Antibody–Nucleic Acid Complexes. Antigenic Domains within Nucleosides As Defined by Solid-Phase Immunoassay†

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ABSTRACT: The usefulness of solid-phase immunoassays for the characterization of anti-nucleoside antibodies was investigated. Antibodies specific for guanosine (G), 7-methylguanosine (m^7G), and cytidine (C) were obtained from the serum of rabbits immunized with nucleoside–KLH (keyhole limpet hemocyanin) conjugates. Solid-phase assays consisted of measuring the ability of these antibodies to be retained by microtiter wells containing immobilized nucleoside–BSA (bovine serum albumin) conjugates. Nucleosides employed as haptens included adenosine (A), N^6-methyl-A (m^6A), guanosine (G), N^2,N^2-dimethyl-G (m_2^2G), 1-methyl-G (m^1G), O^6-methyl-G (m^6G), 7-methyl-G (m^7G), cytidine (C), 5-methyl-C (m^5C), uridine (U), and ribothymidine (T). Spectral analysis of these conjugates revealed that 15–20 nucleosides were coupled to each BSA molecule. Quantitative information regarding the various reactions associated with these assays was obtained by employing antigen and antibody (IgG) preparations radiochemically labeled via reductive methylation using NaB^3H_4 and formaldehyde (specific activities 0.6–2.1 $\times 10^6$ cpm/μg). Data obtained with ^3H-labeled antigens indicated that the adsorption of all nucleoside–BSA conjugates was uniform and irreversible with respect to the assay conditions used. Assays designed to measure antibody binding in the presence of excess antigen revealed that (i) nonspecific binding to immobilized BSA was negligible, (ii) as little as 0.5 ng of bound antibody could be detected, (iii) antibody retention was directly proportional to antibody concentration, and (iv) each anti-nucleoside antibody cross-reacted to a considerable extent with nonhomologous haptens. For example, at all serum concentrations tested, anti-G antibody cross-reacted with A–, C–, and U–BSA antigens to an extent of 30, 60, and 80%, respectively. Cross-reactivity, however, was diminished and, in most instances, abolished by reducing the quantity of immobilized nucleoside–BSA conjugates by 1–3 orders of magnitude (i.e., from 60 to 0.06 ng). Further, data obtained by plotting cross-reactivity as a function of the quantity of antigen immobilized suggested that each homologous hapten possesses a major epitope (antigenic surface) within its heterocyclic ring system. For example, anti-G antibody cross-reacted *poorly* with m^6G, *moderately* with m^7G and m^1G, and *excessively* with m_2^2G. This indicated that the major epitope of guanine encompassed the N-1, C-6, O-6, and N-7 atoms. A similar analysis of anti-C antibodies suggested that the C-4 and N-4 atoms dominate the major structural determinant. Collectively, these results illustrate the unprecedented sensitivity of this solid-phase assay to detect antibody binding to previously unrecognized structurally related haptens. Further, by progressive reduction of the amount of immobilized haptens, sufficient information is obtained to make logical conclusions regarding the identity of the major antigenic domain within a low molecular weight hapten. Last, the application of this immunoadsorbent assay and its conversion to enzyme-linked immunoadsorbent assays (ELISA) for characterizing and selecting specific monoclonal anti-m^7G antibodies are examined.

Numerous immunoassays have been described for the characterization of antibodies specific for various constituents present in nucleic acids (Stollar, 1975; Munns & Liszewski, 1980). Generally, such assays represent modified forms of complement-fixation techniques (Sawicki et al., 1971), quantitative precipitin analyses (Munns et al., 1977), and radioimmunoassays (Levine & Gjika, 1974; Riley et al., 1979). More recently, and with the advent of hybridoma technology, numerous solid-phase immunoadsorbent assays have been developed for the rapid screening of monoclonal antibodies. The most common of these assays is that developed by Engvall and colleagues (Engvall et al., 1971a,b, 1972) and termed enzyme-linked immunosorbent assay or ELISA.[1] One particularly useful form of ELISA detects antibody bound to immobilized antigen by the use of an enzyme-linked (e.g., alkaline phosphatase, peroxidase, etc.) anti-globulin antibody. Variation of this technique includes radiolabeled or fluorescent-linked antibody as well as similarly modified forms of protein A (Miller et al., 1981; Kanai et al., 1982). While the above assays are used frequently in detecting for the presence of monoclonal antibodies, their utilization for characterizing both mono- and polyclonal antibody populations in general and anti-nucleoside antibodies in particular remains to be ascertained.

Our attempts to systematically define this assay for such characterization purposes stem from three observations. First, immunoadsorbent assays are simple, sensitive, reproducible, and adaptable for the processing of large sample populations. Second, they provide a method for characterizing spontaneously occurring anti-DNA antibodies with respect to their conformational (single- vs. double-stranded) and/or individual base specificities (Weisbart et al., 1982, 1983; Munns et al., 1982a, 1984). Third, preliminary studies in our laboratory designed to characterize the specificity of an anti-m^7G antibody preparation (Munns & Liszewski, 1980) via both ELISA and radioimmunoassay yielded conflicting results. Whereas the radioimmunoassay data indicated that anti-m^7G antibody

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[1] Abbreviations: ELISA, enzyme-linked immunosorbent assay; PO_4/NaCl, phosphate-buffered saline; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; A, adenosine; m^6A, N^6-methyl-A; G, guanosine; m^1G, 1-methyl-G; m_2^2G, N^2,N^2-dimethyl-G; m^6G, O^6-methyl-G; m^7G, 7-methyl-G; C, cytidine; m^5C, 5-methyl-C; U, uridine; m^5U, 5-methyl-U or ribothymidine; A–KLH, A–BSA, G–KLH, G–BSA, etc., nucleosides conjugated to KLH and to BSA; IgG, immunoglobulin G; Tris, tris(hydroxymethyl)aminomethane.

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cross-reacted minimally (G) or not at all (A, C, m^5C, U, and T) with structurally related haptens, significant cross-reactivity for all of these nucleosides was observed when examined via ELISA. Because of these observations, we have examined in detail the suitability of ELISA for characterizing experimentally induced anti-nucleoside antibodies. The results presented herein indicate the feasibility of ELISA for (i) quantitating such antibody populations, (ii) providing an extensive analysis as to their cross-reactivity toward other structurally related, nonhomologous haptens, (iii) selecting appropriate monoclonal anti-nucleoside antibodies with the desired specificity, and (iv) identifying antigenic domains within nucleosides responsible for antibody recognition.

Materials and Methods

Materials. Nucleosides employed in the present investigation were obtained from Sigma Chemical Co. and/or P-L Biochemicals. Nucleosides included adenosine (A), N^6-methyladenosine (m^6A), cytidine (C), 5-methylcytidine (m^5C), guanosine (G), 1-methylguanosine (m^1G), N^2,N^2-dimethylguanosine (m_2^2G), 7-methylguanosine (m^7G), uridine (U), and ribothymidine (T). The synthesis of O^6-methylguanosine (m^6G) was accomplished according to the procedure of Briscoe et al. (1978). Alkaline phosphatase conjugated goat anti-rabbit IgG and *p*-nitrophenyl phosphate were obtained from Sigma as were other phosphatase-conjugated antibodies specific for mouse IgG. Ninety-six well microtiter plates (Costar) were from Bellco Glass, Inc. Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were products from Miles and Sigma, respectively.

Nucleoside(tide) Conjugations. All of the nucleosides listed above were coupled to KLH and/or BSA via periodate oxidation techniques (Erlanger & Beiser, 1964). Spectral analysis (230–310 nm) of the resulting conjugates revealed no detectable degradation of nucleosides as a result of the coupling reaction. The extent of conjugation ranged from 15 to 20 and 70 to 200 nucleosides per BSA and KLH molecule, respectively.

Antibody Populations. Experimentally induced anti-nucleoside antibodies were obtained by immunizing rabbits (New Zealand White females) and mice (Balb/CyJ, females) with nucleoside–KLH immunogens. Immunization protocols and preliminary characterization of anti-nucleoside antibodies via quantitative precipitin and/or radioimmunoassays have been described (Munns & Liszewski, 1980). IgG preparations from antiserums and ascites fluid containing murine monoclonal anti-m^7G antibodies (see below) were prepared by precipitation with 50% ammonium sulfate, dialysis (10 mM PO_4, pH 7.4), and DEAE-Sephacel chromatography (Fahey & Horbett, 1959).

To obtain monoclonal anti-m^7G antibodies, approximately 10^8 spleen cells from a Balb/c mouse previously immunized with m^7G–KLH were fused with 10^7 myeloma cells (nonsecreting MOPC-21, line Sp2/0-Ag14). Procedures related to cloning hybrid cells have been described (Munns et al., 1982) as has the [^{3}H]m^7G binding assay used initially in selecting individual clones (Munns & Liszewski, 1980). Preparative quantities of monoclonal antibodies were obtained by injection of cloned hybrid cells into the intraperitoneal cavity of pristane-treated mice (5×10^7 cells/mouse). About 8 days after inoculation, 5 mL of ascites fluid was collected at 2-day intervals. Ascites fluid containing 0.02% (w/v) sodium azide was centrifuged to remove cellular debris and stored at −20 °C prior to use.

Details regarding the selection and physiochemical properties of four monoclonal anti-m^7G antibodies will be described elsewhere (T. W. Munns and M. K. Liszewski, unpublished results). Briefly, clones initially selected by radioimmunoassay with [^{3}H]m^7G were recloned in soft agar to ensure monoclonicity (Munns et al., 1982b). Clones were reselected on the basis of their binding to immobilized m^7G–BSA antigen. IgG fractions were assessed via ELISA for isotype, employing alkaline phosphatase coupled, goat anti-mouse IgG 1, IgG 2a, etc. reagents. All four of the monoclonal anti-m^7G antibodies described here were IgG 1,κ, as evaluated by this technique. Isoelectric focusing gels of these monoclonal antibodies indicated the presence of multiple IgG bands possessing closely related p*I* values [see Munns et al. (1982b)].

Tritium Labeling of Nucleoside–Protein Conjugates and IgG Preparations. IgG and nucleoside–BSA preparations (see above) were labeled via reductive methylation with NaB^3H_4 (specific activity 6.23 Ci/mmol, Amersham-Searle) and formaldehyde (Sigma) as described by Tack et al. (1980). Approximately 0.2 mg of protein was radiochemically labeled for 15 min in a volume of 0.2 mL. The molar ratio of NaB^3H_4 to formaldehyde was 0.37. The resulting ^{3}H-labeled proteins were subjected to gel filtration chromatography (Sephadex G-50) and dialyzed with phosphate-buffered saline (PO_4/NaCl: 10 mM PO_4 and 150 mM NaCl, pH 7.4). The specific activity of these ^{3}H preparations ranged from 0.6×10^6 to 2.1×10^6 cpm/μg of protein.

Immobilization of Antigens. Antigens consisting of nucleoside–BSA conjugates and BSA were diluted with PO_4/NaCl to a final concentration of 10 μg/mL and these solutions pipetted into individual wells of microtiter plates (0.15 mL/well, i.e., 1.5 μg of antigen). Antigens were allowed to interact with the surface of the well for 12–16 h (overnight) at 4 °C. Just prior to use, microtiter plates were inverted to remove unbound antigen and individual wells washed twice with PO_4/NaCl buffer containing 0.05% Tween-20 (PO_4/NaCl/Tween).

The quantity of antigen immobilized to each well was determined with ^{3}H-labeled antigen, e.g., G–[^{3}H]BSA, [^{3}H]BSA, etc. After immobilization (see above) individual wells were immersed in scintillation vials containing 10 mL of aqueous scintillant (PCS, Amersham-Searle). To reduce the quantity of hapten (nucleoside) immobilized, nucleoside–BSA conjugates (10 μg/mL in PO_4/NaCl) were diluted with PO_4/NaCl buffer containing 10 μg/mL BSA. This procedure permits the amount of protein (BSA) adsorbed to each well to remain constant but reduces quantitatively the amount of immobilized hapten.

Detection of Antibodies Retained by Antigen-Coated Wells. Serum and IgG fractions containing the appropriate antibody populations were diluted with PO_4/NaCl/Tween buffer containing 2 mg/mL BSA (PO_4/NaCl/Tween/BSA). Diluted samples (0.05–0.15 mL) were incubated with antigen-coated wells for 30 min at 37 °C. Kinetics studies (not shown) indicated that maximum antibody binding occurred within 15 min. Incubations were terminated by inverting microtiter plates and washing each well twice with PO_4/NaCl/Tween.

Species-specific, anti-IgG antibodies (previously conjugated with alkaline phosphatase) were employed to provide a semiquantitative measure of the amount of bound antibody. These enzyme-linked, second antibodies were diluted in PO_4/NaCl/Tween (usually a 3000–5000-fold dilution from stock according to the recommendation of the commercial supplier) and 0.15-mL aliquots incubated in each well for 30 min at 37 °C. Upon termination of this reaction, plates were again inverted and wells washed 3 times with Tris/NaCl/Tween prior to adding 0.15 mL of alkaline phosphatase substrate

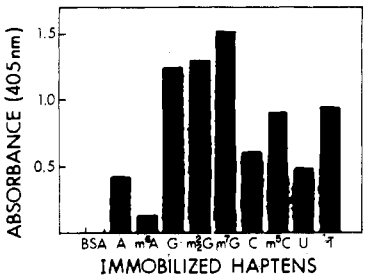

FIGURE 1: Binding of rabbit anti-m^7G antibody to immobilized nucleoside–BSA antigens. Antiserum was diluted 5000-fold and 0.15-mL aliquots were incubated in wells containing immobilized nucleoside–BSA antigens. Antigens included unconjugated BSA and A–, m^6A–, G–BSA conjugates, etc. Antibody retained by each antigen was detected via ELISA using alkaline phosphatase conjugated, goat anti-rabbit IgG and *p*-nitrophenyl phosphate. Incubation conditions, A_{405} measurements, etc. are described under Materials and Methods.

(*p*-nitrophenyl phosphate at 1 mg/mL in 1.0 M diethanolamine, 0.1 mg/mL $MgCl_2$, pH 9.8). The enzymatic reaction was terminated after 30 min by the addition of 0.1 mL of 0.3 N NaOH. Color development (absorbance) was recorded with an ELISA spectrophotometer (Dynatech, Model MR 580) at 405 nm.

Quantitation of Antibodies Retained by Antigen-Coated Wells. To provide a quantitative measure as to the absolute amount of antibody retained, various [^{3}H]IgG fractions of predetermined specific activities were employed. These incubation conditions were identical with those described above for unlabeled preparations. The retention of ^{3}H antibodies was determined both spectrophotometrically (i.e., alkaline phosphatase conjugated anti-IgG and *p*-nitrophenyl phosphate) and radiochemically (cpm bound/well; determined as described above for quantitation of immobilized antigen). Such data provided the requisite information to determine the absolute quantity (nanograms) of IgG bound per well as a function of absorbance (A_{405}). Details regarding the quantitation of ELISA are presented below in connection with Figures 2 and 3.

Results

Characterization of Anti-m^7G Antibodies via ELISA. Previously we have evaluated the specificity of anti-m^7G antibodies in both ^{3}H-labeled hapten binding (Munns & Liszewski, 1980) and quantitative precipitin (Munns et al., 1977) assays. These data indicated that anti-m^7G antibody cross-reacted to a limited extent with G but not with other major nucleosides (A, C, U, and T) present in nucleic acids. To reexamine this specificity in ELISA, we assessed the ability of anti-m^7G antiserum to bind to various immobilized nucleoside–BSA conjugates. These results are presented in Figure 1 and revealed that while anti-m^7G antibody did not bind to unconjugated BSA, significant binding was observed not only to m^7G–BSA but also to all other hapten constituents as well. Further, the cross-reactivity associated with such structurally dissimilar haptens as C and T was maintained even when anti-m^7G antiserum was diluted 6000- and 12 000-fold (data not shown).

The inconsistency of these results when compared with other specificity assays, together with a lack of quantitation associated with ELISA, prompted us to examine in detail the complexities of these immunoadsorbent assays. To accomplish this, we employed both ^{3}H-labeled antigen and antibody preparations. Whereas the former was used to determine the quantity of antigen adsorbed to each microtiter well, the latter ^{3}H probe was used to quantitate the absolute amount of antibody retained by immobilized antigen. To minimize potential

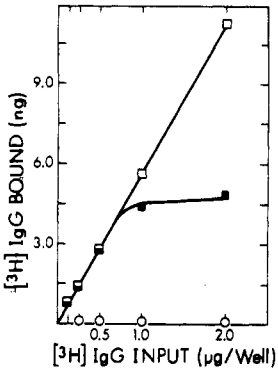

FIGURE 2: Binding of anti-C antibody ([^{3}H]IgG fraction, specific activity 1.3×10^6 cpm/μg) to C–BSA antigen. Open and closed squares represent the binding of antibody to 60 and 0.6 ng of immobilized C–BSA, respectively, and open circles to 60 ng of unconjugated BSA. Incubation conditions and measurement of bound [^{3}H]IgG are described under Materials and Methods. Each data point presented is the mean from three independent experiments. Individual values varied less than 10% of their mean value.

variatons attributable to the degree of conjugation of nucleosides to BSA, only those conjugates containing 15–20 nucleosides per BSA molecule were employed throughout this investigation.

Quantitation of Antigen Immobilization. This parameter was investigated by employing nucleoside–[^{3}H]BSA conjugates and [^{3}H]BSA. The conditions employed for antigen immobilization and the determination of adsorbed radioactivity are described under Materials and Methods. The results of these investigations (not shown) revealed the following information. First, microtiter wells incubated in the presence of 0.15 mL of nucleoside–BSA conjugate (10 μg/mL) or BSA (10 μg/mL) adsorbed 60 ± 10 ng of each antigen. Immobilization was independent of the type of hapten present as well as their variation in the extent of conjugation, i.e., 15–20 nucleosides per BSA molecule as well as none for unconjugated BSA. Second, the quantity of hapten immobilized, but not BSA, was reduced quantiatively by diluting nucleoside–BSA solutions with 10 μg/mL BSA. Last, antigens were adsorbed irreversibly with respect to the various conditions (washings, incubations, etc.) imposed upon the microtiter well throughout the course of the assay.

Quantitation of Antibody Binding. ELISA methodology by definition depends upon an enzyme-linked anti-IgG antibody (or protein A) to detect antibodies previously retained by immobilized antigen. Enzyme-linked reagents, however, do not provide rigorous quantitation with respect to the absolute amount of antibody bound. To obtain this information, we employed anti-nucleoside [^{3}H]IgG preparations of predetermined specific activities ($0.9–1.5 \times 10^6$ cpm/μg). Binding was evaluated as a function of both the concentration of [^{3}H]IgG incubated per microtiter well and the quantity of nucleoside–BSA or antigen immobilized. Representative of our findings are those presented in Figure 2 for anti-C antibody (specific activity 1.3×10^6 cpm/μg). Assays performed to measure antibody binding in the presence of excess antigen (i.e., 60 ng of C–BSA/well, open squares) indicated that (i) as little as 0.5 ng (650 cpm) of bound anti-C antibody was detectable, (ii) binding to unconjugated BSA was negligible and never exceeded 0.02% of the input radioactivity, and (iii) the quantity of ^{3}H-labeled antibody bound was proportional to the concentration of [^{3}H]IgG employed.

By reducing the quantity of immobilized antigen by 2 orders of magnitude (from 60 to 0.6 ng/well), we established conditions whereby the binding of anti-C antibody to C–BSA was

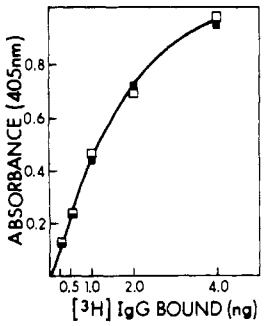

FIGURE 3: Relationship between nanograms of bound anti-C antibody to C–BSA antigen (open and closed squares represent 60 and 0.6 ng of C–BSA, respectively) and absorbance at 405 nm. Duplicate wells were incubated with increasing amounts of antibody ([^{3}H]IgG). One well was processed directly for determination of retained radioactivity and the other for absorbance at 405 nm (see Materials and Methods). Wells used for absorbance measurements were subsequently processed for radioactivity measurements as well. These results (not shown) indicated no significant loss of retained ^{3}H-labeled antibody as a result of the additional incubation steps with alkaline phosphatase coupled, anti-rabbit IgG and *p*-nitrophenyl phosphate. Results are expressed as the mean from three independent experiments (individual values varied less than 12% of their mean).

saturable. These results, also presented in Figure 2 (closed squares), indicate that a maximum of 5 ng of anti-C antibody (0.033 pmol) was capable of binding to 0.6 ng of C–BSA or 0.17 pmol of C. The latter value was obtained by determining the quantity of C–BSA immobilized (0.6 ng) and its extent of nucleoside conjugation (20 C/BSA molecule). Thus, we estimate that, at saturation, the molar ratio of hapten to antibody is approximately 5. Further, when we consider that each antibody contains two combining sites and that perhaps only 30–50% of the conjugated hapten is exposed on the surface of immobilized BSA, the ratio of available hapten to antibody combining sites approximates a stoichiometric relationship (i.e., 1 hapten/antibody combining site).

While the utilization of both ^{3}H-labeled antigens and antibodies provides a quantitative assessment of antibody binding, measurement of bound radioactivity in each microtiter well is cumbersome relative to ELISA. To convert to a quantitative ELISA, absorbance measurements (A_{405}) were correlated with bound antibody. These data (Figure 3) revealed that absorbance (between 0.0 and 0.7 A_{405} unit) is directly related to the quantity of antibody bound. Not shown in Figure 3 was the finding that all anti-nucleoside antibody preparations yielded identical results.

ELISA: Assessment of Antibody Concentration. The data in Figure 2 indicated that, in the presence of excess antigen, binding of ^{3}H-labeled antibody was directly proportional to its concentration. The question remained, however, whether excess antigen was capable of quantitatively retaining its corresponding anti-nucleoside antibody. To obtain this information, anti-C and -G antiserums were incubated for 30 min in the presence of immobilized antigen and the supernatants (i.e., preadsorbed serum) reassessed via ELISA for the detection of unbound antibody. These results are presented in Figure 4 and revealed that preadsorption of antiserum removed greater than 90% of the anti-nucleoside antibody. These results suggest that ELISA can be used to estimate antibody concentration. For example, the data presented in Figure 4 indicate that 4 nL of anti-C and -G antiserums contains 1 and 2 ng of antibody, respectively, i.e., 0.25 and 0.50 mg/mL. These results are in accord with quantitative precipitin measurements (Munns et al., 1977), the latter yielding values of 0.27 (anti-C antibody) and 0.56 ng/mL (anti-G antibody).

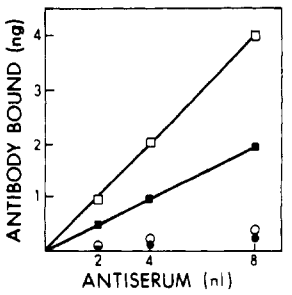

FIGURE 4: Retention of antibody by excess immobilized antigen. The equivalent of 2, 4, and 8 nL of anti-C and -G antiserum (i.e., 0.2 mL of a 10^5-fold dilution of antiserum represents 2 nL) was incubated with 60 ng of C– and G–BSA antigen, respectively. The quantity of antibody bound was determined from absorbance measurements and converted to nanograms by using the standard curve in Figure 3 (open squares, anti-G antibody; closed squares, anti-C antibody). Individual variation from the mean of three independent experiments was less than 9%. To detect for the presence of unbound antibody, antiserum previously incubated with antigen-coated wells (i.e., preadsorbed) was reincubated with additional antigen-coated wells in an identical manner. These data are illustrated above by open and closed circles for preadsorbed anti-G and anti-C antiserum, respectively.

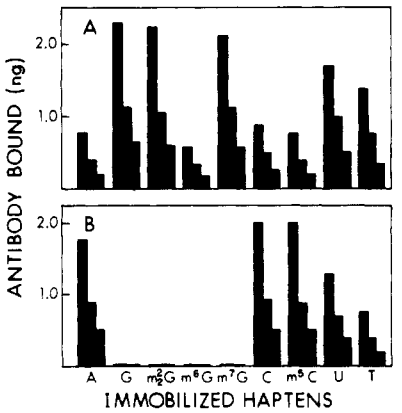

FIGURE 5: Binding of anti-G (panel A) and anti-C (panel B) antibodies to structurally related haptens. Antiserum was incubated with each antigen-coated well. Antigens included BSA, A–BSA, G–BSA, etc. Antibodies retained by each well were detected by ELISA and converted to nanograms by using the standard curve in Figure 3. Each value represents the mean of a minimum of three independent determinations. Variation from the mean was less than 15% (mean values between 0.2 and 0.5 ng) and 8% (mean values greater than 0.5 ng). The decrease in antibody binding reflects the diminishing volume of antiserum used per assay, i.e., from left to right, 16, 8, and 4 nL of anti-C antiserum and 8, 4, and 2 nL of anti-G antiserum.

ELISA: Assessment of Antibody Specificity. Specificity studies were conducted by assessing the ability of each antibody to bind to other structurally related haptens. These results, presented in Figure 5, indicate that both anti-C and -G antibodies bind to varying extents with a considerable number of nonhomologous haptens. Thus, anti-C antibody cross-reacted with m^{5}C and A (extensive) and with U and T (moderate) but not with m^{6}A, G, or the various methylated forms of G (m$^2_2$G, m^{6}G, and m^{7}G). Similarly, anti-G antibodies cross-reacted with m$^2_2$G, m^{7}G, and U (extensively) and with A, C, m^{5}C, and T (moderately) but only minimally or not at all with m^{6}G and m^{6}A. In an attempt to reduce or minimize cross-reactivity, the concentration of antibody was reduced. However, as the data in Figure 5 illustrate, the extent of cross-reactivity appeared independent of antibody concentration. For example, anti-C antibody cross-reacted with A to an extent of 80% at all concentrations tested (Figure 5B).

While these results were unexpected, it is important to stress that the above assays were conducted in the presence of excess antigen (hapten), i.e., conditions not normally encountered in

Table I: Anti-G and -C Antibody Binding to Immobilized Haptens. Effects of Diminishing Quantities of Hapten[a]

hapten–BSA	quantity of antibody retained (ng) for hapten immobilized (ng)[b] 5.0	0.5	0.05	0.005
anti-G antibody				
G	2.3	2.3	2.1	0.5
m^2G	2.1	2.2	2.0	0.4
m^7G	2.2	0.8	0.2	0
m^1G	1.7	0.7	0	0
U	1.6	0.6	0	0
T	1.3	0.3	0	0
C	0.7	0	0	0
m^5C	0.6	0	0	0
A	0.5	0	0	0
m^6G	0.3	0	0	0
anti-C antibody				
C	1.2	1.3	1.2	0.6
m^5C	1.2	1.0	0.4	0.1
A	0.9	0.3	0	0
U	0.6	0.1	0	0
T	0.2	0	0	0
m^6A	0	0	0	0
G	0	0	0	0
m^2G	0	0	0	0
m^6G	0	0	0	0
m^7G	0	0	0	0

[a] The equivalent of 4 nL of anti-C and -G antiserum was incubated in microtiter wells containing immobilized C– and G–BSA antigen. Antibodies retained by each antigen were detected via ELISA and A_{405} measurements converted into nanograms of retained antibody (see Figure 3). [b] The quantity of hapten immobilized was determined according to procedures described under Materials and Methods. On the basis of the extent of conjugation of C and G to BSA (20 C and 18 G/BSA) we determined that approximately 8% of the weight of each conjugate is nucleoside. Thus, the adsorption of 60 ng of C–BSA would result in immobilization of 5 ng of hapten.

antigen–antibody binding reactions. In most circumstances, such reactions are conducted by employing conditions in which the antigen concentration is limiting relative to antibody. To simulate these conditions in ELISA, we progressively decreased through 3 orders of magnitude the quantity of nucleoside–BSA conjugate adsorbed to microtiter wells and reevaluated antibody binding as a function of the quantity of hapten immobilized.

The results of these investigations for anti-G and -C antibodies are presented in Table I. In each instance, the cross-reactivity observed when excess antigen is used rapidly decreases as the quantity of hapten is reduced. Further, an evaluation of cross-reactivity as a function of the quantity of immobilized hapten suggested that the homologous hapten possessed a major epitope (antigenic surface) within its heterocyclic ring system. In regard to anti-G antibody, the data in Table I indicate that this polyclonal antibody reacts *minimally* with m^6G, *moderately* with m^1G and m^7G, and *excessively* with m_2^2G. These findings imply that the major epitope of G emcompasses the N-1, C-6, O-6, and N-7 atoms with a major focal point centered at C-6 and O-6. While the data presented for anti-C antibodies is incomplete, comparison with structurally related haptens suggests that the major epitope in C is dominated by the C-4 and N-4 atoms (Table I). Of particular importance is the NH_2 group attached to C-4, since its replacement with an oxygen atom (e.g., G, U, and T) reduces antibody binding significantly. Further, the ability of anti-C antibodies to cross-react extensively with m^5C implies that C-5 and probably C-6 atoms contribute minimally to the epitope surface.

Table II: Murine Poly- and Monoclonal Anti-m^7G Antibody Binding to Immobilized Haptens. Effects of Diminishing Quantities of Hapten[a]

hapten–BSA	quantity of antibody retained (ng) for hapten immobilized (ng) 5.0	0.5	0.05	0.005
polyclonal				
m^7G	2.3	2.1	2.0	0.6
G	2.0	1.8	1.0	0.2
m^2G	1.8	0.5	0.2	0
m^5C	0.5	0.2	0	0
T	0.3	0	0	0
C	0.1	0	0	0
m^6G	0	0	0	0
monoclonal				
m^7G	2.5	2.3	2.3	0.6
m^2G	0.1	0	0	0
G	0	0	0	0
m^6G	0	0	0	0
m^5C	0	0	0	0
T	0	0	0	0
C	0	0	0	0

[a] Data obtained in the same manner as described in the legend of Table I. The equivalent of 3 nL of mouse anti-m^7G antiserum was used per assay. IgG monoclonal preparations were prepared by $(NH_4)_2SO_4$ fractionation and DEAE-Sephacel chromatography. A similar polyclonal IgG preparation was prepared, labeled with $[^3H]NaBH_4$ (1.2×10^6 cpm/μg), and used to construct a standard curve relating A_{405} measurements to nanograms of antibody bound in a manner identical with that illustrated in Figure 3 for rabbit anti-nucleoside antibodies.

ELISA: Assessment of Mono- and Polyclonal Anti-m^7G Antibodies. A similar assessment of the specificity of polyclonal anti-m^7G is presented in Table II. While this particular antibody population was derived from a mouse, its binding to nucleoside conjugates was similar to rabbit polyclonal anti-m^7G antibodies (see Figure 1). The data contained in Table II indicate that by introducing a methyl group at the N-7 position of G, the major epitope region becomes centered about this modification. However, the ability of G, m^1G, and m_2^2G to be recognized by anti-m^7G antibodies but not m^6G suggests that either (i) the major epitope is extended to include the C-6/O-6 region or (ii) a minor antibody pouplation recognizes the C-6/O-6 domain preferentially.

In view of these complexities we focused our attention on characterizing various murine monoclonal anti-m^7G antibodies initially selected by their ability to bind with $[^3H]m^7G$. Subsequent evaluation of four clones reselected by ELISA revealed that three of these preparations bound to m^7G–, G–, and m_2^2G–BSA in a manner similar to the polyclonal preparation presented in Table II. The remaining clone, however, reacted almost exclusively with m^7G, cross-reacting only minimally with an excess of m_2^2G–BSA (i.e., 60 ng of immobilized m_2^2G–BSA).

These results reveal that the major epitope within the m^7G hapten is dominated by atoms C-6, O-6, and N-7 and, most important, the methyl group attached to N-7. Further, by use of hybridoma technology and ELISA technology we have been able to isolate a particular monoclonal anti-m^7G antibody with a specificity directed almost exclusively toward m^7G.

Discussion

The parameters involved in the binding of antibodies to an immobilized polyhaptenic surface are numerous and complex. Most pertinent to this investigation is whether the binding of an anti-nucleoside antibody is influenced significantly by the

quantity and distribution of immobilized nucleoside. This concern is justified in view of the variable extent of conjugation of nucleosides to proteins (Erlanger & Beiser, 1964; Manns & Liszewski, 1980). To minimize this effect, we selected only those conjugates that contained between 15 and 20 nucleosides per BSA molecule. Further, by employing nucleoside–[^{3}H]-BSA probes, we determined that each conjugate was adsorbed equally to the surface of microtiter wells, irrespective of the nature of the haptenic group.

By employing ^{3}H-labeled antibodies of relatively high specific activity, we were able to quantitate the ELISA in terms of nanograms of antibody bound as a function of adsorbance at 405 nm (Figures 2 and 3). These data were quite useful in determining the stoichiometric relationship between antibody combining sites and the number of haptens available for binding (Figure 2). Accounting for the fact that approximately 50% of the haptens attached to BSA are not accessible for binding, we estimated that antibody saturation of available haptens approached a stoichiometric relationship, i.e., 1 hapten per antibody combining site.

It is generally accepted that the physical dimensions of an antibody combining site can accommodate a determinant the size of a tetra- or pentanucleotide sequence (Seaman et al., 1972; Munns et al., 1979). This does not mean, however, that the determinant cannot be of a smaller size as evidenced by the extensive literature regarding the use of small molecular weight molecules as haptens. While it is usually assumed that antibodies specific for small haptens encompass the entire molecule, our data indicate that domains representative of only a small portion of a nucleoside frequently represent the major epitope surface. Antibodies recognizing such restricted epitopes react therefore with other structurally related haptens, especially when the structural difference resides outside the major antigenic domain of the homologous hapten.

Because immobilized nucleoside–BSA conjugates provide a polyhaptenic matrix, both combining sites of a single IgG molecule participate in the binding reaction. Since the strength of this type of binding (avidity) greatly exceeds that associated with a single combining site (affinity) [see Karush (1970)], it is understandable why ELISA relative to hapten binding assays accentuate the cross-reactivity of structurally related haptens. Moreover, this avidity-enhanced binding to structurally related haptens suggests that nucleosides possess domains within their heterocyclic ring systems that are preferentially recognized by polyclonal antibodies in contrast to other regions that are preferentially ignored. Thus as the data in Table I illustrate, the bulk of anti-G antibodies recognize G by virtue of the spacial arrangement of N-1, C-6, O-6, and N-7 atoms. Further, the ability of m_2^2G to cross-react extensively with anti-G antibodies suggests that the C-2 and N-2 atoms contribute minimally to the antibody recognition of G.

Last, an understanding of cross-reactivities is important when isolation of nucleic acids on the basis of a single modified nucleoside (Munns & Liszewski, 1980) is attempted. For example, the employment of anti-m^7G antibodies to isolate m^7G-capped mRNAs may be difficult when the fact that many mRNAs possess a G/m^7G ratio greater than 500 is considered. Moreover, whereas mRNA is polyhaptenic with respect to G, it is monohaptenic for m^7G. The latter implies that both binding sites of an anti-m^7G antibody can participate in the binding (avidity) to the cross-reacting hapten (G); yet only a single site is available for binding (affinity) the homologous hapten (m^7G). This situation is exemplified by Castleman et al. (1980), who observed via electron microscopy an average of three anti-m^7G antibodies attached to avian sarcoma virus RNA. Whereas a single antibody bound to the 5′-terminal m^7G cap (thereby distinguishing 5′- from 3′-termini), additional antibody molecules (from one to five) were bound randomly. The authors attributed this latter binding to several G residues reacting with the same antibody, i.e., avidity-enhanced binding of a cross-reacting hapten. While the random binding of anti-m^7G antibody in the above study was not critical in defining the 5′-terminus, such binding would be quite detrimental when isolation of m^7G-capped mRNA in the presence of other uncapped RNA species is attempted. On the basis of these considerations and the results presented herein, we have begun to redirect our efforts toward selecting monoclonal anti-nucleoside antibodies that lack the inherent cross-reactivities associated with all polyclonal and some monoclonal preparations.

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Antibody–Nucleic Acid Complexes. Conformational and Base Specificities Associated with Spontaneously Occurring Poly- and Monoclonal Anti-DNA Antibodies from Autoimmune Mice†

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ABSTRACT: An enzyme-linked immunosorbent assay (ELISA) was developed to characterize spontaneously occurring, mono- and polyclonal anti-DNA antibodies. The assay consists of adsorbing single- (ss) and double-stranded (ds) DNA and various nucleoside–bovine serum albumin conjugates (e.g., A–, G–BSA, etc.) to microtiter wells and assesses the ability of various antibodies to bind to these immobilized antigens. The conformational and base specificity of two monoclonal antibodies (designated MRss-1 BWds-3) was examined in this manner. The exclusive binding of MRss-1 to ssDNA and guanosine–BSA (G–BSA) confirms our previous findings [Munns, T. W., Liszewski, M. K., Tellam, J. T., Ebling, F. M., & Hahn, B. H. (1982) *Biochemistry 21*, 2929–2936] that this antibody recognizes single-stranded nucleic acids by virtue of their guanine content. The extensive binding of BWds-3 to dsDNA, its limited binding to ssDNA, and complete absence of binding to nucleoside–BSA antigens implied a double-stranded conformational specificity. Further, competitive studies with naturally occurring and synthetic alternating copolymers indicated that BWds-3 preferentially recognized the native dsDNA antigens. ELISA analysis of the spontaneously occurring, polyclonal anti-DNA antibodies from MRL/lpr and NZB/NZW-F1 mice revealed that the majority of anti-ssDNA antibodies bound to nucleoside–BSA conjugates. Anti-G antibodies were most prominent in both strains of mice, yet lesser and more variable quantities of anti-A, -C, -U, and -T antibodies were also detected. Preadsorption of serum with G–BSA/Sepharose resulted in the complete removal of anti-G antibodies and a 60% reduction in anti-ssDNA antibodies. Anti-ssDNA antibodies were completely removed by preadsorption of serum with a mixture of A–, G–, C–, and T–BSA/Sepharose. Anti-dsDNA antibodies accounted for approximately 30 and 60% of the total antibody population in the serums of MRL/lpr and NZB/NZW-F1 mice, respectively. Last, time–course studies with five individual MRL/lpr mice revealed that the appearance of all anti-DNA antibodies in their serum was coincident and occurred in all animals at the age of 10–11 weeks.

Various strains of mice, particularly NZB/NZW-F1 and MRL/lpr, have been used extensively as experimental models of human systemic lupus erythematosus (SLE)[1] (Seegal et al., 1969; Andrews et al., 1978). The spontaneous appearance of anti-DNA antibodies in the serum of these mice represents one of the most prominent features of this autoimmune disease. Collectively, such serums possess a multiple of anti-DNA antibodies with diverse antigenic specificities (Tan, 1982; Hahn et al., 1980; Marion et al., 1982; Rauch et al., 1982; Eilat et al., 1980). Specificity is frequently defined by the ability of an antibody to recognize either single- (ss) or double-stranded (ds) nucleic acid antigens (Hahn et al., 1980). Antigenic subsets of anti-DNA antibodies have been reported and include individual bases (Munns et al., 1982a), nucleosides (Weisbart et al., 1982, 1983; Munns et al., 1982b), oligonucleotide sequences (Lee et al., 1981), Z and B configurations of dsDNA (Lafer et al., 1981a, 1983), and sugar–phosphate "backbone" structures (Stollar, 1975). The latter appear to recognize both ss and ds conformations to varying degrees and are believed to be specific for either sugar–phosphate backbones and/or portions of base structures exposed at the surface of dsDNA sequences in Z configurations (Lafer et al., 1983).

Investigations designed to evaluate antibody specificity frequently rely upon various competition–inhibition studies. For example, Hahn et al. (1980) classified various monoclonal preparations into anti-ss DNA and anti-dsDNA antibody categories on the basis of inhibiting their binding to [^{125}I]DNA with defined, unlabeled ss- and dsDNA competitors. Utilizing other competition assays and direct binding studies with nucleoside–BSA/Sepharose adsorbents, we recently determined that one of the above monoclonal antibodies (designated MRss-1) recognized ssDNA by virtue of the antigen's content of guanine residues, i.e., MRss-1 bound to ssDNA–agarose and G–BSA/Sepharose but not to A–, C–, U–, or T–BSA/Sepharose adsorbents (Munns et al., 1982a). Most recently,

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[1] Abbreviations: SLE, systemic lupus erythematosus; ds- and ssDNA, double- and single-stranded DNA; ELISA, enzyme-linked immunosorbent assay; A, adenosine; m^6A, N^6-methyl-A; G, guanosine; m_2^2G, N^2,N^2-dimethyl-G; m^7G, 7-methyl-G; C, cytidine; m^5C, 5-methyl-C; U, uridine; m^5U, 5-methyl-U or ribothymidine; BSA, bovine serum albumin; A–BSA, G–BSA, etc., nucleosides covalently linked to BSA; A–BSA/Sepharose, G–BSA/Sepharose, etc., nucleoside–BSA conjugates coupled to Sepharose; PO_4/NaCl, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane; Tris/NaCl, Tris-buffered saline; IgG, immunoglobulin G.

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