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RADIOIMMUNOASSAY FOR N^6(METHYLNITROSO)ADENOSINE: PRODUCTION AND CHARACTERIZATION OF ANTIBODIES[1]

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SUMMARY

Antibodies specific for N^6(methylnitroso)adenosine have been produced in rabbits and a sensitive radioimmunoassay was developed. The nitroso group is immunodominant; 50% inhibition of the binding of [^{3}H]N^6(methylnitroso)adenosine to antibody was obtained with 9.6 pmoles of N^6(methylnitroso)adenosine and 200 nmoles of N^6-methyladenosine. Adenosine was essentially inactive. After nitrosation, N^6(methylnitroso)adenosine can be detected only in those RNA molecules known to contain N^6-methyladenosine.

INTRODUCTION

N^6-Methyladenosine (m^6A)[2] is present in very small amounts and at highly restricted positions in certain RNA molecules (1-4). A recent report indicates that m^6A can be nitrosated under conditions found in the stomach to yield m^6(NO)A (5). This derivative is carcinogenic in Swiss CD-1 mice and thus joins a list of approximately 100 other N-nitroso compounds that have been shown to be carcinogenic in experimental animals (6). Several of these compounds are under intensive study since the possibility exists

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[2] Abbreviations used are: m^6A, N^6-methyladenosine; m^6(NO)A, N^6(methylnitroso)adenosine; $tRNA^{val\ I}$, transfer RNA valine specific type I; $tRNA^{phe}$, transfer RNA phenylalanine specific; HSA, human serum albumin; $m^7G(5')ppp(5')m^6Am$, 7-methylguanosine(5')ppp(5')N^6-O^2-dimethyladenosine; $m^7G(5')ppp(5')A$, 7-methylguanosine(5')ppp(5')adenosine.

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that they can be formed when naturally occurring secondary amines react with dietary or environmental sources of nitrites.

In this communication we report the production of antibodies directed toward $m^6(NO)A$ and the development of a sensitive and specific radioimmunoassay for this compound. This efficient method will facilitate the detection of this compound in physiological fluids and tissues. It will also aid in determining the factors that influence its formation from m^6A. The assay can also be used to detect m^6A in RNA molecules after conversion to the N-nitroso derivative by treatment of the polymers with nitrous acid. Although other reactions may occur with RNA during this treatment (e.g. deamination of adenosine to inosine), m^6A with its secondary amino group is fairly unique in forming a N-nitroso derivative that can react with the antibody.

MATERIALS AND METHODS

The following compounds were purchased from the sources cited: m^6A (Aldrich, Milwaukee, Wi.); $m^7G(5')ppp(5')m^6Am$ and $m^7G(5')ppp(5')A$ (P and L Biochem., Milwaukee, Wi.); $tRNA^{val\ I}$ (*Escherichia coli*) and $tRNA^{phe}$ (yeast) were gifts from Dr. A. Redfield and Paul Johnston of this department. $[^3H]m^6A$ (10 Ci/mmole) was labeled by Amersham-Searle (Arlington Heights, Ill.) by catalytic exchange in solution with tritium gas. All other chemicals used were of reagent grade.

m^6A was nitrosated according to the procedure of Giner-Sorolla *et al.* (7). At the end of the reaction the pH was adjusted to 7.0 with NH_4OH at 0° with stirring. The solvent was removed by lyophilization and the solid taken up in cold H_2O. The pale yellow crystals which were poorly soluble in cold H_2O were filtered, washed with cold H_2O and cold absolute ethanol, and dried overnight at 50° under vacuum. The product gave one spot on TLC: silica gel G with ethyl acetate:1-propanol:H_2O (4:1:2) as the mobile phase, $m^6(NO)A$ (Rf 0.58), m^6A (Rf 0.36). Its spectrum agrees with published data (7): λ max 225, 265, 295 nm (ϵ 9800, 6000, 9600). $[^3H]m^6A$ was nitrosated by stirring with 5 mg sodium nitrite in 0.5 ml of 50% acetic acid for 24 hrs at 4° and purified by TLC in the above solvent system (yield based on radioactivity = $\geq$ 90%).

$m^7G(5')ppp(5')m^6Am$ and $m^7G(5')ppp(5')A$ (100 μg) were taken up in 0.5 ml of 50% acetic acid, 5 mg sodium nitrite was added, and the mixture shaken for 24 hrs at 4°. The material was diluted into buffer containing an equal amount of 8.7 N NaOH for

assay. Controls in which sodium nitrite was omitted were run for each sample. In a second experiment, the reaction mixture was lyophilized twice to remove the acetic acid and then dissolved in buffer for assay. Similar results were obtained in both cases. $tRNA^{val\ I}$ and $tRNA^{phe}$ (50 μg) were treated in the same way in a 1 ml volume, isolated by passing through a Sephadex G-50 column (Pharmacia), lyophilized, dissolved in 0.5 ml buffer and 0.1 ml (10 μg) assayed directly.

To prepare the conjugate for immunization, m^6A was coupled to HSA by the method of Erlanger and Beiser (8). In typical preparations, 6-12 moles of nucleoside were incorporated per mole of protein. The conjugate (12 mg) was nitrosated by dissolving it in 8 ml 50% acetic acid and adding 8 mg sodium nitrite per hour for 4 hours. After stirring for an additional 24 hours, the conjugate was dialyzed. Female New Zealand albino rabbits were immunized by injection of $m^6(NO)A$-HSA conjugate (1 mg/rabbit) in complete Freund's adjuvant into the toe pads and intramuscularly once per week for three weeks. During the next five weeks the animals were bled periodically. The course of immunization was then repeated.

The radioimmunoassay which utilized a second antibody to separate free labeled hapten from antibody-bound hapten was similar to that described in previous work (9). In some cases, 50% $(NH_4)_2SO_4$ (10) was used for this separation. For this assay 0.6 ml of buffer (0.01 M Tris pH 7.5, 0.15 M NaCl, 0.1% gelatin) and 0.1 ml each of normal rabbit serum, inhibitor, labeled ligand, and diluted antibody were incubated at 37° for 1 hr then cooled in an ice bath. Saturated $(NH_4)_2SO_4$ (1.0 ml) was added with mixing and the tubes were allowed to stand at 0° for 20 min. After centrifugation the precipitate was dissolved in 1.0 ml H_2O. It was counted in 10 ml of 12% BBS-3 counting fluid with a Packard Tri-carb liquid scintillation spectrometer.

RESULTS

All four rabbits immunized with the $m^6(NO)A$-HSA conjugate produced antibodies. The sera from one rabbit were used throughout these studies. The antibodies are highly specific for $m^6(NO)A$ (Fig. 1 and Table 1) and can detect as little as 1.2 pmoles of this compound. m^6A is appreciably less effective than $m^6(NO)A$ in inhibiting the antigen-antibody reaction (A concentration greater than 2×10^4 is required to give equivalent inhibition). Adenosine, adenosine-5'-phosphate and adenosine-3'-phosphate are essentially inactive. Less than 10% inhibition at the 100 nmoles level was observed with these compounds and with

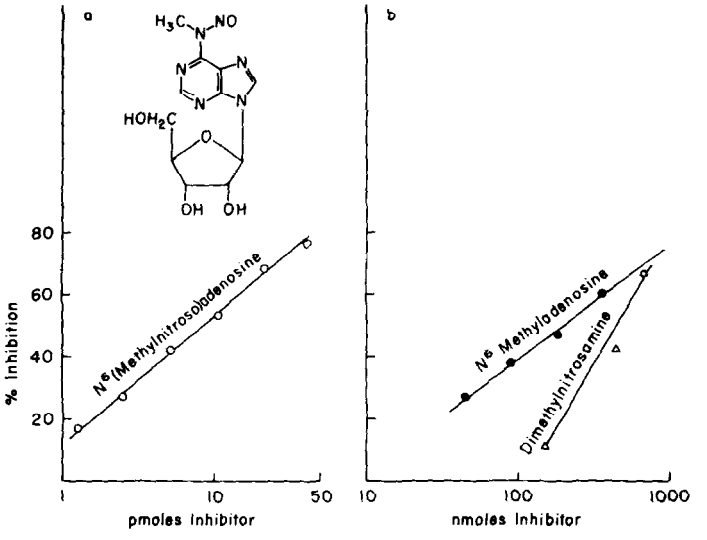

Fig. 1 Inhibition of the binding of [^{3}H]N^6(methylnitroso)-adenosine by N^6(methylnitroso)adenosine antibodies in the presence of (a) N^6(methylnitroso)adenosine (o) and (b) by N^6-methyladenosine (●), and dimethylnitrosamine (△). The immune sera (at a 1/100 dilution) precipitated 4800 of the 23,000 cpm of labeled hapten added to each tube, normal rabbit sera precipitated 400 cpm non-specifically.

deoxyadenosine, guanosine, thymidine, cytosine, inosine, uridine and/or their 5'-phosphates. Dimethylnitrosamine and N-nitroso-pyrrolidine are poor inhibitors of the antigen-antibody reaction (Table 1).

$tRNA^{val\ I}$ from E. coli has been sequenced and is known to contain one residue of m^6A (11). This methylated nucleoside is absent in $tRNA^{phe}$ from yeast. After treatment with nitrous acid, these RNAs were passed through Sephadex G-50 to remove low molecular weight reagents and assayed. When the serological activity was related to the standard curve for the free nucleoside (Fig. 1), each mole of $tRNA^{val\ I}$ was found to contain the equivalent of 0.10 moles of m^6(NO)A/mole RNA (Table 1). For $tRNA^{phe}$ this value was 0.004 moles/mole RNA indicating that other bases and products formed during nitrosation of this polymer do not cross react with the antibodies to an appreciable extent.

Table 1

A. Inhibition of the binding of [^{3}H]N^6(methylnitroso)adenosine to N^6(methylnitroso)adenosine antibody

Inhibitor	nmoles required for 50% inhibition
N^6(Methylnitroso)adenosine	0.0096
N^6-Methyladenosine	200
N^6-(Δ^2-Isopentenyl)adenosine	298
Dimethylnitrosamine	570
N-Nitrosopyrrolidine	3000

B. N^6(methylnitroso)adenosine equivalents in RNA samples treated with nitrous acid (7)

Sample	moles m^6(NO)A/mole RNA Expected	Found
E. coli tRNA$^{val\ I}$	1	0.10
Yeast tRNA^{phe}	0	0.004
m^{7}G(5')ppp(5')m^{6}Am	1	0.12
m^{7}G(5')ppp(5')A	0	0.0

m^{7}G(5')ppp(5')m^{6}Am and m^{7}G(5')ppp(5')A were treated with nitrous acid. When the serological activity was related to the standard curve for the free nucleoside (Fig. 1), the equivalent of 0.12 moles of m^6(NO)A (Table 1) was found for each mole of m^{7}G(5')ppp(5')m^{6}Am that was nitrosated. No activity could be detected with m^{7}G(5')ppp(5')A which had been treated similarly or with the controls.

DISCUSSION

The Erlanger-Beiser procedure (8) for the preparation of antibodies to ribonucleosides and nucleotides depends upon the oxidation by periodate of the vicinal 2' and 3' hydroxyls in ribose to aldehyde groups. The aldehydic derivatives react with the lysine groups of the protein at slightly basic pH. These addition products are stabilized by reduction with sodium borohydride to the tertiary amine. Specific antibodies for

several minor constituents, including methylated nucleosides and inosine (12), pseudouridine (13), N^6-(Δ^2-isopentenyl)-adenosine (14), and more relevant to this paper m^6A (15-16) have been prepared using this procedure. The antibodies to m^6A (15-16) were used primarily for the preparation of affinity columns. When determined by a quantitative precipitin reaction, 1.2 and 1000 μmoles of m^6A and adenosine, respectively, per mg antibody were required for 50% inhibition of the antigen-antibody reaction (15). In the radioimmunoassay the ability of $m^6(NO)A$ and adenosine to compete for the antibody binding site varies by a factor of $> 10^6$. The direct determination of m^6A as the N-nitroso derivative offers a distinct advantage over current techniques requiring labeling of newly synthesized RNA and isolation of m^6A after enzyme hydrolysis (2-4, 15, 16).

The finding of 0.10 mole of $m^6(NO)A$ in $tRNA^{val\ I}$ rather than 1.0 moles may be due to the fact that an assumption made in the calculations (i.e. that $m^6(NO)A$ inhibits equally well whether it exists as the free nucleoside or as part of the polymer) may require revision. Oligonucleotides in which the m^6A occupies a terminal position ($m^7G(5')ppp(5')m^6Am$) or an internal position (e.g. $tRNA^{val\ I}$) and which are known to be completely nitrosated should be used as inhibitors to construct standard curves. Under experimental conditions employed to nitrosate RNA in these studies, conversion of m^6A to $m^6(NO)A$ was only 60%. Modification of the procedure also may be required to improve the quantitation. The environment of $m^6(NO)A$ in the nitrosated polymer may also influence the quantitation. Unless cross linking has taken place, $m^6(NO)A$ should be accessible to the antibody since the reaction disrupts the secondary and tertiary structure of the RNA molecule.

In summary, the radioimmunoassay is applicable to the detection of $m^6(NO)A$ in RNA molecules treated with nitrous acid. Other products formed as a result of this reaction do not interfere with this assay. From the quantitative differences

observed when $m^6(NO)A$ and m^6A are used as inhibitors, the N-nitroso group appears to be immunodominant. The immunological approach to detect other N-nitroso compounds is therefore worth pursuing.

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