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Immunological Detection of O^6-Methylguanine in Alkylated DNA†

William T. Briscoe,‡ John Spizizen,* and Eng M. Tan§

ABSTRACT: Antibodies to O^6-methyldeoxyguanosine were produced in rabbits and utilized in a radioimmunoassay to detect this nucleoside at picomole levels. The specificity of the antibodies was demonstrated by the use of nucleoside analogues as inhibitors in the radioimmunoassay. The antibodies cross-reacted with O^6-methylguanosine, O^6-methylguanine, and O^6-ethylguanosine. There was 10^4 to 10^6 times less sensitivity to inhibition by deoxyadenosine, deoxyguanosine, and guanosine than by O^6-methyldeoxyguanosine. The radioimmunoassay also detected O^6-methylguanine in DNA alkylated by agents known to produce O^6-methylguanine, such as N'-methyl-N-nitrosourea. DNA alkylated with dimethyl sulfate, which does not produce O^6-methylguanine in DNA, cross-reacted with the antibodies to a very limited extent. Such an assay system for modified nucleic acid components would be very useful in following the production, persistence, and repair of these lesions in a variety of cells and tissues treated with a broad spectrum of carcinogens and suspected carcinogens.

In the investigation of the mode of action of carcinogens, much work has centered around the effects of alkylating agents on DNA (Singer, 1975). Recently, a specific alkylation product, O^6-methylguanine, has been found in DNA treated with N'-methyl-N-nitrosourea (MNU)[1] (Loveless, 1969; Kirtikar & Goldthwait, 1974). O^6-Methylguanine accumulated preferentially in rat brain DNA upon in vivo administration of this potent neurocarcinogen (Goth & Rajewsky, 1974; Kleihues & Margison, 1974; Margison & Kleihues, 1975). This modified base also appears to be mutagenic at the level of transcription (Gerchman & Ludlum, 1973). However, O^6-methylguanine is relatively unstable under conventional DNA hydrolysis procedures (Singer, 1976) and is difficult to

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‡ Present address: Department of Biochemistry, School of Medicine, Oral Roberts University, Tulsa, Oklahoma.
§ Present address: Division of Rheumatology, University of Colorado Medical Center, Denver, Colorado.

[1] Abbreviations used: MNU, N'-methyl-N-nitrosourea; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; TS buffer, 0.14 M NaCl, 0.01 M Tris, pH 7.5.

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detect in a quantitative manner (Lawley & Thatcher, 1970; Friedman et al., 1965).

Studies utilizing immunological methods for the detection of modified bases, nucleosides, and nucleic acids have been reported. Antibodies to various normal, modified, or damaged bases in DNA have been produced (Erlanger & Beiser, 1964; Halloran & Parker, 1966; Levine et al., 1966; Natali & Tan, 1971) as well as to modified nucleosides (Levine & Gjika, 1974; Levine et al., 1975) and radioimmunoassays developed that are sensitive to the picomole level. For example, anti-7-methylguanine specific antibodies were used to detect 7-methylguanine in dimethyl sulfate treated poly(guanylic acid) and DNA (Sawicki et al., 1976).

Thus, it was of considerable interest to investigate the possibility of developing an immunological procedure for detecting O^6-methylguanine in methylated DNA. We found that O^6-methylguanosine was antigenic and this served as the basis for the development of a radioimmunoassay. Data showing the immunological specificity of the antibodies employed in this assay are presented.

Experimental Procedure

O^6-Methylguanosine (O^6-me-rG) Production. O^6-Methylguanosine was prepared by the method of Gerchman et al. (1972) in which 2-amino-6-chloropurine riboside (6-Cl-rG) was allowed to react with sodium methoxide. In a typical synthesis, 1 g of sodium metal was placed in 7 mL of absolute methanol under an anhydrous nitrogen blanket. The formation of sodium methoxide was carried to completion with gentle heating. Two milliliters of the sodium methoxide (NaOMe) was added to 300 mg of 6-Cl-rG in 14 mL of absolute methanol at 70 °C, incubated at that temperature for 2 h, and then at room temperature overnight. The reaction mixture was neutralized with concentrated HCl and cooled to 4 °C upon which white needle-like crystals settled out. After evaporation of the methanol, the crystals were dissolved in 10 mL of hot H_2O. The resulting product was >98% O^6-me-rG (O^6-methylguanosine). Spectral analysis of the product revealed two maxima at 248, 280 nm and two minima at 225, 251 nm; $A_{250}/A_{260} = 1.78$; $A_{280}/A_{260} = 1.70$ at pH 7.

O^6-Ethyl- and O^6-isopropylguanosine (O^6-et-rG and O^6-iprop-rG) were made as described above employing the corresponding alcohol rather than methanol. These products were purified by passage over a Sephadex G-10 column.

O^6-Methyl[^{3}H]guanosine of a low specific activity was produced by alkylation of [^{3}H]guanosine by diazomethane (Arndt, 1943; Friedman et al., 1965) and the product purified by Sephadex G-10 chromatography.

Column Chromatography. Chromatographic separation and purification of nucleotides, nucleosides, and bases were carried out using a 1.5 × 100 cm column of Sephadex G-10 (Sweetman & Nyhan, 1968). Applied samples were eluted with 0.05 M ammonium bicarbonate buffer (pH 7.5) at a flow rate of 42 mL/h. The elution volumes of several related compounds were as follows: blue dextran, 72 mL; 2′-deoxyguanosine 5′-monophosphate, 105 mL; O^6-methyldeoxyguanosine 5′-monophosphate, 172 mL; 1-methylguanosine, 240 mL; deoxyguanosine, 300 mL; 7-methylguanosine, 360 mL; guanine, O^6-methylguanosine, and O^6-methyldeoxyguanosine, 475 mL; O^6-methylguanine, 780 mL.

Preparation of O^6-Methylguanosine–Bovine Serum Albumin Complex. The antigen employed to immunize rabbits was O^6-methylguanosine complexed to bovine serum albumin by the method of Erlanger & Beiser (1964). O^6-me-rG (50 mg) and 50 000 cpm [^{3}H]-O^6-me-rG were mixed in 7 mL of H_2O to a known specific activity. Sodium periodate (0.5 mmol) was added and the reaction mixture incubated at 37 °C for 30 min. Excess periodate was removed by addition of 0.4 mL of 1 M ethylene glycol. To this was added 140 mg of bovine serum albumin in 3 mL and the pH adjusted to 9–9.5 with 5% potassium carbonate and incubated at 20 °C for 45 min. Seventy-five milligrams of $NaBH_4$ dissolved in 5 mL of H_2O was added and allowed to react overnight. This results in the reduction of a hydroxide group on the 2′ or 3′ carbon of the ribose residue. Excess borohydride was removed by addition of 2.5 mmol of formic acid for 60 min. The pH was adjusted to pH 8.5 and the solution dialyzed against cold running water for 24 h. Spectral analysis of the product as well as radioactive determinations revealed 12–15 nucleosides covalently linked to each bovine serum albumin molecule.

Immunizations. New Zealand albino rabbits (2–2.5 kg, male and female) were immunized with the O^6-me-rG–bovine serum albumin complex (5.5–6 mg in 1 mL of 0.1 M phosphate buffer (pH 7.0)/rabbit) homogenized in an equal volume of complete Freund's adjuvant and injected im and sc in two to four sites. Injections were given weekly. Serum was collected at weekly intervals.

Preparation of ^{3}H-Labeled Nucleoside-ε-Aminocaproate. [^{3}H]-O^6-Methylguanosine-ε-aminocaproate of a high specific activity (up to 1.5 Ci/mmol) was made by a modification of the procedure described by Levine & Gjika (1974). Five milligrams of synthesized O^6-me-rG was dissolved in a tenfold excess of water. In place of bovine serum albumin, 6 mg of ε-aminocaproic acid was added and the reaction mixture adjusted to pH 9–9.5 as before. An aliquot of this mixture, 0.55 mL (1.4 μmol), was added in a fume hood to approximately 20 mCi of $NaB[^3H]_4$ in 34 μL of H_2O and the mixture incubated overnight in the dark at 20 °C. Excess sodium borohydride was destroyed with 20 mmol of formic acid as before, the pH adjusted to 8.5, and the product purified by Sephadex G-10 chromatography. Three peaks of radioactivity were eluted with 0.05 M ammonium bicarbonate buffer, pH 7.5. The first peak contained most of the radioactivity and did not bind to antibodies produced against O^6-me-rG–bovine serum albumin. The second and third peaks did bind to the antibodies with the third peak binding three to four times better than the second. The material of the third peak was concentrated and employed as radioactive antigen in the radioimmunoassay.

Radioimmunoassay. The radioimmunoassay system employed was similar to one described by Levine & Gjika (1974). Serum at the appropriate dilution was incubated at 56 °C for 30 min to inactivate complement components. The assay consisted of duplicate samples of 100 μL of serum, 50 μL of inhibitor or buffer, and 100 μL of [^{3}H]-O^6-methylguanosine-ε-aminocaproic acid (2500 cpm in inhibition studies and 1000 cpm in antisera titering experiments), mixed in that order. The reaction mixture was incubated at 37 °C for 1 h and overnight at 4 °C. The antigen–antibody complexes were precipitated by addition of an equal volume of saturated ammonium sulfate at 4 °C (Minden & Farr, 1967; Minden et al., 1969) and after 15 min centrifuged at 2000 rpm for 30 min. The supernatant layer was removed by careful aspiration and the precipitate washed with 1 mL of 50% saturated ammonium sulfate at 4 °C. Following centrifugation and aspiration as before, the precipitate was dissolved in 0.6 mL of H_2O and 0.5 mL was transferred to a scintillation vial. Five milliliters of scintillation fluid (1000 mL of toluene + 16 g of Omnifluor + 500 mL of Triton X-100) was added and the radioactivity determined by liquid scintillation counting.

Characterization of the Antibody. DEAE-cellulose chromatography was performed on a 0.9 × 21 cm column (Whatman DE-52) equilibrated in 0.01 M phosphate buffer (pH 8.0).

A 1-mL sample of undiluted serum was dialyzed overnight against the same buffer and 0.75 mL applied to the column. The column was eluted with a 0.01–0.3 M phosphate buffer (pH 8.0) with a concave gradient of 150 mL (Friedman et al., 1965). The A_{280} of the eluent was monitored and peak fractions were pooled and dialyzed against 0.14 M NaCl, 0.01 M Tris-Cl, pH 7, before assaying.

Molecular weight determination of the antibody fraction was made on a Sepharose 6B column (2 × 85 cm) standardized with thyroglobulin and bovine serum albumin. The starting material was the O^6-methylguanosine-ε-aminocaproate binding fractions from the DEAE-cellulose column after concentration by lyophilization. The material was eluted from the column with 0.2 M KCl, 0.01 M Tris-Cl, pH 7.8, 0.001 M EDTA collecting 2.5-mL fractions. Aliquots of 150 μL of each fraction were assayed for the ability to bind 100 μL of [^{3}H]-O^6-methylguanosine-ε-aminocaproic acid (1000 cpm) in a standard radioimmunoassay.

Alkylation of DNA. Alkylation of calf thymus DNA by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was performed as described by Lawley & Thatcher (1970) and Lawley & Shaw (1972). Ten milligrams of DNA in 15 mM phosphate buffer (pH 7) or 0.2 M Tris-HCl buffer (pH 7.8) with 8 mM glutathione was mixed with 12–100 mg of MNNG and incubated 1 h at 37 °C. DNA was precipitated by addition of 0.1 volume of 2.5 M sodium acetate and 2 volumes of cold ethanol. Following centrifugation the DNA was washed with cold ethanol and dissolved in TS buffer (0.14 M NaCl, 0.01 M Tris, pH 7.5).

DNA was methylated by dimethyl sulfate as described by Sawicki et al. (1976). Five milligrams of denatured DNA (12 min in a boiling water bath followed by rapid cooling in an ice–water bath) in 5 mL of 0.8 M sodium cacodylate buffer (pH 6.8), 0.2 M NaCl, 0.001 M EDTA was added to 271 μmol of dimethyl sulfate and incubated 1 h at 37 °C. Excess dimethyl sulfate was neutralized by addition of 0.6 mmol of sodium thiosulfate. The mixture was dialyzed against TS buffer with 1 mM EDTA.

MNU methylation of DNA was accomplished according to Lawley & Shaw (1972). Two milliliters of 0.2 M Tris-Cl (pH 8.2) containing 5 mg of calf thymus DNA was added to 265 mg of MNU and incubated at 37 °C for 1 h. DNA was precipitated with ethanol as before and redissolved in 1 mM EDTA.

Analysis of me-DNA. Methylated DNA was subjected to mild acid hydrolysis (0.1 N HCl at 37 °C for 16 h or 70 °C for 1 h) for depurination. The hydrolysate was neutralized with NaOH, applied to a Sephadex G-10 column (1.5 × 100 cm), and eluted with 0.05 M ammonium bicarbonate (pH 7.5). The A_{254} of the effluent was monitored and peak areas were concentrated by lyophilization. The resulting apurinic acid and bases were identified and quantitated by UV spectroscopy in 0.1 M phosphate (pH 7.0). The molar extinction coefficients employed were as follows: apurinic acid, ϵ_{260} = 6623; 7-methylguanine, ϵ_{280} = 7250; guanine, ϵ_{260} = 7200; adenine, ϵ_{260} = 13 300.

Materials. Sephadex G-10-120, calf thymus DNA, bovine serum albumin, ε-amino-*N*-caproic acid, guanosine, 2-deoxyguanosine, guanine, 2′-deoxyguanosine 5′-monophosphate, 1-methylguanosine, 7-methylguanosine, 7-methylguanine, N^2-methylguanine, 6-methoxypurine riboside (O^6-methyladenosine), 6-methylpurine riboside, 2-amino-6-chloropurine riboside (6-chloroguanosine), 2′-deoxyadenosine, thymidine, 2′-deoxycytidine, and uridine were obtained from Sigma Chemical Co., St. Louis, Mo. The Het-Chem Co., Harrisonville, Mo., supplied 2-aminopurine riboside and purine riboside; O^6-methyl-2′-deoxyguanosine was made by Dr. P. B. Farmer, Chester Beatty Research Inst., London, U.K. Complete Freund's adjuvant was from Difco Labs, Detroit, Mich. Sodium boro[^{3}H]hydride (6 Ci/mmol) was supplied by Amersham/Searle Corp. *N*-Methyl-*N*-nitrosourea was from ICN Life Sciences Group, K & K Fine Chem. Div., Irvine, Calif., and dimethyl sulfate and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine came from Aldrich Chemical Co., Milwaukee, Wis. Omnifluor and [^{3}H]-2′-deoxyguanosine 5′-monophosphate came from New England Nuclear, Boston, Mass., and Triton X-100 came from Atlas Chemical and Manufacturing Co., San Diego, Calif.

Results

Rabbits were bled at weekly intervals following antigen inoculation. Sera obtained from these bleedings were titered for their ability to bind ^{3}H-labeled antigen in a radioimmunoassay at varying dilutions. Antibody activity was detected in the serum of rabbit 6460 3 weeks after the immunizations began. By the eighth week, serum at a 1:20 dilution bound about 50% of the antigen in the assay (437 of 939 cpm) and the serum collected after the booster immunizations could bind 50% at 1:80 dilution. Assay of the serum obtained prior to immunization demonstrated only background levels of antigen binding (<25 cpm). Nine weeks after the start of weekly immunizations, sera from two other rabbits, 0019 and 0020, could bind 50% of the ^{3}H-labeled antigen in a radioimmunoassay at dilutions of 1:7 and 1:40, respectively.

The identity of the antigen-binding component of the serum as being a true antibody is based on: (1) its appearance in sera of rabbits only after immunization; (2) the elution of this component from a DEAE-cellulose column with the IgG fraction; and (3) the estimation of its molecular weight by Sepharose 6B chromatography to be approximately 150 000.

The immunospecificity of the antibodies was determined by incorporation of O^6-methylguanosine analogues into the radioimmunoassay as inhibitors of the normal antigen–antibody binding. In most of these assays, serum from rabbit 6460 obtained 8 weeks after the start of immunization was employed at a 1:20 dilution. The background radioactivity for such an assay (i.e., complete mixture with preimmune serum) was less than 20 cpm. A control 100% binding (no inhibition) from complete mixture with no inhibitor represented 750–900 cpm of the 2200–2500 cpm included in the assay. This dilution of the antibody ensured that the assay was performed in the linear range of antigen–antibody reactions. Duplication of an assay yielded results within 3–5%. O^6-Methyldeoxyguanosine was the most potent inhibitor requiring only 0.045 nmol (45 pmol) of the nucleoside to inhibit the antigen–antibody interaction by 50%. O^6-Methylguanosine was the next most potent inhibitor followed by the ethyl analogue, O^6-ethylguanosine (Figure 1). To inhibit the interaction to the same degree, it required 26 times as much free base (O^6-methylguanine) as O^6-methyldeoxyguanosine, over 15 500 times as much deoxyadenosine, and nearly 90 000 times more deoxyguanosine. Table I shows the results of a number of nucleosides and analogues tested and indicates the importance of substitution at the 6 position of guanosine in the antigen–antibody interaction. The presence of groups such as ethoxy, isopropyl, chlorine, aminomethyl at the 6-position permits ready reaction with these antibodies. The importance of the amino group at the C-2 position of guanine is indicated by the lowered reactivity of O^6-methylpurine riboside (O^6-methylguanosine without the 2-amino group) and in the greater reactivity of the 2-aminopurine riboside relative to purine riboside. The importance of

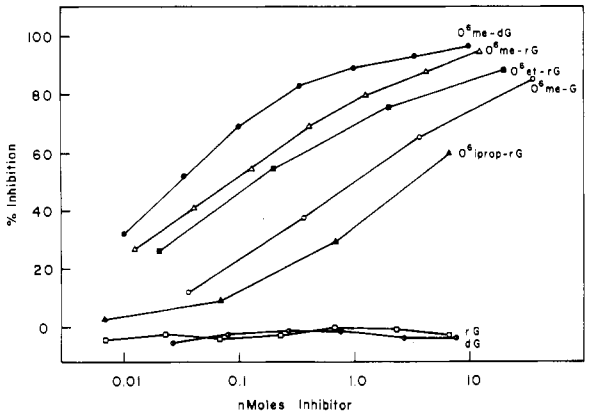

FIGURE 1: Assay of inhibition of anti-O^6-methylguanosine and O^6-methylguanosine-ε-aminocaproic acid binding by various compounds. O⁶-me-dG = O^6-methyldeoxyguanosine; O⁶-me-rG = O^6-methylguanosine; O⁶-et-rG = O^6-ethylguanosine; O⁶-me-G = O^6-methylguanine; O⁶-iprop-rG = O^6-isopropylguanosine; rG = guanosine; dG = deoxyguanosine. Assay conditions are described in text.

TABLE I: Inhibition of O^6-Methylguanosine-ε-Aminocaproic Acid Binding by Immune Serum.[a]

	nmol necessary to inhibit reaction by 50%	Amount rel to O^6-methyldeoxyguanosine
O^6-Methyldeoxyguanosine	0.045	1
O^6-Methylguanosine	0.065	1.4
O^6-Ethylguanosine	0.18	4
6-Chloroguanosine	0.75	16.7
O^6-Methylguanine	1.18	26
O^6-Isopropylguanosine	2.75	61
O^6-Methylpurine riboside	2.0	44
N^6-Methyladenosine	53	1 178
6-Methylpurine riboside	92	2 044
2-Aminopurine riboside	155	3 444
Purine riboside	235	5 222
1-Methylguanosine	500[c]	11 000
Deoxyadenosine	700[d]	15 500
Deoxyguanosine	~4 000[e]	90 000
Guanosine	~20 000[f]	4.4×10^5
7-Methylguanosine	>3 000	
7-Methylguanine	>3 000	
dGMP	>3 000	
N^2-Methylguanosine	ND[b]	
Uridine	ND	
Thymidine	ND	
Deoxycytidine	ND	

[a] Assay conditions as described in Methods. [b] ND = not detectable. Less than 20% inhibition at >500 nmol of inhibitor. [c] 44% at 336 nmol. [d] 44% at 398 nmol. [e] 21% at 374 nmol. [f] 11% at 353 nmol.

the ribose moiety and in particular the 2′-deoxyribose moiety is demonstrated by the relative reactivities of O^6-methyldeoxyguanosine, O^6-methylguanosine, and O^6-methylguanine. It is possible that the presence or absence of a hydrogen at the N-1 position is of great significance as compounds that show a greater reactivity with the antibodies apparently do not contain a hydrogen at the N-1 position, while those of low reactivity (guanosine and 7-methylguanosine) do.

The higher specificity of O^6-methyldeoxyguanosine vs. O^6-methylguanosine is due to the fact that the immunogen employed as hapten is a modified O^6-methylguanosine that was reduced at the 2′ or 3′ carbon of the ribose and thus more closely resembled O^6-methyldeoxyguanosine.

Because antibody specificity often changes with time and varies between rabbits, the specificities of the sera from rabbit 6460 obtained 6 and 8 weeks after the start of immunizations and a week after two booster immunizations were compared and found to be the same. (The specificities were compared on the basis of the degree of inhibition obtained with five inhibitors: O^6-methylguanosine, O^6-methyldeoxyguanosine, 6-chloroguanosine, deoxyguanosine, and guanosine.) Comparison of serum from rabbit 0020 revealed almost identical specificity. Preliminary results from the serum of rabbit 0019 show that it may be more specific for the O^6-methylguanine base with the ribose moiety exhibiting little influence on the reaction with the antibody.

Studies were carried out to determine if these antibodies would react specifically with O^6-methylguanine in methylated DNA. For this purpose, a radioimmunoassay employing antiserum and [^{3}H]-O^6-methylguanosine-ε-aminocaproic acid with methylated DNA as an inhibitor was used. In performing such an assay, several questions must be answered: Will methylated DNA behave as an inhibitor in this assay? Is the response a true antigen-antibody interaction or is it due to a nonspecific DNA-protein interaction? Will the antibody have access to the antigen in native DNA or must the DNA be denatured? Will the antibodies recognize O^6-methylguanine in the DNA chain but not cross-react with other components such as adenine or other methylguanines?

The first question was answered in that calf thymus DNA treated with MNNG and heat denatured was found to be an inhibitor in the assay at reasonably low levels. Control experiments revealed that nonmethylated DNA, either native or heat denatured, would give a nonspecific interference when employed at levels greater than 100–200 nmol of DNA-P. An increase in the slope of inhibition by methylated DNA at comparably high levels indicated that it, too, gave nonspecific interference. At concentrations below 150 nmol of DNA-P/assay, the results appeared to follow normal antibody-inhibition kinetics.

To answer the question of whether antibodies would have access to the antigen in native DNA, as well as to determine what effect the length of the DNA might have on the reaction, the following experiment was performed. Calf thymus DNA was methylated with MNNG. Portions of this material were sonicated for 0, 10, and 120 s and half of each of these heat denatured. Assay results revealed that 11.7 nmol of DNA-P of any of the heat denatured DNAs would inhibit the reaction by 48% while the same amount of the double standard DNA sonicated 120 s inhibited only 29%, that sonicated 10 s only 27%, and the unsonicated double-stranded DNA inhibited only 12%. Thus, these results indicate that the degree of inhibition by double-stranded me-DNA increased with sonication but was not as high as the inhibition by heat-denatured me-DNA, which was independent of sonication. From this, it was concluded that inhibition of the antigen-antibody reaction is related to the exposure of the bases in the DNA and that sonication of native DNA possibly results in regions of single strandedness.

The question as to whether antibodies recognize O^6-methylguanine exclusively or cross-react with other bases or even react with any type of methylated base is a difficult one. To begin to answer this, DNA methylated by an O^6-methylguanine-producing carcinogen (MNU) and DNA methylated by a non-O^6-methylguanine-producing alkylating agent (dimethyl sulfate) were compared with regard to inhibitory activity (Kleihues & Cooper, 1976; Lawley et al., 1972; Loveless, 1969). While alkylation by these two agents occurs optimally under different pH and buffer conditions, these conditions

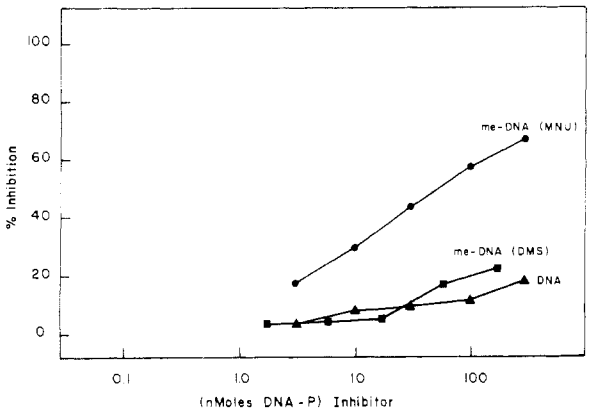

FIGURE 2: Assay of inhibition of antigen–antibody binding by heat-denatured DNA, DMS (dimethyl sulfate)-treated DNA and MNU-treated DNA. For dimethyl sulfate treated DNA, 5 mg of DNA was treated with 271 μmol of dimethyl sulfate resulting in methylation of approximately 27% of the guanine bases. For MNU-treated DNA, 5 mg of DNA was added to 2.56 mmol of MNU resulting in methylation of approximately 28–30% of the guanine bases.

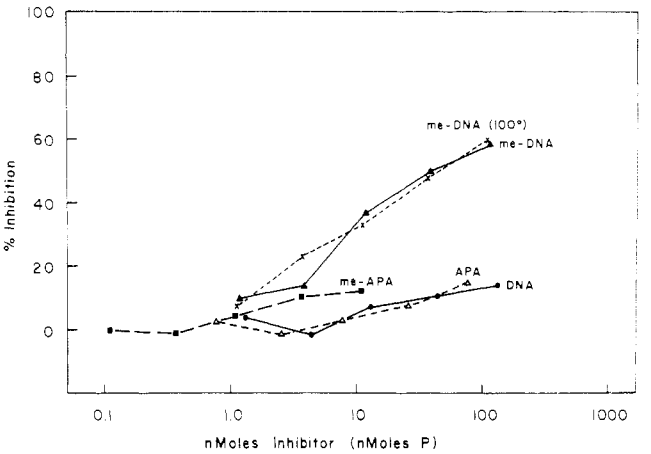

FIGURE 3: Assay of inhibition of antigen–antibody binding by single-stranded DNA samples. DNA = DNA; APA = apurinic acid derived by mild acid hydrolysis; me-DNA = methylated DNA (methylated with MNU); me-DNA (100 °C) = me-DNA treated at 100 °C to remove 7-methylguanine and 3-methyladenine; me-APA = apurinic acid derived by mild acid hydrolysis of me-DNA (100 °C).

should not affect the radioimmunoassay since excess alkylating agent and buffer were removed and the assays performed under identical conditions. Differences in the assay results should reflect differences in the pattern of alkylation of DNA by these two agents. Aliquots of DNA reacted with each of these methylating agents were acid hydrolyzed, the products separated by Sephadex G-10 chromatography and the percentage of total guanine that was alkylated to 7-methylguanine determined spectrophotometrically. For 5 mg of DNA treated with 271 μmol of dimethyl sulfate 27% of the total guanine was found to be in the form of 7-methylguanine. For 5 mg of DNA treated with 2.56 mmol of MNU, 28–30% of the total guanine was found to be 7-methylguanine. The amount of O^6-methylguanine in the MNU-treated DNA was too little to detect by this chromatographic procedure. Employing theoretical calculations to estimate the amount of O^6-methyldeoxyguanosine in these two alkylated DNAs, based on Kleihues & Cooper (1976) and Lawley & Thatcher (1970), the MNU treated DNA contains approximately 2–3% of its guanosine as O^6-methyldeoxyguanosine while the dimethyl sulfate treated DNA contains less than 0.01%. Thus, these two preparations of DNA contain comparable amounts of methylated bases, the important difference being that the dimethyl sulfate treated material lacked O^6-methylguanine. As shown in Figure 2, unmethylated DNA produced only background levels of inhibition (<15%); dimethyl sulfate treated DNA gave little inhibition, while MNU treated DNA inhibited the antigen–antibody binding many times greater (approximately five times greater at 100 nmol of DNA-P) than the dimethyl sulfate treated DNA. Extrapolation of the slopes reveals that it would require about 50 times as much dimethyl sulfate treated DNA as MNU-treated DNA to give the same degree of inhibition.

In order to ascertain whether the antibodies were detecting O^6-methylguanine in the DNA or were combining with depurinated sites, the following experiment was performed. Alkaline denatured DNA was methylated with MNU. An aliquot of the me-DNA was heated at 100 °C for 20 min in 1 mM phosphate (pH 7) to release the 7-methylguanine and 3-methyladenine (Lawley & Thatcher, 1970) and the partially depurinated me-DNA (100 °C me-DNA) collected by ethanol precipitation. Apurinic acid was produced from a portion of this material by mild acid hydrolysis and the resulting material collected by ethanol precipitation. These materials, as well as alkaline denatured unmethylated DNA and apurinic acid made from it, were compared for inhibitory effects by the radioimmunoassay system as shown in Figure 3. Unmethylated DNA and apurinic acid produced from it are not inhibitory. The me-DNA and the partially depurinated me-DNA are equally good inhibitors, indicating that 7-methylguanine and 3-methyladenine are unlikely to be involved in the antibody binding. The loss of inhibitory action of the me-DNA following complete depurination implicates O^6-methylguanine as the site of binding with antibody.

Discussion

It is apparent from the data presented that the antisera described above are highly specific for O^6-methylguanosine and could readily be employed to detect O^6-methyldeoxyguanosine, O^6-ethylguanosine, and O^6-methylguanine. The cross-reactivity of these antisera with other methylguanines found in methylated DNA and with the normal nucleosides was four to six logs less than with O^6-methylguanosine.

The antibodies obtained in these studies are capable of binding alkylated DNA, and it is apparent that the reaction is specific for the O^6-methylguanine moiety in the polynucleotide. It should be noted that in these early in vitro tests, the levels of alkylation are substantially higher than would be found under in vivo situations. However, subsequent purification of the antibodies and further refinements of the assay system should enhance the sensitivity of the assay to a more practical level for use with biological systems.

An assay system such as this will be a very useful probe for detecting O^6-methylguanine in DNA treated with certain alkylating agents, including carcinogens. This would be extremely useful in studies of the role of O^6-methylguanine in carcinogenesis and the nature of the repair enzyme systems that are operative in vivo.

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Adenosine Incorporation by Unfertilized Mouse Ova: Adenylation of RNA and Adenosine Diphosphate Ribosylation of Protein†

R. J. Young* and K. Sweeney

ABSTRACT: Unfertilized mouse ova incorporated [^{3}H]adenosine into cold trichloroacetic acid insoluble material at a level which was highest 3–5 h after ovulation but which decreased sharply thereafter. Not all of the [^{3}H]adenosine-labeled material was released into the aqueous layer by chloroform–phenol extraction of labeled ova lysates. The labeled material in the aqueous layer was hydrolyzed by ribonucleases A and T_1 to [^{3}H]adenosine and a fragment which was converted by 0.3 M KOH to 3′-[^{3}H]AMP and [^{3}H]adenosine in the ratio of 12.5/1. [^{3}H]Adenosine-labeled material associated with protein was isolated by virtue of its resistance to ribonucleases T_2 and U_2. This material was released into the supernatant following incubation of the protein fraction with hydroxylamine, alkaline buffers, or 0.3 M KOH and was identified as oligo(ADP-ribose) with average chain length of 4–5 units by its hydrolysis to 5′-[^{3}H]AMP and phosphoribosyl[^{3}H]AMP with snake venom phosphodiesterase. 5′-[^{3}H]AMP was also released from the labeled protein fraction by alkali and snake venom phosphodiesterase suggesting ovum proteins are also mono(ADP-ribosylated). At the time of maximum [^{3}H]-adenosine incorporation, 24% of the label is present as oligo(ADP-ribose), 9% as the monomer, 47% as $(A)_{14}$ segments, and 20% is present at the 3′ terminus of RNA. [^{3}H]Adenosine, [^{3}H]ADP-ribose, and [^{3}H]cAMP were absent from the supernatant fraction of ova lysates but [^{3}H]NAD, the precursor of poly(ADP-ribose), [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]AMP were present. The kinetics of [^{3}H]adenosine incorporation suggest that a relationship may exist between adenosine metabolism in the unfertilized ovum and its fertilizability.

Biochemical studies of fertilization and embryonic development have concentrated on the echinoderm and amphibian rather than mammals because of the difficulty in collecting large numbers of mammalian ova and embryos in a short time, and the lack of suitable media and methods for their in vitro culture. The technique for superovulation (Gates, 1971) has enabled the collection of larger numbers of ova and embryos, and the development of culture methods using chemically defined media which support embryonic development through the preimplantation stages (Brinster, 1972) has led to increasing numbers of studies on mammalian (usually mouse and rabbit) embryonic development (see Schultz & Church, 1975; Epstein, 1975). As yet, however, there have been few biochemical studies on the mammalian one-cell embryo or the unfertilized ovum.

At ovulation, the mouse ovum contains a store of maternal products synthesized during oogenesis. These include polysaccharides, a variety of enzymes, rRNA, tRNA (see Biggers & Stern, 1973; Engel & Franke, 1976) and, based on the presence of poly(A) (G. Stull, personal communication) and incorporation of guanosine into the 5′-terminal cap (Young, 1977), mRNA. After fertilization, a low level of RNA synthesis can be detected (Young et al., 1978), but the pronuclei

† From the Reproductive Biology Unit, Department of Obstetrics and Gynecology, Cornell University Medical College, New York, New York 10021. *Received January 4, 1978.* Supported by The Rockefeller Foundation and National Institutes of Health Grant HD 10230.

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