Antibodies to guanosine

Fractionation and specificities

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Bovine serum albumin conjugates of guanosine prepared by the periodate method was used as immunogen to elicit guanosine antibodies in rabbits. The specificities of the antibodies were studied by the inhibition of their binding to [^{3}H]G$^{ox\text{-}red}$, [^{32}P]DNA and [^{3}H]RNA by related non-radioactive compounds. A population of antibodies is specific to G$^{ox\text{-}red}$ with an average association constant of around $10^7 M^{-1}$ at 4°C. There are a population of antibodies which bind to [^{32}P]ssDNA and [^{3}H]RNA specifically at guanosine residues. RNA binding antibodies were separated into two populations by affinity chromatography.

Guanosine *Antibody binding*

1. INTRODUCTION

Nucleic acid reactive antibodies with restricted heterogeneity, high affinity and specificity have many potential applications [1–9]. We were succesful in preparing antibodies specific to pG which bind to RNA at G residues but not to dsDNA or ssDNA [10]. Antibodies raised against protein conjugates of guanosine prepared by the periodate method which modifies the ribose can be expected to have a different type of sugar specificity in polynucleotide binding. It was of interest to study such antibodies.

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Abbreviations: G, guanosine; I. inosine; A, adenosine; C, cytosine; U, uracil; pG, guanosine 5′-phosphate; G$^{ox\text{-}red}$, guanosine oxidized with $NaIO_4$ and reduced with $NaBH_4$; similarly A$^{ox\text{-}red}$, U$^{ox\text{-}red}$, C$^{ox\text{-}red}$, I$^{ox\text{-}red}$; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

2. MATERIALS AND METHODS

The materials used were obtained from the following sources: nucleotides, bovine serum albumin (BSA), hexanediamine (Sigma Chemical Co., St. Louis, MO USA), nitrocellulose filters, 25 mm diameter, 0.45 μm pore size (Microdevices, Ambala cantt., India).

Protein estimation was done by the method in [11]. [^{32}P]DNA was prepared from colitis phage according to the procedure in [12]. DNA was denatured by heating at 100°C for 10 min and rapidly cooling in ice–salt mixture. [^{3}H]RNA was prepared from *Neurospora crassa* grown in a medium containing [^{3}H]uracil according to the procedure in [13].

Guanosine was coupled to BSA and RSA by the periodate method [14]. An average of 15 molecules of guanosine were coupled per molecule of BSA.

Rabbits were immunized with 1 mg of BSA-G (0.5 ml) emulsified in complete Freund's adjuvant (0.5 ml) thrice at weekly intervals on foot pads and intradermally at multiple sites. Rabbits were bled by the marginal ear vein 10 days after the third in-

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jection. The rabbits were boosted after 10 days with 1 mg of the immunogen emulsified in incomplete Freund's adjuvant and again bled after 10 days. Boosting was repeated from time to time.

γ-Globulins were prepared from the antisera by a combination of ammonium sulphate precipitation and DEAE-cellulose chromatography [15]. Nitrocellulose filter assay was used for studying the binding of antibodies to [^{3}H]G$^{ox\text{-}red}$, [^{32}P]DNA, [^{3}H]RNA [16].

Cyanogen bromide was prepared according to the procedure in [17]. AH-Sepharose was prepared according to the method in [18]. AH-Sepharose-G was prepared by the periodate oxidation method [14] using 1 mmol of guanosine, 2 mmol of $NaIO_4$ and 2 mmol of $NaBH_4$ for 10 ml of AH-Sepharose (packed volume). Amount of guanosine coupled was estimated by UV-absorption after solubilizing the gel in 1% $NaBH_4$ in 1 N NaOH at 70°C for 4–6 h and found as 4.5 μmol of guanosine/ml of AH-Sepharose.

3. RESULTS

Guanosine antibodies were elicited in rabbits using BSA-conjugate of guanosine, prepared by the periodate method as the immunogen. The response of the rabbits to the immunogen was first checked by the formation of precipitin bands against BSA-G in Ouchterlony double diffusion test. Amount of antibodies detected by quantitative precipitation of the antisera with RSA-G was between 0.2 and 0.54 mg/ml of antisera from different bleeds of three rabbits used. The amount of antibodies that bind to [^{3}H]G$^{ox\text{-}red}$ was between 0.004 and 0.375 mg/ml of antisera. Binding of [^{3}H]G$^{ox\text{-}red}$ to the antiserum from one of the rabbits which had the highest titer is shown in fig.1a. The average association constant was 1.78×10^7 M^{-1} (fig.1b). All the further studies were done with this sample. Fig.2 shows the inhibition of binding to [^{3}H]G$^{ox\text{-}red}$ by non-radioactive compounds. The molar and relative concentrations required for 50% inhibition, computed from the data are given in table 1. G$^{ox\text{-}red}$ is the best inhibitor showing that these antibodies are specific for the sugar modified form of guanosine. Guanosine is needed 7900 times the concentration of G$^{ox\text{-}red}$. Inhibition data for I$^{ox\text{-}red}$, A$^{ox\text{-}red}$, U$^{ox\text{-}red}$ and C$^{ox\text{-}red}$ show the high base-specificity of the antibodies. pG$^{ox\text{-}red}$ is a very inefficient inhibitor. It is not clear why an extra phosphate makes such a big difference in inhibition.

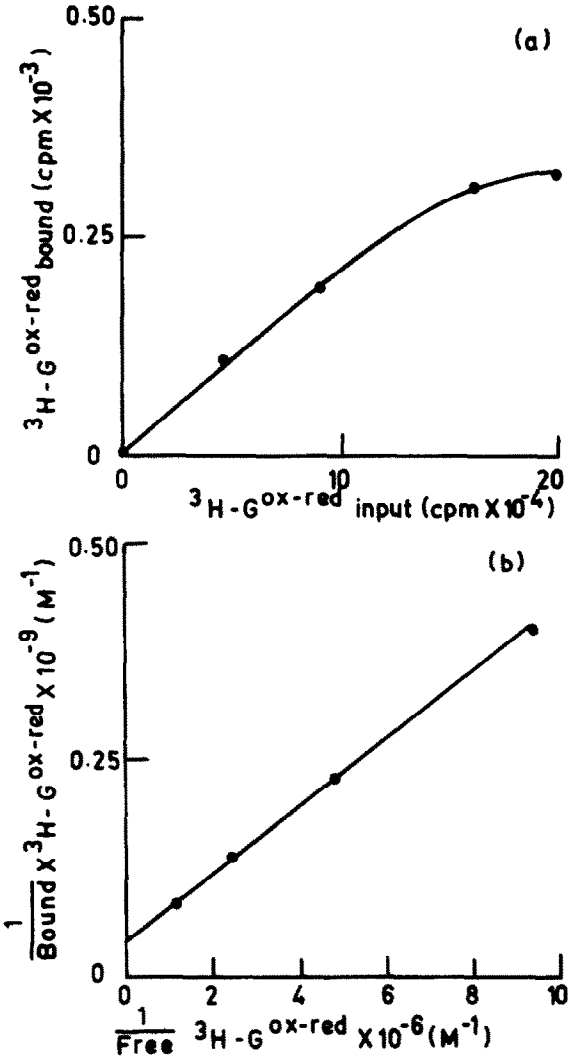

Fig. 1. Binding to [^{3}H]G$^{ox\text{-}red}$. (a) Reaction mixtures containing 200 μg of anti-G γ-G and varying amounts of [^{3}H]G$^{ox\text{-}red}$ in 0.3 ml TBS (0.01 M Tris–HCl, pH 7.5, 0.15 M NaCl and 0.02% NaN_3) were incubated at 0°C for 10 min and passed through nitrocellulose filters. Filters were washed with 10 ml TBS, dried and counted for radioactivity in 0.5% 2,5-diphenyloxazole in toluene. Control experiments had normal rabbit γ-G instead of antibodies and the retention was between 60 and 150 cpm at different inputs of [^{3}H]G$^{ox\text{-}red}$. (b) Double reciprocal plot of the data in fig. 1a. K_a was calculated from it.

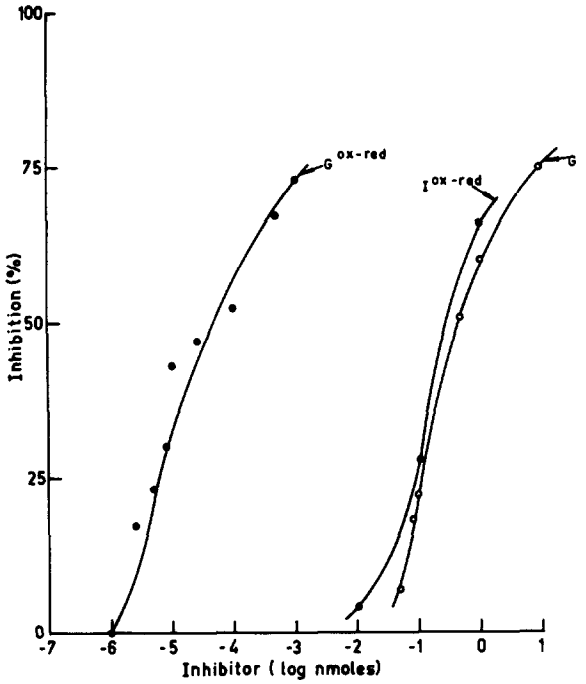

Fig. 2. Specificity of [^{3}H]G$^{ox\text{-}red}$ binding to anti-G τ-globulins. Reaction mixtures containing 25 μg of anti-BSA-G τ-G and [^{3}H]G$^{ox\text{-}red}$ (18 000 cpm) with or without inhibitors in 0.3 ml TBS. Experiment as described in fig.1. Net binding without inhibitor: 2800 cpm. Retention with normal τ-G: 66 cpm.

Table 1

Specificity of [^{3}H]G$^{ox\text{-}red}$ binding to BSA-G τ-globulins

Inhibitor	Conc. needed for 50% inhibition (M)	Relative conc. for 50% inhibition
G$^{ox\text{-}red}$	1.86×10^{-8}	1
G	1.489×10^{-6}	7 900
pG$^{ox\text{-}red}$	1.32×10^{-5}	70 000
dG	2.359×10^{-5}	126 000
I	2.64×10^{-5}	141 000
pG	1.05×10^{-4}	564 000

A$^{ox\text{-}red}$, U$^{ox\text{-}red}$ and C$^{ox\text{-}red}$ did not bring about 50% inhibition at a concentration of 0.33×10^{-6} M. A, U, C and T did not bring about 50% inhibition even at 3.3×10^{-3} M concentration

3.1. *Binding to DNA*

Guanosine antibodies did not retain a significant amount of [^{32}P]dsDNA (highest input of [^{32}P]dsDNA – 10 000 cpm for 400 μg of τ-G) on nitrocellulose filters. Fig.3 shows the binding of

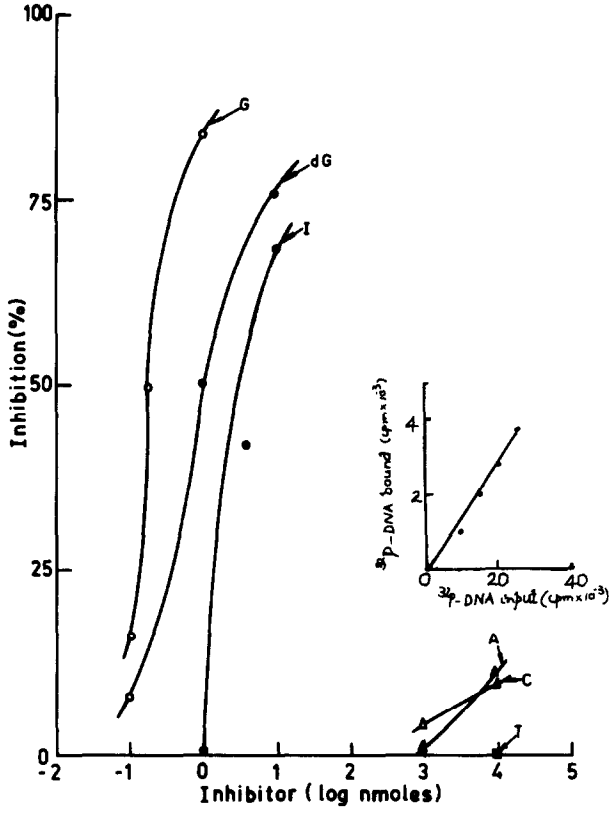

Fig. 3. Specificity of [^{32}P]ssDNA binding to BSA-G antibodies. Reaction mixtures contained 400 μg of anti-BSA-G τ-G and 26 000 cpm of [^{32}P]DNA in 0.3 ml TBS with or without inhibitors. Net binding without inhibitor: 4298 cpm. Retention with normal rabbit τ-G: 1068 cpm. Inset: binding to ^{32}P-ssDNA. Reaction mixtures contained 400 μg of anti-BSA-G τ-G and varying inputs of [^{32}P]ssDNA in 0.3 ml TBS. Experiments as in fig.1.

guanosine antibodies to [^{32}P]ssDNA and the specificity of the binding. Guanosine is the best inhibitor, followed by deoxyguanosine and inosine. A, C and T are vey poor inhibitors, showing that the antibodies bind specifically at G residues.

3.2. *Binding to RNA*

Guanosine antibodies bind to [^{3}H]RNA. The binding follows the typical hyperbolic pattern. Specificities of the binding are shown in fig.4. Guanosine is the best inhibitor, followed by dG, pG, and I. G$^{ox\text{-}red}$ is a poorer inhibitor than

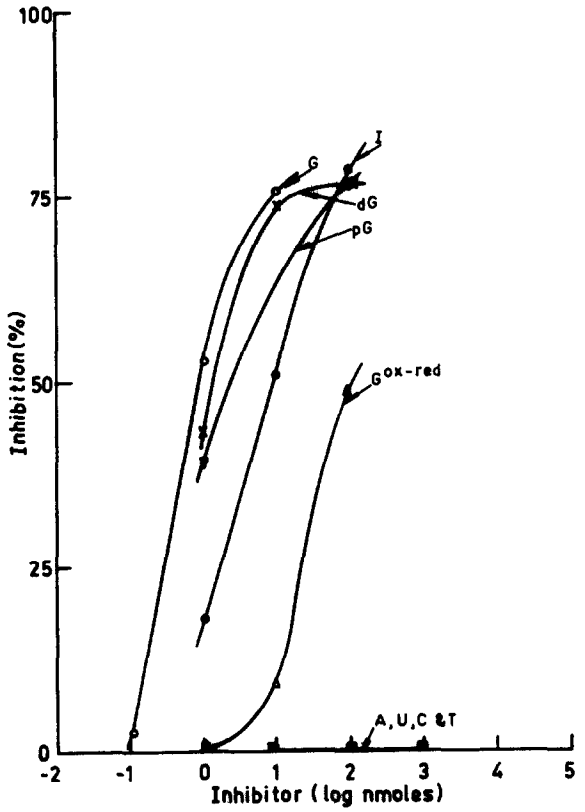

Fig. 4. Specificity of [^{3}H]RNA binding to BSA-G antibodies. Reaction mixtures contained 20 μg of anti-BSA-G and 14000 cpm of [^{3}H]RNA with or without inhibitors in 0.3 ml TBS. Net binding without inhibitor: 2883 cpm. Retention with normal τ-G: 225 cpm. Experiment as in fig.1.

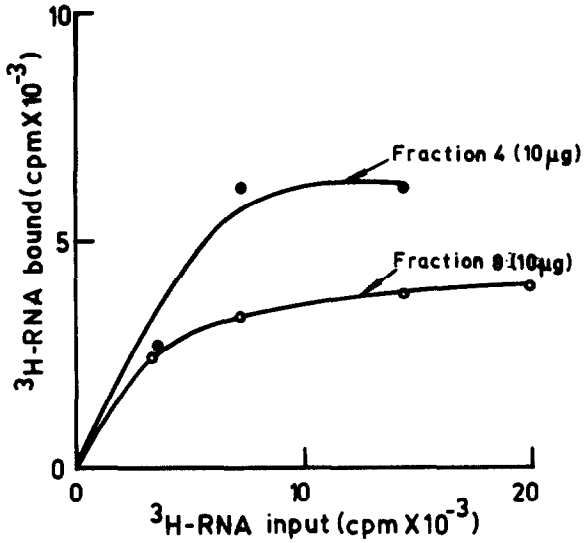

Table 2

Fractionation of anti-BSA-G τ-G on AH-Sepharose-G column

Sample	Fraction	Volume (ml)	Protein (mg)	[^{3}H]G^{ox-red} bound (cpm/μg)
Anti-BSA-G τ-G		23.5	190	82
Unbound	1	23	179.4	Nil
G^{ox-red} (2%) elution:				
	2	2	0.78	260
	3	2	1.02	5882
	4	2	0.63	9523
	5	2	0.43	2857
	6	2	0.24	1750
Acetic acid (1 M) elution:				
	7	4	1.16	Nil
	8	4	0.96	Nil
	9	4	0.04	Nil

Column vol. 6 ml; flow rate: 6 ml/h. TBS with 1 M NaCl was used throughout the operations. Anti-BSA-G τ-G were loaded on the affinity column and thoroughly washed. The bound antibodies were eluted with 2% G^{ox-red} and after washing, with 1 M AcOH. G^{ox-red} eluted fractions were dialyzed against TBS (thrice), 5% pyridine in TBS (twice) and TBS (thrice). Unbound and acetic acid eluted fractions were dialyzed against TBS. All fractions were estimated for protein and tested for binding to [^{3}H]G^{ox-red}. Assay as in fig. 1

guanosine. This shows that these populations of antibodies are different from those picked up by the assay for [^{3}H]G^{ox-red} binding. A, U, C and T do not compete for the binding at the highest concentrations tested, showing that these antibodies bind to RNA specifically at guanosine residues.

3.3. *Fractionation*

Anti-G τ-globulins were passed through

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Fig. 5. Binding of [^{3}H]RNA to purified BSA-G antibodies. Assay mixtures contained 10 μg of fraction 4 (table 2), or 30 μg of fraction 8 (table 2) and different amount of [^{3}H]RNA in 0.3 ml TBS. Experiment as in fig.1. Retention with normal rabbit τ-G with different inputs of [^{3}H]RNA: 150–250 cpm.

Table 3

Specificity of [^{3}H]RNA binding to fractionated G-antibodies

Inhibitor	Relative conc. for 50% inhibition	
	Fraction 3 (table 2)	Fraction 8 (table 2)
G	1	1
dpG	0.17	3.09
pG	1.99	77.7
dG	1.41	97.89
I	-	251.5
$G^{ox\text{-}red}$	5.6	1953
DNA	12.5	24.8
RNA	22.38	28.18

A, U, C and T did not bring about any inhibition at the highest concentrations tested

AH-Sepharose-G column. The adsorbed antibodies were first eluted with 2% $G^{ox\text{-}red}$ and then with 1 M acetic acid. All fractions were tested for binding to [^{3}H]$G^{ox\text{-}red}$. Results are given in table 2. Fractions eluted by $G^{ox\text{-}red}$ bind to [^{3}H]$G^{ox\text{-}red}$, whereas fractions eluted by acetic acid do not bind to [^{3}H]$G^{ox\text{-}red}$. Both, the $G^{ox\text{-}red}$ and acetic acid eluted fractions bind to [^{3}H]RNA (fig.5). The specificities of RNA binding are given in table 3. All guanosine containing compounds (dpG, G, dG, pG and $G^{ox\text{-}red}$) are good inhibitors of binding, for fractions eluted by $G^{ox\text{-}red}$, whereas only G and dpG efficiently inhibit binding of the acetic acid eluate to RNA. $G^{ox\text{-}red}$ is the least efficient in inhibiting RNA binding among guanosine containing compounds, in both $G^{ox\text{-}red}$ and acetic acid eluted fractions. These results show that the two fractions of antibodies contain different populations that bind to RNA specifically at G residues.

4. DISCUSSION

BSA-G was used as the immunogen to elicit guanosine antibodies in rabbits. The specificities of the antibodies were analyzed by their binding to radioactive ligands ([^{3}H]$G^{ox\text{-}red}$, [^{3}H]RNA and [^{32}P]DNA), and inhibition of the binding by nonradioactive compounds using nitrocellulose filter assay. At the concentrations studied the assay establishes the specificities of antibodies with high affinity to the radioactive ligand used and these are the populations of interest to us. dsDNA is not retained on nitrocellulose filters and so have been used by many laboratories for studying the interaction of dsDNA with proteins. The nitrocellulose filters from Microdivices, Ambala, India could be used for studying the binding of antibodies to ssDNA also, since they did not retain significant amount of ssDNA. These filters could be used conveniently for studying antibody binding to RNA also since RNA retention was very low at pH 7.5. We have used 10 min incubation at 0°C for our studies on hapten and polynucleotide binding since we found approximately the same amount of binding up to 12 h incubation. Antibodies that bind to [^{3}H]$G^{ox\text{-}red}$ are specific to the sugar modified form of guanosine. Different populations of guanosine antibodies bind to RNA, as is evident from the results obtained from competition studies in [^{3}H]RNA binding, before and after fractionation. Authors in [19,20] could not detect the binding of guanosine antibodies (raised against BSA-G) to RNA by double antibody technique, even though they had taken care to eliminate nuclease activity. However, using the same technique they could show the binding of antibodies to ssDNA and the binding was inhibited maximum by guanosine, as shown in the present studies. The studies reported here show that by using BSA-G as the immunogen it is possible to elicit in rabbits, antibodies to guanosine which bind to ssDNA and RNA specifically at guanosine residues.

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