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3-METHYLADENINE-DNA GLYCOSYLASE: A PROBE FOR DETERMINING ALKYLATION DAMAGE AND REPAIR IN HUMAN FIBROBLASTS

Thomas P. Brent

Division of Biochemical & Clinical Pharmacology
St. Jude Children's Research Hospital, 332 North Lauderdale
Memphis, Tennessee 38101

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SUMMARY: 3-Methyladenine-DNA glycosylase was partially purified from human lymphoblasts and used as an enzymatic probe to assay the amounts of 3-methyladenine in DNA from cultured human fibroblasts after treatment with dimethyl sulfate. Aside from this specific alkylation product, the total number of alkylated bases was estimated after depurination by heating. Both enzyme-induced and heat-induced apurinic sites were converted to strand breaks and estimated after alkaline sucrose-gradient sedimentation. The results indicate that 3-methyladenine in cultured human fibroblasts is rapidly excised, with a half-life of about 2 hours. The rest of the alkylated purines (mostly 7-methylguanine) are removed much more slowly, with a half-life of about 20 hours.

Mammalian cells treated with alkylating agents manifest many of the reactions that characterize excision-repair of DNA —— namely, rapid loss of labeled alkylated purines from DNA, incorporation of new deoxynucleotides to fill the excised gaps, and rejoining of strand breaks (see Roberts (1) for review). Since most DNA-alkylating agents give rise to a spectrum of reaction products, one must first determine the amount of each product and monitor its repair before attempting to correlate specific biologic effects with specific lesions. Gross measures of excision-repair, such as DNA repair replication or overall loss of labeled alkylation product, do not permit such discrimination of specific lesions in DNA. Direct determination of specific DNA lesions requires isolation, separation, and identification of each alkylation product. This can be achieved only with those alkylating agents that are available at high specific radioactivity and that induce stable products in DNA. Furthermore, relatively large amounts of tissue are needed to provide enough DNA adduct.

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An alternative approach exploits the ability of DNA repair enzymes to recognize specific lesions or classes of lesions in DNA. Such enzymes, either endonucleases or glycosylases, can convert specific lesions to DNA strand breaks that can be measured with considerable sensitivity (2).

Thus, studies on the repair of ultraviolet-induced pyrimidine dimers in bacteria and mammalian cells have been done with dimer-specific endonucleases, highly purified from M. luteus or T_4-infected E. coli (3,4). A relatively crude and poorly characterized preparation from M. luteus has been similarly used to determine the repair of as-yet-unidentified lesions in γ-irradiated DNA from E. coli (5) or human cells (6). In a similar study, Shackleton and Roberts (7) measured sites in DNA from dimethyl sulfate-treated Chinese hamster cells that were sensitive to an M. luteus extract.

The current study determined the amount of dimethyl sulfate-induced damage in human fibroblast DNA; the assay probe was a 3-methyladenine-DNA glycosylase partially purified from human lymphoblasts. This preparation (8) cleaved 3-methyladenine in DNA, but did not appear to act on 1-methyladenine, 7-methyladenine, 7-methylguanine or O^6-methylguanine. Strand breaks, produced by this enzyme and alkaline hydrolysis of the resultant apurinic sites (9), were taken as a measure of 3-methyladenine in the DNA. The gross alkylation damage was measured after heating the isolated DNA to 70°C at neutral pH to depurinate all methylated purines and then converting apurinic sites to strand breaks by alkaline treatment.

MATERIALS AND METHODS

Cell culture and labeling: Normal human foreskin fibroblasts were maintained in monolayer cultures in Dulbecco's modified Eagles Minimal Essential Medium, supplemented with 10% fetal calf serum (K.C. Biologicals). Each confluent culture was subcultured in four new plastic culture flasks (75 cm^2) and labeled with either [^{14}C]thymidine (0.67 μCi/ml; 50 mCi/mmol) or [^{3}H]thymidine (2.5 μCi/ml; 1 Ci/mmol). After 66 hours, the radioactive medium was replaced with fresh medium (30 ml). Dimethyl sulfate, dissolved in 30 μl of ether was rapidly diluted in medium to a concentration of 0.11 mM immediately before addition to ^{14}C-labeled cultures. An equivalent volume of ether alone (0.1%) was mixed with medium before addition to ^{3}H-labeled control cultures.

DNA isolation: At various times after the addition of dimethyl sulfate, one flask of ^{3}H-labeled control cells and one flask of ^{14}C-labeled alkylated cells were washed three times with buffer that contained 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 2 mM EDTA and 1 mM dithiothreitol (Buffer A); the cells were then scraped from the plastic and pooled.

The pooled ^{14}C- and ^{3}H-labeled cells were centrifuged at low speed and resuspended in 100 µl of Buffer A. This cell suspension was added to 1.4 ml of a lysing solution that contained 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.1 M NaCl, 1% Sarkosyl NL30 and 2 mg protease/ml (Sigma Type VI). After incubation for 30 min at 37°C, an equal volume of freshly distilled phenol saturated with Tris-HCl (pH 7.5) was added and the incubation continued on a tube roller at 20 rpm for 30 min at room temperature. After centrifugation to separate the two phases, the aqueous layer was carefully removed and dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 0.02% sodium azide. The isolated DNA was stored frozen at -20°C before being assayed for enzyme or heat-sensitive sites.

Estimation of enzyme- or heat-sensitive sites in DNA: DNA (200 µl), isolated at various times after dimethyl sulfate treatment, was incubated for 60 min at 37°C with 200 µl of 3-methyladenine-DNA glycosylase purified from human lymphoblasts (equivalent to 10,000 units Fraction III) (8). Control incubations, without enzyme, contained 200 µl of enzyme buffer (10 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 10% glycerol, 0.02% sodium azide). The reaction was terminated by adding 100 µl of proteinase K (1 mg/ml in 10 mM Tris-HCl (pH 7.5), 10 mM EDTA) to hydrolyze the large amount of protein in the enzyme extract. After incubation for 5 min at 37°C, 50 µl of 1 N NaOH was added and the incubation continued for 15 min at 37°C to convert all apurinic sites, produced by glycosylase action, to strand breaks (9). A measure of total alkylation was obtained after heating 200 µl of the DNA with 200 µl of enzyme buffer (pH 7.5) at 70°C for 60 min. As described before, NaOH was added to convert apurinic sites to strand breaks. The reaction mixture was carefully layered onto a 5-20% alkaline sucrose gradient and centrifuged in the SW 40.1 rotor of a Beckman L5-50 ultracentrifuge (30,000 rpm for 135 min at 20°C), and then fractionated by upward displacement in an ISCO Gradient Fractionator. Each fraction was neutralized with HCl before addition of scintillation fluid (P.C.S. Amersham) and assay of radioactivity by liquid scintillation spectometry. PM2 DNA that contained about one strand break per molecule (achieved by partial depurination at 70°C, pH 5 and subsequent alkali treatment) was used as a molecular weight marker to calibrate the gradients. Form II PM2 DNA (3.25×10^{6} daltons) had a sedimentation coefficient of 21S. Weight-average molecular weight ($\bar{M}w$) of fibroblast DNA was calculated as:

$$\bar{M}w = \frac{\Sigma(C_i \times M_i)}{\Sigma C_i}$$

where C_i and M_i were the dpm and molecular weight, respectively, of the ith fraction.

Number-average molecular weight ($\bar{M}n$) was taken as one-half of $\bar{M}w$ and the number of strand breaks in 10^{8} daltons of DNA was taken as $(1/\bar{M}n \times 10^{8})-1$.

RESULTS AND DISCUSSION

Figure 1 shows the sucrose gradient profiles of ^{14}C-labeled DNA from dimethyl sulfate-treated cells and ^{3}H-labeled DNA from untreated controls at various times after alkylation. The DNA was analyzed after treatment with

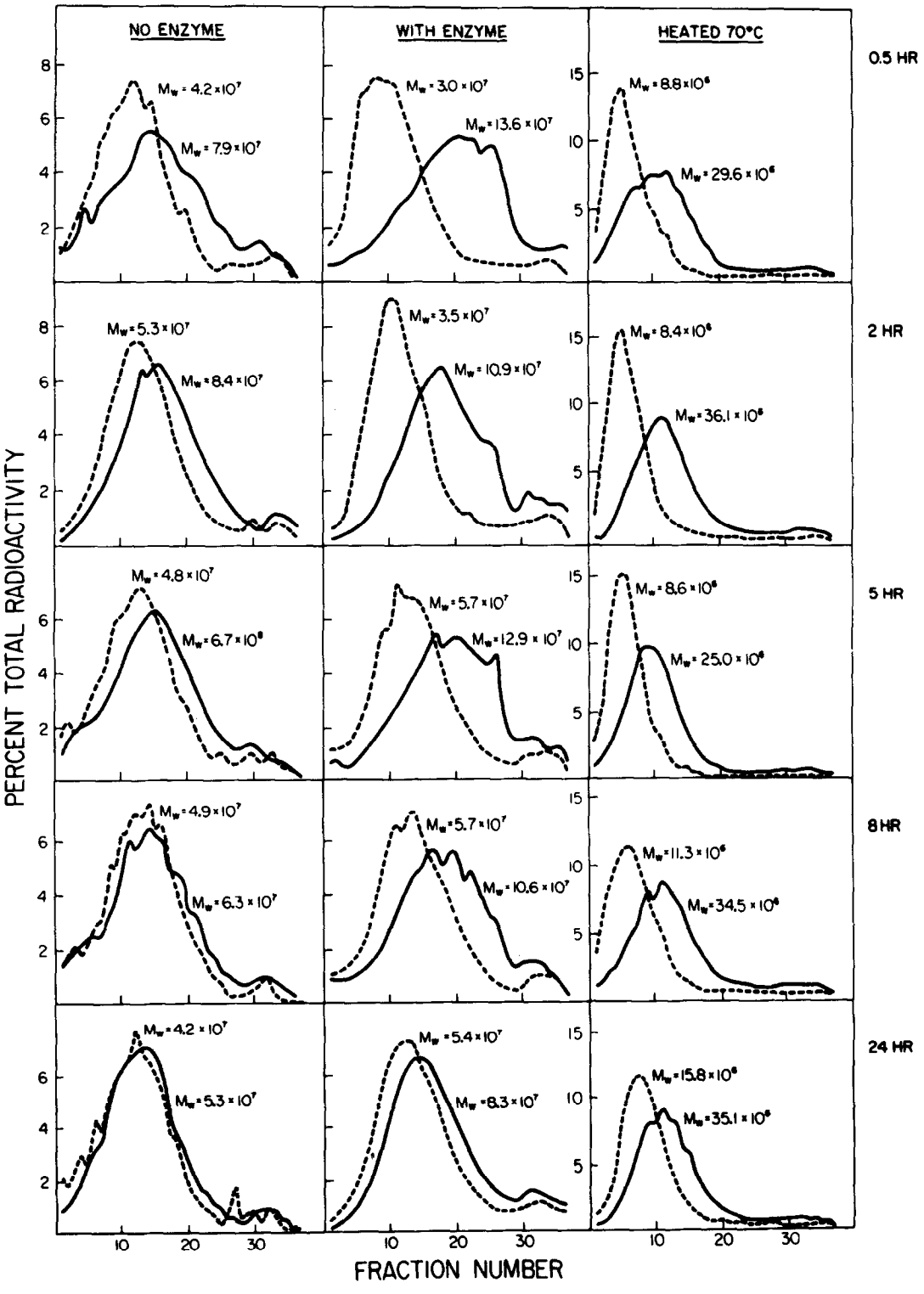
NO ENZYME
WITH ENZYME
HEATED 70°C
0.5 HR
2 HR
5 HR
8 HR
24 HR
$M_w = 4.2 \times 10^7$
$M_w = 7.9 \times 10^7$
$M_w = 3.0 \times 10^7$
$M_w = 13.6 \times 10^7$
$M_w = 8.8 \times 10^6$
$M_w = 29.6 \times 10^6$
$M_w = 5.3 \times 10^7$
$M_w = 8.4 \times 10^7$
$M_w = 3.5 \times 10^7$
$M_w = 10.9 \times 10^7$
$M_w = 8.4 \times 10^6$
$M_w = 36.1 \times 10^6$
$M_w = 4.8 \times 10^7$
$M_w = 6.7 \times 10^7$
$M_w = 5.7 \times 10^7$
$M_w = 12.9 \times 10^7$
$M_w = 8.6 \times 10^6$
$M_w = 25.0 \times 10^6$
$M_w = 4.9 \times 10^7$
$M_w = 6.3 \times 10^7$
$M_w = 5.7 \times 10^7$
$M_w = 10.6 \times 10^7$
$M_w = 11.3 \times 10^6$
$M_w = 34.5 \times 10^6$
$M_w = 4.2 \times 10^7$
$M_w = 5.3 \times 10^7$
$M_w = 5.4 \times 10^7$
$M_w = 8.3 \times 10^7$
$M_w = 15.8 \times 10^6$
$M_w = 35.1 \times 10^6$
PERCENT TOTAL RADIOACTIVITY
FRACTION NUMBER

Figure 1

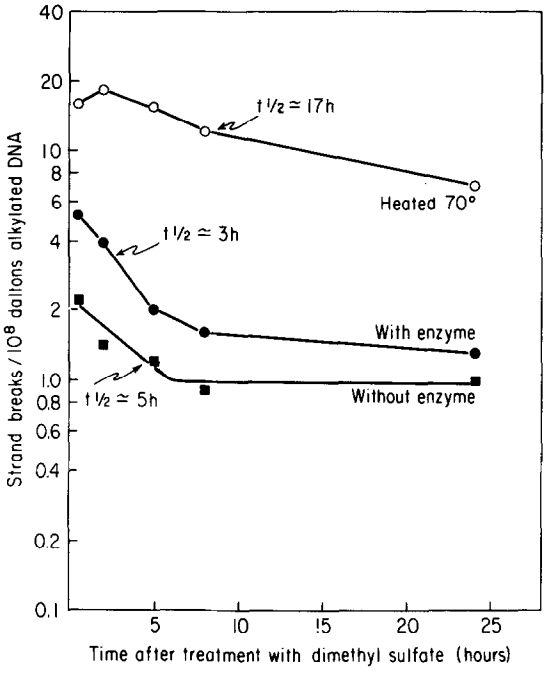

Figure 2. Time-course for repair of lesions in alkylated DNA. Strand breaks occurring spontaneously (■ ——— ■), produced by DNA-glycosylase (● —— ●), or heating to 70°C (0 —— 0) were determined after treatment with alkali. Each point represents the difference between strand breaks in alkylated and untreated DNA in a single alkaline sucrose gradient in Figure 1.

3-methyladenine-DNA glycosylase or buffer, or after heating to 70°C. Variations in molecular weight between different DNA preparations were eliminated after the difference in the number of strand breaks (per 10^8 daltons of DNA) in the ^{14}C-labeled alkylated- and ^{3}H-labeled control DNA was calculated for each individual gradient. This difference measurement was not subject to any variability other than that due to alkylation treatment.

When the data in Figure 1 are expressed in this way, several features emerge (Figure 2). First, about 75% of the enzyme-sensitive sites were lost rapidly, within 6 hours after dimethyl sulfate treatment (half-life of about 3 hours). Second, heat-sensitive sites were lost at a relatively slow and constant rate (half-life of 17 hours); by 24 hours after treatment, almost 50%

Figure 1. Alkaline sucrose gradients of isolated DNA after incubation at 37°C with buffer, with 3-methyladenine-DNA glycosylase, or after heating at neutral pH to 70°C. Dashed line represents ^{14}C-labeled methylated DNA. Solid lines represent ^{3}H-labeled control DNA. Sedimentation was from left to right.

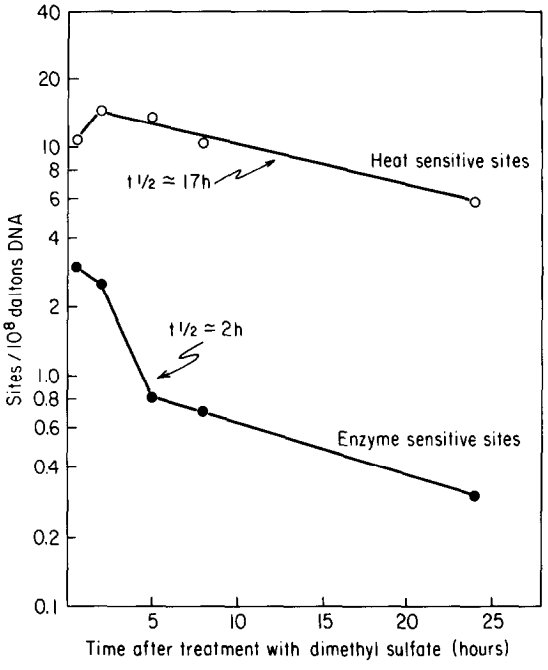

Figure 3. Time-course for loss of 3-methyladenine from dimethyl sulfate-treated DNA. The level of 3-methyladenine was calculated from the difference between the numbers of enzyme-sensitive and spontaneous sites, described in Figure 2. Similarly, the levels of heat-sensitive sites represent the difference between the total heat-sensitive sites and DNA-glycosylase-sensitive sites represented in Figure 2.

of these sites still remained. Lastly, the "spontaneous" strand breakage in the alkylated DNA incubated with buffer alone essentially paralleled that for enzyme-sensitive sites during the first 6 hours after alkylation (half-life, of 5 hours). This suggests that these sites also represent 3-methyladenine that was cleaved either enzymatically *in vivo* or spontaneously both *in vivo* and during isolation of the DNA.

Figure 3 shows the time course for loss of enzyme-sensitive sites after the results were corrected for spontaneously occurring strand breaks. Similarly, the loss of heat-sensitive sites is shown after the data were corrected for spontaneous and enzyme-induced strand breaks. Thus, in Figure 3, the half-life for 3-methyladenine during the first 6 hours after treatment was 2 hours, while that for heat-sensitive sites remained constant at about 17 hours over a 24-hour period.

These results are consonant with previous studies of other mammalian cell lines. Enzymatically determined 3-methyladenine constituted about 15-20% of the total alkylations, in close accord with the expected methylation pattern of dimethyl sulfate (10,11). The 2- to 3-hour half-life for 3-methyladenine sites in DNA also corresponds closely with that reported for rodent cells (7,12,13).

Apart from 3-methyladenine, the only major alkylation product due to dimethyl sulfate is known to be 7-methylguanine (10,11) thus, the heat-sensitive sites are presumed to represent this product. Although it has been generally accepted that 7-methylguanine is not actively excised in mammalian (mainly rodent) cells, the current study has shown that the rate of loss (half-life of less than 20 hours) is over five times faster than the spontaneous rate in vitro (12,13). This suggests that an active process is indeed involved. Similar observations have been made recently for loss of 7-methylguanine for human cells (14,15) Furthermore, although the half-life for 7-methylguanine is over 5 times longer than that for 3-methyladenine, the rates of excision are comparable on a molar basis, since there is over 5 times more 7-methylguanine than 3-methyladenine in the DNA.

In summary, this study has shown that the partially purified human 3-methyladenine-DNA glycosylase is a useful enzymatic probe for dissection of aklylation products in DNA and for determination of their fate in cultured human cells. Hence, these methods appear to be suitable for determining whether any of the so-called human "DNA-repair syndromes" are caused by the inability to repair this specific alkylation damage. The increased sensitivity to methyl methanesulfonate of E. coli (Tag) mutants (16), which are defective in 3-methyladenine-DNA glycosylase, suggests that the sensitivity of human cells to methylating or ethylating agents (17) might result from a similar defect.

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