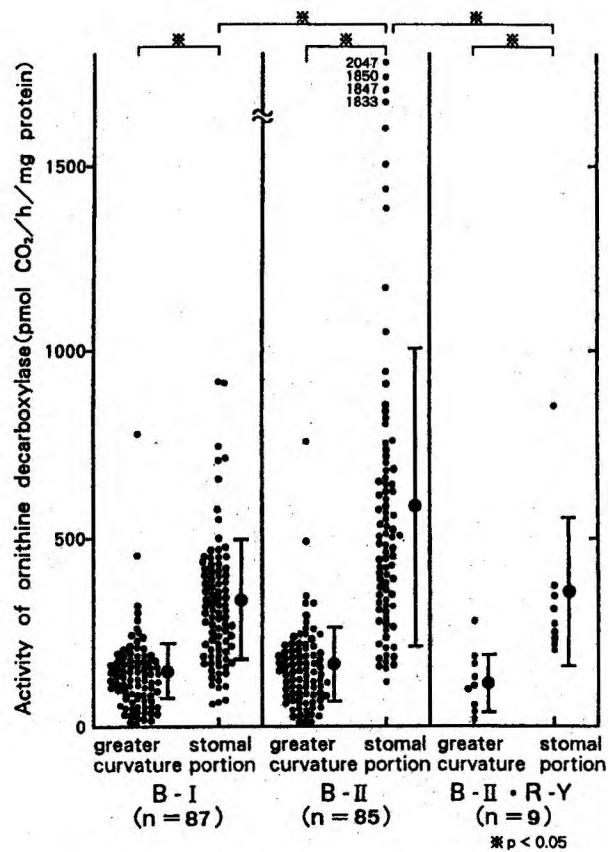
versus 5.8% and 50.0 versus 2.3%) and were significantly more frequent in the stomal mucosa following a B-II than following a B-I (91.9 versus 13.5% and 78.4 versus 2.7%). They were seldom recognized at the greater curvature in at least one-half of the whole specimens studied.

Discussion

The frequency of primary cancer developed in the gastric remnant surgically treated for peptic ulcer varies from one reported study to another. Toftgaad (2) showed the fact that the risk of carcinogenesis of the gastric remnant more than 20 years after gastrectomy is severalfold higher than was expected. Sowa et al. (3), Tokudome et al. (4), and Kondo (5) demonstrated that stomal cancer develops more frequently following a B-II than following a B-I and that duodenogastric reflux might play a role in the carcinogenesis. The endoscopic finding of a remnant stomach is redness, while that of the stomal mucosa is rubor, edema, or erosion. The positive rates of these findings increased according to postoperative duration and were especially high in patients who had undergone the B-II method. Biopsy of sections revealed a high frequency of glandular dilatation and irregular glandular structure. These changes were much more frequently recognized at the stomal portion in cases having a long postoperative period after B-II operation. In this study, the frequencies of these changes at the stomal portion following a