Study of DNA Methylation by Tobacco-Specific N-Nitrosamines

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An enzyme-linked immunosorbent assay (BA-ELISA) involving use of biotin-labeled anti-rabbit IgG and avidin-labeled horseradish peroxidase was developed for the measurement of O'-methyl-2'-deoxyguanosine (O'-MedGuo). Up to 5 μg of methylated DNA was enzymatically hydrolyzed, and the extent of inhibition of binding of immobilized O'-MedGuo-bovine serum albumin to rabbit anti-O'-MedGuo was measured. Fifty percent inhibition of antigen-antibody binding was achieved with 2.5 pmole of O'-MedGuo.

Separation of O'-MedGuo from unmodified nucleosides by high-performance liquid chromatography (HPLC-BA-ELISA) allowed detection of 700 fmole O'-MedGuo in 1 μg of DNA.

Among the tobacco-related carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is one of the most potent. In F344 rats it induces nasal cavity, lung and liver tumors. Four hours after a single IV injection of NNK to F344 rats (87 mg/kg body weight), O'-MedGuo was present in target organs (μmole O'-MedGuo/mole dGuo) (nasal mucosa, 219; lung, 13.2; and liver, 34.5) but was not detectable in nontarget organs. F344 rats receiving daily IP injections of NNK (40 mg/kg body weight) for 14 days were sacrificed 24 hr after the last injection. The levels of (O'-Medguo/dGuo) were 7.9 and 11.4 μmole/mole in the nasal mucosa and lung, respectively. In the liver no O'-MedGuo was detected, but 1050 μmole of 7-MeGua/mole Gua was measured by HPLC-fluorimetry. No DNA methylation was observed in the nasal mucosa or liver of F344 rats treated with the nicotine-derived carcinogen N'-nitrosonornicotine. Reduction of the carbonyl of NNK is a major metabolic pathway, giving rise to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butan-1-ol (NNAI). Nasal mucosae were cultured in vitro with NNK or NNAI. After 1 hr, methylation at the O' of Gua and 7-dGuo sites were observed with NNK but not with NNAI. Methylation by NNAI after 24 hr was associated with the conversion of NNAI to NNK. These results suggest that NNAI is not associated with the activation of NNK to DNA methylating species.

Introduction

The high incidence of lung cancer among smokers is well documented (1). Studies carried out during the last 10 years have shown that N-nitrosamines present in tobacco smoke are relatively potent carcinogens in animals. The same studies have suggested that N-nitrosamines are important etiologic factors in tobacco smoking-related cancer (2). Among the 13 N-nitrosamines detected in cigarette smoke, the tobacco-specific N-nitrosamines are among the most abundant (3). Prospective and retrospective studies have demonstrated a link between snuff exposure and development of oral cancer (4,5). At present, tobacco-specific N-nitrosamines are the only carcinogens to have been isolated in significant amounts from snuff (6). The levels of absorption of tobacco components by respiratory tract tissues vary among smokers and intrinsic exposure to N-nitrosamines during tobacco smoking is difficult to assess. The tobacco-specific N-nitrosamines: N'-nitrosonornicotine (NNN) and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are derived from nicotine and are metabolized rapidly in vivo to metabolites identical to those of nicotine (2). Consequently, an assessment of exposure to nicotine-derived N-nitrosamines by quantification of their metabolites is not feasible.

However, alkylation species are generated only from NNN and NNK, and levels of their persistent adducts in DNA and proteins could be used as an index of human exposure. Alternatively, assessment of repair of pro-mutagenic DNA damage would measure the ability of an individual to respond to carcinogen insults. The objective of the present study was to develop a safe, inexpensive and sensitive method to measure the levels of O'-methyl-2'-deoxyguanosine (O'-MedGuo) in DNA damaged by activated NNK.

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Materials and Methods

Chemicals

Synthesis of NNK has been described, and its purity was greater than 99% by high performance liquid chromatography (HPLC) (7). Sodium borohydride reduction of NNK gave 4-(methylthiosamino)-1-(3-pyridyl)-butan-1-ol (NNAl) which was free of any traces of NNK by reverse phase HPLC.

Culture of Rat Nasal Mucosa. Removal of the nasal septum and its covering mucosa from the rat nasal cavity and the culture technique of the septum have previously been described (8). Each septum was cultured in a 35-mm culture dish (Falcon, Bethesda, MD) with 2 mL of medium containing either 100 μg of NNK or 101 μg of NNAl/mL. After 1, 3, or 24 hr of culture, the septa were harvested, and DNA was extracted from three combined explants. The medium was harvested and stored frozen. After filtration of the media, levels of NNK and NNAl were measured by HPLC on an octodecylsilane-bonded phase column (4.6 mm × 12.5 cm) as described elsewhere (9).

Rat Treatment with NNK and NNN. Five F344 rats each weighing approximately 280 g at the start of the experiment were fed an NIH-07 diet (Ziegler Brothers, Inc., Gardners, PA). They were injected IV with a solution of NNK in 0.9% NaCl/H2O (87 mg/kg body weight) and decapitated 4 hr later. Livers, lungs, kidneys, esophagi, spleens, and hearts were excised and immediately frozen over dry ice. The nasal cavity was opened as described previously (8), and the mucosa covering the nasal septa and ethmoturbinates of the five rats was scraped off with a scalpel, pooled, and the DNA extracted as described previously (10).

Two F344 rats each weighing about 248 g received daily IP injections of 10 mg of NNN dissolved in 0.9% NaCl/H2O (20 mg/mL). Each rat received 14 injections. Two other rats each received 10 mg of NNK dissolved in 0.9% NaCl/H2O (20 mg/mL). The four rats were exsanguinated by heart puncture 24 hr after the last injection and the tissues mentioned above were excised and stored frozen until DNA extraction.

DNA samples were dissolved in 50 mM pH 7.2 Tris HCl and DNA concentration was determined by fluorimetry (11). DNA was hydrolyzed according to the method of Müller and Rajewsky (12).

Antibody Production and Purification

O6-Methylguanosine (O6-MeGuo) was conjugated to bovine serum albumin (BSA) (Sigma, St. Louis, MO) or keyhole limpet hemocyanin (KLH) (Calbiochem, La Jolla, CA) according to the method of Erlanger and Beiser (13). New Zealand White rabbits were immunized with an emulsion of the conjugate in complete Freuds adjuvant (Calbiochem). The inoculum was injected intradermally at approximately 20 sites on the back of each rabbit. Four booster injections were given at 8-week intervals. The 2-week bleeding after the last booster injection was used in the present study. The anti-O6-MeGuo antibodies were purified by affinity chromatography as previously described for the anti-O6-ethyl-2'-deoxyguanosine (12).

BA-ELISA. The biotin-avidin enzyme-linked immunosorbent assay (BA-ELISA) was performed entirely at room temperature in 96-well polystyrene microtiter plates (Costar, Cambridge, MA). Each experimental value was determined in triplicate. The amount of O6-MeGuo in a sample was determined by the use of two standard curves. The first set of standards was used to measure the extent of inhibition of binding of the primary antibody. The primary antibody was diluted 1:1000. Further dilution of 4:5, 3:5 and 2:5 (corresponding to 20, 40 and 60% inhibition) served to construct the standard curve. The second curve consisted of five 6-fold dilutions of O6-MeGuo ranging between 0.1 and 25 pmole per well and was used to equate a given amount of O6-MeGuo with a given degree of inhibition.

All wells were coated with 100 μL of O6-MeGuo-BSA at a concentration of 100 ng/mL in 50 mM Tris-HCl buffer, pH 7.2, for 1 hr. After the removal of unbound O6-MeGuo–BSA, unoccupied protein binding sites were saturated by the addition of 100 μL of a 1% (w/v) solution of BSA in phosphate-buffered saline (PBS) for 30 min. Wells were then emptied and washed. The standard washing procedure was to rinse twice with PBS–TWEEN (Sigma) (0.5 mL Tween 20/L PBS) and four times with distilled water.

To set up the standard curves and experimental points on the plate the following procedure was followed. All reagents were diluted in PBS containing 0.1% (w/v) BSA. O6-MeGuo standards or experimental samples as 50 μL aliquots were added to the appropriate wells. Wells used for the primary antibody standards received 50 μL of diluent. Then wells containing O6-MeGuo standards or experimental samples received 50 μL of primary antibody (1:1000 dilution) and wells for the primary antibody standards received 50 μL of the appropriate antibody dilution. After incubation for 1 hr the wells were emptied and washed with PBS as above.

Bound antibody was detected with biotin-labeled antirabbit IgG and avidin-labeled horseradish peroxidase (Vector Labs, Burlingame, CA). Then 100 μL of biotin-labeled antibody was incubated in the wells for 1 hr, then removed and the wells washed as before; 100 μL of avidin-horseradish peroxidase was then incubated for 30 min and washed as before. Enzyme activity was measured with the substrate 1,2-diaminobenzene (o-phenylenediamine, Sigma) at 500 μg/mL in 100 mM pH 6.2, phosphate buffer with 0.006% H2O2 (v/v) or with 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (Sigma) at 200 μg/mL in 0.01 M phosphate buffer, pH 6.0, with 0.001% H2O2 (v/v). Freshly prepared substrate solution, 100 μL, was added to each well and incubated until the absorbance was approximately 0.5. The reaction was stopped with 100 μL of 2 N HCl. The absorbance was read at 490 nm for 1,2-
Diaminobenzene and at 405 nm for 2',2'-azino-di-(3-ethyl benzthiazoline sulfonyl acid) diaminonitromethyl.

**HPLC-BA-ELISA.** The HPLC apparatus has been described previously (14). Separation of deoxyribonucleosides obtained by DNA hydrolysis was performed with a µ-Bondapak-C18 column (4.9 mm × 30 cm) (Waters Associated, Milford, MA) and with a gradient of 0 to 35% aqueous methanol in 50 min. The flow rate was 1 mL/min, and 1-mL fractions were collected. The fractions were then lyophilized and reconstituted with 0.5 mL of PBS. Individual fractions were then analyzed by BA-ELISA as described above. The recovery of O6-MeGuo from HPLC vary between 70 and 100%. HPLC-fluorimetry was carried out as described by Herron and Shank (15).

**Results**

Figure 1 demonstrates the specificity and sensitivity of the antibody used in the BA-ELISA for O6-MeGuo. Fifty percent inhibition of antibody binding to immobilized O6-MeGuo-BSA was achieved with 2.5 pmole of O6-MeGuo. If a probability grid was used to plot the inhibition curves, inhibition was linear between 15 and 85%. Fifteen percent inhibition is equivalent to 100 fmole of O6-MeGuo per well. Since, of the compounds tested, those containing a methyl group attached to the O6 position of guanine gave the highest levels of inhibition, the specificity of the antibody was clearly directed towards this group. N6-Methyl-2'-deoxyadenosine (N6-MeAdo) was at least 1000-fold less efficient than O6-MeGuo in inhibiting antigen-antibody binding. This indicates that the antibody may recognize the amino group at the 2 position of the O6-MeGuo. The poor inhibition of binding of 7-methyl-2'-deoxyguanosine (7-MeGuo) versus O6-MeGuo and of 3-methylguanine versus O6-MeGuo emphasizes the importance of the methyl group being attached to the O6 position of the guanine moiety. All five nucleosides naturally occurring in DNA were found to inhibit binding only at concentrations 100,000-fold higher than those required for O6-MeGuo binding.

The maximum amount of hydrolyzed DNA that could be used per well was 5 μg. This maximum limit sets the lower limit of methylation which can be detected for 20 pmole O6-MeGuo/mg DNA. At 25 μg of DNA per cell this level of methylation corresponds to 316,000 O6-MeGuo residues per diploid genome (16). To circumvent this limitation, reverse-phase HPLC was used to separate O6-MeGuo from unmodified and other methylated nucleosides. As shown in Figure 2 this technique was used to measure the level of O6-MeGuo in DNA isolated from the nasal mucosa of NNK treated rats. Only those fractions having an elution volume equal to that of O6-MeGuo gave substantial inhibition (64%) of antibody binding in the BA-ELISA. By coupling HPLC with the BA-ELISA the amount of DNA analyzed can be increased from 5 μg to 1 mg or more. Following this procedure a 1 mg sample containing as little as 700 pmole, which corresponds to 10,500 residues of O6-MeGuo per diploid genome, can be analyzed in duplicate (six wells).

**Figure 1**. Inhibition of anti-O6-MeGuo antibody binding to immobilized O6-MeGuo-BSA by various nucleobases and nucleosides. At a concentration of 100,000 pmole per well, 2'-deoxyadenosine gave 39% inhibition, dGuo, adenine, and thymidine gave less than 20% inhibition and 2'-deoxyctytosine, 5-methyl-2'-deoxyctytosine, 3-methylguanine, and 7-methyladenine gave less than 10% inhibition.

**Figure 2**. HPLC separation of deoxyribonucleosides obtained by enzyme hydrolysis of 367 μg of DNA isolated from the nasal mucosa of F344 rats sacrificed 4 hr after IV injection of NNK (87 mg/kg body weight). Numbered peaks were identified as follows: (1) hydrolyzing enzymes; (2) 2'-deoxyctytosine; (3) dGuo and thymidine; (4) 2'-deoxyadenosine. The retention volume of O6-MeGuo under these conditions is 49 mL. The peak at this retention volume corresponds to 5.2 pmole of O6-MeGuo: (-) UV (254 nm); (——) inhibition of antigen-antibody binding.

The accuracy of BA-ELISA and HPLC-BA-ELISA methods was compared to that of radiochromatographic and HPLC-fluorimetric methods. Calf thymus DNA was methylated with [14C]Methyl-N-nitrosourea and analyzed by the four methods. The methods agree well with each other, giving correlation coefficients by linear regression analysis greater than 0.99.
The metabolic pathways of NNK are illustrated in Figure 3. Carbonyl reduction of NNK gives NNAI. As with most N-nitrosamines, hydroxylation of the carbon α to the N-nitroso group of NNK and NNAI could be considered a crucial and rate-limiting step in their bioactivation to alkylating species. For instance, α-methylene hydroxylation of NNK and NNAI would lead to the generation of methyl diazohydroxide (7). The keto acid and hydroxy acid (compounds 10 and 11 of Fig. 3) are two major urinary metabolites of F344 rats treated with NNK (17).

The methylating capacities of NNK and NNAI were compared by culturing rat nasal mucosa with these two N-nitrosamines. As shown in Table 1, NNK was rapidly reduced to NNAI in this system. Only 14% of the initial amount of NNK remained at the end of the 24-hr culture period. In contrast, NNAI was not extensively metabolized, and 77% of the initial amount remained unchanged after 24 hr.

Table 1. NNK-NNAI interconversions during culture of rat nasal mucosa with either NNK or NNAI.

| Time of culture, hr | NNK, nmole/mL | NNAI, nmole/mL | NNK, nmole/mL | NNAI, nmole/mL |
|---------------------|---------------|----------------|---------------|----------------|
| 1                   | 440 ± 7       | 35 ± 5         | ND            | 480 ± 11       |
| 3                   | 323 ± 8       | 80 ± 5         | 2.6 ± 0.1     | 398 ± 10       |
| 24                  | 68 ± 9        | 39 ± 1         | 4.2 ± 0.3     | 369 ± 11       |

*The initial concentration of NNK or NNAI was 482 nmole/mL. Values are mean ± SE obtained from four cultured media.

After 24 hr of culture, the total level of NNK + NNAI was 107 nmole/mL and 373 nmole/mL when NNK and NNAI were used as starting N-nitrosamines, respectively. The extent of DNA methylation was higher when NNK rather than NNAI was added to the culture medium (Table 2). With NNAI, DNA methylation after 24 hr was associated with the oxidation of a small fraction of NNAI to NNK.

Table 2. Levels of methylation of rat nasal mucosa cultured in vitro with NNK or NNAI.

| Time of culture, hr | O6-MeGua, μmole/mole Gua | 7-MeGua, μmole/mole Gua | O6-MeGua, μmole/mole Gua | 7-MeGua, μmole/mole Gua |
|---------------------|---------------------------|-------------------------|---------------------------|-------------------------|
| 1                   | 39                        | 600                     | ND                        | ND                      |
| 3                   | 65                        | 1060                    | ND                        | ND                      |
| 24                  | 138                       | —                       | 52.1                      | 745                     |

*Mean of two samples of DNA extracted from three nasal septa each. The initial concentrations of NNK or NNAI were 482 nmole/mL.

*Not detected. Limit of detection is 30 μmole/mole dGuo.

*Not determined.

F344 rats were either treated with a single dose of NNK and sacrificed 4 hr later or were administered 14 daily doses of NNK and sacrificed 24 hr later. As shown in Table 3, O6-MedGuo was found only in nasal mucosa, lung and liver.
Table 3. Susceptibility of F344 rat organs to develop tumors and levels of O⁶-MedGuo after treatment with NNK.

| Organ            | Susceptibility | O⁶-MedGuo/dGuo, μmol/mole |
|------------------|----------------|---------------------------|
| Nasal mucosa     | + +            | 219 ± 9.9, 7.9 ± 1.4      |
| Lung             | + + +          | 13 ± 1.6, 11.4 ± 3.8      |
| Liver            | +             | 84 ± 2.6, ND              |
| Kidney           | -              | ND/                        |
| Esophagus        | -              | ND                        |
| Spleen           | -              | ND                        |
| Heart            | -              | ND                        |

\* F344 rats were injected SC with 60 subdoses of NNK during 20 weeks. Total dose was 1 mmole/kg body weight (19).
\* Values are mean ± SE (n = 3) as determined by HPLC-BA-ELISA.
\* Five F344 rats were injected IV with a solution of NNK (87 mg/kg body weight) and were sacrificed 4 hr later.
\* Two F344 rats received each daily IP injections of NNK (40.3 mg/kg body weight) for 14 days. The rats were sacrificed 24 hr after the last injection.
\* Level of 7-MeGua/mole Gua as measured by HPLC-fluorimetry was 1050 μmol.
\* ND detected. Limit of detection was 3 μmol O⁶-MedGuo/mole dGuo.

Discussion

The BA-ELISA described in this report is a sensitive, rapid, and simple method for the detection of O⁶-MedGuo in DNA modified by methylating agents. The use of biotin-avidin reagents reduces nonspecific binding of the second antibody and results in lower background readings. The BA-ELISA can easily be performed in one day and does not require the use of radiolabeled tracers. The sensitivity of BA-ELISA was increased considerably by chromatographic separation of methylated and naturally occurring nucleosides obtained by enzyme hydrolysis.

The HPLC-BA-ELISA is more time-consuming than the BA-ELISA, taking 2 days to complete, but is limited in the amount of DNA which can be analyzed only by the amount of hydrolyzed DNA which can be loaded on the HPLC column. In HPLC-fluorimetry it is critical to achieve good separation of the modified nucleobases from other components that could also fluoresce at the particular excitation and emission wavelengths used. Good separation of the nucleoside mixture is not as critical in HPLC-BA-ELISA because of the high specificity of the antibody for the carcinogen modified nucleoside.

As shown in Figure 3, reduction of NNK to NNAI is a major metabolic pathway which was observed in vivo, in organ cultures and during incubation of NNK with rat liver microsomal fraction (3). Interestingly, this reduction was also observed in mouse tissue early during fetal life (18). In human tissues excised from the oral cavity and upper respiratory tract and cultured in vitro, reduction of NNK to NNAI was also the major metabolic pathway. α-Carbon hydroxylation of NNK by cultured human tissues was also observed, but was not as extensive as in cultured animal tissue (19).

The reoxidation of NNAI to NNK was observed with explants of A/J mouse lung (a target tissue of NNK and NNAI), although the equilibrium favored NNAI (20). The ability of NNK and NNAI to generate methylating species was compared in cultured rat nasal mucosa (Table 1). Levels of O⁶-MeGua and 7-MeGua were higher with NNK than with NNAI, suggesting that NNK is a better substrate for the activating enzymes. This hypothesis was supported by a study of the interconversion of NNK and NNAI by these explants. NNK was efficiently reduced to NNAI but the reverse reaction was not favored. Furthermore, the low level of oxidation of NNAI to NNK paralleled a low level of DNA methylation observed with NNAI after 24 hr of culture. These results suggest that NNAI is not associated with the activation of NNK to DNA methylaing species.

NNK, along with NNN and N'-nitrosanatabine, are formed by N'-nitrosation of either nicotine or anatabine during curing and/or smoking of tobacco (21, 22). All three N'-nitrosamines induce tumors in F344 rats, but NNK is the most potent. Even at a dose of 1 mmole/kg body weight NNK injected SC to male F344 rats induces a high percentage of nasal tumors (74%) and lung tumors (85%) but a low percentage of liver tumors (11%). In contrast, NNN induces a lower percentage of nasal tumors (56%), a low percentage of lung tumors (14%), and no liver tumors (23).

After treatment of F344 rats with NNK, methylation of the DNA at the O⁶-Gua site was observed only in organs that develop tumors (Table 3). In NNN-treated rats no O⁶-MeGuo was observed in nasal mucosa or liver DNA. These results suggest that the relatively higher carcinogenic potency of NNK compared to NNN could be due to the formation of the promutagenic lesion O⁶-MeGuo by NNK. However, the formation of other adducts could also mediate the carcinogenicity of NNK. A recent mutagenicity study of compounds analogous to NNK supports this hypothesis (24).

As shown in Figure 3, methyl hydroxylation of NNK would lead to the pyridyloxobutyldiazohydroxide (Compound 5). According to a whole-body autoradiographic study of rats treated with NNK, alkylation by compound 5 would take place in the mucosa of the ethmoturbinates, lateral nasal gland, bronchial mucosa, and liver (25). Whether compound 5 (Fig. 3) leads to persistent and mispairing adducts is currently being studied with monospecific antibodies and BA-ELISA.

Methodology developed in the present study will certainly be instrumental in assessing damage to human DNA by NNK and other methylating substances present in tobacco smoke and chewing tobacco.

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REFERENCES

1. U.S. Department of Health, Education and Welfare. Smoking and Health: A Report of the Surgeon General, DHEW, Washington, DC, 1979 PHS79-50666, Chapter 5, pp. 9–28.
2. Hecht, S. S., Castonguay, A., Rivenson, A., Mu, B., and Hoffmann, D. Tobacco specific nitrosamines: carcinogenicity, metabolism, and possible role in human cancer. J. Environ. Sci. Health Cl. 1–54 (1983).
3. Hoffmann, D., Adams, J. D., Brunneman, K. D., and Hecht, S. S. Assessment of tobacco-specific N-nitrosamines in tobacco products. Cancer Res. 39: 2505–2509 (1979).
4. Azell, T., Mörnstad, H., and Sanderström, B. Snusning och munhåle cancer. En retrospektiv studie. Tandl-tdn. 70: 1048–1052 (1978).
5. Winn, D. M., Blot, W. J., Shy, C. M., Pickle, L. W., Toledo, A., and Fraumeni, J. F. Snuff dipping and oral cancer among women in the southern United States. N. Engl. J. Med. 304: 745–749 (1981).
6. Hoffmann, D., and Adams, J. D. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. Cancer Res. 41: 4305–4308 (1981).
7. Hecht, S. S., Chen, C. B., Dong, M., Ornaf, R. M., Hoffmann, D., and Tso, T. C. Chemical studies on tobacco smoke. LI. Studies on non-volatile nitrosamines in tobacco. Beitr. Tabakforsch. 9: 1–6 (1977).
8. Brittebo, E. B., Castonguay, A., Furuya, K., and Hecht, S. S. Metabolism of tobacco-specific nitrosamines by cultured rat nasal mucosa. Cancer Res. 43: 4843–4848 (1983).
9. Carmella, S. G., and Hecht, S. S. High performance liquid chromatographic analysis of metabolites of the nicotine derived nitrosamines, N'-nitrosornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Anal. Biochem. 145: 239–244 (1985).
10. Daoud, A. H., and Irving, C. C. Methylation of DNA in rat liver intestine by dimethylnitrosamine and N-methylnitrosoureas. Chem.-Biol. Interact. 16: 135–143 (1977)
11. Labara, C., and Paigen, K. A simple, rapid and sensitive DNA assay procedure. Anal. Biochem. 102: 344–352 (1980).
12. Müller, R., and Rajewsky, M. F. Immunological quantification by high-affinity antibodies of O'-ethyldeoxyguanosine in DNA exposed to N-ethyl-N-nitrosourea. Cancer Res. 40: 887–896 (1980).
13. Erlanger, B. F., and Beiser, S. M. Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc. Natl. Acad. Sci. (U.S.) 52: 68–74 (1964).
14. Hecht, S. S., Lin, D., and Chen, C. B. Comprehensive analysis of urinary metabolites of N'-nitrosornicotine. Carcinogenesis 2: 833–838 (1981).
15. Herron, D. C., and Shank, R. C. Quantitative high-pressure liquid chromatography analysis of methylated purines in DNA of rats treated with chemical carcinogens. Anal. Biochem. 100: 58–63 (1979).
16. Maslansky, C. J., and Williams, G. M. Ultraviolet light-induced DNA repair synthesis in hepatocytes from species of differing longevities. Mech. Ageing Devel. 20: 191–203 (1985).
17. Hecht, S. S., Young, R., and Chen, C. B. Metabolism in the F344 rats of 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. Cancer Res. 40: 4144–4150 (1980).
18. Castonguay, A., Tjalvé, H., Trushin, N., and Hecht, S. S. Perinatal metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in C57B1 mice. J. Natl. Cancer Inst. 72: 1117–1126 (1984).
19. Castonguay, A., Stoner, G. D., Schut, H. A. J., and Hecht, S. S. Metabolism of tobacco-specific N-nitrosamines by cultured human tissues. Proc. Natl. Acad. Sci. (U.S.) 80: 6694–6697 (1983).
20. Castonguay, A., Lin, D., Stoner, G. D., Radok, P., Furuya, K., Hecht, S. S., Schut, H. A. J., and Klaunig, J. E. Comparative carcinogenicity in A/J mice and metabolism by cultured mouse peripheral lung of N'-nitrosornicotine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and their analogues. Cancer Res. 43: 1223–1229 (1983).
21. Adams, J. D., Lee, S. J., Vinehuski, N., Castonguay, A., and Hoffmann, D. On the formation of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone during smoking. Cancer Letters. 17: 339–346 (1983).
22. Hoffmann, D., Dong, M., and Hecht, S. S. Origin in tobacco smoke of N'-nitrosornicotine, a tobacco-specific carcinogen. J. Natl. Cancer Inst. 58: 1841–1844 (1977).
23. Hoffmann, D., Rivenson, A., Amin, S., and Hecht, S. S. Dose-response study of the carcinogenicity of tobacco-specific N-nitrosamines in F344 rats. J. Cancer Res. Clin. Oncol. 108: 81–86 (1984).
24. Hecht, S. S., Lin, D., and Castonguay, A. Effects of alpha-deuterium substitution on the mutagenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Carcinogenesis 4: 305–310 (1983).
25. Castonguay, A., Tjalve, H., and Hecht, S. S. Tissue distribution of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and its metabolites in F344 rats. Cancer Res. 43: 630–638 (1983).