Nucleic Acids Research

Antibodies distinguishing between intact and alkali-hydrolyzed 7-methylguanosine

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ABSTRACT

Antibodies specific for intact 7-methylguanosine (m^7G) were induced in rabbits and mice by immunization with nucleoside-BSA or nucleoside-hemocyanin conjugates. Since m^7G undergoes alkali-catalyzed hydrolytic fission of the purine ring, modifications were made in the procedure for conjugation of m^7G to proteins. After periodate oxidation, m^7G was incubated with protein at pH 9.1 at 4°C for one hour during which the nucleoside was found to be stable. Reduction of the Schiff base was done with t-butylamine borane for 30 minutes, and the conjugated protein was isolated quickly by gel filtration at pH 7.2. Both rabbits and mice produced antibodies that readily distinguished between the intact and hydrolyzed m^7G. Antibody specificity depended largely on the presence of an intact 7-substituted imidazole ring and some cross-reaction occurred with 7-methylinosine. A weaker reaction occurred with ribothymidine and thymidine. Mouse antibodies induced by m^7G-hemocyanin showed the highest specificity. They also recognized m^7G in the isolated mRNA cap structure $m^7G(5')ppp(5')A$.

INTRODUCTION

A variety of eukaryotic and viral mRNA's contain 7-methylguanosine (m^7G) as a part of a 5'-terminal 'cap' structure (1-5) which is thought to play an important role in translation (6,7). Since the presence of methylated bases in mRNA caps could provide a basis for isolating mRNA populations by affinity chromatography, antibodies specific for these constituents of the cap have been investigated as potential affinity reagents.

Antibodies specific for m^7G have been described (8,9) and purified from rabbit antisera (10). These antibodies have been used to detect m^7G in synthetic nucleic acid polymers (11) and to fractionate oligonucleotides containing methylated bases (12). However, attempts to use antibodies specific for m^7G to purify cap-containing mRNA's have been largely unsuccessful. This may be due to two reasons: 1) antibodies to m^7G-BSA may not have sufficient affinity to bind very low concentrations of the monovalent mRNA cap, and 2) previously developed antisera may contain significant amounts of antibody specific for

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hydrolyzed m^7G rather than m^7G itself.

7-methylguanosine is highly susceptible to an alkali-catalyzed ring fission which occurs above pH 8.5 (13) and which is accompanied by the change in absorption spectrum shown in Fig. 1. Previous studies of antibodies specific for m^7G have used nucleoside-protein conjugate immunogens synthesized at pH 9.5 at room temperature (14, 8-12) or m^7G purified by thin layer chromatography with solvents containing NH_4OH (8); these immunogens may have contained significant amounts of hydrolyzed m^7G.

In this report, we describe the synthesis of m^7G-protein conjugates under pH and temperature conditions that minimize the alkali hydrolysis of the purine. Our studies indicate that rabbit and mouse antibodies induced by conjugates made under these conditions are highly specific for the intact form of m^7G and readily distinguish between the intact and alkali-degraded m^7G. The antibodies can recognize m^7G in the isolated mRNA cap structure $m^7G(5')-ppp(5')A$, but with lower affinity than they show for free m^7G. The affinity of these antibodies still may not be high enough to allow their use for isolation of small amounts of capped mRNA.

MATERIALS AND METHODS

Chemicals

7-methylguanosine, guanosine, 7-methylinosine, and adenosine were purchased from Sigma Chemical Co. Remaining nucleosides were purchased from Calbiochem. 7-methylguanosine-5'-triphosphate and RNA 5'-terminal fragment (cap), $m^7G(5')ppp(5')A$, were purchased from P-L Biochemicals, Inc. CM BioGel A was obtained from Bio-Rad Laboratories. N-2(aminoethyl)carbamylmethylated-Ficoll (AECM-Ficoll), synthesized by the method of Inman (15) was kindly supplied by Dr. M. I. Johnston.

Preparation of m^7G-protein conjugates

Ten mg of m^7G was oxidized with 0.6 ml of 0.1 M $NaIO_4$ in water (final pH = 6.6) for 30 minutes at room temperature. Oxidized m^7G was added dropwise to 12 mg of rabbit serum albumin (RSA), bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH) in 2 ml cold 0.3 M $NaHCO_3$, and the pH adjusted to 9.1 with 5% K_2CO_3. The remainder of the preparation was conducted at 4°C. Conjugation was allowed to proceed for one hour at pH 9.1 in the cold. The Schiff base conjugate was reduced with 50 mg t-butylamine borane (Eastman Organic Chemicals) rather than sodium borohydride in order to reduce the bubbling and pH change due to rapid H_2 production. The preparation was then chromatographed in the cold on a Sephadex G-25 column and washed through with PBS (0.01 M

potassium phosphate, pH 7.2, 0.14 M NaCl).

Preparation of [^{3}H]-m^{7}G-RSA and [^{3}H]-guanosine-RSA

Two mg of m^{7}G or guanosine was oxidized with 100 μl 0.1 M NaIO$_4$ in water for 30 minutes at room temperature. Oxidized nucleoside was added to 2 mg RSA in 0.3 ml of 0.3 M NaHCO$_3$, the pH was adjusted to 9.1, and the reaction was incubated in the cold for one hour. Approximately 2 mg of [^{3}H]-KBH$_4$ (New England Nuclear, 25 mCi/4.5 mg) was added, and the conjugate was reduced over-night at 4°C. The volume of the reaction mixture was brought to 2 ml with PBS and dialyzed in the cold against several changes of PBS.

Guanosine-BGG-CM BioGel

For preparation of an adsorbent to remove antibodies that cross-reacted with guanosine, bovine γ-globulin (BGG) was linked to CM Bio-Gel A by means of the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiim-ide (EDAC). Fifty milliliters of CM Bio-Gel A was washed with 250 ml of PBS and 250 ml coupling buffer (3 mM KPO$_4$, pH 6.3). BGG (1 gm/50 ml coupling buf-fer) was added to the washed gel, the pH was adjusted to 6.3, and the reaction was shaken gently for 30 minutes at room temperature. EDAC (5 gm) was added, the pH was again adjusted to 6.3, and the gel was allowed to react for 24 hours at room temperature. The coupled gel was then washed with PBS. 77% of the added BGG was found to be bound to the gel. Guanosine was then linked to the BGG-BioGel using the technique of Erlanger and Beiser (14).

m^{7}G-AECM-Ficoll-Sepharose

For preparation of an immunoabsorbant for antibody purification, AECM-Ficoll, prepared as described by Inman (15), was linked to CNBr-activated Sepharose 6B by the method of Porath, et al. (17). Three hundred milligrams of m^{7}G were oxidized with 15 ml 0.1 M NaIO$_4$ in water for 30 minutes at room temperature. Oxidized m^{7}G was mixed with 50 ml AECM-Ficoll-Sepharose in 50 ml 0.15 M NaHCO$_3$. pH and temperature were maintained at 9.1 and 4°C respectively for three hours. The Schiff base conjugated gel was reduced with 400 mg t-butylamine borane overnight at 4°C. The gel was then washed with 1600 ml cold PBS. It was found that 61% of the added m^{7}G was bound to the gel.

Spectrophotometric measurements alkali-hydrolyzed m^{7}G

All spectrophotometric measurements were made with a Cary 14 spectro-photometer. 7-methylguanosine (4 mg/ml H$_2$O) was diluted with an equal volume of 0.6 M sodium carbonate (pH 10, 9.5 or 9.3) or 0.6 M sodium phosphate (pH 8.15 or pH 7.2). Final pH's were 10, 9.5, 9.1, 8.1, and 7.2. Aliquots were taken at various intervals, diluted 1:9 with cold 0.2 M sodium phosphate pH 7.0, and frozen. For spectrophotometric readings, samples were diluted 1:9

with PBS.

Thin layer chromatography of hydrolyzed m^7G

20 mg of m^7G were dissolved in 0.5 ml 1 N NaOH (final pH = 13). After one hour at 37°C, an aliquot of the solution was spotted on cellulose-coated acetate TLC sheets (Eastman Chromogram sheets) and developed in methanol:H_2O (7:3). A uv-absorbent spot at Rf=0.6 was eluted from the gel with PBS. The compound isolated in this manner gave a spectrum identical to fully hydrolyzed m^7G. An intact m^7G standard gave a fluorescent spot at Rf=0.5.

Immunization

Three female New Zealand white rabbits (numbers 417, 418, and 419) were immunized at multiple intradermal sites with 1.25 mg of m^7G-BSA in complete Freund's adjuvant on day 0 and the same amount of m^7G-BSA in incomplete Freund's adjuvant on days 7 and 14. Sera were obtained on day 19. Late sera were obtained five days after intradermal boosts in incomplete Freund's.

A second group of three rabbits (numbers 383, 384, and 394) were injected intradermally with 1.5 mg m^7G-BSA in complete Freund's adjuvant on day 0 and the same amount of m^7G-BSA in incomplete Freund's on days 7 and 14. Five days prior to each bleed, animals were given intravenous boosts of 1.5 mg m^7G-BSA in PBS.

Six BALB/c mice were immunized intraperitoneally with 200 µg of m^7G-KLH in complete Freund's adjuvant on days 0 and 21 and were bled five days after the second injection. Sera from the six animals were pooled.

Quantitative precipitin assays

For quantitative precipitation, 0.1 ml of serum, 2 to 80 µg of antigen, and PBS were incubated in a final volume of 0.3 ml at 37°C for two hours and 4°C overnight. Precipitates were washed with 300 µl cold PBS and dissolved in 300 µl 0.1 N NaOH. Protein concentration was determined by the method of Lowry, et al. (16).

Radioimmunoassays

To measure binding of labeled antigen, 100 µl of antiserum or purified antibody and 20 µl (2000 to 4000 cpm) of either [^3H]-m^7G-RSA or [^3H]-guanosine-RSA were incubated for two hours at 37°C and overnight at 4°C. Immunoglobulin was precipitated with 75 µl of gamma globulin (an ammonium sulfate fraction) of goat anti-rabbit IgG for one hour at 37°C. Precipitates were centrifuged in a Brinkmann microfuge for 2 minutes and dissolved in 100 µl 0.1 N NaOH, the tubes washed with 100 µl 0.1 N NaOH, and both were counted in 3 ml Beckman Ready-Solv.

For competitive radioimmunoassay, 100 μl of appropriately diluted serum or 50 μg purified antibody was incubated with 50 μl of inhibitor or PBS for ten minutes at 37°C. Twenty μl (approx. 13.2 μg, 4000 cpm) of [^{3}H]-m^{7}G-RSA was added and the reaction was allowed to continue for one hour at 37°C and overnight at 4°C. Immunoglobulin was precipitated for one hour at 37°C with 50 μl of the gamma globulin fraction of goat anti-rabbit γ-globulin serum and precipitates were analyzed as described above.

RESULTS

Effects of pH and temperature on the rate of m^{7}G degradation

The alkali-catalyzed ring fission of m^{7}G to 2-amino-4-hydroxy-5-(N-methyl)carboxamide-6-ribosylamino-pyrimidine (hydrolyzed m^{7}G) can be monitored spectrophotometrically (Figure 1) and quantitated from changes in the 275/260 absorbance ratio. Ring fission was accelerated by increasing pH and temperature (13) (Figure 2). At pH 9.1, the m^{7}G had a half-life of 9 hours at room temperature. At pH 9.5 and 10.0, the corresponding half-life values were five hours and one hour respectively. A nearly neutral solution (pH 7.2) of m^{7}G incubated at 37°C was hydrolyzed at the same slow rate as a pH 8.1 solution maintained at room temperature, and the spectrum of intact m^{7}G remained unchanged at pH 9.1 at 4°C for 10 hours. Therefore, the original procedure for the conjugation of nucleosides to proteins (11) was modified so that all reactions occurred at 4° and conjugation of the periodate-oxidized nucleoside to protein took place at pH 9.1 rather than at pH 9.5. An additional modification was the use of t-butylamine borane for reduction of the Schiff base linkage; this evolved much less gas than did the use of sodium borohydride, and allowed the rapid separation of conjugated protein from free nucleoside by gel filtration on a Sephadex G-25 column. The reduction step was only 30 minutes. The difference spectrum between free protein and protein-nucleoside conjugates produced by this modified procedure was that corresponding to intact m^{7}G. The protein conjugates were stable at pH 7.4 at 37°C over a period of 10 hours so that the alkaline hydrolysis would not be expected to occur in vivo.

Quantitative precipitation and cross-reactions with guanosine

Rabbit anti-m^{7}G antisera contained antibody populations that cross-reacted with G-RSA (Table 1). The cross-reacting antibodies could be removed by absorption of serum with a G-BGG-Sepharose column. The absorbed serum no longer precipitated with G-RSA and, whereas 50 μl of unabsorbed #418 serum bound 70% of a 33 μg sample of [^{3}H]-G-RSA, the same amount of absorbed serum bound less than 1%. Absorbed serum still precipitated with m^{7}G-RSA and bound radioactive
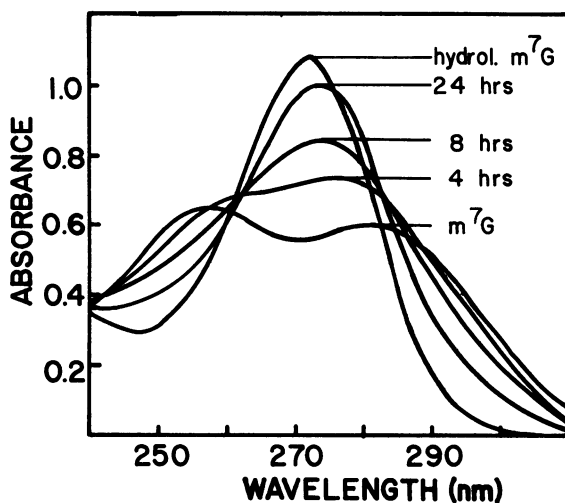
Figure 1. Alkali hydrolysis of m^7G at 23°C, pH 9.0. 50 mg m^7G was dissolved in 1 ml water. Samples were diluted with an equal volume of 0.6 M $NaHCO_3$, pH 9.2 (final pH = 9.0) and incubated for 24 hours at 23°C. At various intervals 100 μl aliquots were diluted 1:10 in 0.2 M KPO_4, pH 7.0. A control sample of m^7G was diluted 1:20 directly into pH 7.0 buffer. Another control sample was hydrolyzed for 24 hours at 23°C with an equal volume of 2 N NaOH and then diluted 1:10 in pH 7.0 buffer. All samples were further diluted 1:100 in PBS for spectrophotometric measurements.

m^7G-RSA.

The route of antigen administration affected antibody titers and cross-reactivity (Table 1). Early sera (a and b) from intravenously boosted animals contained little precipitable antibody; later sera (c and d) had more antibody but were highly cross-reactive with G-RSA. In contrast, sera from intradermally boosted rabbits showed less cross-reactivity and contained five to thirty times the amount of precipitable antibody found in the sera of intravenously injected animals.

Specificity for undegraded m^7G

Competitive radioimmunoassays showed that antibodies from rabbits immunized with undegraded m^7G-RSA conjugates could distinguish between intact and alkali-degraded m^7G. Either whole rabbit serum or immunospecifically purified anti-m^7G antibodies bound labeled m^7G-RSA, and 2-100 nmoles of unlabeled free m^7G competed for this binding (Fig. 3). (A serum with little guanosine cross-reactivity, #418, was used.) A 150-fold higher concentration of hydrolyzed m^7G was required for similar competition for whole serum, and a 600-fold higher concentration in competition for purified antibody. Further, the kinetics of degradation of m^7G at pH 9.1 were similar as measured either
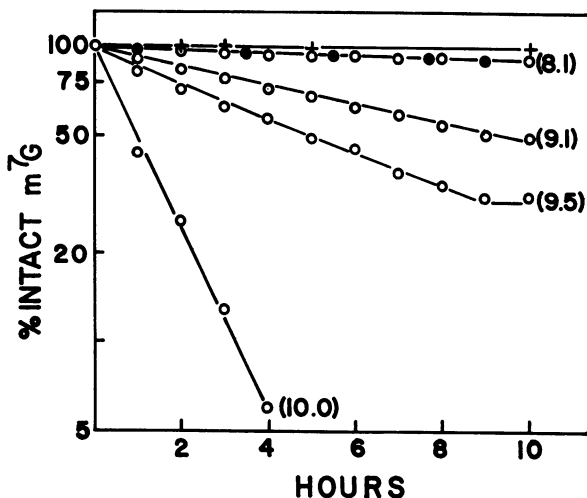
Figure 2. Alkali hydrolysis of m^7G. m^7G (4 mg/ml H_2O) was diluted with an equal volume of appropriate buffer: 0.6 M $NaHCO_3$, pH 10.1, 9.5, 9.3 or 0.6 M KPO_4, pH 8.1 or 7.2 and incubated at the designated temperatures. Final pH's were 10, 9.5, 9.1, 8.1 and 7.2. At various times, 100 µl aliquots were diluted 1:10 in 0.2 M KPO_4, pH 7.0. Samples were further diluted 1:10 in PBS for spectrophotometric measurements. (O) 23°C at the pH in parentheses, (●) 37°C, pH 7.2, (✛) 4°C, pH 9.1.

Table 1. Quantitative precipitation of anti-$(m^7G$–BSA) sera with m^7G–RSA and guanosine–RSA. Expressed as mg protein precipitated at equivalence/ml serum.

	$*m^7G$–RSA	$*$guanosine–RSA
Group I: Intradermal boost		
417 a	1.87	0.15
418 a	2.55	0.26
419 a	1.02	0.10
Group II: Intravenous boost		
383 a	0.08	0.06
384 a	0.09	0
394 a	0.23	0.43
383 b	0.25	0.21
394 b	0.21	0.36
383 c	0.55	0.42
394 c	1.85	1.54
383 d	0.52	0.63

$*$ The nucleoside/protein substitution ratios for m^7G–RSA and guanosine–RSA were 15:1 and 17:1 respectively. Guanosine–RSA was prepared by the technique of Erlanger and Beiser (14).
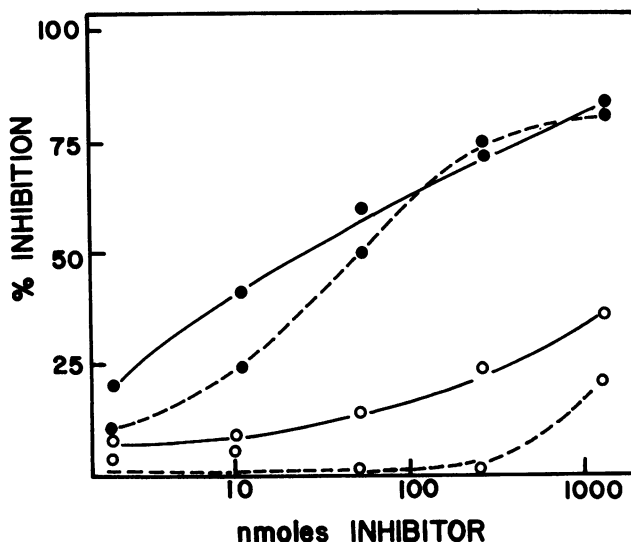
Figure 3. Radioimmunoassay of rabbit antiserum and purified rabbit antibody. Hydrolyzed m^7G was made by dissolving 20 mg m^7G in 0.5 ml 1 N NaOH and incubating it at 37°C for one hour. The reaction mixture was neutralized with HCl and diluted 1/5 in water. The product had a spectrum characteristic of fully hydrolyzed m^7G (270/260 = 2.10) and migrated as a single spot (Rf = 0.60) in TLC. (----) purified anti-m^7G, (____) whole serum #418b: serum dilution, 1/5, (●) m^7G, (O) hydrolyzed m^7G. Without competitor, serum and purified antibody bound 48% and 41% of labeled m^7G-RSA respectively.

spectrophotometrically or by loss of competitive serological reactivity in the radioimmunoassay (Fig. 4).

Specificity for intact m^7G was even more apparent in mouse sera induced by m^7G-KLH conjugates prepared by the modified procedure (Fig. 5). A 400-fold higher concentration of hydrolyzed m^7G than intact m^7G was needed for competitive binding to a serum pool from six mice that received two i.p. doses of antigen in complete Freund's adjuvant. The amount of native nucleoside required for 50% inhibition was 12 nmoles as compared to 25 nmoles required with rabbit anti-(m^7G-BSA) antisera.

Detection of m^7G in a mRNA cap

The binding of labeled m^7G-BSA was inhibited by m^7G present in the RNA cap structure m^7GpppA (Fig. 6). The entire cap was not as good an inhibitor as m^7G alone when the two forms were compared on a molar basis. 7-Methylguanosine-5'-triphosphate was compared to intact m^7G and mRNA cap in radioimmunoassay with mouse (anti-m^7G-KLH) antisera. A 10-fold greater concentration of the trinucleotide was needed to effect the same amount (50%) of inhibition as intact m^7G. m^7GTP was only a slightly better inhibitor than m^7GpppA.
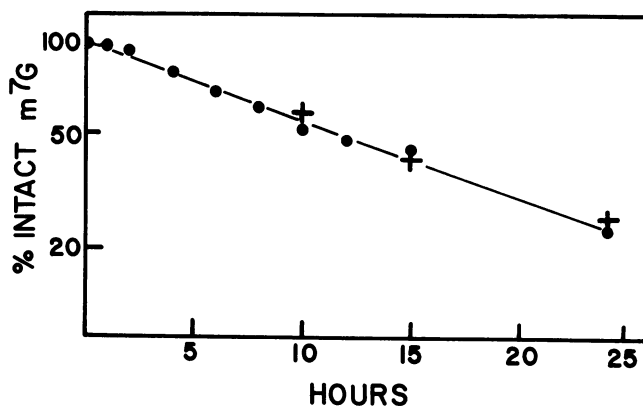
Figure 4. Determination of per cent intact m^{7}G in solutions of m^{7}G hydrolyzed at pH 9.1 at 23°C. m^{7}G (50 mg) was hydrolyzed at pH 9.1 at 23°C for 24 hours. Aliquots were neutralized at various times and used for spectrophotometric measurements and as inhibitors in radioimmunoassay. Per cent m^{7}G remaining determined from spectra (●) and from radioimmunoassay with purified antibody (✛).

Competition by other nucleosides

Anti-m^{7}G antibodies have shown specificity for methylated purines over non-methylated purines, and have distinguished among 1-, 3-, and 7-methylpurine
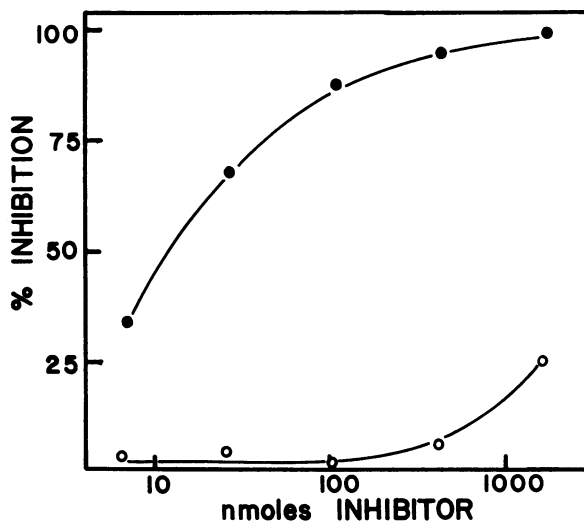

Figure 5. Radioimmunoassay of pooled mouse antisera. (●) m^{7}G, (○) hydrolyzed m^{7}G. Serum dilution, 1/50. Without competitor, serum bound 28% of labeled m^{7}G-RSA.
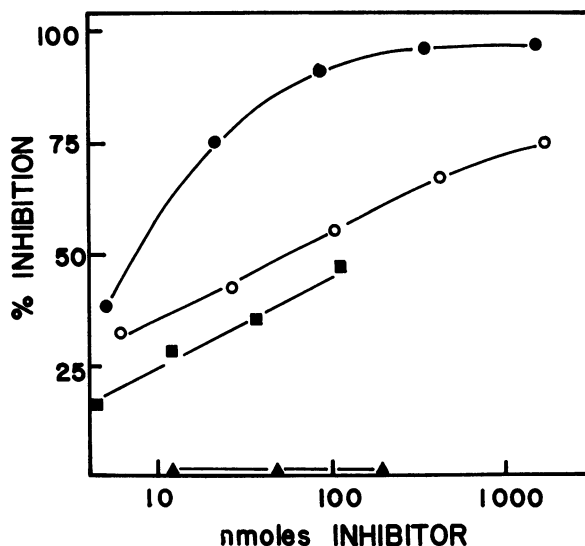
Figure 6. Radioimmunoassay of pooled mouse antisera. (●) m^7G, (O) m^7GTP, (■) $m^7G(5')ppp(5')A$, (▲) guanosine. Serum dilution, 1/50. Without competitor, serum bound 34% of labeled m^7G-RSA.

derivatives (8). Extending such comparisons, we found that 7-methylosine (m^7I) could inhibit the binding of m^7G-RSA by antibody, and that the pyrimidine nucleosides thymidine and ribothymidine also competed at high concentration (Table 2). The non-methylated purine ribosides adenosine and inosine and the pyrimidine riboside uridine were less inhibitory at the same high concentrations.

Table 2. Binding inhibition of rabbit (anti-m^7G-BSA) antibodies by nucleosides.

Inhibitor	Purified anti-m^7G: nmoles required for 50% inhibition	Anti-(m^7G-BSA) serum: nmoles required for 30% inhibition
m^7G	55	4
hydrolyzed m^7G	(20% at 1300 nmoles)	700
guanosine	*	(0 at 200 nmoles)
m^7I	180	500
thymidine	*	(29% at 1700 nmoles)
ribothymidine	5000	(24% at 1800 nmoles)
inosine	*	(17% at 1600 nmoles)
uridine	*	(16% at 1700 nmoles)
adenosine	*	(12% at 1700 nmoles)

*
 not done

DISCUSSION

Previous reports of anti-m^7G antibodies either did not test whether the antibodies could react with alkali-hydrolyzed m^7G (8,11) or suggested that m^7G and hydrolyzed m^7G were equally reactive (10). This was an unexpected result since anti-nucleoside antibodies are usually able to detect even small changes in base structure (14). We therefore explored the possibility that more specific antibodies could be developed if precautions were taken to prevent any hydrolysis of m^7G during conjugation to protein. We examined the pH and temperature dependence of hydrolysis, found it was negligible at pH 9.1 and 4°C, and modified the standard coupling procedure accordingly. We have also found that the use of a milder reducing agent permitted a more rapid isolation of the conjugate. The antibodies induced by these nucleoside-protein conjugates did distinguish clearly between native and alkali-treated m^7G. Meredith and Erlanger, recognizing the same problem found that the mono-nucleotide, m^7GMP, is more resistant than the nucleoside to hydrolysis and have used it rather than the nucleoside to form an immunogen (18).

Our whole rabbit sera contained a population of anti-guanosine antibodies that could be removed by absorption. We found the amount of this population could be reduced and the amount of hapten-specific antibody increased if an intravenous immunization route was avoided.

Our studies of the reactivity of anti-m^7G antibodies towards hydrolyzed m^7G as well as other pyrimidine and purine nucleosides suggested that antibody specificity depends largely on the whole nucleoside with substantial contribution by the modified imidazole ring. m^7I competed with hapten in RIA even though its pyrimidine ring has different substituents. Ribothymidine and thymidine were only slightly reactive but were better inhibitors than uridine possibly indicating that some antibody populations recognize the pyrimidine ring particularly in the region of N3 to C5 and the 5-methyl substituent. This may explain why reactivity toward m^7G is not completely abolished when it is in the fully hydrolyzed form. An additional basis for antibody recognition may be charge distribution. Both m^7G and m^7I exist in a betaine configuration (19) in which the imidazole and pyrimidine rings carry partial positive and negative charges respectively. The corresponding unmethylated nucleosides do not carry this charge distribution.

Although the antibodies can recognize m^7G in mRNA cap, the degree of reactivity is less than with free m^7G. This may reflect the constrained configuration of m^7G as it exists in mRNA cap (10). NMR studies of mRNA cap show that m^7G is in a stacked configuration with respect to the adjacent base which

may prevent maximum recognition of m^7G by antibody. It is interesting to note that m^7GTP was a less effective competitor than m^7G but slightly better than cap in the radioimmunoassay. The terminal phosphate of the m^7GTP may be interacting with the purine ring, partially obscuring anti-m^7G recognition sites.

Relatively high concentrations of m^7G and mRNA cap were required for inhibition of anti-m^7G antibodies indicating that antibody affinity may be limiting for their use in isolating mRNA. An antibody with an affinity of 10^7 could measure cap-containing mRNA 1000 bases long (M.W. 350,000) at an RNA concentration of 35 μg/ml. If the RNA concentration were far lower, then only a small percentage of it could be bound even by an excess of antibody (21). In many experiments with labeled mRNA, lower concentrations are in fact present, so that high affinity antibodies will be required. One general approach to producing high affinity antibodies is the use of KLH as a carrier (22,23). This is consistent with the finding that lower amounts of free m^7G inhibited anti-(m^7G-KLH) antibodies with a standard amount of labeled antigen (Figures 5 and 6). A second approach would be the use of a larger hapten, such as the nucleotide or whole mRNA cap.

ACKNOWLEDGEMENTS

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