Dose-Response Studies With Concurrent Administration of Piperazine and Sodium Nitrite to Strain A Mice

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SUMMARY—Male strain A mice were given 0.69–18.75 g piperazine/kg food and 0.05–2.0 g sodium nitrite (NaNO₂)/liter drinking water for 20–25 weeks, and were killed 10–13 weeks later. The yield of lung adenomas was significantly greater than in untreated controls when as little as 0.69 g piperazine/kg plus 1.0 g NaNO₂/liter, or 6.25 g piperazine/kg plus 0.25 g NaNO₂/liter, were given. A maximum of 30.7 adenomas/mouse more than the controls was observed with 6.25 g piperazine/kg plus 2.0 g NaNO₂/liter. Mononitrosopiperazine (MNP) at 34.5 mg/liter drinking water induced 3.8 adenomas/mouse more than the controls. Negative results were obtained with NaNO₂ alone, piperazine alone, 12.3 g sodium nitrate (NaNO₃)/liter alone, or 12.3 g NaNO₃/liter plus 18.75 g piperazine/kg. The latter result indicated that nitrate was not reduced to nitrite under conditions suitable for reaction with the piperazine. When the piperazine concentration was raised from 0.69–18.75 g/kg food in 3 threefold increments at a constant level of nitrite, the adenoma yield was increased between successive doses by ratios of 1.4 to 2.5. A ratio of 3.0 was predicted by application of the kinetic equation for MNP formation (adenoma yield was assumed to be proportional to MNP production). When the nitrite concentration was raised from 0.25–2.0 g/liter water in 3 twofold increments at a constant level of piperazine, the adenoma yield was increased by ratios of 3.7 to 3.8, in agreement with the predicted ratio of 4.0.—J Natl Cancer Inst 49: 119–124, 1972.

IN PREVIOUS STUDIES, Sander et al. (1–4) and Ivankovic and Preussmann (5) reported that the concurrent administration of sodium nitrite (NaNO₂) and secondary amines or alkylureas can induce tumors in rats. In our studies (6, 7), Swiss mice given 5–10 g morpholine, methylaniline, piperazine, methyleurea, or ethyleurea/kg food with 1 g NaNO₂/liter drinking water showed a significant increase in lung adenomas. The doses of the secondary amines, ureas, and nitrite were high because we wished to confirm the limited data available on tumorigenicity by this type of treatment.

We now report studies of lung adenoma enhancement in male strain A mice by the concurrent administration of piperazine in the food and NaNO₂ in the drinking water. In one experiment, the piperazine concentration was maintained at a constant level and the nitrite concentration was varied. In a second experiment, the nitrite concentration was kept constant and the piperazine concentration was varied. We also administered high concentrations of sodium nitrate (NaNO₃) with piperazine to a single group of mice, to determine whether nitrate might be reduced to nitrite in vivo under conditions which would make it available for nitrosamine formation.

The lowest concentrations of the chemicals were comparable with possible human exposure levels. Increments of dose were chosen for their usefulness in correlating chemical kinetic studies.

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in vitro with quantitative assessment of tumorigenesis in vivo.

MATERIALS AND METHODS

NaNO₂ and NaNO₃ were obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey, and piperazine (practical grade, mp 108–109° C) from Eastern Organic Chemicals, Rochester, New York. Mononitrosopiperazine (MNP) (bp 80–85° C/0.1 mm) was synthesized (8) by Dr. Larry Keefer, National Cancer Institute, Bethesda, Maryland.

Strain A mice, bred in our animal colony, were housed in groups of 10 per polycarbonate cage measuring 8 X 7 X 5 inches. Ground corn cobs (Sanicel) were used as bedding. Stock solutions of all compounds in distilled water were prepared at 20 X the final concentrations, with a separate stock solution for each concentration. The stock piperazine solution, brought to pH 7 with HCl, was added twice weekly to a portion of Wayne rat and mouse powdered food, ground with a mortar and pestle, mixed with the remainder of the powdered food in an oscillating paint-mixing machine, and fed ad libitum. The NaNO₂, NaNO₃, and MNP stock solutions were diluted 20 X with distilled water at least once weekly, and given as drinking water in brown bottles at the end of the cage opposite from the food. All stock solutions and the piperazine-containing food were stored at 4°C in the dark. At the end of the experiment, all survivors (>90% of the initial number) were killed and autopsied; only these mice were included in the results. We counted all adenomas on the surface of the lungs by the Shimkin technique (9). Lung adenomas were distinct, noncoalescent 0.5- to 2-mm nodules. Histologic examination of the lungs from 1 of every 5 mice confirmed the diagnosis. The accuracy of the counts was checked by a recounting of the lungs preserved in formalin. There was a minimum variation of ±10%, with the greatest variation in lungs containing >20 adenomas. In lungs containing <10 adenomas, duplicate counts were usually identical.

RESULTS

The results for 18 groups of mice, divided into 2 series of experiments, are given in table 1 and text-figures 1 and 2. In most groups the mean body weight was similar to that of the untreated controls; but in group 7, which had the highest level of piperazine plus nitrite, the mean body weight was 22 g at the end of treatment compared with 25 g for the control group, and the mice appeared in poor condition. Mice given varying doses of nitrite with a fixed dose of piperazine (groups 14–18) had a decrease in mean body weight with increasing levels of nitrite. After treatment was stopped, the weights rose to nearly equal values for all groups (text-fig. 3).

The mean food consumption for each group was 3.0–3.7 g/mouse/day, with an average for all groups of 3.1 g/mouse/day. The mean water consumption for each group was 4.3–6.0 ml/mouse/day, with an average for all groups of 5.1 ml/mouse/day (excluding values of 4.0 ml water/day for group 7, which had the highest level of piperazine, and 3.2 ml/day for group 14, which had the highest dose of nitrite).

Group 6, the “positive” control given MNP (total dose, 22 mg) in the drinking water, had 3.8 adenomas/mouse, 7 times the control incidence of 0.5 adenomas/mouse.

NaNO₃ given alone at 1 and 2 g/liter drinking water to groups 2 and 13, did not increase the lung adenoma response when compared with the con-
### Table 1.—Lung adenoma induction in strain A mice

<table>
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<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Concentration* (g/kg food or g/liter water)</th>
<th>Effective No. of mice</th>
<th>Adenoma-bearing mice Number</th>
<th>Percent</th>
<th>Total No. of adenomas</th>
<th>Adenomas/adenoma-bearing mouse Mean</th>
<th>Adenomas/mouse</th>
<th>Adenoma yield† Value</th>
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*Piperazine was given in the food; MNP, NaN0, and NaN0 were given in the drinking water.
†Probability of chance occurrence compared with untreated controls of the same species.
‡Adenomas/mouse minus value in untreated controls.
TEXT-Figure 1.—Mean lung adenomas/mouse at different piperazine doses and at a constant level of NaNO₂. Groups 7–10 were given 0.69–18.75 g piperazine/kg food plus 1.0 g NaNO₂/liter water. Vertical lines show 95% confidence limits.

TEXT-Figure 2.—Mean lung adenomas/mouse at different NaNO₂ doses and at a constant level of piperazine. Groups 14–18 were given 0.05–2.0 g NaNO₂/liter water plus 6.25 g piperazine/kg food. Vertical lines show 95% confidence limits.

Controls. Group 12, in which 6.25 g piperazine/kg food was administered alone, had 0.4 adenomas/mouse—significantly greater than the control value of 0.1 adenomas/mouse (P<0.02). The percent of tumor-bearing animals was not significantly increased. In group 4, piperazine at a higher concentration of 18.75 g/kg produced no effect, even though the mice lived 8 weeks longer, which suggests that the response in group 12 was without significance.

TEXT-Figure 3.—Body weight of mice given different levels of NaNO₂ and a constant level of piperazine. Mean body weight of untreated control group 11 (solid line) is compared with that of groups 14–16 (broken lines), which were given 6.25 g piperazine/kg food plus 0.05, 0.1, and 0.2% NaNO₂ in drinking water. The mice were treated for 20 weeks and killed 10 weeks later.

No effect on adenoma yield was observed in group 5, given 12.3 g NaNO₂/liter drinking water, or in group 5, given the same level of NaNO₂ with 18.75 g piperazine/kg. The total dose during the experiment was estimated to be 7.9 g NaNO₂/mouse (both groups) and 7.8 g piperazine/mouse (group 5).

Significant increases in adenoma yield occurred in 8 of the 9 groups given piperazine in the food and NaNO₂ in the drinking water, when compared with the untreated controls. Increase in the concentration of piperazine, with NaNO₂ maintained at a constant level of 1.0 g/liter, increased the yield to 18.7 adenomas/mouse for the highest level of piperazine (18.75 g/kg) (text-fig. 1). Even the lowest concentration of piperazine (0.69 g/kg) in group 10 produced a significant (P<0.001) response; this group had estimated total doses of 284 mg piperazine and 640 mg NaNO₂ per mouse.

The relationship between adenomas/mouse and increasing nitrite concentration, with piperazine maintained at 6.25 g/kg, is illustrated in text-figure 2. A maximum of 30.7 adenomas/mouse occurred at the highest level of nitrite (2.0 g/liter). A significant response (P<0.001) compared with untreated controls was observed in group 17 when only 0.25 g NaNO₂/liter (250 ppm) was given; this group had...
estimated total doses of 410 mg piperazine and 128 mg NaNO₂ per mouse. At the lowest dose (0.05 g NaNO₂/liter) the effect was not statistically significant, although the adenomas/mouse and percent tumor-bearing mice were about twice the control values.

**DISCUSSION**

The negative response in group 5, which received 6.25 g piperazine/kg food and 12.3 g (145 mmoles) NaNO₂/liter water, may be compared with the positive response of 0.6 adenomas/mouse in group 17, which received the same dose of piperazine and 0.25 g (3.62 mmoles) NaNO₂/liter water. We conclude that <3.62/145×100 (i.e., <2.5%) of the administered nitrate was reduced to nitrite in vivo under conditions which would make it available for reaction with piperazine (10–13). This observation that nitrate ingestion did not produce in vivo nitrosation of piperazine in mice may not be applicable to other species because of species variation in gastrointestinal microflora or other physiologic factors.

Group 8 (series 1) and group 16 (series 2), treated with 6.25 g piperazine/kg food plus 1 g nitrite/liter water, yielded 12.9 and 8.1 adenomas/mouse, respectively. This difference was probably because the mice of series 1 were treated for 5 weeks longer and killed when they were 8 weeks older than the mice of series 2. Similarly, the untreated control groups had 0.5 adenoma/mouse in series 1 (group 1) and only 0.1 adenoma/mouse in series 2 (group 11). For both series, the adenoma response was higher than that previously reported for similarly treated Swiss mice, which yielded only 1.6 adenomas/mouse (6). This illustrates the greater sensitivity of the strain A mice used here.

Group 6, given 34.5 mg MNP/liter drinking water, had 3.8 adenomas/mouse. This is the first report showing that MNP is carcinogenic in the mouse. In view of the short duration of the experiment, it is not surprising that tumors outside the lung were not observed. The lung tumorigenic activity of MNP appears to be less than that of dinitrosopiperazine and nitrosomorpholine; doses of 40 and 80 mg/liter, respectively, induced 4–5 lung adenomas/mouse in the relatively insensitive Swiss mice (6). Similarly, MNP induced tumors in the rat at many sites late in life (6), but its activity was less than that of dinitrosopiperazine.

The nitrosation of piperazine in vitro to produce MNP proceeds at a maximum rate at pH 3, and is governed by the equation:

\[
\text{rate} = k \cdot [\text{piperazine}] \cdot [\text{nitrite}]^2 \tag{1}
\]

where bracketed words refer to molar concentrations. For stoichiometric concentrations (i.e., total concentrations irrespective of the state of ionization), the rate constant \(k\) at 25°C and pH 3 is 5000 moles⁻²liter⁻¹min⁻¹ (14, 15). Since MNP is nitrosated to give dinitrosopiperazine with a rate constant of only 400 moles⁻³liter⁻²min⁻¹ under similar conditions (14), the chief initial product of piperazine nitrosation is MNP, and not the dinitroso derivative. If the extent of nitrosation is small, the amount of MNP produced is approximately proportional to the amount of MNP produced, the same relationship should apply. The yield of lung adenomas in strain A mice is known to be proportional to the dose of the classic lung carcinogen urethan (9, table 6); MNP and urethan may have similar dose-response curves, since both are water soluble and of small molecular weight.

When the piperazine concentration in the food was raised from 0.69–18.75 g/kg in 3 threefold increments at a constant level of nitrite, the adenoma yield increased by ratios of 2.5, 2.3, and 1.4 between successive doses (table 1). The first 2 ratios (between results for the 3 lowest doses) are less than the ratio of 3.0 predicted from equation [1] on the basis of the assumption in the previous paragraph.

When nitrite concentration in the drinking water was raised from 0.25–2.0 g/liter in 3 twofold increments at a constant level of piperazine, the adenoma yield increased by ratios of 3.7, 3.7, and 3.8 between successive doses (table 1), in agreement with the ratio of 4.0 predicted by application of equation [1]. The ratio of 3.0 between the adenoma yield for the 2 lowest doses of 0.72 and 3.62 mmoles nitrite/liter is far from the theoretical ratio of 25, but in this case the adenomas/mouse for the lowest dose were not significantly different from those in the untreated controls; consequently,
the actual increment in response is difficult to ascertain. In this biologic model, we found that 250 ppm of NaNO₂ in the drinking water, given with 6.25 g piperazine/kg food, produced a significant increase in lung adenomas. This concentration of NaNO₂ is similar to the maximum permitted residual nitrite level (200 ppm) in meat and fish.

Piperazine salts are widely used as antihelmintic drugs for pinworms (Enterobius) and roundworms (Ascaris) (16). The maximum daily recommended dose is 3.5 g/day for 2 days for ascariasis, and 2.0 g/day for 7 days for enterobiasis. No special attention to diet is recommended.

Although unknown factors could affect possible MNP production in man when piperazine and food containing residual nitrite are taken together, a limited intake of nitrite-containing foods in individuals receiving piperazine seems prudent. It also appears important to evaluate possible hazards due to the intragastric formation of N-nitroso compounds from other drugs which contain nitrosatable groups, e.g., secondary amines (11, 14, 17), tertiary amines (18), alkylureas (14), and N-alkylcarbamates (14).

REFERENCES


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