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INTERACTIONS BETWEEN *N*-ACETYLCYSTEINE AND ASCORBIC ACID IN MODULATING MUTAGENESIS AND CARCINOGENESIS

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Both ascorbic acid (AsA, vitamin C) and *N*-acetylcysteine (NAC), a precursor and analogue of glutathione, possess a broad array of biological properties underlying their protective role in a variety of pathophysiological conditions. However, under certain circumstances, AsA behaves as a pro-oxidant rather than an anti-oxidant and produces adverse effects. This prompted us to evaluate whether NAC could interact with AsA in preventing mutation and cancer. AsA significantly increased spontaneous revertants in the *Salmonella typhimurium* strains TA102 and TA104, which are sensitive to oxidative mutagens. In contrast, NAC lowered the spontaneous background in TA104 and neutralized the negative effects of AsA. Moreover, NAC and AsA showed additive effects in reducing chromium(VI) and in reverting its mutagenicity. A single i.p. injection of urethane (1 g/kg body weight) to 120 A/J mice resulted, after 4 months, in the formation of a total of 1,532 lung tumors, 425 in the 30 mice treated with the carcinogen only, 404 in those treated with urethane plus AsA, 365 in those treated with urethane plus NAC and 338 in those treated with urethane plus the combination of AsA and NAC (both given daily with drinking water at the dose of 1 g/kg body weight). Compared to positive controls, tumor multiplicity was poorly affected by AsA, whereas it was significantly decreased by NAC and even more so by its combination with AsA. The overall volumes of lung tumors in the 4 groups were 107.5, 89.3, 61.3 and 49.7 mm^3, respectively. Tumor sizes were slightly but significantly decreased in mice treated with AsA and more so in those treated with NAC and NAC plus AsA, their combination being significantly more effective than each individually. All protective effects elicited by combining the 2 drugs were additive. Therefore, NAC prevents the adverse effects of AsA on spontaneous mutagenicity; at the same time, this thiol behaves in an additive fashion with AsA, inhibiting the mutagenicity of chromium(VI) and the lung tumorigenicity of urethane in mice. These findings suggest that NAC and AsA could conveniently be combined in cancer chemoprevention and other pharmacological interventions. *Int. J. Cancer* 88:702–707, 2000.
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Ascorbic acid (AsA, vitamin C) and *N*-acetylcysteine (NAC) possess pleiotropic properties, which explain their protective role in certain pathophysiological conditions. Both compounds inhibit mutation and cancer in a variety of experimental test systems and are thus considered to be among the most promising cancer chemopreventive agents.

In particular, AsA, which was isolated more than 70 years ago by Szent-Gyorgyi (1928), is a major water-soluble anti-oxidant present in cells and plasma (Frei *et al.,* 1989). The anti-mutagenic, anti-clastogenic and anti-carcinogenic effects of AsA (Cameron *et al.,* 1979; Shamberger, 1984; Fraga *et al.,* 1991; Krinsky, 1993) have been ascribed to several mechanisms, including its anti-oxidant properties (Frei *et al.,* 1989), the ability to react with nitrite as a prototype inhibitor of the nitrosation reaction (Mirvish *et al.,* 1972), the regulatory role in transcription and translation (Lyons and Schwarz, 1984; Huang *et al.,* 1993; Sullivan *et al.,* 1994) and cell-cycle arrest at the G_2/M DNA damage checkpoint during oxidative stress (Gautam *et al.,* 1999).

NAC, which has extensively been used as a mucolytic drug for almost 4 decades, has also been shown to exert anti-genotoxic and anti-carcinogenic effects in a variety of test systems (reviewed by Kelloff *et al.,* 1994; De Flora *et al.,* 1995*a,b*). This thiol works extracellularly and intracellularly as a precursor of reduced glutathione (GSH, γ-glutamylcysteinylglycine). A number of mechanisms of action have been postulated, such as trapping of electrophiles and scavenging of reactive oxygen species (De Flora *et al.,* 1995*a,b*), inhibition of nitrosation (De Flora *et al.,* 1988), enhancement of thiol concentrations in intestinal bacteria (Camoirano *et al.,* 1988), enhancement of detoxification in non-target cells (De Flora *et al.,* 1995*a,b*), stimulation of metabolic activation coordinated with enhanced detoxification and block of reactive metabolites (De Flora, 1998), inhibition of spontaneous mutations related to DNA repair background (De Flora *et al.,* 1994), protection of nuclear enzymes and enhancement of repair of DNA damaged by carcinogens (Cesarone *et al.,* 1991), correction of hypomethylation (Lertratanangkoon *et al.,* 1996), signal-transduction modulation (Bergelson *et al.,* 1994; Ho *et al.,* 1999), induction of cyclin-dependent kinase inhibitors and G_1 prolongation (Liu *et al.,* 1999), inhibition of neovascularization (Cai *et al.,* 1999), inhibition of type IV collagenases involved in degradation of basement membranes (Albini *et al.,* 1995) and inhibition of chemotaxis and invasion of malignant cells (Albini *et al.,* 1995).

Unfortunately, under certain conditions, AsA functions as a pro-oxidant rather than an anti-oxidant. As shown by the literature, AsA can thus enhance both spontaneous and chemically induced mutations in prokaryotes and eukaryotes and up-regulate chemically induced tumors in rodents (reviewed by Shamberger, 1984). Mechanistically, AsA has been proposed to act as a reducing agent for chelate-complexed Fe^{3+}, thereby favoring production of hydroxyl radicals from hydrogen peroxide through a Fenton-type reaction (Winterbourn, 1979; Benatti *et al.,* 1983). NAC is a scavenger of reactive oxygen species, particularly of hydroxyl radicals generated in Fenton-type reactions (Aruoma *et al.,* 1989; Doelman and Bast, 1990; Izzotti *et al.,* 1998). Based on several lines of evidence, it has been postulated that cellular GSH may be essential for the function of AsA (Meister, 1992). However, GSH is not transported efficiently into cells (Meister, 1989). In contrast, NAC is readily taken up and de-acetylated inside cells to yield cysteine, which is the rate-limiting amino acid in the ATP-dependent synthesis of this tripeptide (De Flora *et al.,* 1995*a*).

These considerations prompted us to evaluate whether the combination of NAC with AsA could produce beneficial effects in mutagenicity and carcinogenicity test systems. Firstly, we evaluated the combined effects of NAC and AsA on spontaneous mutagenicity in *Salmonella typhimurium* since we had previously observed that NAC selectively lowers the spontaneous background of revertants in strain TA104 whereas AsA enhances the spontaneous mutagenicity in both TA102 and TA104 (De Flora *et al.,* 1994), which are typically reverted by oxidative mutagens (Levin *et al.,* 1982). Then, based on the knowledge that AsA and GSH are the major intracellular reductants of chromium(VI) (reviewed by De Flora and Wetterhahn, 1989) and that AsA, GSH and NAC are

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capable of reverting the mutagenicity of this metal (De Flora *et al.*, 1989*a*), we evaluated the interaction between AsA and NAC in accomplishing this process. Finally, we used the lung tumor assay for assessing the interaction between NAC and AsA, with urethane as a test carcinogen. Previously, oral NAC had been shown to inhibit urethane-induced lung tumors in Swiss albino mice (De Flora *et al.*, 1986*a*), BALB/c mice (Balansky and De Flora, 1998) and A/J mice (Witschi *et al.*, 1998). The data reported here provide evidence that NAC and AsA have additive anti-mutagenic and anti-carcinogenic properties and that NAC prevents the adverse effects of AsA on spontaneous mutagenicity in bacterial strains sensitive to oxidative mechanisms.

MATERIAL AND METHODS

Chemicals

AsA (L-ascorbic acid) and urethane (ethyl carbamate) were purchased from Sigma (St. Louis, MO) and sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) from Merck (Darmstadt, Germany). NAC (Zambon, Vicenza, Italy) was used as a pure reagent in *in vitro* experiments and in the form of a commercial product, containing 200 mg of drug per confection (Fluimucil, Zambon), in *in vivo* experiments.

Animals

A total of 120 female A/J inbred mice (Harlan, Milan, Italy), aged 4 to 6 weeks and weighing 12 to 14 g at the start of the experiment, were used. Animals were housed in plastic cages (10/cage) on sawdust bedding and maintained on standard rodent chow (MIL; Morini, Reggio Emilia, Italy) and drinking water *ad libitum.* The temperature of the animal room was 23 ± 2°C, with a relative humidity of 55% and a 12 hr day–night cycle. The housing and treatment of mice were in accordance with our national and institutional guidelines.

Mutagenicity assays

Evaluation of spontaneous and chromium(VI)-induced mutagenicity was made in the Ames' reversion test. The *S. typhimurium his*$^-$ strains TA102 and TA104, both carrying the *hisG428* mutation (Levin *et al.*, 1982), were used. Test compounds (sodium dichromate, AsA and NAC) were dissolved in PBS (pH 7.4) and used at the doses indicated under Results. Several preliminary assays were carried out to find the optimal doses of AsA and NAC, which needed to be in the linear part of dose-response curves and sufficient to decrease the chromium(VI)-induced mutagenic response but not too high to determine complete inhibition when the 2 agents were combined.

Mutagenicity was evaluated according to the plate incorporation test (Maron and Ames, 1983), with the modifications suggested by De Flora *et al.* (1994) for assessing in parallel modulation of the mutagenic response and survival of test bacteria.

Evaluation of chromium(VI) reduction

Chromium(VI) reduction by AsA and NAC, in 10 μl volumes, was evaluated by mixing a fixed dose of chromium(VI) with varying doses of AsA and NAC. These were pre-determined in preliminary assays prior to performing the definitive experiments reported under Results. After 30 min of contact at room temperature, 2 ml of a reagent containing *s*-diphenylcarbazide (Carlo Erba, Milan, Italy; 40 mg in 100 ml of 19% ethanol and 8% sulfuric acid in water) were added. After 10 min at room temperature, the resulting chromium–diphenylcarbazonium complex was measured at 540 nm in a spectrophotometer (model U-3200; Hitachi, Tokyo, Japan). Residual chromium(VI) was calculated using detailed calibration curves prepared with sodium dichromate.

Mouse lung tumor assay

Mice were acclimatized for 10 days, after which all of them received a single i.p. injection of urethane, at a dose of 1 g/kg body weight. Animals were divided into 4 groups, each composed of 30 mice, including mice treated with urethane only, mice treated with urethane and AsA, mice treated with urethane and NAC and mice treated with a combination of AsA and NAC. Both drugs were given with drinking water, starting 7 days before injection of the carcinogen and continuing until the end of the experiment. Concentrations of AsA and NAC were calibrated according to body weight and water consumption, to yield a calculated daily intake of 1 g/kg body weight each. The body weight of each mouse was measured at weekly intervals.

Four months after injection of urethane, all mice were anesthetized with diethyl ether and killed by cervical dislocation. Tellyesniczky's fixative (1 ml) was instilled into the trachea, and lungs were removed and immersed in the same fixative. After 7 days of fixation, the number of surface tumors was scored using a stereomicroscope. Urethane-induced tumors are localized beneath the pleura, and there is no advantage in examining serial lung sections (Shimkin and Stoner, 1975; Balansky, 1995). The 2 main diameters of each tumor were measured with a 10 mm/0.1 linear micrometer. Ten tumors per group were subjected to standard histopathological analysis after staining with hematoxylin–eosin.

Statistical analysis

The significance of variations in mutagenicity was evaluated by Student's *t*-test. The parallelism of curves relating either chromium(VI) reduction or inhibition of its mutagenicity to doses of AsA or AsA plus NAC was evaluated by the test for equality of slopes of regression lines. Differences in multiplicity and size of lung tumors were evaluated with ANOVA and Fisher's PLSD *post-hoc* test.

The effect of interaction between AsA and NAC on the parameters pertaining to lung tumors (total number, multiplicity, mean diameter and total volume) was assessed by calculating an index *(S)* according to the following formula:

$$S = \frac{A - D}{2A - B - C}$$

where A, B, C and D are the values observed for each parameter in mice treated with urethane only (A), urethane plus AsA (B), urethane plus NAC (C) and urethane plus AsA and NAC (D). This formula was adapted from the calculation proposed by Rothman (1976) for assessing interactions between 2 risk factors in epidemiological studies.

RESULTS

Effects of AsA and/or NAC on "spontaneous" mutagenicity

Varying amounts of AsA, NAC and the combination of both were assayed in the Ames' plate incorporation test, using *S. typhimurium his*$^-$ strains TA102 and TA104. Figure 1 shows, as an example, the results of an experiment aimed at evaluating modulation of spontaneous mutagenicity. Similar indications were provided by the other 3 experiments (not shown).

When tested alone, AsA produced an increase of mutagenicity in both strains TA102 and TA104. Also due to the high background of revertants in these strains, the observed increases did not meet 1 of the criteria for positivity in the Ames test, *i.e.*, at least a doubling of spontaneous revertants. However, these results were dose-dependent, reproducible in different experiments and statistically significant in TA104 at the doses of 2 ($p < 0.05$), 4 to 6 ($p < 0.01$) and 8 to 10 ($p < 0.001$) μmol/plate and in TA102 at the doses of 6 ($p < 0.05$) and 8 to 10 ($p < 0.001$) μmol/plate.

In contrast, NAC did not affect mutagenicity in TA102 and produced a dose-dependent decrease of spontaneous revertants in TA104, which was evident and statistically significant ($p < 0.001$) at the lowest tested dose (2 μmol/plate). Combination with NAC in the same test system resulted in complete prevention of the adverse effects of AsA. Indeed, the NAC–AsA association counteracted the enhancement by AsA of spontaneous mutagenicity in TA104 and decreased the number of revertants to the same levels

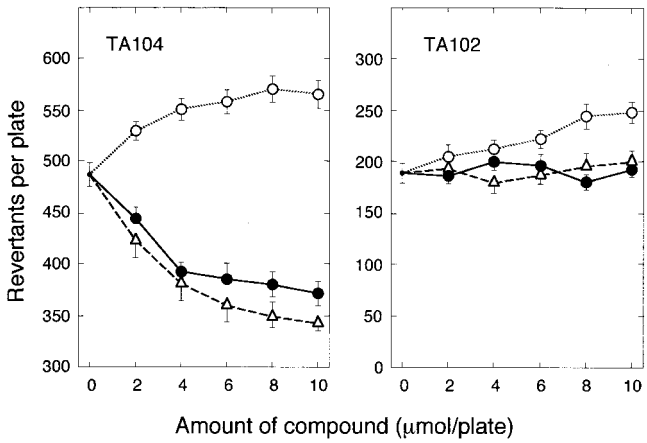

FIGURE 1 – Effect of varying amounts of AsA (open circles), NAC (open triangles) or their combination (solid circles) on spontaneous mutagenicity in *S. typhimurium his*$^-$ strains TA104 and TA102. Results are means ± SD of triplicate plates. See text for statistical analysis.

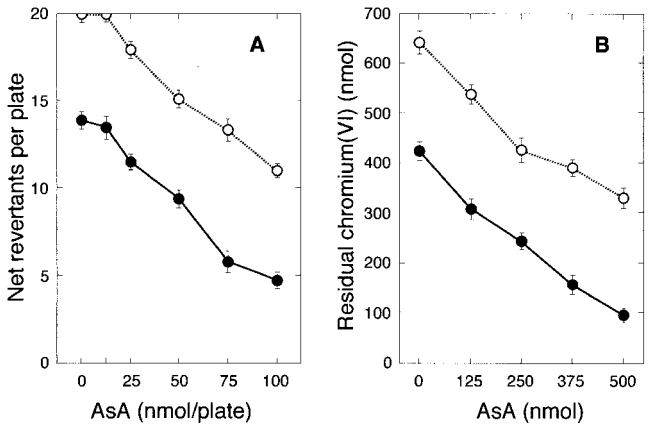

FIGURE 2 – *(a)* Inhibition by varying amounts of AsA of net revertants (total less spontaneous revertants) induced by sodium dichromate (0.5 μmol/plate) in *S. typhimurium his*$^-$ strain TA102, either in the absence (open circles) or in the presence (solid circles) of NAC (5 μmol/plate). *(b)* Reduction by varying amounts of AsA of sodium dichromate [20 nmol chromium(VI)], either in the absence (open circles) or in the presence (solid circles) of NAC (250 nmol). See text for statistical analysis.

recorded with NAC alone, with significant ($p < 0.001$) decreases at all doses compared to untreated controls. In TA102, the combination of NAC with AsA inhibited the increase of spontaneous revertants produced by AsA alone to such an extent that revertant levels were indistinguishable from those of untreated controls.

Effects of AsA and/or NAC on chromium(VI) stability and mutagenicity

As assessed in preliminary experiments, aimed at optimizing the doses of test compounds, both NAC and, even more potently, AsA reduced the hexavalent chromium salt sodium dichromate and inhibithed its mutagenicity in *S. typhimurium* TA102.

The combination of a fixed amount of NAC with varying amounts of AsA significantly enhanced inhibition of chromium(VI) mutagenicity (Fig. 2*a*) and reduction of chromium(VI) (Fig. 2*b*). As demonstrated by the parallelism of the regression lines relating either chromium(VI) reduction or inhibition of its mutagenicity by AsA only (empty circles) or AsA plus NAC (full circles), the combination of the 2 reducing agents yielded additive effects. Reduction of chromium(VI) and inhibition of its mutagenicity by the AsA–NAC combination were significantly greater ($p < 0.001$) than the effects produced by either drug individually. Similar results were obtained in 3 additional experiments (not shown). Survival of bacteria was not affected at any tested dose.

TABLE I – MULTIPLICITY AND SIZE OF LUNG TUMORS IN VARIOUSLY TREATED A/J MICE

Treatment	Total number of tumors	Multiplicity (tumors/mouse) (mean ± SD)	Diameter of tumors (mm) (mean ± SD)
Urethane	425	14.17 ± 3.21	0.73 ± 0.21
Urethane + AsA	404	13.47 ± 3.52	0.70 ± 0.20[3]
Urethane + NAC	365	12.17 ± 3.56[1]	0.63 ± 0.19[4]
Urethane + AsA + NAC	338	11.27 ± 3.41[2]	0.59 ± 0.20[4,5]

[1]$p = 0.03$ compared to urethane alone.–[2]$p = 0.001$ compared to urethane alone, $p = 0.01$ compared to urethane + AsA.–[3]$p = 0.02$ compared to urethane alone.–[4]$p < 0.0001$ compared to urethane alone.–[5]$p < 0.0001$ compared to urethane + AsA, $p = 0.01$ compared to urethane + NAC.

Effects of AsA and/or NAC on urethane-induced lung tumors in A/J mice

Administration with drinking water of NAC, AsA or their combination did not significantly affect body weight gain in A/J mice treated with an i.p. injection of urethane (curves not shown). Seven days before injection of urethane, weights of mice treated with either urethane only, urethane plus AsA, urethane plus NAC or urethane plus AsA and NAC (means ± SD) were 12.4 ± 0.5, 12.8 ± 0.8, 13.9 ± 0.4 and 13.5 ± 0.3 g, respectively. At the end of the experiment, weights were 22.6 ± 1.2, 22.3 ± 0.9, 22.3 ± 0.6 and 23.0 ± 0.4 g, respectively. This demonstrates the lack of gross toxicity of both drugs at the tested doses.

Four months after administration of urethane, all 120 mice developed lung tumors. The total number of tumors was 1,532, including 425 tumors in the 30 mice treated with the carcinogen only, 404 in those treated with urethane plus AsA, 365 in those treated with urethane plus NAC and 338 in those treated with urethane plus AsA and NAC. All tumors exhibited a homogeneous appearance at the stereomicroscope, and histological analysis of 10 tumors per experimental group showed that all were classifiable as adenomas.

As shown in Table I, the slight decrease of tumor multiplicity in mice treated with AsA was not statistically significant, whereas NAC and, even more so, NAC plus AsA produced a significant decrease.

Table I also shows that AsA, NAC and NAC plus AsA, in increasing order of potency, decreased the mean diameter of lung tumors. The effects were not marked but statistically significant. Moreover, the decrease in size produced by the combined drugs was significantly greater than that produced by either drug individually.

The size distribution of tumors in the 4 treatment group is illustrated in Figure 3. Besides lowering the total number of tumors, treatment with AsA and, even more so, with NAC and the NAC–AsA combination produced a shift to the left in the size of tumors; *i.e.*, there was some increase in the number of smaller tumors and a pronounced decrease in the number of larger tumors. In particular, in the 4 treatment groups (urethane, urethane plus AsA, urethane plus NAC, urethane plus AsA and NAC), there were 172, 184, 213 and 223 tumors with a diameter ≤0.6 mm, whereas there were 253, 220, 152 and 115 with a diameter ≥0.7 mm, respectively. Arbitrarily assuming a spherical shape, the total volumes occupied by lung tumors in the 30 mice of each group were 107.5, 89.3, 61.3 and 49.7 mm^3, respectively.

To evaluate whether the interaction between NAC and AsA was synergic, additive or antagonistic, we applied an *S* index, calcu-

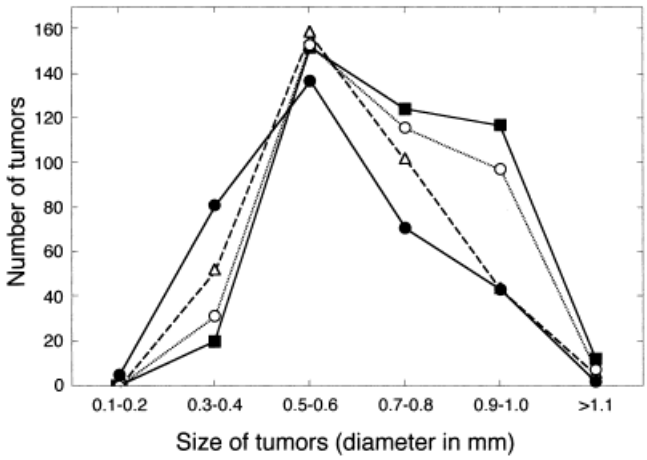

FIGURE 3 – Number of lung tumors of varying sizes in mice treated either with urethane only (solid squares), urethane plus AsA (open circles), urethane plus NAC (open triangles) or urethane plus NAC and AsA (solid circles).

lated as described in Material and Methods. For the different available parameters, we obtained the following results:

—Total number of tumors: $S = \dfrac{425 - 338}{2\times425 - 404 - 365} = 1.07$

—Multiplicity of tumors: $S = \dfrac{14.17 - 11.27}{2 \times 14.17 - 13.47 - 12.17} = 1.07$

—Mean diameter of tumors: $S = \dfrac{0.73 - 0.59}{2 \times 0.73 - 0.70 - 0.63} = 1.08$

—Total volume of tumors: $S = \dfrac{107.5 - 49.69}{2 \times 107.5 - 89.3 - 61.3} = 0.94$

Thus, for all parameters, the *S* index is near unity, which means that the effects of NAC and AsA were additive in the mouse lung tumor assay.

DISCUSSION

The results of the present study provide evidence for the individual effects of AsA and NAC in experimental models of mutagenesis and carcinogenesis and shed new light on their interaction in these biological systems.

Modulation of so-called spontaneous mutagenesis is of particular interest since the exact nature of this process and the relative contribution of different mechanisms remain to be defined and explored more deeply. The present data confirm, for the individual compounds, the results of a previous study evaluating the ability of 34 compounds, many of which were classified as putative chemopreventive agents, to affect the background of revertants in *S. typhimurium* strains TA102 and TA104 (De Flora *et al.*, 1994). Indeed, AsA enhanced the number of revertants in both strains, to a modest (less than 2-fold) but significant extent, in a dose-related and reproducible fashion. Due to the sensitivity of these strains to oxidative mutagens, it is likely that AsA behaves in this system as a pro-oxidant, according to the mechanism hypothesized above. However, like other thiols (De Flora *et al.*, 1994), NAC was confirmed to selectively inhibit spontaneous mutagenicity in TA104 but not in TA102. Both strains carry the ochre mutation *hisG428*, but the nonsense mutation TAA is present in a single copy on the TA104 chromosome, whereas TA102 contains the same mutation on a multicopy plasmid (Levin *et al.*, 1982). Moreover, although both TA102 and TA104 contain plasmid pKM101, which channels DNA repair through an error-prone pathway (Perry and Walker, 1982), only TA104 has a deletion of the *uvrB* gene, which codes for an error-free DNA excision-repair system (Ames *et al.*, 1973). Therefore, the finding that NAC and other thiols selectively lower spontaneous mutagenicity in TA104 suggests that supply of SH groups may corroborate some DNA-repair function that is unmasked in the strain lacking the *uvrB* background. As an alternative hypothesis, SH groups may block possible components present in the test system micro-environment, which may induce mutations reparable *via* the *uvrB* mechanism (De Flora *et al.*, 1994).

Another *in vitro* end point evaluated in the present study was the ability of the 2 agents to reduce chromium(VI) and inhibit its mutagenicity. Chromium(VI) is a human carcinogen, but carcinogenicity is limited to the respiratory tract and to the high exposures encountered in certain occupational settings (International Agency for Research on Cancer, 1990). These patterns are due to the fact that, in the organism, chromium(VI) tends to be reduced to chromium(III), which is inactive as a consequence of the poor ability to cross cell membranes (De Flora and Wetterhahn, 1989). AsA and GSH are the major chromium(VI) reductants not only in target cells but also in body fluids, such as secretions of the gastrointestinal tract, plasma and the epithelial lining fluid covering the lower respiratory tract, as well as in long-lived non-target cells, such as pulmonary alveolar macrophages and red blood cells (De Flora and Wetterhahn, 1989; De Flora *et al.*, 1997). Administration of AsA to humans enhances the chromium(VI)-reducing capacity of plasma (Korallus *et al.*, 1984) and, both experimentally and clinically, AsA is the most efficacious antidote for treatment of chromium(VI) poisoning (Hathaway, 1986). Administration of NAC to rats enhanced the amounts of chromium(VI) reduced in pulmonary alveolar macrophages (De Flora *et al.*, 1986*b*). In the present study, individual treatment with NAC and, even more potently, with AsA reduced chromium(VI) and inhibited its mutagenicity in bacteria, thus confirming the results of a previous study (De Flora *et al.*, 1989*a*).

The lung tumor assay in mice (Shimkin and Stoner, 1975) is one of the most extensively used bioassays for evaluating the ability of chemopreventive agents to modulate the chemical induction of lung tumors. In previous studies, a significant decrease of lung tumors was observed in mice treated with a single i.p. injection of urethane and receiving oral NAC. In particular, this protective effect was observed in Swiss albino mice treated with 1 g/kg body weight urethane and receiving a diet supplemented with 0.2% NAC (De Flora *et al.*, 1986*a*), in BALB/c mice treated with 1 g/kg body weight urethane and receiving NAC with drinking water at doses of either 0.1 or 0.5 g/kg body weight (Balansky and De Flora, 1998) and in A/J mice treated with 0.25 g/kg body weight urethane and receiving a diet supplemented with 0.2% NAC (Witschi *et al.*, 1998). In the last study, however, NAC was not effective in mice treated with either 0.1 or 1 g/kg body weight urethane (Witschi *et al.*, 1998). NAC was also successful at significantly decreasing the frequency of micronucleated erythrocytes in both bone marrow (data not shown) and peripheral blood (Balansky and De Flora, 1998) of mice treated with urethane.

In the present study, administration of NAC with drinking water, at a dose of 1 g/kg body weight, significantly decreased both multiplicity and size of lung tumors induced in A/J mice by urethane at 1 g/kg body weight. At the same dose, AsA did not significantly decrease the multiplicity of lung tumors but significantly reduced their size, which may tentatively be ascribed to inhibition of tumor growth by this compound. A series of experiments performed in BALB/c mice treated with an i.p. injection of urethane (1 g/kg body weight) provided evidence that continuous administration of AsA with drinking water, at doses up to 0.75 g/kg body weight, does not affect the multiplicity of lung tumors (data not shown). Post-treatment with AsA, starting 30 days after injection of the carcinogen, was conversely successful at significantly decreasing lung tumor multiplicity when tested at doses higher than 2.5 g/kg body weight (data not shown). Further ex-

periments, using equivalent doses of AsA under either continuous or post-treatment conditions, are needed to complete this study, which so far has provided evidence for an anti-promoting effect of AsA at high doses.

The major goal of the present study was to evaluate, under well-controlled experimental conditions, the outcome of the interaction between NAC and AsA in the 3 test systems described above. The results were quite encouraging in that they provided evidence for the ability of NAC to counteract an adverse effect of AsA and to produce additive effects regarding those end points which were favorably modulated by AsA itself. Recycling of AsA from its oxidized forms is required to maintain intracellular stores (May *et al.,* 1998), and GSH has been shown, both *in vitro* and *in vivo,* to maintain a reducing milieu in the cell, which can reduce dehydroascorbic acid (Meister, 1992, 1994; Rose and Bode, 1992; Winkler *et al.,* 1994). Most likely, the same function can be provided by NAC, either *per se* or intracellularly, as a precursor of cysteine and GSH. This explains the ability of NAC to prevent the pro-oxidant action of AsA leading to an increase in background levels of revertants in bacterial strains sensitive to oxidative mechanisms. We previously showed that NAC is a scavenger of mutagenic oxygen species generated by either electron-transfer reaction (De Flora *et al.,* 1989*b*) or energy-transfer reaction (Camoirano *et al.,* 1993). Interestingly, the combination of NAC with AsA not only neutralized the enhancing effect of AsA on spontaneous mutagenicity but even down-regulated spontaneous revertants to the same extent as NAC alone did. Anti-oxidant mechanisms are also likely to play a major role in modulation by NAC and/or AsA of chromium(VI)-induced mutagenicity and urethane-induced lung tumorigenicity. Indeed, oxidative mechanisms are involved in the genotoxicity and carcinogenicity of chromium(VI) (reviewed by De Flora and Wetterhahn, 1989) as well as in the carcinogenicity of urethane, which involves the formation of ethene adducts of adenosine and cytidine in the initiation step (Guengerich and Kim, 1991; Park *et al.,* 1993). Both chromium(VI) reduction, accompanied by reversal of its mutagenicity, and the decrease in size of urethane-induced lung tumors following treatment with the AsA–NAC combination were significantly more pronounced compared not only to controls but also to individual treatment with each drug.

Based on the *in vivo* evidence for a physiological role of the AsA–GSH system, it would have been of interest to design an experiment involving chronic administration of large doses of AsA combined with compounds that increase tissue levels of GSH (Meister, 1992). As supported by several decades of clinical experience in a variety of therapeutic applications, both AsA and NAC are highly tolerable drugs, which can be used in humans at large doses and for long periods of time. The convenience of combining AsA and NAC, as demonstrated by the experiments performed in the present study, offers encouraging perspectives for both prophylactic and therapeutic purposes.

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