Antibody–Nucleic Acid Complexes. Oligo(dG)$_n$ and -(dT)$_n$ Specificities Associated with Anti-DNA Antibodies from Autoimmune MRL Mice†

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Received May 19, 1989; Revised Manuscript Received August 4, 1989

ABSTRACT: The specificity of anti-DNA antibodies in the sera of unimmunized autoimmune MRL mice was initially assessed via an enzyme-linked immunosorbent assay (ELISA). Antibody binding profiles to a panel of immobilized antigens (AMP–, GMP–, CMP–, UMP–, and TMP–BSA, ss- and dsDNA) demonstrated high levels of immunoglobulins reacting with GMP and ssDNA and intermediate levels with AMP, TMP, and dsDNA. Fractionation of serum anti-DNA antibodies into subsets on the basis of their binding to GMP– and TMP–agarose indicated that the resulting GMP- or TMP-reactive antibodies bound to their homologous nucleotides and ssDNA. Competition–inhibition studies with soluble mono-, oligo-, and polynucleotides revealed that GMP- and TMP-reactive antibodies were highly specific for oligo(dG)$_n$ and -(dT)$_n$ sequences, respectively. Whereas the relative affinity of TMP-reactive autoantibodies to oligo(dT)$_n$ increased with oligonucleotide length (n = 2, 4, 6, 8, 10, 15), GMP-reactive antibodies preferentially recognized oligo(dG)$_{10}$ ($K_a \simeq 1 \times 10^7$ M^{-1}). While neither antibody recognized oligo(dA)$_8$ and -(dC)$_8$ competitors, mixed-base oligonucleotides were inhibitory at concentrations approximately 10-fold greater than similarly sized oligo(dG)$_n$ and -(dT)$_n$ sequences. Similar characterizations of both pooled and individual MRL sera indicated that anti-DNA antibodies represent 8–10% of the total serum IgG. More importantly, GMP-reactive autoantibodies predominated and accounted for 60–70% of the entire unbound anti-DNA antibody population.

The spontaneous appearance of autoantibodies with specificities directed toward nucleic acids and other nuclear antigens is a major diagnostic feature of systemic lupus erythematosus (SLE).[1] Investigations as to the structure, specificity, and pathogenicity of these immunoglobulins are the subject of reviews (Tan, 1982; Schwartz & Stollar, 1985; Schattner, 1987). While there is agreement that SLE reflects abnormalities within the immune system, neither the origin of these autoantibodies nor the etiology of the disease is known. Numerous observations have implicated both anti-DNA antibodies and DNA antigens in the pathology of SLE. Significant findings include their localization (deposition) within damaged tissues and/or organs, e.g., kidneys (Koffler, 1974), and a direct correlation between circulating levels of anti-DNA antibodies and disease activity (Tan, 1982).

The sequence and conformational complexities of nucleic acid antigens imply the presence of an exceedingly large number of potential determinants (epitopes). Likely epitopes include bases, nucleosides (-tides), oligonucleotides, repeating units of (deoxy)ribose phosphate, and various conformations induced by nucleotide sequences (A-, B-, Z-DNA) and/or related proteins (RNP particles). Specificity studies with both mono- and polyclonal anti-DNA antibodies suggest that many of the above epitopes participate in these binding reactions (Eilat, 1986; Stollar, 1986). However, whether one or several of these antigenic structures predominate has never been established with certainty. This uncertainty can in part be attributed to the fact that serum anti-DNA antibodies are heterogeneous and presumably polyspecific (Lafer et al., 1981; Eilat, 1986). The specificity of monoclonal preparations is also suspect inasmuch as they may not be representative of the polyclonal population from which they were selected.

In an attempt to resolve this dilemma, we began to devise strategies whereby the polyspecific nature of serum autoanti-DNA antibodies could be fractionated into monospecific subsets. One approach involves an ELISA which measures antibody binding not only to immobilized ss- and dsDNA but to a panel of nucleoside (-tide)-protein conjugates as well (Weisbart et al., 1983; Munns et al., 1984a). By use of this ELISA system it was previously determined that the distribution of nucleotide-reactive anti-DNA antibodies in SLE sera (both human and murine) was not random but highly ordered, i.e., anti-GMP > TMP ≥ AMP > UMP > CMP (Munns et al., 1984a, 1987). More recently, purified GMP-reactive autoantibodies from human SLE sera demonstrated a preferential recognition for GMP-enriched oligonucleotide sequences (Munns et al., 1987). Because of these findings, an extensive analysis of anti-DNA antibody populations in murine SLE sera was initiated. Here we document that the majority of anti-DNA antibodies (>60%) in the autoimmune MRL mice are GMP-reactive and can be isolated free of other nucleotide-reactive antibodies by adsorption to GMP–agarose. Subsequent characterization of these purified antibodies revealed both size- and sequence-defined oligonucleotide specificities with affinities approaching 1×10^7 M^{-1} for oligo(dG)$_n$ sequences. A second minor population of anti-DNA antibodies reactive with TMP was similarly characterized and appears to be highly specific for oligo(dT)$_n$ sequences.

Materials and Methods

Materials. Nucleosides, nucleotides, and immunochemical reagents associated with ELISA testing were obtained from Sigma. Nucleic acids representative of single-stranded (ss) and double-stranded (ds) DNA (genomes of bacteriophages

† This research was supported by Grant GM 39217 awarded by the National Institutes of Health and by the Lottie Caroline Hardy Trust for Arthritis Research.

[1] Abbreviations: SLE, systemic lupus erythematosus; ds- and ssDNA, double- and single-stranded DNA; ELISA, enzyme-linked immunosorbent assay; RID, radial immunodiffusion; PBS, phosphate-buffered saline; AMP–, GMP–, CMP–, UMP–, and TMP–BSA, 5′-nucleotides covalently linked to bovine serum albumin; PBS–TB, PBS containing 0.05% Tween 20 and 2 mg/mL BSA; oligo(dN)$_n$, oligonucleotides containing a defined number (n) of nucleotides (N) yet undefined with respect to sequence.

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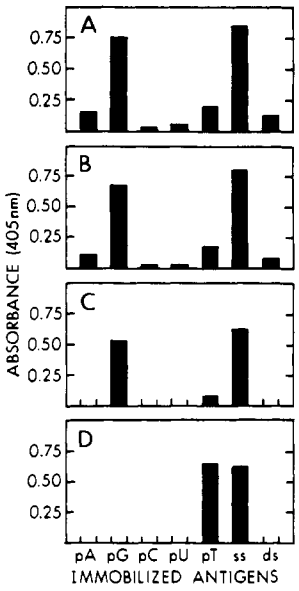

FIGURE 1: ELISA analysis of MRL autoantibodies present in unfractionated sera (panel A) and those retained by DNA–agarose (panel B), GMP–agarose (panel C), and TMP–agarose (panel D). Immobilized antigens include the 5′-phosphates of A, G, C, U, and T (i.e., pA, pG, pC, pU, and pT) and ss- and dsDNA. Details regarding ELISA and purification schemes are presented under Materials and Methods. On the basis of ELISA analyses, recoveries of antibody activities after DNA–, GMP–, and TMP–agarose adsorption were 92, 80, and 58%, respectively. These data in conjunction with radial immunodiffusion were used to determine antibody concentration in MRL sera (see Table II).

fd and λ, respectively) were from Miles, and DNA– and GMP–agarose were from Bethesda Research Laboratories and Sigma, respectively. The 5′-phosphates of adenosine (AMP), guanosine (GMP), cytidine (CMP), uridine (UMP) and ribothymidine (TMP) were coupled to BSA via periodate oxidation (Stollar, 1980), and only those conjugates possessing 20–24 haptens/BSA molecule were utilized as immobilized antigens (see Figure 1). Oligo- and polynucleotides used in competition–inhibition assays were obtained from Pharmacia and/or synthesized as described below. Concentrations of each competitor were determined by spectral analysis and the extinction coefficients provided by the manufacturer.

Mixed-Base Oligonucleotides. Size-defined, mixed-base oligonucleotides [oligo(dN)$_n$, where n = 6, 10, and 15] were synthesized by using an Applied Biosystems DNA synthesizer (Model 380B). For example, oligo(dN)$_{10}$ was prepared with four pore glass columns each containing a single 3′-deoxynucleoside present in DNA. Thereafter, equimolar quantities of nucleoside phosphoramidites (dA, dG, dC, and T) were added to each of nine cycles to produce oligo-d(N_9A), -d(N_9G), -d(N_9C), and -d(N_9T). Aliquots of these 10-mers were subsequently mixed to produce a mixed-base oligo(dN)$_{10}$. Oligo(dN)$_6$ and -(dN)$_{15}$ were similarly prepared by using the appropriate number of cycles. Oligonucleotide lengths were confirmed by gel electrophoresis using oligo(dT)$_n$ (n = 6, 10, and 12–18; Pharmacia) as standards. Oligo(dG)$_{10}$ was also prepared as above. An additional cycle with Aminolink 2 (Applied Biosystems, Bulletin 49) was included. This reagent covalently links an aliphatic primary amine (aminohexyl group) at the 5′-end of oligonucleotides (Chollet & Kawashima, 1985).

Laboratory Animals and Sera Collection. Multiple MRL/lpr breeding pairs were obtained from Jackson Laboratory (Bar Harbor, ME) and bred at the animal facilities at Washington University. F1 generations were screened at monthly intervals for the spontaneous appearance of serum antinucleotide and anti-DNA antibodies as evaluated via ELISA testing (see below). Blood samples were obtained from the retroorbital venous plexus, and the resulting sera were heat inactivated (56 °C, 30 min) and stored at −20 °C.

ELISA. A detailed description of ELISA as it relates to measurements of anti-DNA antibodies has been presented elsewhere (Munns et al., 1984b, 1987). Absorbance measurements (A_{405nm}) were determined directly from microtiter wells with a Dynatech MR 580 spectrophotometer. ELISA analysis of serially diluted serum revealed that the quantity of antibody bound to immobilized antigens was directly proportional to absorbance when the latter did not exceed 0.60 A_{405} unit (see Table I). When feasible, each serum or antibody preparation subjected to ELISA was diluted sufficiently to yield absorbance values within this linear range. The immobilization of nucleotide–BSA conjugates to microtiter wells has been described (Munns et al., 1984b). For immobilization of polynucleotide antigens, wells were initially coated with poly(L-lysine) (10 μg/mL in H_2O, overnite at 4 °C) prior to washing in phosphate-buffered saline (PBS, 10 mM PO_4 and 150 mM NaCl, pH 7.3) and incubation of antigen (10 μg/mL in PBS; 2 h; 24 °C). Microtiter wells were subsequently washed in PBS–TB (PBS containing 0.05% Tween 20 and 2 mg/mL BSA) prior to incubation with antisera or various immunoglobulin preparations.

Isolation of Specific Antibody Populations with DNA– and Nucleotide–Agarose Adsorbents. For this isolation of DNA-reactive autoantibodies, 2.0 mL of pooled MRL sera (diluted to 10 mL in PBS–TB) was incubated with DNA–agarose (2.0 mL bed volume) overnite at 4 °C. The adsorbent was washed by repeated centrifugation–resuspension (3×) with 10-mL aliquots of PBS–TB. Retained antibodies were subsequently eluted with four aliquots (2.5 mL each) of acidified BSA (2 mg/mL BSA, 0.05% Tween 20, pH 2.8–3.0), neutralized, and diluted in PBS–TB for ELISA assessments and IgG concentration (see below). Aliquots of affinity-purified anti-DNA antibodies were further fractionated by their successive adsorption to GMP– and to TMP–agarose (i.e., antibodies not retained by GMP–agarose were reincubated in the presence of TMP–agarose). Antibodies retained by each of these adsorbents were processed (elution, ELISA, and IgG concentration determinations) in a manner identical with that used for purified anti-DNA antibodies. Whereas DNA– and GMP–agarose were obtained from commercial sources (see above), TMP–agarose was prepared in our laboratory via a carbodiimide condensation reaction between the 5′-phosphate of TMP and the primary amino groups of aminohexyl-Sepharose (Stollar, 1980).

Radial Immunodiffusion (RID). RID kits available from Miles/ICN were used to assess IgG levels in MRL sera and in preparations of affinity-purified anti-TMP and anti-GMP antibodies. The procedures employed were those provided by the manufacturer. Repeated analysis with standard solutions of mouse IgG indicated that these assays were reproducible inasmuch as the variance was less than 10% of the calculated mean value.

Competition–Inhibition ELISA. For assessment of antibody specificity a series of competition–inhibition assays were conducted by utilizing a modified ELISA. These assays consisted of preincubating various soluble competitors (nucleotides and oligo- and polynucleotides) with affinity-purified antibody preparations in PBS–TB buffer (30 min, 24 °C) prior to ELISA testing. Inhibition of antibody binding by various competitors was determined by measuring their ability to reduce the resulting absorbance (A_{405}) relative to control assays

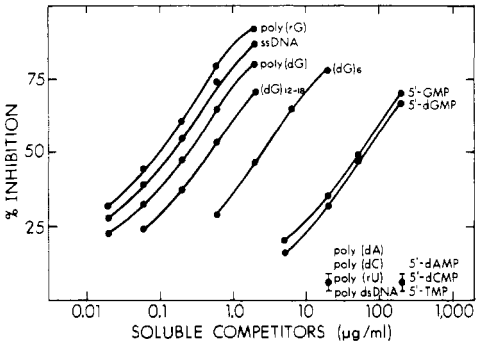

FIGURE 2: Competition–inhibition assays employing guanine-containing mono-, oligo-, and polynucleotides as competitors to inhibit purified anti-GMP antibody binding to immobilized ssDNA. Control assays without competitors yielded an absorbance value of 0.70 ± 0.05 (A_{405nm}). By plotting percent inhibition versus competitor concentration, the quantity of each competitor required for 50% inhibition was determined via extrapolation (see Tables III–V). Each competitor was tested in triplicate at three to six different concentrations. Deviation from mean values (listed above) were less than 12%.

lacking competitor, e.g., percent inhibition = $100[A_{405}$ (competitor)$/A_{405}$ (control)]. Each competitor was tested in triplicate at three or four different concentrations. By plotting percent inhibition (linear scale) versus competitor concentrations (log scale), the quantity of each competitor required to achieve a 50% inhibition in antibody binding toward immobilized ssDNA was determined (see Figure 2, Tables III and IV). The presence of Tween and BSA in the PBS–TB buffer precludes the binding (immobilization) of competitors to the microtiter well.

Radioimmunoassays. Oligo$(dG)_{10}$ was synthesized according to Applied Biosystems technology (see above) with an aminohexyl group covalently linked to the 5′-terminus (Chollet & Kawashima, 1985). Reductive methylation of the amino group with $[^3H]NaBH_4$ resulted in a labeled product with a specific activity of 20000 cpm/μg (Tack et al., 1980). $[^3H]$Oligo$(dG)_{10}$ was separated from unincorporated radioactivity by gel filtration using Sepharose G-25. The radioimmunoassay consisted of incubating either MRL sera (1.0 μL) or purified GMP-reactive antibody with increasing quantities of [^{3}H]hapten for 45 min at 24 °C (total volume of 0.5 mL in PBS–TB). The reaction was continued for an additional 30 min in the presence of anti-mouse IgG–agarose (50 μL bed volume). At the conclusion of these incubation periods free and antibody-bound [^{3}H]hapten were separated by repeated centrifugation–resuspension (3×) of the agarose adsorbent. The washed adsorbent was solubilized in NCS (Amersham Searle) and processed for determination of the quantity of [^{3}H]hapten bound to GMP-reactive autoantibodies. Various control assays (e.g., without antibody or with anti-rabbit IgG–agarose) were included to assess nonspecific binding (negligible, <0.1%).

Results

Nucleotide-Reactive and Nucleic Acid Reactive Autoantibodies in MRL Sera. The usefulness of ELISA in characterizing spontaneously occurring anti-DNA antibodies is illustrated in Table I. In contrast to the lack of significant quantities of nucleotide-reactive and nucleic acid reactive autoantibodies in normal mice sera (Balb/c), relatively high titers of these antibodies are present in autoimmune MRL mice, especially those reactive with GMP–BSA and ssDNA antigens. Numerous findings of interest appear in Table I. For example, MRL sera had to be diluted greater than 100-fold more than Balb/c sera to detect comparable levels of both

Table I: Serum Autoantibody Binding to Nucleotide–BSA and DNA Antigens in ELISA[a]

sera (sex) and dilutions	absorbance (405 nm)						
	nucleotide–BSA					DNA	
	AMP	GMP	CMP	UMP	TMP	ss	ds
MRL (male)							
1:6000	0.08	0.38	0.00	0.02	0.11	0.54	0.12
1:3000	0.16	0.82	0.02	0.06	0.25	0.85	0.22
1:1500	0.25	1.40	0.04	0.10	0.45	1.50	0.38
MRL (female)							
1:6000	0.10	0.56	0.00	0.05	0.17	0.62	0.14
1:3000	0.18	1.05	0.04	0.11	0.30	1.11	0.28
1:1500	0.38	>1.50	0.07	0.21	0.62	>1.50	0.61
Balb/c (female)							
1:50	0.09	0.34	0.02	0.04	0.08	0.04	0.03
1:25	0.16	0.50	0.00	0.09	0.15	0.10	0.05

[a] Pooled sera from six mice (20–24 week old) were diluted as indicated, and 100 μL was used in ELISA to assess autoantibody binding to designated antigens. Absorbance values represent the mean of three experiments with variation of any individual value less than 10% of the calculated mean (for values greater than 0.1 A_{405} unit). See Materials and Methods for additional details.

nucleotide- and DNA-reactive autoantibodies. However, in contrast to the large differences in their titers, the relative distribution of antibodies binding to a panel of nucleotide haptens is remarkably similar for both species, i.e., GMP > TMP ≥ AMP > UMP > CMP. Also to be noted are the elevated levels of antibodies (20–25%) in the sera of female MRL mice relative to their male counterparts. Finally, the levels of antibodies binding to GMP–BSA approximate those binding to ssDNA antigen but not dsDNA.

Fractionation of Anti-DNA Antibodies. On the basis of their reactivity toward specific nucleotide haptens, we attempted to isolate subsets of these autoantibodies utilizing DNA– and nucleotide–agarose adsorbents. Antibodies retained by these adsorbents were eluted and subjected to ELISA (specificity) and RID (IgG content) analyses. The ELISA results are presented in Figure 1 and illustrate the pattern of antibody binding associated with unfractionated sera (panel A) as well as those retained by DNA– (panel B), GMP– (panel C), and TMP–agarose (panel D). The almost identical binding patterns exhibited by unfractionated sera and the DNA–agarose bound fraction (panels A and B) imply that the bulk of autoantibodies reactive with DNA cross-react to a large extent with individual nucleotide haptens. This finding is further supported by the results presented in panels C and D. In these instances antibodies retained by GMP– and TMP–agarose reacted with their homologous hapten–BSA conjugates and ssDNA but minimally or not at all with other nucleotides or with dsDNA. These data suggest that both nucleotide-reactive antibodies are monospecific and recognize ssDNA by virtue of this antigen's content of GMP and TMP residues. Lastly, electrophoretic characterization of these affinity-purified preparations revealed the presence of two major peptides corresponding to the heavy (50000) and light (25000) chains of IgG (data not shown).

MRL Serum Levels of Anti-DNA Antibodies. Unfractionated sera as well as those antibody populations retained by specific agarose adsorbents were subjected to radial immunodiffusion. Such analyses permit an approximation of levels of various circulating antibodies in MRL sera. Our findings (Table II) reveal that of the total serum IgG approximately 10% binds to DNA agarose. Further, about two-thirds of this anti-DNA population binds to GMP–agarose, i.e., GMP specific. Similar analysis of TMP-reactive antibodies indicated that their concentration was considerably less and more variable (Table II), with higher levels being

Table II: Serum Concentration of DNA- and Nucleotide-Reactive Autoantibodies (IgG) from MRL Mice[a]

preparations	MRL pooled sera (mg/mL IgG) male (% unfractionated)	female (% unfractionated)
unfractionated sera	22.4 (100)	25.7 (100)
DNA–agarose	1.9 (8.5)	2.4 (9.3)
GMP–agarose	1.3 (5.8)	1.4 (5.4)
TMP–agarose	<0.2	0.4 (1.6)

[a] Pooled sera from six mice were subjected to affinity chromatography procedures for isolation of anti-DNA, -GMP, and -TMP antibodies. These preparations together with unfractionated sera were subjected to radial immunodiffusion analysis for IgG content.

Table III: Inhibition of Binding of GMP- and TMP-Reactive Autoantibodies to Immobilized ssDNA by Soluble Nucleotides (-sides)[a]

nucleotides (-sides)	competitor concentration at 50% inhibition: anti-GMP (μg/mL) (mM)	anti-TMP (μg/mL) (mM)
5′-AMP	>2000	>2000
5′-GMP	58 (0.16)	>2000
5′-CMP	>2000	>2000
5′-UMP	>2000	>2000
A	>2000	>2000
G	85 (0.30)	>2000
C	>2000	>2000
U	>2000	>2000
5′-dAMP	>2000	>2000
5′-dGMP	65 (0.19)	>2000
5′-dCMP	>2000	>2000
5′-TMP	>2000	68 (0.21)
dA	>2000	>2000
dG	82 (0.31)	>2000
dC	>2000	>2000
T	>2000	82 (0.34)

[a] Refer to the legend of Figure 2 for details.

associated with female mice (also see Table I). Collectively, these results and those presented in Table I and Figure 1 reveal that the majority of anti-DNA antibodies in MRL sera preferentially recognize GMP.

Specificities Associated with GMP- and TMP-Reactive Autoantibodies via Competition–Inhibition ELISA. To obtain additional information regarding autoantibody specificity, we adopted a competition–inhibition assay that affords the opportunity to screen a variety of potential antigens. The results of an abbreviated study using mono-, oligo-, and polynucleotide competitors with anti-GMP antibody are presented in Figure 2. These data demonstrate the effectiveness of all GMP-containing competitors (except dsDNA) to inhibit GMP-reactive antibody binding to ssDNA. Further, the degree of competition observed between these GMP competitors appears to be dependent upon size (length), nucleotide composition, and, to some extent, their structure (i.e., ribose versus deoxyribose). Other competitors lacking GMP residues are not inhibitory even at concentrations 10–100-fold higher than their corresponding GMP-containing counterparts. These and other findings regarding antibody specificities are considered in detail below.

Mononucleotide (-side) Antigens. All nucleotides (-sides) present in nucleic acids were examined for their ability to inhibit GMP- and TMP-reactive autoantibodies. The data in Table III report the concentration of each competitor required to inhibit antibody binding by 50%. Of those nucleotides (-sides) listed, only those possessing a guanine and thymine base moiety significantly inhibited anti-GMP and -TMP antibody binding, respectively. While only minor differences were apparent between deoxy and ribonucleoside structures, nucleotides possessing a 5′-phosphate were 2-fold better competitors than their nucleoside counterparts (e.g., compare millimolar concentrations between 5′-GMP and G). Most significant was the lack of inhibition by nucleotides (-sides) containing adenine, cytosine, and uracil moieties. These observations provide important evidence that the base component of GMP and TMP represents the major antigenic structure. Even though the negative charge imparted by the PO_4 moiety increases the apparent affinity by about 2-fold (see above), the presence of ribose and/or PO_4 moieties within other nucleotides (AMP, dAMP, CMP, dCMP, and UMP) did not result in anti-GMP or -TMP antibody recognition of these molecules.

Table IV: Inhibition of Binding of GMP- and TMP-Reactive Autoantibodies to Immobilized ssDNA by Soluble Oligonucleotides[a]

oligonucleotides	competitor concentration at 50% inhibition: anti-GMP (μg/mL) (μM)	anti-TMP (μg/mL) (μM)
$(dN)_2$	65 (99.4)	62 (94.8)
$(dN)_4$	25 (19.1)	26 (19.9)
$(dN)_6$	12 (6.1)	10 (5.1)
$(dN)_{10}$	9.1 (2.8)	4.1 (1.3)
$(dN)_{15}$	3.9 (1.3)	2.0 (0.7)
$(dG)_2$	22 (30.3)	>50
$(dG)_4$	6.0 (4.13)	>50
$(dG)_6$	2.3 (1.06)	>50
$(dG)_8$	0.25 (0.09)	>50
$(dG)_{10}$	0.25 (0.07)	>50
$(dG)_{12-18}$	0.52 (0.10)	>50
$(dT)_2$	>50	18 (28.0)
$(dT)_4$	>50	7.0 (5.43)
$(dT)_6$	>50	2.8 (1.45)
$(dT)_8$	>50	1.3 (0.50)
$(dT)_{10}$	>50	0.70 (0.22)
$(dT)_{12-18}$	>50	0.21 (0.04)
$(dA)_8$	>50	>50
$(dC)_8$	>50	>50

[a] Refer to the legend of Figure 2 for details.

Oligonucleotide Antigens. These studies initially focused upon the use of *length-defined* but *sequence-undefined* oligonucleotides that presumably contain equimolar quantities of dA, dG, dC, and T. While each oligo$(dN)_n$ competitor inhibited anti-GMP and -TMP antibodies by approximately equal extents, their concentration required for 50% inhibition was markedly reduced with increasing oligonucleotide length. Thus, as the data in Table IV report, about 20- and 100-fold higher molar concentrations of $(dN)_2$ were required to achieve the same degree of antibody inhibition as that observed for $(dN)_6$ and $(dN)_{15}$, respectively.

These results together with the assumed base specificity of each antibody preparation prompted us to examine other sets of oligonucleotides comprised entirely of GMP and TMP residues. While TMP-containing oligonucleotides were unable to inhibit GMP-reactive antibodies, they inhibited TMP-reactive antibodies in a manner dependent upon oligonucleotide length. Further, their molar concentration required for 50% inhibition was significantly reduced when compared with the corresponding $(dN)_n$ oligonucleotides. Results with the oligo$(dG)_n$ series were similar inasmuch as they failed to inhibit TMP-reactive antibodies. Of interest was the finding that the most potent inhibitor within the oligo$(dG)_n$ series was $(dG)_{10}$. Further increases (12–18-mers) or decreases (2–8-mers) in

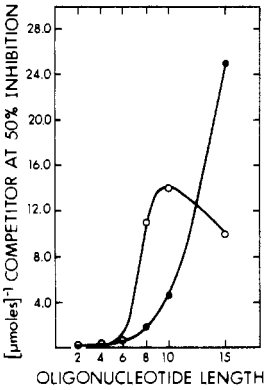

FIGURE 3: Relative affinities of anti-GMP and -TMP antibodies for oligo(dG)$_n$ and oligo(dT)$_n$ sequences, respectively. Relative affinity represents the reciprocal of the concentration (micromolar) of each oligomer required to inhibit antibody binding by 50% (see Table IV for micromolar concentrations of each oligonucleotide required for 50% inhibition). Open circles represent the relative K_a of anti-GMP antibody for the above designated oligo(dG)$_n$ sequences. Solid circles represent the relative K_a of anti-TMP antibody for oligo(dT)$_n$ sequences.

Table V: Inhibition of Binding of GMP- and TMP-Reactive Autoantibodies to Immobilized ssDNA by Soluble Polynucleotides[a]

	competitor concentration at 50% inhibition	
polynucleotides	anti-GMP (μg/mL)	anti-TMP (μg/mL)
poly(dG)	0.25	>50.0
poly(dI)	0.31	>50.0
poly(dT)	2.50	0.05
poly(dC)	>50.0	>50.0
poly(dA)	>50.0	>50.0
poly[d(CG)]	0.25	>50.0
poly[d(AT)]	25.0	2.4
poly[d(AC)]	>50.0	>50.0
ssDNA	0.35	2.0
dsDNA	>50.0	>50.0
poly(rG)	0.30	>50.0
poly(rA)	>50.0	>50.0
poly(rC)	>50.0	>50.0
poly(rU)	>50.0	>50.0
mRNA	1.0	>50.0

[a] Refer to the legend of Figure 2 for details.

oligonucleotide length resulted in a moderate and progressive reduction in the inhibition of the antibody binding reaction. Also noteworthy was the additional finding that oligo(dA)$_8$ and -(dC)$_8$ were not effective competitors in these competition–inhibition assays, i.e., a result comparable with that achieved with mononucleotides (Table III). Figure 3 summarizes the data in Table IV by plotting the *relative* affinities each antibody has for their respective oligo(dG)$_n$ and -(dT)$_n$ sequences as a function of oligonucleotide length.

Polynucleotide Competitors. A series of synthetic homo- and copolymers as well as naturally occurring polynucleotides were similarly assessed (Table V). In regard to TMP-reactive antibodies, only those competitors containing a thymine moiety inhibited the antibody binding reaction. Of interest was the finding that as little as 0.05 μg/mL of poly(dT) was required for 50% inhibition, a concentration 40-fold less than that required for either ssDNA or poly[d(AT)]. This latter result implies the importance of consecutive thymine residues (tracts) within polynucleotides for maximal antibody recognition.

The inhibition patterns associated with GMP-reactive autoantibodies were more complex. While all guanine-containing polynucleotides were inhibitory, extensive and moderate levels of inhibition were observed with poly(dI) and poly(dT), respectively. The cross-reactivity of poly(dI) is not surprising

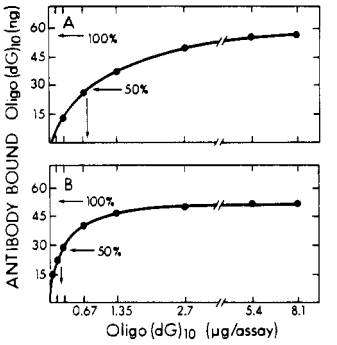

FIGURE 4: Binding of [^{3}H]oligo(dG)$_{10}$ to unfractionated (panel A) and affinity-purified (panel B) anti-GMP antibodies for assessment of antibody affinity (K_a). Arrows denote oligo(dG)$_{10}$ saturation of antibody combining sites (i.e., 100% binding) and the concentration of unbound hapten at 50% saturation. The reciprocal of the unbound hapten concentration is 2.5 × 10^6 M^{-1} (panel A) and 1 × 10^7 M^{-1} (panel B), i.e., the affinity constants for unfractionated and purified anti-GMP antibody, respectively. Oligo(dG)$_{10}$ concentrations were determined from its specific activity, i.e., 20 000 cpm/μg. These and other details regarding this binding assay are described under Materials and Methods.

when the structural features of inosine and guanosine are compared (i.e., the former lacking only the 2-NH$_2$ group of the latter). Also, the cross-reactivity associated with poly(dT) antigen has been noted by others (Zouali & Stollar, 1986). This observation is consistent with our findings that GMP-reactive antibodies bind to a limited extent with TMP–BSA in ELISA (Figure 1D). Other minor discrepancies in inhibition patterns observed between poly(dG), ssDNA, poly(rG), and mRNA reflect in all likelihood variable degrees of base pairing, conformations assumed in solution, and polynucleotide size. Finally, neither affinity-purified antibody was inhibited by dsDNA, a result previously noted during ELISA testing (Figure 1C,D).

Affinity Constants (K_a) Associated with GMP-Reactive Autoantibodies and Oligo(dG)$_{10}$ Hapten. Noting that anti-GMP antibodies represent the largest concentration of nucleotide-reactive antibodies in MRL sera (Table II) and appear to be specific for oligo(dG)$_{10}$ (Table IV), a series of binding studies were conducted with [^{3}H]oligo(dG)$_{10}$ to assess antibody affinity. This information was obtained by incubating increasing quantities of [^{3}H]oligo(dG)$_{10}$ with a constant amount of antibody until all antibody combining sites were saturated with hapten. These results appear in Figure 4A and reveal that a maximum of 56 ng (15.0 pmol) of [^{3}H]hapten could be bound to anti-GMP antibody (in 1.0 μL of pooled MRL sera). Further extrapolation of these data revealed that serum GMP-reactive autoantibodies possess an approximate affinity constant (K_a) of 2.5 × 10^6 M^{-1} for oligo(dG)$_{10}$, i.e., the reciprocal of the *unbound* hapten concentration at 50% saturation. In contrast, the K_a for antibodies purified via GMP–agarose was estimated to be 1 × 10^7 M^{-1} (Figure 4B). This 4-fold increase in K_a between sera and purified antibodies reflects in all likelihood the preferential binding of high-affinity antibodies to GMP–agarose. Identical binding studies using six individual sera from MRL mice (20–24 week old) yielded K_a values of 1–7 × 10^6 M^{-1} (K_a for the corresponding purified preparations were 20–40% higher). Interestingly, the relative K_a of affinity-purified anti-GMP antibodies for oligo(dG)$_{10}$ (1.4 × 10^7 M^{-1}, see Figure 3) was in excellent agreement with that obtained by radioimmunoassay (1.0 × 10^7 M^{-1}).

DISCUSSION

Investigations designed to assess antibody specificity are important for numerous reasons. First, they permit diagnostic

tests to be developed to detect and monitor disease activity (Tan, 1982). Second, they provide insights into potential therapeutic approaches not discernible without such knowledge, e.g., the use of tolerogens for suppression of specific antibody activities (Borel et al., 1986). Third, monoclonal autoantibodies have become useful as biological reagents for isolating and characterizing nucleic acids and/or related proteins, e.g., RNP particles (Pettersson et al., 1984), tRNA synthetases (Bernstein et al., 1984), and Z-DNA (Stollar, 1986). Equally and perhaps more important, specificity studies will provide insights as to the nature of autoantigens that conceivably are required for eliciting spontaneously occurring antibodies (Schwartz & Stollar, 1985; Schattner, 1987).

Notwithstanding the numerous advantages of ELISA to detect and characterize autoantibodies, data derived from such assays should be interpreted with caution. For example, it is often impossible to distinguish between low- and high-affinity antibodies as a result of their avidity-enhanced (bivalent) binding to polyhaptenic antigens (Eilat et al., 1986). Further, immobilized polynucleotide antigens may lack the varied conformations these molecules assume in solution. For these reasons a modified ELISA (competition–inhibition) was utilized to further assess the specificity of affinity-purified anti-GMP and TMP-reactive antibodies. Results obtained with mono- and polynucleotide competitors (Tables III and V) confirmed the base-dependent specificity of both GMP- and TMP-reactive antibodies (Figure 1). This was demonstrated by the inability of mono- or polynucleotide competitors lacking a guanine or thymine base moiety to effectively inhibit anti-GMP and -TMP antibodies, respectively. Competition studies with oligonucleotides (Table IV) revealed that both antibodies preferentially recognized oligonucleotide sequences possessing consecutive tracks of GMP and TMP residues and that inhibition was dependent upon oligonucleotide length (Figure 3). Whereas the relative affinities of anti-TMP antibodies for oligo$(dT)_n$ competitors increase with oligonucleotide length, anti-GMP antibodies preferentially recognize oligo$(dG)_{10}$. Further, the average affinity of anti-GMP antibodies for oligo$(dG)_{10}$ approximated 1×10^7 M^{-1} (Figures 3 and 4), values in excellent agreement with those reported for monoclonal antibodies specific for repeating d$(GC)_n$ sequences (Stollar et al., 1986) and for oligo$(dT)_n$ (Lee et al., 1982).

ELISA analyses of human SLE sera also support our findings regarding the dominance of GMP-reactive antibodies. For example, using nucleoside–protein conjugates as immobilized antigens, Weisbart et al. (1983) concluded that of 40 SLE sera screened, antibodies reacting with guanosine and to a lesser extent cytidine were most prominent. Disproportionate increases in anti-GMP antibodies were also detected in active and inactive SLE patients relative to those found in the sera of asymptomatic controls (Munns et al., 1987). Upon purification, these anti-GMP antibodies also possessed specificities toward oligo$(dG)_n$ sequences, other GMP-enriched polynucleotides, and unknown antigens restricted to the nucleolar organelles of fixed HEp-2 cells. Recently, three human myeloma proteins possessing anti-ssDNA antibody activity were shown to be specific for guanine and/or thymine (Zouali & Stollar, 1986). While the above specificity studies were conducted with autoantibodies derived from sera, characterization of several monoclonal antibodies originally derived from autoimmune mice has also been reported to be specific for GMP- (Koike et al., 1985) or TMP-containing polynucleotides (Ballard & Voss, 1985; Lee et al., 1982). Most notable were the latter antibodies since they reacted with oligo$(dT)_n$ sequences in a manner similar to that of the polyclonal TMP-reactive autoantibodies reported herein (Table IV).

It remains to be determined why the bulk of anti-DNA antibodies in SLE sera react with GMP- and GMP-enriched oligo- and polynucleotides (Table II). These data, however, are consistent with the observation that DNA obtained from SLE immune complexes was significantly higher in GC content relative to that in total human DNA (Sano & Morimoto, 1982). These authors further noted that GC-enriched DNA forms unique conformations (Podder, 1971; Selsing et al., 1979; Wang et al., 1981) that conceivably permit it to be preferentially recognized by a variety of nucleases. These types of cleavages would result in DNA fragments whose termini would be enriched with G and/or C nucleotides. Other investigations pertaining to the crystal structure of d(GGGGCCCC) also suggest that sequences containing tracts of consecutive guanines adopt a structure rather different from that of mixed-sequence dsDNA (McCall et al., 1985). For example, such structures appear to be more sensitive to S_1-nuclease and DNase II (Nickol & Felsenfeld, 1983; Drew & Travers, 1984). Further, poly(dG) poly(dC) assumes an A-like conformation that unlike other dsDNAs does not form nucleosomes (Simpson & Kungler, 1979). From an immunological viewpoint it is important to note that of the four nucleosides (A, G, C, and T) tested as immunogens (nucleoside–KLH conjugates) and tolerogens (nucleoside–IgG, where IgG was obtained from the Balb/c host) guanosine was the dominant hapten both for immunization and for induction of tolerance (Stollar & Borel, 1976).

In conclusion, we have employed a multiple antigen screening system (ELISA) to initially characterize the specificity of autoanti-DNA antibodies present in MRL mice. The results of these studies allowed us to develop strategies for the isolation of specific subsets of anti-DNA antibodies, namely GMP- and TMP-reactive antibodies. On the basis of competition–inhibition assays these affinity-purified antibodies were highly specific for oligonucleotide sequences comprised entirely of GMP and TMP residues, i.e., a base-dependent oligonucleotide specificity. Quantitative estimates of anti-DNA antibodies indicated that they represent approximately 10% of the total serum IgG fraction and that about two-thirds of the anti-DNA antibody population is GMP reactive.

Registry No. 5′-AMP, 61-19-8; 5′-GMP, 85-32-5; 5′-CMP, 63-37-6; 5′-UMP, 58-97-9; A, 58-61-7; G, 118-00-3; C, 71-30-7; U, 58-96-8; 5′-dAMP, 653-63-4; 5′-dGMP, 902-04-5; 5′-dCMP, 1032-65-1; 5′-TMP, 365-07-1; dA, 958-09-8; dG, 961-07-9; dC, 951-77-9; T, 50-89-5; $(dG)_2$, 15180-30-0; $(dG)_4$, 32327-38-1; $(dG)_6$, 58626-19-0; $(dG)_8$, 58626-20-3; $(dG)_{10}$, 58626-21-4; $(dG)_{12-18}$, 25656-92-2; $(dT)_2$, 1969-54-6; $(dT)_4$, 2476-57-5; $(dT)_6$, 2642-44-6; $(dT)_8$, 1270-05-9; $(dT)_{10}$, 55508-38-8; $(dT)_{12-18}$, 25086-81-1; $(dA)_8$, 72673-13-3; $(dC)_8$, 81742-55-4; poly(dG), 25656-92-2; poly(dI), 27732-54-3; poly(dT), 25086-81-1; poly(dC), 25609-92-1; poly(dA), 25191-20-2; poly[d(CG)], 29855-95-6; poly[d(AT)], 25464-54-4; poly[d(AC)], 29855-94-5; poly(rG), 25191-14-4; poly(rA), 24937-83-5; poly(rC), 30811-80-4; poly(rU), 27416-86-0.

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Structure of Bovine Adrenal Dopamine β-Monooxygenase, As Deduced from cDNA and Protein Sequencing: Evidence That the Membrane-Bound Form of the Enzyme Is Anchored by an Uncleaved Signal Peptide[†,‡]

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Received August 23, 1989

ABSTRACT: A full-length cDNA for dopamine β-monooxygenase (DβM) from bovine adrenal glands has been cloned and sequenced. The soluble and membrane-derived forms of DβM have also been sequenced from their N-termini. While the observed sequences for the soluble protein correspond to those previously reported [Joh, T. H., & Hwang, O. (1986) *Ann. N.Y. Acad. Sci. 493*, 343-350], the heavy subunit of membrane-derived enzyme is found to contain a unique N-terminus. Alignment of this N-terminus with that deduced from cDNA cloning indicates identity at 22 (and possibly 26) out of 27 residues. This comparison leads us to conclude that the membranous form of bovine DβM retains an uncleaved N-terminal signal peptide as the source of membrane anchoring.

Dopamine β-monooxygenase (DβM, EC 1.14.17.1) catalyzes the conversion of dopamine to the neurotransmitter and hormone norepinephrine within the catecholamine-secreting vesicles (chromaffin granules) of the adrenal medulla. The presence of DβM as both a soluble and membrane-bound protein is well documented, with an approximately equal distribution of enzyme activity between these forms (Winkler & Carmichael, 1982). DβM is not unique in this regard, since two additional vesicular proteins—enkephalin convertase (Fricker et al., 1986) and peptidylglycine α-amidating monooxygenase (Diliberto et al., 1987)—have also been demonstrated to exist in dual forms. A major unresolved question in the area of DβM catalysis has been the structural difference and precursor-product relationship between the soluble and membranous forms of the enzyme.

Early investigations of DβM from bovine adrenal glands had suggested that both enzyme forms were comprised of four, identically glycosylated subunits with a molecular size of ca. 75 kDa (Wallace et al., 1973; Foldes et al., 1972; Hortnagel et al., 1972; Craine et al., 1973). More extensive investigations revealed *two* major protein bands at 72 and 75 kDa, with the membranous enzyme showing a higher proportion of the 75-kDa band (Saxena & Fleming, 1983; Speedie et al., 1985).

[†] Supported by a grant from the National Institutes of Health (to J.P.K., GM 25765).
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02890.
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0006-2960/89/0428-10054$01.50/0