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Application of the gel shift assay to study the affinity and specificity of anti-DNA autoantibodies

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Abstract

We have demonstrated that the gel shift assay, a powerful method to study protein · DNA interactions under equilibrium conditions, is both an accurate and precise method to measure the affinity of anti-DNA · DNA immune complexes. One difficulty in performing gel shift assays is disruption of protein · DNA equilibria during the time needed for complexes to enter the gel matrix. However, we have found that highly cross-linked polyacrylamide gels, which are known to form non-restrictive matrices, do not perturb anti-DNA · DNA complexes. Using anti-ssDNA BV04-01 as a model antibody, we find good agreement between the dissociation constants (K_d) measured in the gel shift assay using a 5.4% polyacrylamide gel cross-linked with 0.6% (bis)acrylamide, and those obtained previously by fluorescence quenching. Because gel shift assays require only nanogram quantities of analyte and can be performed in several hours, it is well suited for a range of anti-DNA binding studies.

Keywords: Systemic lupus erythematosus; Anti-DNA autoantibody; Gel shift assay; Equilibrium dissociation constant

1. Introduction

A principal characteristic of the autoimmune disorder systemic lupus erythematosus (SLE) is antibodies that recognize both double-stranded and single stranded DNA (dsDNA and ssDNA, respectively) (Koffler et al., 1967, 1971, 1983; Voss and Casperson, 1988; Tan, 1989; Carson, 1991). The hypothesis that anti-DNA · DNA immune complexes can mediate the renal damage associated with SLE has stimulated efforts to determine the fine specificity of anti-DNA and to elucidate the autoreactive epitopes on nucleic acid antigens (Swanson et al., 1994). On a fundamental level such information should provide insight into the mechanism of clonal activation, and practically it may aid the development of new treatments for SLE, like antagonists of anti-DNA · DNA complexation (Ben-Chetrit et al., 1988).

Although anti-DNA have been studied extensively, the affinity of these antibodies for DNA antigens is not well understood (Koffler et al., 1967, 1971; Schwartz and Stollar, 1985; Pankewycz et al., 1987; Voss and Casperson, 1988; Sanford and Stollar, 1992). In part, this can be attributed to the methods used to characterize anti-DNA ·

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DNA complexes. For example, the enzyme-linked immunosorbent assay (ELISA), perhaps the most common technique used to measure the affinity of anti-DNA for DNA antigens, is prone to a range of artifacts many of which stem from the fact that solid-phase binding measurements enhance weak (non-specific) interactions through cooperativity and statistical additivity (Jencks, 1981; Stevens, 1987; Pesce and Michael, 1992; Seligman, 1994).

Several laboratories have demonstrated that assays conducted at equilibrium circumvent many of the problems associated with ELISA and related solid-phase assays. For example, Lee has found that by solid-phase radioimmunoassay monoclonal anti-dsDNA Jel 241 possesses roughly equal affinity for ssDNA and dsDNA, but when the measurements are repeated by equilibrium gel filtration, (non-specific) recognition of ssDNA is not observed (Lee et al., 1982). In addition, equilibrium assays have been used to evaluate the importance of bivalent cooperative binding (Papalian et al., 1980; Sanford and Stollar, 1992). For example, Braun and Lee have provided evidence that at least for some anti-DNA, bivalent binding affords a small energetic contribution to the stability of anti-DNA · DNA complexes at equilibrium (Braun and Lee, 1987).

While equilibrium binding techniques have provided the clearest understanding of the stability of anti-DNA · DNA complexes, many equilibrium assays like fluorescence quenching, gel filtration, and dialysis are limited by the amount of analyte required and the time needed to perform the measurements (Lee et al., 1982; Braun and Lee, 1987; Tetin et al., 1993). The gel shift assay independently introduced by both Crothers and Revzin is an attractive technique to study the affinity of proteins for DNA ligands under equilibrium conditions (Fried and Crothers, 1981, 1984; Revzin et al., 1986; Revzin, 1990; Carey, 1991). This assay can be performed in several hours, requires only nanogram quantities of both protein and DNA, and can measure dissociation constants (K_d) below micromolar directly, or in the millimolar range by competition.

The method entails application of a pre-equilibrated protein · DNA complex onto a non-denaturing polyacrylamide gel. The free DNA rapidly enters the matrix while the migration of the complex is retarded due to the molecular weight of the protein. To determine K_d values using the gel shift technique, a fixed concentration of DNA is titrated with an excess of protein, the bound and free DNA are separated, and the DNA concentrations are subsequently quantified. In addition, by varrying the salt concentration in the initial binding buffer (e.g., from 1 mM to 1 M NaCl), the ionic strength dependence on binding can also be measured (Weeks and Crothers, 1992; Swanson et al., 1994). Although the gel shift assay has proved useful for quantifying the stability of anti-DNA · DNA complexes (Chmielewsky and Schultz, 1991), disruption of the antibody · DNA equilibrium while the complex first enters the gel matrix has limited the use of this method. Here we describe a new gel matrix that overcomes this problem and demonstrate our results using anti-ssDNA BV04-01 (Ballard and Voss, 1985) as a representative test antibody. We find that the K_d values measured using the gel shift assay are in quantitative agreement with the equilibrium K_d values measured by fluorescence quenching.

2. Materials and methods

2.1. Antibody and DNA ligands.

Affinity purified anti-ssDNA BV04-01 (IgG2b,κ; Ballard and Voss, 1985) was generously provided by E.W. Voss, Jr. DNA ligands were synthesized on a MilliGen/Biosearch Cyclone Plus instrument and were purified by reversed-phase HPLC as previously described (Stevens et al., 1993; Swanson et al., 1994). DNA samples were 5′ end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and were purified on 20% polyacrylamide non-denaturing gels (Maxam and Gilbert, 1980).

2.2. Gel shift assay.

Non-denaturing polyacrylamide gels consisting of acrylamide (5.4%), *N,N′*-methylene-(bis)acrylamide (0.6%), ammonium persulfate (0.05%), and

N,N,N',N'-tetramethylethylenediamine (0.057%) were cast in a Hoefer Tall Mighty Small apparatus (10 cm × 12 cm × 0.75 mm). One hour after polymerization, the gels were pre-electrophoresed (4°C) at 106 V until the current was constant with time, which was typically 1 h. Dissociation constant titrations were conducted by incubating samples of BV04-01 (ten-fold on either side of the dissociation constant) with labeled DNA (0.1 nM) at 22°C for 1 h in TBE binding buffer (89 mM Tris · HCl, 89 mM boric acid, 2.5 mM EDTA, 150 mM NaCl, 5% sucrose (w/v), pH 8.3, 5 μl total volume). The reactions were then loaded onto a gel at 300 V for 90 s during which time the samples entered the matrix. Electrophoretic separation of the bound and free DNA was then conducted at 106 V for 30 min at 4°C. The gels were autoradiographed at −80°C for 16 h and DNA concentrations were quantified by densitometry using a molecular dynamics personal densitometer running the IMAGEQUANT software package. The data were fit via nonlinear least-squares regression to the single site binding isotherm:

$$\%\text{free DNA} = K_d/(K_d + [\text{antibody}]) \quad (1)$$

From equation 1, the apparent K_d corresponds to the protein concentration at which half the DNA is bound (Revzin, 1990). All measurements were performed in triplicate and the error represents the error to the curve fit (Stevens et al., 1993).

3. Results and discussion

One problem often encountered in gel shift assays is disruption of protein · DNA equilibria as the complex enters the gel matrix (Carey, 1991). This phenomenon arises because of unfavorable steric interactions between the complex and the gel matrix and leads to 'smearing' of the free DNA band, making data analysis difficult. The most common way to circumvent this problem is to decrease the degree of cross-linking in the gel to afford a less restrictive matrix. Yet we have found that gels containing very low amounts of cross-linking reagent (e.g., 63:1 acrylamide to (bis)acrylamide) still disrupt anti-DNA · DNA complexes. Stollar has addressed this problem using a system analogous to the 'stacking-resolving' gels used for protein separations (Sanford et al., 1988). Specifically, a 4% polyacrylamide gel cross-linked at a ratio of 32:1 acrylamide to (bis)acrylamide was poured on top of a 15% gel cross-linked at 19:1 ratio. Here the upper gel is designed to provide a matrix that allows the complex to enter the gel. However, in our hands this system still disrupts anti-DNA · DNA equilibria and K_d values cannot be measured accurately (Fig. 1).

Interestingly, at very high cross-linking ratios the fibers in the polyacrylamide gels supercoil and present less of a hindrance to mobility (Chrambach, 1985). For example, a 1 MDa protein migrates freely in a 6% polyacrylamide gel containing a 9:1 ratio of acrylamide to (bis)acrylamide. Indeed, we have found that gels with a high degree of cross-linking do not perturb anti-DNA · DNA equilibria and in principle are ideal to measure K_d values of anti-DNA · DNA complexes using the gel shift assay. For example, in contrast to the data given in Fig. 1, the densit-

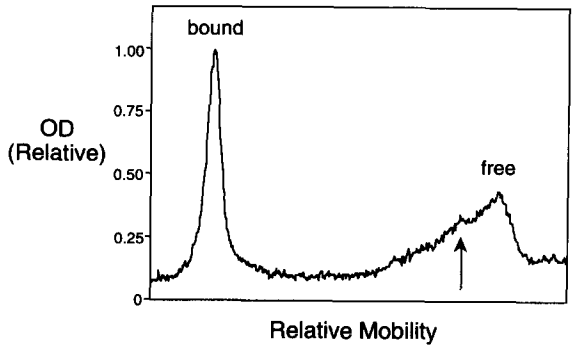

Fig. 1. Densitometry trace of a BV04-01 · DNA complex electrophoresed using the stacking-resolving gel system (Sanford et al., 1988). Although the bound DNA runs as a Gaussian-shaped peak, the free DNA appears as two unresolved peaks, which results from dissociation of the complex. The area under the arrow denotes DNA from dissociation of the complex. In principle, differences between the ionic strength of binding buffer and the running buffer/gel matrix can affect protein · DNA complexes during the assay dead time. However, this is generally not a problem (Carey, 1991; Weeks and Crothers 1992; Swanson et al., 1994). This would also give rise to smearing which is readily apparent.

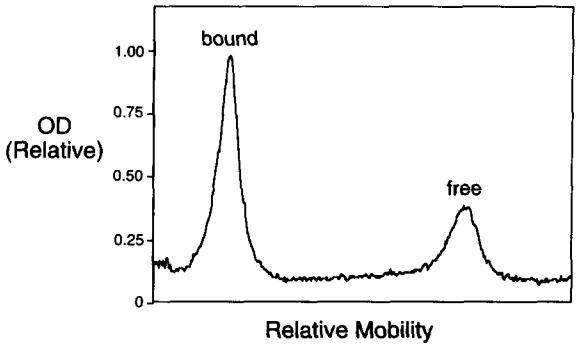

Fig. 2. Densitometry trace of BV04-01·DNA complex electrophoresed on a 5.4% polyacrylamide gel containing a 9:1 ratio of acrylamide to (bis)acrylamide. In contrast to the data presented in Fig. 1, both the bound and free DNA bands run as well-resolved Gaussian-shaped peaks which suggests that the gel matrix does not dirupt the complex.

ometry plot shown in Fig. 2 shows that both the free and bound DNA appear as distinct Gaussian-shaped peaks which can be cleanly integrated.

We next employed a highly cross-linked polyacrylamide matrix in gel shift assays to examine the specificity and affinity of anti-ssDNA BV04-01 toward a panel of ssDNA homopolymers. We chose this monoclonal antibody for our initial experiments because its binding properties have previously been examined in detail, both by solid-phase techniques (Ballard and Voss, 1985) and by fluorescence quenching conducted at equilibrium (Tetin et al., 1993). The results from the gel shift affinity titrations indicate that BV04-01 binds oligo(T) tighter than the other three homopolymers and is consistent both with solid-phase and equilibrium binding data (Table 1 (**A**), Fig. 3). However, our findings also indicate that BV04-01 possesses a modest affinity for oligo(dG), a result that is not found in solid-phase assays, but is observed by equilibrium fluorescence quenching (Tetin et al., 1993).

The affinity differences between the homopolymer ligands may reflect either a large intrinsic preference for thymidine or small variations in affinity for the four bases that are amplified through statistical binding additivity (Jencks, 1981). This latter hypothesis is supported by the finding that BV04-01 binds short thymidine

Table 1
Apparent dissociation constants for BV04-01·DNA complexes (K_d values are μM per IgG binding site)

A		**B**	
A_{21}	> 20	$A_8A_5A_8$	> 20
C_{21}	15 ± 2	$A_8C_5A_8$	> 20
G_{15}	0.14 ± 0.014	$A_8G_5A_8$	6.1 ± 0.4
T_{21}	0.0044 ± 0.00042	$A_8T_5A_8$	3.7 ± 0.3

The data were obtained as described in the materials and methods section. **A**: BV04-01·homopolymer complexes. G_{15} was used because G_{21} is not synthetically accessible. **B**: BV04-01·epitope complexes. Binding to just the N_5 epitope was confirmed by DNA footprinting (data not shown). See text for discussion.

oligomers (Ballard and Voss, 1985), so that the N_{21} ligands must possess multiple overlapping binding sites with respect to BV04-01 recognition. To distinguish between these two possibilities we measured binding to ligands that contain only a single pentanucleotide DNA epitope, specifically, A_8-N_5-A_8. In designing these constructs we selected to flank the binding epitope (N_5) with A_8 because it does not bind to BV04-01. In addition,

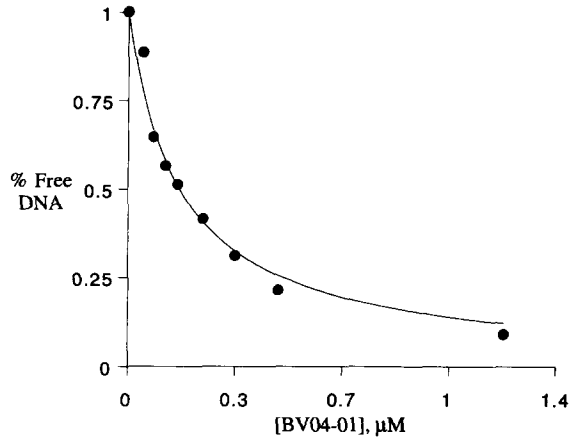

Fig. 3. Representative binding isotherm. Each data point (in this case for BV04-01·G_{15}) represents the average from three different binding titrations. These data were fit using equation 1, and afforded a K_d of 137 ± 14 nM ($\chi^2 = 0.03$). Since the K_d values obtained using equation 1 assume that the concentration of the complex is small, the lowest protein concentration used in the binding titrations is 100-fold greater than the DNA concentration (Carey, 1991).

21-mers were chosen rather than just N_5 so the number of phosphate groups would be identical to the 21-base long homopolymers, thereby accounting for any non-specific electrostatic interactions. The data in Table 1 (**B**) show that affinity of BV04-01 for the N_5 epitopes is greatest for T_5 followed by $dG_5 > dC_5 \gg dA_5$, in good agreement with the equilibrium K_d values measured by fluorescence quenching (Tetin et al., 1993). With the exception of adenosine, the differences in affinity between the bases is relatively small compared to binding of the N_{21} ligands. These results suggest that the large differences in affinity between the homopolymer ligands arise from small differences in binding free energy that are amplified by statistical additivity (Jencks, 1981).

To summarize, we have demonstrated that the gel shift assay can be used to study the affinity and specificity of anti-DNA. Using anti-ssDNA BV04-01 as a representative antibody, we find that the affinities observed using the gel shift assay are in qualitative agreement with previous ELISA data for this antibody and are in close quantitative agreement with K_d values measured by fluorescence quenching. In more recent experiments we have used the gel shift assay to measure equilibrium binding constants for a panel of ten IgG murine monoclonal anti-DNA of varrying isotype, possessing affinity for either ssDNA or dsDNA (Swanson and Glick, manuscript in preparation). Again we find that the K_d values obtained with the gel shift assay (between 0.3 nM and 1 μM) correspond well with binding constants measured by fluorescence quenching. Thus we believe that the gel shift assay compliments the exisiting methodology to study anti-DNA · DNA interactions, and should be quite useful in addressing more quantitative aspects of immune complex formation and stability.

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References

Ballard, D.W. and Voss, Jr., E.W. (1985) Base specificity and idiotype of anti-DNA autoantibodies reactive with synthetic nucleic acids. J. Immunol. 135, 3372–3380.

Ben-Chetrit, E.D., Eliat, D. and Ben-Sasson, S.A. (1988) Specific inhibition of the DNA-anti-DNA immune reaction by low molecular weight anionic compounds. Immunology 65, 479–485.

Braun, R.P. and Lee, J.S. (1987) Equilibrium binding parameters of an autoimmune monoclonal antibody specific for double-stranded DNA. J. Immunol. 139, 175–179.

Carey, J. (1991) Gel retardation. Methods Enzymol. 208, 103–117.

Carson, D.A. The specificity of anti-DNA antibodies in systemic lupus erythematosus. (1991) J. Immunol. 146, 1–2.

Chmielewsky, J. and Schultz, P.G. (1991) Monoclonal antibodies with sequence specific affinity for a stem - loop structure in DNA Tetrahedron 47, 2563–2572.

Chrambach, A. (1985) The practice of quantitative gel electrophoresis. V. Neuhoff and A. Maelicke (Eds.), VCH. Deerfield Beach, FL, pp. 62–68.

Fried, M.G. and Crothers, D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucl. Acids Res. 9, 6505–6522.

Fried, M.G. and Crothers, D.M. (1984) Kinetics and mechanism in the reaction of gene regulatory proteins with DNA. J. Mol. Biol. 172, 263–282.

Jencks, W.P. (1981) On the attribution and additivity of binding free energy. Proc. Natl. Acad. Sci. USA 78, 4046–4050.

Koffler, D., Schur, P.H. and Kunkel, H.G. (1967) Immunological studies concerning the nephritis of systemic lupus erythematosus. J. Exp. Med. 126, 607–624.

Koffler, D., Carr, R., Agnello, V., Thoburn, R. and Kunkel, H.G. (1971) Antibodies to polynucleotides in human sera: antigenic specificity and relation to disease. J. Exp. Med. 134, 294–312.

Koffler, D., Miller, T.E. and Faiferman, I. (1983) Antipolynucleotide antibodies: the rheumatic connection. Hum. Pathol. 14, 406–418.

Lee, J.S., Dombroski, D.F. and Mosmann, T.R. (1982) Specificity of autoimmune monoclonal Fab fragments binding to single-stranded deoxyribonucleic acid. Biochemistry 21, 4940–4945.

Maxam, A.M. and Gilbert, W. (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65, 499–560.

Pankewycz, O.G., Migliorini, P. and Madaio, M.P. (1987) Polyreactive autoantibodies are nephritogenic in murine lupus nephritis. J. Immunol. 139, 3287–3294.

Papalian, M., Lafer, E., Wond, R., and Stollar, B.D. (1980) Reaction of systemic lupus erythematosus antinative DNA antibodies with native DNA fragments from 20 to 1,200 base pairs. J. Clin. Invest. 65, 469–477.

Pesce, A.J. and Michael, J.G. (1992) Artifacts and limitations of enzyme immunoassay. J. Immunol. Methods 150, 111–119.

Revzin, A. (1990) Analyzing DNA-protein interactions by gel electrophoresis. Concepts Biochem. 67, 749–753.

Revzin, A., Ceglarek, J.A. and Garner, M.M. (1986) Comparison of nucleic Acid-protein interactions in solution and in polyacrylamide gels. Anal. Biochem. 153, 172–177.

Sanford, D.G. and Stollar, B.D. (1992) Assay of anti-DNA antibodies. Methods Enzymol. 212, 355–371.

Sanford, D.G., Kortkow, K.J. and Stollar, B.D. (1988) Immunochemical detection of multiple conformations within a 36 base pair oligonucleotide. Nucleic Acids Res. 16, 10643–10655.

Schwartz, R.S. and Stollar, B.D. (1985) Origins of anti-DNA autoantibodies. J. Clin. Invest. 75, 321–327.

Seligman, S.J. (1994) Influence of solid-phase antigen in competition enzyme-linked immunosorbent assays (ELISAs) on calculated antigen-antibody dissociation constants. J. Immunol. Methods 168, 101–110.

Stevens, F. (1987) Modification of an ELISA-based procedure for affinity determination: Correction necessary for use with bivalent antibody. Mol. Immunol. 24, 1055–1060.

Stevens, S.Y., Swanson, P.C., Voss, Jr., E.W. and Glick, G.D. (1993) Evidence for induced fit in autoantibody·DNA complexes. J. Am. Chem. Soc. 115, 1585–1586.

Swanson, P.C., Cooper, B.C., and Glick, G.D. (1994) High resolution epitope mapping of an anti-DNA autoantibody using model DNA ligands. J. Immunol. 152, 2601–2612, and references therein.

Tan, E.M. (1989) Anti nuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. Adv. Immunol. 44, 93–151, and references therein.

Tetin, S.Y., Rumbley, C.A., Hazlett, T.L. and Voss, Jr., E.W. (1993) Elucidation of anti-ssDNA autoantibody BV04-01 binding interactions with homooligonucleotides. Biochemistry 32, 9011–9017.

Weeks, K.M. and Crothers, D.M. (1992) RNA binding assays for Tat-derived peptides: implications for specificity. Biochemistry 31, 10281–10287.

Voss, Jr., E.W. and Casperson, G.F. (1988) In: E.W. Voss, Jr. (Ed.), Anti-DNA Antibodies in SLE. CRC Press, Boca Raton, FL, p. 1.