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CHEMOPREVENTION BY *N*-ACETYLCYSTEINE OF URETHANE-INDUCED CLASTOGENICITY AND LUNG TUMORS IN MICE

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A major goal in pre-clinical cancer chemoprevention research is to assess the predictive value of intermediate biomarker modulation towards tumor prevention. With this aim, BALB/c mice were treated with 10 daily i.p. injections of urethane (ethyl carbamate), each of 400 mg/kg body weight. Groups of mice received with drinking water either a drug containing the thiol *N*-acetylcysteine (NAC), at 0.1 or 0.5 g/kg body weight, or its excipient, starting 27 days before the first injection of the carcinogen until the end of the experiment. Out of the 30 mice, 10 per group were identified and individually monitored for 8 sequential times in order to assess the course of micronucleated normochromatic erythrocytes in peripheral blood. This systemic genotoxicity biomarker increased during the 10-day period of treatment with urethane, reached a peak 2 to 6 days after the last injection, and was still significantly higher than the baseline after 10 additional days. Clastogenicity was significantly inhibited by NAC, with a dose-related effect, but not by the drug excipient. As evaluated 4 months after the first injection of urethane, most mice developed lung tumors, whose multiplicity was not affected by the drug excipient but was significantly decreased in the presence of NAC. Correlation between the frequency of micronucleated normochromatic erythrocytes at peak levels and lung-tumor multiplicity was highly significant when evaluated in the context of all 40 mice undergoing cytogenetic analyses ($r = 0.561$, $p = 0.0002$). It was similarly high, but did not reach the significance threshold, within each treatment group, due to the lower number of animals and some deviations from the regression line. Therefore, the prediction of lung-tumor yield based on the intensity of the early genotoxicity biomarker is justified when formulated within a sufficiently large group of animals, but is not absolute at individual level. ***Int. J. Cancer*** **77:302–305, 1998.**
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One of the main goals in cancer-chemoprevention research is to establish a parallelism between modulation of intermediate biomarkers and induction of tumors by standard carcinogens, and to assess the efficacy of chemopreventive agents in inhibiting both endpoints (Kelloff *et al.,* 1992). In this domain, pre-clinical studies in rodents provide useful experimental models for evaluating the predictive value of intermediate endpoints towards occurrence and/or prevention of the disease.

With this main objective in mind, we designed a study investigating the protective effects of the thiol *N*-acetylcysteine (NAC), a known cancer-chemopreventive agent (Kelloff *et al.,* 1994; De Flora *et al.,* 1995*a*,*b*), in the mouse lung-tumor assay. Urethane (ethyl carbamate) was used as a mutagenic and carcinogenic agent. The ability of this compound to induce lung tumors in mice was first reported more than 50 years ago (Nettleship *et al.,* 1943), and this bio-assay has been extensively used as a model for lung tumorigenesis and its modulation (Mirvish, 1968; Shimkin and Stoner, 1975; Balansky, 1995; De Flora *et al.,* 1986). We have shown that supplementation of the diet with 0.2% NAC, before and after an i.p. injection of urethane, resulted in significant inhibition of the lung tumor yield in Swiss albino mice (De Flora *et al.,* 1986).

In the present study we re-assessed, with some methodological changes, the ability of oral NAC to prevent lung tumors in mice. In parallel, we evaluated the efficacy of NAC to inhibit urethane-induced clastogenicity in the same animals. Besides being a strong clastogen in mouse bone marrow (Wild, 1978; Heddle *et al.,* 1983; Ashby *et al.,* 1990), urethane has been shown to cause a time-related increase in the number of micronucleated normochromatic erythrocytes (MN NCE) in mouse peripheral blood (Balansky *et al.,* 1992). The assessment of this endpoint is particularly useful in case of repeated exposure to clastogens, since induced MN accumulate to significant levels in mouse peripheral-blood NCE (Schlegel and MacGregor, 1983). This biomarker cannot be evaluated in most species, including humans, since peripheral-blood erythrocytes carrying these chromosomal alterations are sequestered by the spleen (Schlegel and MacGregor, 1983). Nevertheless, it can be conveniently exploited in the mouse model as a general indicator of clastogenicity and, compared with most other biomarkers, has the advantage of being non-invasive, since it requires only a drop of blood. In this way, as shown in the present study, it is possible to monitor the time course of MN NCE frequency and the subsequent yield of lung tumors in a single animal, as related to treatment with the carcinogen and administration of the chemopreventive agent.

MATERIAL AND METHODS

Animals

A total of 120 male BALB/c mice (Animal Laboratory, National Center of Oncology, Sofia, Bulgaria), aged 8 to 12 weeks and weighing 22 to 26 g, were used. This mouse strain has been shown to be sensitive to urethane tumorigenicity by developing, in 4 months, a very high incidence of lung tumors, identified at histology as alveolar and papillary adenomas (Balansky, 1995). Lung-tumor multiplicity was sufficiently high to evaluate the effect of chemopreventive agents (Balansky, 1995).

The animals were housed in plastic cages (10 mice per cage) on sawdust bedding, and maintained on standard rodent chow and tap water *ad libitum.* The animal-room temperature was 25 to 27°C, with a relative humidity of 55%, and a 12-hr day-night cycle. Housing and all treatment of mice were in accordance with national and institutional guidelines.

Treatment

After 10 days of acclimatization, the mice were divided into 4 groups (30 mice each), including: (a) mice treated with urethane (Sigma, St. Louis, MO) only; (b) mice treated with urethane plus the excipient of the drug (Fluimucil, Zambon, Vicenza, Italy); (c) mice treated with urethane plus the complete drug containing NAC at a calculated daily intake of 0.1 g/kg body weight; and (d) mice treated with urethane plus the complete drug containing NAC at a calculated daily intake of 0.5 g/kg body weight.

Urethane was injected i.p. into all mice at the daily dose of 400 mg/kg body weight for 10 consecutive days, starting on day 27, taking the earliest collection of blood (see later) as time 0 of the experiment. The NAC-containing drug or its excipient were

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administered daily with drinking water, starting on day 0 of the experiment until the mice were killed. Since the NAC formulation used is stable in drinking water for at least 2 days at room temperature (data not shown), the drug-containing water was changed every 2 days, and dilution of the drug in drinking water was adjusted weekly on the basis of *ad libitum* consumption. In the group of mice treated with the excipient only, its dilution in drinking water was the same as for the complete drug at the highest NAC intake (0.5 g/kg body weight).

The mice were observed daily for general appearance, and weighed at weekly intervals.

Clastogenicity analyses

Ten mice from each experimental group were given an identification number, to allow multiple clastogenicity analyses and assessment of lung-tumor yield in individual animals. On days 0, 27, 33, 37, 43, 50, 60 and 70 of the experiment, peripheral blood was collected from the lateral tail vein of mice treated either with urethane or with the drug containing NAC at 2 different doses. The mice treated with urethane plus the drug excipient were monitored only on day 43 of the experiment. Therefore, in all, 250 blood samples were available. Blood smears were stained with May-Grünwald-Giemsa, and NCE were scored at a magnification of 1,000× for frequency of MN, according to the standard criteria (Schmid, 1975). A minimum of 20,000 NCE were scored for each blind sample by a single reader.

Lung-tumor yield

Four months after the first injection of urethane, all surviving mice were killed by diethyl ether. The lungs were removed, fixed in 10% buffered formalin, and the number of superficial tumors was scored with the aid of a magnifying lens. Urethane-induced tumors are localized beneath the pleura, and there is no advantage in examining serial lung sections (Shimkin and Stoner, 1975), as also evaluated in BALB/c mice (Balansky, 1995). The lung-tumor yield was expressed in terms of tumor incidence (frequency of tumor-bearing mice) and of multiplicity (mean number of tumors per mouse) within each experimental group.

Statistical analyses

Comparisons between treatment groups concerning frequencies (survival and tumor incidence) were made by χ^2 analysis. Those concerning mean values (body weight, MN NCE and tumor multiplicity) were made by Student's *t*-test. Correlations between the intermediate biomarker and the tumor multiplicity were evaluated by regression analysis and calculation of r values.

RESULTS

Time course of MN induction in peripheral blood NCE

Figure 1 shows the individual frequencies of MN NCE in all tested mice and their mean values at various times before and after 10 daily i.p. injections of urethane. The effect of the oral administration of NAC at 2 different dose regimens is also shown.

The baseline frequency in mice receiving urethane only was 1.0 ± 0.1 MN NCE/1000 NCE (mean ± SD of the 40 data recorded at days 0, 27, 60 and 70 of the experiment). An evident induction of MN, accounting for an average 2.9-fold increase over the baseline ($p < 0.001$), was observed after 6 doses of urethane. MN frequency further grew 2 days after the last injection of the carcinogen (4.7-fold, $p < 0.001$), *i.e.*, on day 38 of the experiment, and 6 days later reached a peak (6.0-fold, $p < 0.001$). Thereafter the curve declined, but MN levels were still elevated (1.7-fold, $p < 0.001$) 14 days after the last injection of urethane.

Administration with drinking water of the NAC-containing drug did not affect the frequency of ''spontaneous'' MN NCE. However, treatment with the chemopreventive agent attenuated urethane-induced MN, as shown by an evident flattening of time-course curves (Fig. 1). In particular, treatment with the drug containing NAC at a dose of 0.1 g/kg body weight significantly inhibited the

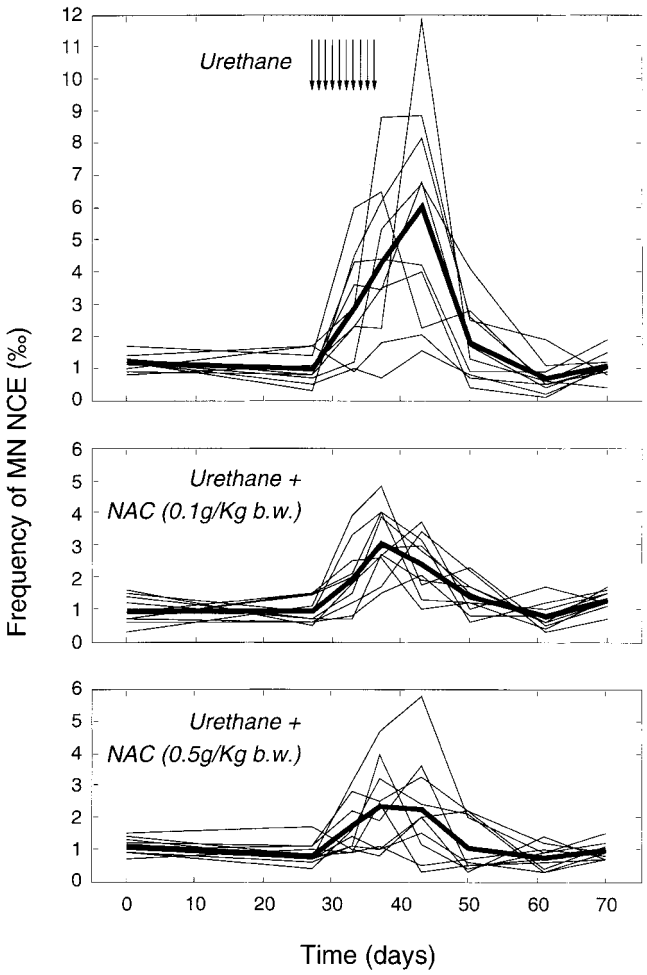

FIGURE 1 – Time course of the frequency of MN NCE in mice treated either with urethane only or with urethane plus the complete drug containing NAC at either 0.1 or 0.5 g/kg body weight. MN NCE were evaluated in peripheral blood at days 0, 27, 33, 43, 50, 60 and 70 of the experiment. Urethane was injected i.p. at the daily dose of 400 mg/kg body weight for 10 consecutive days, starting on day 27. The NAC-containing drug was administered with drinking water throughout duration of the experiment. The lines indicate the time course of the frequency of MN PCE in each one of the 10 mice examined for this parameter within each experimental group. The bold lines indicate the mean values at each monitoring time.

formation of MN 8 days after the last injection of urethane ($p < 0.01$), whereas inhibition by NAC at 0.5 g/kg body weight was significant 2 days ($p < 0.05$) and 8 days ($p < 0.01$) after the last injection of urethane. As monitored at one time only, i.e., 8 days after the last injection of urethane, administration of the drug excipient, in the absence of NAC, did not significantly affect the frequency of MN NCE (5.5 ± 0.9‰) as compared with positive controls treated with urethane only (6.0 ± 1.0‰). Inhibition of MN induction in the mice receiving the complete drug, irrespective of the NAC dose, was significant ($p < 0.01$) also when compared with that of mice receiving the drug excipient only.

Body weight and lung-tumor yield

Body growth in urethane-treated mice was not affected by treatment with the NAC-containing drug or its excipient. At the start of the study, body weights (means ± SD) were 25.5 ± 0.7, 26.9 ± 0.8, 27.6 ± 0.7 and 28.6 ± 0.9 g in mice treated with urethane only, urethane plus the drug excipient, urethane plus the drug containing NAC at 0.1 g/kg body weight and urethane plus the

drug containing NAC at 0.5 g/kg body weight respectively. At the end of the study, body weights were 31.6 ± 1.0, 33.3 ± 1.2, 34.9 ± 0.9 and 32.0 ± 1.0 g respectively.

As shown in Table I, the large majority of mice developed lung tumors 4 months after the first injection of urethane. Administration of the drug excipient did not affect the tumor yield. Administration of the drug containing NAC failed to affect tumor incidence but was successful in decreasing tumor multiplicity. In particular, the decrease of tumor multiplicity in mice treated with NAC at 0.5 g/kg body weight was significant as compared with mice treated either with urethane only ($p < 0.01$) or with urethane plus the drug excipient ($p < 0.01$). Inhibition by NAC at 0.1 g/kg b.w. was only significant as compared with urethane ($p < 0.05$) but did not reach the significance threshold as compared with the drug excipient.

Correlation between induction of MN PCE and lung-tumor multiplicity

Figure 2 shows the correlation between the frequency of MN NCE and the corresponding lung-tumor multiplicity. The frequency of MN PCE was inferred from the peak, occurring 12 to 18 days after the first injection of urethane, of time-course curves recorded in each one of the 40 mice tested for this parameter (Fig. 1). The tumor multiplicity was assessed about 3.5 months later, *i.e.,* 4 months after the first injection of urethane. It does not overlap with the data shown in Table I, since correlation was evaluated in a sub-set of 10 out of 30 mice per group, randomly selected for clastogenicity analyses.

The correlation indices between the early biomarker and tumor multiplicity within each experimental group were rather high, but only approached the significance threshold (Fig. 2). In particular, in mice treated with urethane only (open circles), r was 0.597 ($p = 0.068$); in mice treated with urethane plus the drug excipient (filled circles), r was 0.531 ($p = 0.115$); in mice treated with urethane plus the drug containing NAC at 0.1 g/kg body weight (open triangles), r was 0.558 ($p = 0.094$); in mice treated with urethane plus the drug containing NAC at 0.5 g/kg body weight (filled triangles), r was 0.302 ($p = 0.397$). The overall r relative to 40 mice was 0.561. Due to the larger number of animals, this value was highly significant ($p = 0.0002$).

DISCUSSION

The results obtained in the present study showed that multiple i.p. injections of urethane transiently induce a systemic clastogenic damage, which is followed 3.5 months later by appearance of lung tumors. The oral administration of a NAC-containing drug resulted in a significant, dose-related inhibition of both endpoints, which could be ascribed to NAC itself and not to the drug excipient.

TABLE I – INCIDENCE AND MULTIPICITY OF LUNG TUMORS

Treatment	Surviving mice		Mice with tumors		Tumors/mouse
	Number	(%)	Number	(%)	Mean ± SD
Urethane	26	(86.7)	24	(92.3)	5.7 ± 0.8
Urethane + drug excipient	28	(93.3)	25	(89.3)	4.9 ± 1.1
Urethane + drug (0.1 g NAC/kg b.w.)	27	(90.0)	22	(81.5)	3.3 ± 0.6[1]
Urethane + drug (0.5 g NAC/kg b.w.)	29	(96.7)	25	(86.2)	2.8 ± 0.4[2]

Lung-tumor yield was evaluated 4 months after the first of 10 daily injections of urethane, and expressed in terms of incidence (mice with tumors) and multiplicity (tumors/mouse). Each experimental group was composed of 30 mice at the start of the study.

[1]Statistically significant as compared with mice treated with urethane only ($p < 0.05$).–[2]Statistically significant as compared with mice treated either with urethane only ($p < 0.01$) or with urethane plus drug excipient ($p < 0.05$).

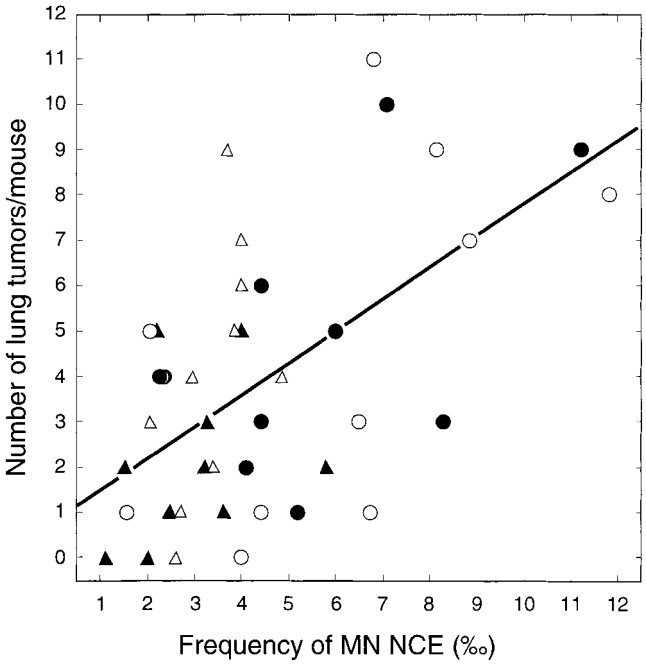

FIGURE 2 – Relationships between the maximum frequency of MN NCE in peripheral blood (Fig. 1) and multiplicity of lung tumors (Table I) evaluated after 3.5 months in each one of the 40 mice belonging to 4 experimental groups, *i.e.,* mice treated with urethane only (○) mice treated with urethane plus the drug excipient (●), mice treated with urethane plus the drug containing NAC at 0.1 g/kg body weight (△), and mice treated with urethane plus the drug containing NAC at 0.5 g/kg body weight (▲). The equation of the overall regression line was: $y = 0.666x + 0.887$. See text for indication of r values and their significance within each experimental group and within pooled groups.

In particular, the frequency of MN NCE rapidly increased during the 10-day period of treatment with the carcinogen, reached a peak 2 to 6 days after the last injection and, compared with the baseline, was still elevated after 10 additional days. The observed chromosomal alterations reflect clastogenic damage occurring in the bone marrow, which during erythropoiesis affects dividing erythroblasts before they become polychromatic erythrocytes (PCE) by extrusion of nuclei. PCE, still containing ribosomal RNA, last in the bone marrow for 12 to 24 hr, then for a similar time in the peripheral blood, where they mature into NCE, which persist for about one month (Mavournin *et al.,* 1990). Thus MN NCE are diluted into the normal NCE population pre-existing in the blood. Nevertheless, especially after repeated exposure to clastogens, as it was the case in the present study, MN tend to accumulate in these relatively long-lived cells, thereby providing a reliable indicator of systemic damage. NAC, which inhibited ''spontaneous'' mutagenicity in DNA repair-deficient bacteria sensitive to oxidative agents (De Flora *et al.,* 1994), failed to affect the ''spontaneous'' levels of MN NCE but significantly inhibited the clastogenic effect produced by urethane.

In agreement with the results of an earlier study (De Flora *et al.,* 1986), the present data provide evidence that, even under more stringent conditions, a continuative regimen with oral NAC prevents the induction by urethane of lung tumors in mice. In fact, in the present study urethane was given in 10 daily i.p. injections, each of 400 mg/kg body weight, whereas in the previous study (De Flora *et al.,* 1986) the carcinogen had been given in a single i.p. injection of 1 g/kg body weight. Dosage of the chemopreventive agent was similar in the 2 experiments, since in the present study NAC was administered with drinking water, in the form of a drug

formulation, at calculated daily intakes of either 0.1 or 0.5 g/kg body weight, whereas earlier (De Flora *et al.*, 1986) NAC had been administered with the diet at a calculated daily intake of 0.12 g/kg body weight.

Besides the preventive effects towards urethane-induced pulmonary tumors in mice, administration of NAC with drinking water has been shown to significantly decrease several molecular, biochemical, cytogenetical and histopathological alterations produced in the lung of Sprague-Dawley rats treated i.t. either with benzo[a]pyrene or with air-particulate extracts, or exposed whole-body to cigarette smoke (Balansky *et al.*, 1996; De Flora *et al.*, 1991*a*, 1995*a;* Izzotti *et al.*, 1994, 1996). On the other hand, the dietary administration of NAC failed to prevent the lung tumors induced in Fischer rats by s.c. injections of a tobacco-specific nitrosamine, *i.e.*, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), although the incidences both of nasal-cavity tumors and of Leydig-cell tumors of the testis were reduced at high doses of NAC in the same animals (Chung *et al.*, 1996).

From a mechanistic point of view, NAC is known to possess an array of multiple protective properties, among which is the ability to work as a nucleophile and a scavenger of reactive oxygen species, both extracellularly *per se* and intracellularly as a precursor of cysteine and reduced glutathione (GSH) (De Flora *et al.*, 1995*a,b*). These mechanisms may be relevant in the observed prevention of urethane clastogenicity and tumorigenicity. Urethane is known to be metabolically activated by dehydrogenation to form vinyl carbamate. This proximate electrophilic metabolite is further oxidized to vinyl carbamate epoxide, which is likely to be the major electrophilic, mutagenic and carcinogenic metabolite of urethane (Park *et al.*, 1993). Trapping of vinyl carbamate epoxide by thiols is demonstrated by the finding that NAC, cysteine and GSH inhibited its bacterial mutagenicity (Park *et al.*, 1993). Since GSH *S*-transferase probably contributes to detoxification of this metabolite (Park *et al.*, 1993), it is noteworthy that oral NAC significantly enhanced GSH S-transferase activity in the liver of the same mice in which lung tumors were inhibited (De Flora *et al.*, 1986). Moreover, as an anti-oxidant (De Flora *et al.*, 1991*b*, 1995*a*), NAC is also expected to interfere with the oxidative metabolism of urethane, which ultimately leads to formation of etheno adducts of adenosine and cytidine (Guengerich and Kim, 1991; Park *et al.*, 1993).

Due to the opportunity to follow the chronological evolution of clastogenic damage in the blood of individual mice and, later on, to measure the tumor yield in the lungs of the same animals, it was important to evaluate how the early biomarker correlated with the late pathological event. The correlation index between frequency of MN NCE at peak levels and multiplicity of lung tumors was highly significant within the whole sub-set of 40 mice undergoing cytogenetic analyses. The r values were similarly high in 3 out of 4 treatment groups, but they did not attain the significance threshold, due to the lower number of animals. It can thus be concluded that the overall predictive value of the biomarker towards lung tumors is satisfactory, in the sense that the majority of mice with intense clastogenic damage also bore a large number of lung tumors. However, there were some exceptions to this trend. Therefore, the prediction of tumor yield as related to the early biomarker of genotoxicity appears to be justified when formulated within a sufficiently large group of animals, but is not absolute at individual level.

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