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Deoxyribonucleic acid methylation in human cells exposed to nitrosocimetidine

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N-Nitroso compounds are, with a few notable exceptions, potent carcinogens in laboratory animal tests [1]. It is now clear that humans are chronically exposed to nitroso compounds from enviromental and dietary sources, and there are indications that nitroso compounds can be formed *de novo* in the gastrointestinal tract due to the reaction of ingested nitrosatable compounds with ingested nitrite or with nitrite formed by the enzymic reduction of nitrate carried out by endogenous micro-organisms [2–4]. It has been suggested that this *in vivo* synthesis may present a significant risk to human health [5, 6]. The role of nitroso compounds in the etiology of human cancer, however, remains to be established.

The compound cimetidine has recently come to our attention. Marketed under the trade name of Tagamet, it is a very effective drug for the clinical treatment of gastrointestinal disorders [7]. Well over eleven million people worldwide have taken, or are now taking, this compound [8] at doses in the range of 1 g/day. Cimetidine is a nitrosatable compound; a report on the chemistry of the *N*-nitrosation process indicates that the reaction occurs at a measurable rate in acidic solution in the presence of nitrite [9]. Thus the possibility exists that under the proper conditions some fraction of the dosed cimetidine may become nitrosated *in vivo*. It is emphasized that no evidence for the *in vivo* formation of nitrosocimetidine has, as yet, been reported. Nitrosocimetidine (NC) has been found to be a direct-acting mutagen in the Amestest [10], to produce strand breaks in intracellular DNA [11, 12], and to stimulate cellular DNA repair processes [12]. We have reported that the compound will methylate DNA *in vitro* to produce the same methylated purine products, 7-methylguanine (7meG), 3-methyladenine (3meA) and O^6-methylguanine (O^6meG), with the same yield distribution as observed using the carcinogens methylnitrosourea (MNU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [13]. We are continuing our study of NC within the context of these known *N*-nitroso methylating carcinogens and report here some of the cellular DNA alkylation characteristics utilizing human cells in culture.

The Raji cell line used in this investigation is a lymphoblastoid line established from a Burkitt's lymphoma patient [14]. These are hardy, rapidly growing cells which can be obtained in high yield from suspension cultures.

Materials and methods

Cell cultures. The Raji cell line was supplied by Drs. M. Levitt and E. E. Henderson, Department of Microbiology, Temple University School of Medicine. The cells were maintained in RPMI 1640 medium plus 10% heat-pretreated fetal calf serum (growth medium) containing penicillin and streptomycin. Medium and additives were obtained from Flow Laboratories. Cells were grown in 250-ml tissue culture flasks (Falcon) at 37° in an atmosphere of 5% CO_2.

Chemicals. MNU was prepared as described previously [15, 16], and NC was synthesized according to the protocol of Bavin *et al.* [9]. MNNG was obtained from the Aldrich Chemical Co., Milwaukee, WI. MNU and MNNG containing ^{14}C in the methyl group were purchased from the New England Nuclear Corp., Boston, MA (16.7 and 16.3 μCi/μmole respectively). Cimetidine carrying ^{14}C in the CH_3-group *alpha* to the amino nitrogen was supplied by Smith Kline & French Laboratories, Welwyn Garden City, Hertfordshire, England, and nitrosated at this nitrogen by Dr. M Abou-Gharbia, Chemistry Department, Temple University, by scaling down the procedure previously described for the synthesis of unlabeled NC [13, 17]. Utilizing this protocol, unlabeled compound was produced with greater than 98 per cent purity [13]. The purity of ^{14}C-labelled NC was checked using a recently developed high pressure liquid chromatography (h.p.l.c.) method which permits the resolution of cimetidine and NC. Ninety-five per cent of the radioactivity in a sample of our [^{14}C]NC preparation demonstrated a column retention time identical to that of NC and 4 per cent had a retention time identical to that of cimetidine. The specific activity of this preparation was 9.8 μCi/μmole. All other chemicals used in this study were reagent grade.

Rate of nitroso compound decomposition in growth medium. Nitroso compound dissolved in dimethylsulfoxide (DMSO) was added to the growth medium (37°) with vigorous mixing (final DMSO, 5%) and a portion was delivered to a cuvette preheated to 37° in the temperature regulated chamber of a Gilford 2400S recording spectrophotometer. The timed chart drive was activated immediately upon mixing, and the recording of optical density changes relative to a growth medium blank was initiated within 15 sec of this event.

Cell treatment and DNA isolation. Raji cells were isolated from the growth medium plus antibiotics by gentle centrifugation and were resuspended in growth medium, (37°) to a final concentration of 3.0×10^7 cells/ml as determined by hemocytometer counts. The trypan blue exclusion test indicated a cell viability of greater than 95 per cent in all preparations. The cells were dosed with a radioactive nitroso compound dissolved in DMSO (final DMSO, 5%) and incubated at 37°. Treatment was terminated after the desired interval by gently pelleting the cells, washing them twice with phosphate-buffered saline (Flow Laboratories), and resuspending the cells in pH 7 phosphate buffer containing 0.2% sodium dodecylsulfate. The cells were lysed by three freeze–thaw cycles, and the lysates were treated concurrently with 100 units/ml T_1 RNase and 100 μg/ml heat-pretreated pancreatic Rnase A (Sigma) for 1 hr at 37° and then twice for 30 min (37°) with 0.6 mg/ml heat-pretreated Pronase (Sigma). The preparations were extracted three times with phenol saturated with pH 8 Tris buffer, dialyzed against 0.1 M K_2HPO_4, 1 mM Na_2EDTA (pH 7.0), and lyophilized.

Quantitation of methylated purine yields using h.p.l.c. Lyophilized samples of DNA from treated cells were dissolved in 0.1 M HCl (pH adjusted to 1) and hydrolyzed by incubating at 70° for 40 min [18]. The hydrolysate was then subjected to h.p.l.c. quantitative analysis as described previously [19].

Results and discussion

Treatment of Raji cells with NC radiolabeled in the methyl group adjacent to the *N*-nitroso moiety results in the covalent transfer of this label to sites on cellular DNA. A high pressure liquid chromatogram of a purified DNA sample from NC-treated cells, which had been depurinated by heating in mild acid, is shown in Fig. 1. This chromatographic technique resolves methylated and unmodi-

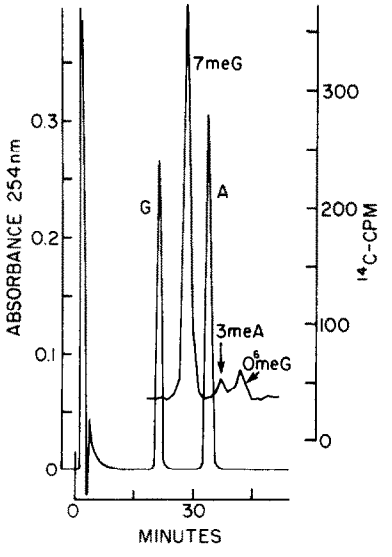

Fig. 1. High pressure liquid chromatogram of mild acid hydrolyzed NC-modified DNA isolated from Raji cells. Treatment: 3×10^7 cells/ml growth medium were incubated for 1 hr at 37°, with 250 μM [^{14}C-methyl]NC; DNA was isolated and hydrolyzed as described in Materials and Methods. Chromatography: 500 μl of hydrolysate was applied to a BioRad Aminex A-9 cation exchange h.p.l.c. column (250 mm × 4 mm) and eluted with 0.8 M ammonium formate (pH 3.0, 60°) at 0.7 ml/min; elution was monitored utilizing a u.v. detector (254 nm), and 1.5-min fractions were collected. Radioactivity in these fractions was determined using standard liquid scintillation counting methods. Eluted peaks were identified by their retention times relative to authentic markers [13, 19]. Quantitation: guanine yield was estimated by comparing the observed u.v. detected peak height with values on a peak height vs guanine concentration standard curve [19]. In the present example, there were 2.4×10^{-8} moles of guanine in the applied sample which is representative of approximately 40 μg DNA. The specific activity of a methylated purine was assumed to be identical to that of the methylating nitroso compound [19].

fied DNA purines; apurinic acid is eluted in the void volume. Three major peaks of radioactivity were observed to have retention times identical to those of the methylated purines 7meG, 3meA and O^{6}meG. The ratio of the counts in the O^{6}meG peak relative to the 7meG peak was 0.11 (s.d., 0.02; four determinations); the 3meA/7meG ratio was low and variable in the several runs, probably due to losses of 3meA during DNA isolation [20]. The observed O^{6}meG/7meG ratio was identical to that found in purified DNA modified *in vitro* with NC, detected using optical methods [13]. Moreover, this product ratio was observed in the DNA isolated from Raji cells treated with MNNG (0.11; S.D., 0.02; five determinations) and MNU (0.09; S.D., 0.01; four determinations) and in DNA modified by these compounds *in vitro* [13]. Finally it is noted that this same O^{6}meG/7meG product ratio was detected in DNA modified *in vitro* with dimethylnitrosamine (DMN) activated by hepatic microsomal enzymes [19]. Since DNA methylation is probably due to independent reactions at the several kinds of DNA nucleophilic sites, each with unique, intrinsic reaction properties [16, 21, 22], the observed identical product yield ratios provide evidence that the same molecular species that ultimately reacts with DNA is generated from NC, MNNG, MNU, and DMN.

It has been proposed that the reactive alkylating species is produced from methylating *N*-nitrosamides and amidines as a consequence of chemically initiated decomposition of these compounds [18, 23, 24]. We thus determined some of the decomposition characteristics of NC and MNNG in aqueous solution [13]. In the present context we give emphasis to the observation that in pH 7.4 phosphate buffer NC was remarkably stable, demonstrating a half-life of approximately 135 hr (23°) compared to the 5.6 hr observed for MNNG. Further, we discovered that, whereas MNNG decomposition was accelerated in the presence of small molecules containing nucleophilic nitrogen or sulfur atoms, an observation consistent with previous reports [18, 25], NC decomposition was stimulated only by sulfhydryl compounds. These findings have been verified recently in our laboratory, utilizing an h.p.l.c. technique that permitted us to monitor the disappearance of intact compound, and extended with the observation that, when the sulfhydryl group was blocked by a methyl moiety (i.e. *S*-methylcysteine), the accelerated decomposition was completely inhibited.*

The chemical stability of NC was evident when the compound was incubated in our Raji cell growth medium at 37° (Fig. 2); after 2 hr the absorbance peak at 390 nm, due to the *N*-nitroso group chromophore [13], had decreased approximately 10 per cent suggesting that about 90 per cent of the NC remained intact. (We take the decay in the intensity of this absorbance band as an indicator of compound decomposition [13, 18, 26].) In contrast, the decomposition of MNNG approached completion within 90 min and of MNU within 15 min. Some fraction of these decomposition reactions will generate the alkylating fragment as evidenced by the ability to methylate DNA *in vitro* under similar conditions [13]. How much of the alkylating fragment generated in the growth medium will be able to travel

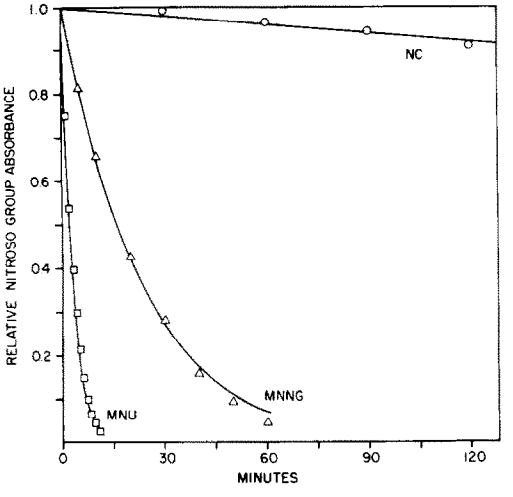

Fig. 2. Decomposition of MNU, MNNG and NC in Raji cell growth medium (37°). Decomposition was monitored by observing the decay of the *N*-nitroso chromophore absorbance peak: 400 nm for MNU and MNNG, 390 nm of NC [13]. Nitroso compound concentrations were 1 mM, and extrapolated zero time absorbances were in the range of 0.15 absorbance units. Curves are drawn through the data points assuming first-order kinetics; estimated half-lives of MNU, MNNG and NC in growth medium (37°) are 2.1 min, 15.7 min and 17.6 hr respectively.

* D. E. Jensen, manuscript in preparation.

through the medium and through the cell to alkylate nuclear DNA is unknown. Based on the observations that the methylating fragment is adept at modifying weakly nucleophilic oxygen sites on DNA [16, 21, 22], however, it can be assumed that the fragment is quite reactive and that there will be a high probability that it will be consumed in a reaction with a local nucleophile shortly after it is generated.

Some of our experiments considering the rate of cellular DNA methylation are illustrated in Fig. 3. The concentration of alkylating agent used to dose the cells was the same in each of these experiments. It was found that the alkylation of cellular DNA using MNNG was complete within 30–45 min after treatment was initiated. The data appear to suggest that the DNA alkylation rate is slightly faster than the rate of compound decomposition in the growth medium (Fig. 2), in accord with an observation published previously [18], perhaps indicating that MNNG decomposes more rapidly in the intracellular environment. DNA alkylation by MNU was over shortly after dosing and was seen to produce a low yield relative to that observed using MNNG. This result may suggest that the rate of MNU decomposition in the growth medium is comparable to the rate of transport of the intact compound in the cell. DNA alkylation due to NC treatment was observed to occur at a steady rate during a 6-hr incubation except for an approximately 2-fold faster rate during the first 30 min (Fig. 3A). In a separate experiment we found that DNA methylation yield continued to increase during the 6- to 30-hr treatment interval with a decreased average rate (Fig. 3B). During the same interval, the specific activity of DNA isolated from MNNG-treated Raji cells was observed to decrease. Overall, the degree of cellular DNA alkylation was observed to be somewhat less after NC treatment than after treatment with an equivalent dose of MNNG. The *in vitro* experiments [13] suggest two possible reasons for this effect: (a) a concurrent denitrosation reaction may have been operating to inactivate NC as an alkylating agent, and (b) intracellular thiol concentrations may have limited the rate and thus the degree of NC decomposition. The experiments utilizing this system continue. Finally, the 1-hr DNA alkylation yield vs applied dose curves (data not shown) were linear over a wide range of agent concentration, with NC and MNU demonstrating slopes equal to approximately 10 and 5 per cent of the MNNG slope respectively.* These observations are consistent with the 1-hr time point data shown in Fig. 3A.

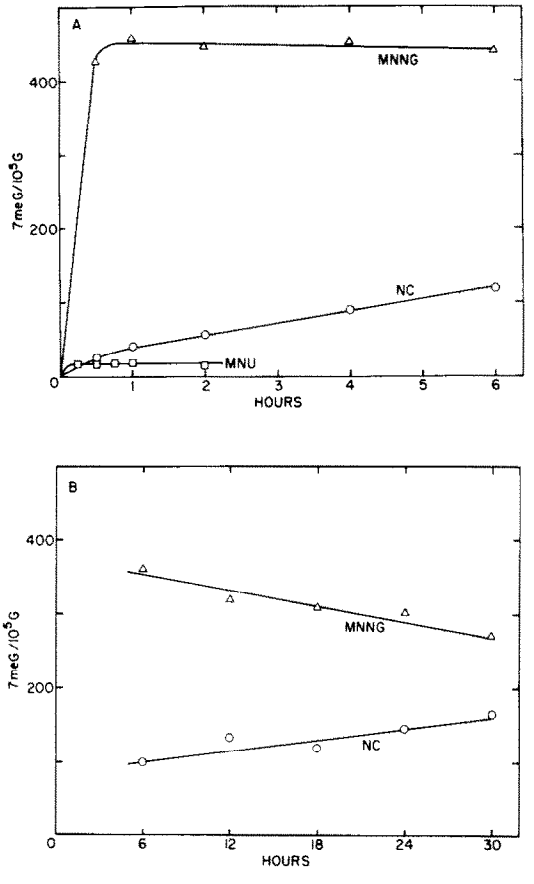

Fig. 3. DNA methylation in Raji cells exposed to MNU, MNNG or NC as a function of time. Treatment: 3×10^7 cells/ml growth medium were incubated for various intervals of time at 37° in the presence of 250 μM ^{14}C-methyl labeled nitroso compound; DNA was isolated and hydrolyzed as described in Materials and Methods. Chromatography and purine yield quantitation were carried out as indicated in the legend of Fig. 1. The degree of DNA alkylation is represented by the 7meG yield per 10^5 unmodified guanine residues; 7meG lesions represent approximately 70 per cent of the total DNA methylation yield [18, 21]. (A) Experiment in which DNA alkylation yields were assessed during the first 6 hr after treatment was initiated. (B) Experiment in which DNA alkylation yields were assessed during the time interval bounded by 6 hr and 30 hr after treatment was initiated.

In summary, NC was found to methylate cellular DNA to produce the same kinds of lesions in the same relative proportions as were observed using MNU and MNNG. NC was unique relative to the other two compounds in that it hydrolyzed only very slowly in cellular growth medium and methylated the DNA in rapidly growing Raji cells slowly but persistently. The degree of NC–DNA modification over 30 hr never reached that achieved by an equal dose of MNNG in 1 hr, but after the first 15 min of treatment became somewhat higher than that attained by an equivalent dose of MNU.

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Effects of smooth muscle calcium antagonists on human basophil histamine release*

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Calcium antagonists including verapamil, D-600, and nifedipine (BAY 1040) can function as potent inhibitors of vascular smooth muscle contractile responses induced by agonists or by potassium (K^+) depolarization [1–3]. They appear to function as competitive Ca^{2+} antagonists [4–6]. Not all smooth muscle responses are sensitive to these antagonists and it appears likely that the potent inhibitory activity of these compounds (IC_{50} value values of 10^{-6} to 10^{-9} M) is directed to one component of Ca^{2+} translocation, that through voltage-sensitive Ca^{2+} channels [1, 2]. Other smooth muscle relaxants, which include a very diverse collection of structures [7], act at different sites, and in a less defined fashion, to reduce Ca^{2+} translocation [8, 9]. Two of these, dantrolene sodium and 8-(*N*,*N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8), have been found to antagonize contractile responses in guinea pig ileum and vas deferens. Not all smooth muscle contractile responses are blocked to the same degree by different Ca^{2+} antagonists [2].

Calcium is also required for stimulus-secretion coupling in histamine release from rat mast cells and human basophils caused by antigens and mitogens [10–12]. Histamine release from these cells can be inhibited by a variety of agents including beta agonists, E series prostaglandins and choleratoxin, and inhibition is associated with an increase in cellular cyclic AMP content [13]. The Ca^{2+} antagonists TMB-8 [14] and dantrolene sodium [9] inhibit in variable fashion the release of histamine from rat mast cells caused by dextran, compound 48/80, and the calcium ionophore A23187 [15–17]. The flavonoid quercetin is also an active inhibitor of antigen-induced histamine release from human basophils [18]. In the latter system the inhibitory effect of quercetin is partly overcome by increased buffer Ca^{2+} concentrations. In the experiments reported here we compared the "classical" smooth muscle Ca^{2+} antagonists verapamil and nifedipine, as well as dantrolene sodium and TMB-8, with quercetin for their inhibitory activities on antigen-induced histament release from human basophils.

Materials and methods

Chemicals. Verapamil and D-600 were obtained from Knoll, A. G., Ludwigshafen, West Germany, nifedipine from Bayer, A. G., Postsach, Federal Republic of Germany, dantrolene sodium from Norwich-Eaton Pharmaceutical, Norwich, NY, and quercetin from the Aldrich Chemical Co., Milwaukee, WI. TMB-8 was synthesized in our laboratory [19]. All compounds were dissolved in dimethylsulfoxide (DMSO) and were diluted in Tris-buffer

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