Dual Reactivity of Several Monoclonal Anti-nucleosome Autoantibodies for Double-stranded DNA and a Short Segment of Histone H3*

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We have shown previously that four IgG monoclonal autoantibodies (mAbs) reacted in ELISA with both double-stranded (ds) DNA and peptide 83–100 of histone H3. The peptide 83–100 contains a cysteine residue at position 96 and readily dimerizes at pH 7–8. We describe here that only the 83–100 dimers, and not the 83–100 monomers, are recognized by the four antibodies and inhibit in ELISA the binding of mAbs to dsDNA. The equilibrium affinity constants (Kₒ) and kinetic rate constants of two of these mAbs were measured in a biosensor system. Kₒ values were significantly higher when these mAbs were tested with dsDNA as compared with the 83–100 dimer. Further higher Kₒ values were measured with mononucleosomes containing DNA and histones. It is proposed that these four mAbs are directed against a topographic determinant formed by DNA and the region 83–100 of H3 present as a dimer at the surface of nucleosome, and that they react, although significantly less well, with DNA and peptide dimer tested separately. This study provides a quantitative and kinetic basis to interaction between several antibodies and distinct antigenic structures and allows us to better understand the structural basis of apparent autoantibody cross-reactivity.

Numerous observations have implicated anti-DNA antibodies and nucleosomal antigens in the pathology of systemic lupus erythematosus (1, 2). Antibodies to DNA play a major role in lupus nephritis, their titers correlate with disease activity, and deposits of anti-DNA-containing immune complexes are found in the kidneys of lupus patients. Histones, and apparently also nucleosomes, can be detected within glomerular deposits (3–5), and anti-DNA, anti-histone, as well as anti-nucleosome antibodies can be eluted from kidneys of (NZB/W)F1 and MRL lpr/lpr lupus mice (6, 7).

The trigger antigen giving rise to anti-DNA antibodies has not been strictly identified but a number of evidences strongly support that nucleosomes (the basic repeating unit of chromatin) may represent a potential immunogen (8, 9). Nucleosomestructure and distinct antigenic structures and allows us to better understand the structural basis of apparent autoantibody cross-reactivity.

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The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid chromatography; RU, resonance units; PBS, phosphate-buffered saline; RAM, rabbit anti-mouse.


Monoclonal Anti-nucleosome Antibodies

Nucleosomes, Histones, and Histone Peptides—Calf thymus nuclei were prepared as described previously (17). They were resuspended in 15 mM Tris buffer (pH 7.4) containing 15 mM NaCl, 60 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 0.25% sucrose and digested at 30 °C with micrococcal nuclease. The nuclei were lysed at 0 °C for 30 min in 1 mM Tris-Cl buffer (pH 7.4) containing 0.2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation (10 min at 4,000 x g at 4 °C), 150–200 A₂₆₀ units of digested chromatin (supernatant fraction) were layered on 5–29% (w/v) sucrose gradients buffered in 10 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 0.2 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride and centrifuged for 21 h at 4 °C in a SW28 Beckman rotor at 25,000 rpm. The gradients were fractionated (0.5 ml/fraction), and the absorbance (260 nm) of each fraction was measured. The preparations were then characterized by 2% agarose gel electrophoresis, and fractions containing mononucleosomes were pooled and kept at 4 °C for a maximum of 5 days. They were never frozen. The content in histones of each nucleosome preparation was checked by 15% polyacrylamide electrophoresis.

Histone H3 used in this study was obtained from calf thymus and purified as described (18). Its purity was assessed by 18% polyacrylamide gel electrophoresis. Peptide 83–100 of H3 has been described in previous studies (18, 19). Peptides partially overlapping the sequence 83–100, namely peptides encompassing residues 79–92, 91–104, and 98–112 were prepared (Table I). Calf thymus sequences were used for all syntheses. The purity of all peptides was assessed by analytical HPLC on a nucleosil C8 column, 5 μm (3.9 x 15 mm), using a triethylammonium phosphate buffer system. Peptides were purified using a medium pressure chromatography apparatus. Amino acid analysis and electrospray mass spectra showed that the purified peptides had the expected composition. Dimers of H3 peptides 83–100, 91–104, and 98–112 were prepared by dissolving peptides in phosphate-buffered saline (PBS) (pH 7.4) containing 2% (w/v) dimethyl sulfoxide as oxidizing agent and keeping the solution at air and at room temperature for 3–4 days before use. The presence of dimers was checked by HPLC, and the products were analyzed by fast atom bombardment mass spectrometry. The dimer solution was stable for at least 2 months.

Enzyme-linked Immunosorbent Assay (ELISA)—The direct ELISA procedure used to measure the binding of mAbs was as described previously (20) using plates coated with 100 ng/ml H3 in 0.05 M carbonate buffer pH 9.6 or with 2 mM NaCl and without P20, were then injected at a flow of 2 μl/min on the streptavidin precoated chip. This procedure allowed about 20 BU (20 pg of DNA/mm²) to be immobilized on the chip. The surface was then washed with 5 μl of HBS containing 0.05% (v/v) SDS. To study the binding of mAbs to mononucleosomes, mAbs were immobilized by trapping them on sensor chips containing covalently bound RAM Fc according to the manufacturer's instructions. The binding experiments (pulses with the various analytes used between 1 and 250 nm on the specific surfaces) were performed at 25 °C and at a flow rate of 5, 10, and 40 μl/min. Antibody concentrations were determined according to Karls son et al. (23). Injection times were from 4 to 8 min, and the postinjection phase duration was 10 min. Conditions used were established to avoid mass transport effects (24). The regeneration step was optimized for each antigen. The surfaces were then regenerated during 1 min with 10 mM acetate buffer (pH 6) in the case of the matrix with peptide dimer, HBS containing 0.05% SDS and P20 in the case of the dsDNA matrix and 0.1 M HCl in the case of the RAM Fc matrix. Both the procedure used to measure the antibody kinetic constants and the theory of kinetic measurements using the BiaCore biosensor system have been reviewed recently (25).

RESULTS

In our former study dealing with the characterization of the four mAbs 2, 42, 53, and 56, direct ELISA format was used in which dsDNA, histones, and histone peptides were directly adsorbed to plastic solid phase. In Fig. 1, we show the results of inhibition experiments performed by using dsDNA as coated antigen and several peptides covering the region 79–112 of H3 (Table I) as fluid phase competitors. These peptides partially overlap the region 83–100. During the course of this study, we found that mainly dimers of peptide 83–100, and not monomers of this peptide, were significantly able to inhibit the reaction of mAbs to dsDNA (as exemplified with mAb 56 in Fig. 1). Dimers of peptide 83–100 were obtained by dimethyl sulfoxide-mediated disulfide formation at 20 °C and pH 7.4 (26). Monomeric...
peptides 79–92, 91–104, and 98–112 and dimers of peptides 91–104 and 98–112 inhibited weakly (<30%) or not at all the antibody reaction to dsDNA (Fig. 1). This inhibition was not observed with mAbs 36 and 51, which react with dsDNA but not with 83–100 dimers (Fig. 1).

Thus, the 83–100 dimer efficiently competes with DNA for the binding of mAbs to dsDNA. Reciprocally, we found that the binding of mAbs 2, 42, 53, and 56 to 83–100 peptide dimers could be very efficiently inhibited by dsDNA (Fig. 2). Up to nearly 100% inhibition was reached with about 5–25 ng/ml competitor dsDNA (according to mAbs), both when dsDNA was used as antigen and competitor (Fig. 2A) and when dsDNA was used to inhibit the binding of antibodies to the 83–100 dimer (Fig. 2B).

These results clearly indicate that four mAbs are able to react with both dsDNA and a unique peptide structure contained in the 83–100 dimer. The fact that 91–104 dimers showed much weaker activity than 83–100 dimers with mAbs supports the conclusion that these mAbs do not only react with the flanking residues of the disulfide bridge.

The two mAbs, 42 and 56, were produced in larger amounts, extensively purified to remove all bound nuclear material, and their capacity to recognize dsDNA and 83–100 dimers was further measured in the BIAcore using either the peptide covalently linked to the dextran matrix through its free NH2 terminus or biotinylated dsDNA fragments immobilized onto the biosensor surface covalently precoated with streptavidin. We also studied the reactivity of mAbs with calf thymus mononucleosomes. The BIAcore biosensor system based on surface plasmon resonance detection permits the quantitative analysis of biomolecular interactions in real time. One of the molecular partners is immobilized on a dextran matrix coupled to a thin gold film, while the other one is introduced in a continuous flow passing over the sensor surface. An optical system detects changes in refractive index close to the metal surface, which allows the concentration of the reactants to be measured. The binding signal is continuously monitored and is translated into a sensorgram, expressed in RU over time. There are at least three major advantages of using biosensors for molecular interaction measurements: (i) molecules don’t have to be labeled, (ii) each step of the reaction can be directly and instantaneously visualized, and (iii) accurate affinity and kinetic constants can be easily measured. As discussed previously (27), the structure of nucleosomes may be considerably altered if they are covalently bound to the dextran matrix on the sensor chip via amino groups, particularly because histone tails, which are very basic, play an important role in the stabilization of the edifice. To overcome this problem, one can either present nucleosomes by a first antibody (for example directed against one of the constitutive histones) and study the binding of antibodies directed against another histone (27) or one can capture the murine mAb under study with a first antibody directed against mouse Ig and then pulse nucleosomes used in this case as analytes in the fluid phase. The latter procedure was used in this study with mAbs 42 and 56. Several concentrations (1–250 nm) of nucleosomes were allowed to react with immobilized mAbs (300 RU), and flow rates of 5, 10, and 40 μl/min were used. Kinetic rate constants and equilibrium affinity constants of mAbs for the 83–100 dimer, dsDNA, and nucleosomes are shown in Table II. It appears clearly that in the BIAcore system, both mAbs 42 and 56 preferentially bound dsDNA compared with the 83–100 dimer. The equilibrium affinity constants, $K_a$, of mAbs 42 and 56 were, respectively, 13 and 7 times higher for dsDNA as compared with the 83–100 dimer. In both cases, this was essentially due to a higher association rate constant, $k_a$. Interestingly, mAbs 42 and 56 were found to strongly react with nucleosomes. Equilibrium affinity values $K_a$ of both mAbs 42 and 56 for calf thymus mononucleosomes were $2.7 \times 10^{10} M^{-1}$, i.e. 25 and 15 times higher than $K_a$ values measured for dsDNA, and 332 and 102 times higher than $K_a$ values measured for the 83–100 dimer. As compared with binding to dsDNA, this increase in $K_a$ values was mainly due to lower $k_a$ values. In a control experiment performed with mAb 56, we found that when presented by RAM Fc, this mAb, as in direct ELISA format, did not recognize H3 or H3 dimers used as ligands. Reciprocally, mAb 66 used as ligand did not bind H3 covalently linked to the dextran matrix.

A few experiments were performed with Fab fragments prepared from mAb 56. We found that the respective $K_a$ values of Fab 56 for the 83–100 dimer and dsDNA were six to seven times lower (i.e. $3.7 \times 10^8$ and $300 \times 10^6 M^{-1}$) than the $K_a$ values ($270 \times 10^8$ and $1870 \times 10^6 M^{-1}$) of mAb 56 for these antigens. Constants $k_a$ were of the same order; the lowered $K_a$ values were due to higher $k_a$ values as to be expected when monovalent binding occurs. In both cases, was thus the association of mAb 56 with the 83–100 dimer and dsDNA essentially bivalent, and strictly speaking, it was "avidity" rather than "affinity" of antibody that was measured in this study.

In a further study using the BIAcore system, we confirmed that the same antibody population bound dsDNA and 83–100 dimer, mAb 56 was first allowed to react with the 83–100 dimer and then recovered by injecting 10 mM acetate buffer (pH 6). These antibodies were immediately reinjected on a sensor chip presenting dsDNA, and expected RU could be measured.

We could exclude the possibility that Fc domains were involved in the antibody binding as in the three assays described (plates with DNA or 83–100 dimer and chips with nucleosome), RAM Fc was used to reveal bound mAbs or to capture bound
mAbs. Furthermore, as described above, Fab 56, as mAb 56, bound both dsDNA and 83–100 dimer.

**DISCUSSION**

DNA antibodies have been shown by several investigators to cross-react with diverse nuclear or non-nuclear components. In some cases, the cross-reaction involves structures where the similarity can be rationalized, such as cardiolipin, which has two phosphate groupings approximately the same distance apart as those on DNA. Even in this case, it has been suggested that only anti-DNA antibodies of relatively low avidity extensively cross-react with cardiolipin, while high-avidity antibodies do not (12). More frequently, DNA antibodies reacting with cell-surface proteins and also with extracellular matrix proteins have been described (e.g. Refs. 28–30). However, no detailed kinetic affinity studies of any of these cross-reactions have been undertaken. Moreover, as pointed out previously, some of these reactivities may be mainly due to the presence of nucleosomal material complexed to the antibody (1).

The results described herein further establish our recent finding that several monoclonal antibodies generated from autoimmune mice and extensively purified react with both dsDNA and peptide 83–100 of H3 present as a dimer involving a disulfide bond linking cysteine residues 96 and provide a quantitative support to this apparent cross-reaction. Several explanations for our observations can be proposed.

First, one can argue that mAbs 2, 42, 53, and 56 can be ranged among the so-called polypeptic antibodies. However, in general, these polyreactive antibodies are of the IgM isotype, have affinities that tend to be low (with $K_a$ values between $10^3$ and $10^4$ m$^{-1}$; Ref. 31), and react with multiple autoantigens.

Second, an alternative explanation is that the antibodies bind with dsDNA and 83–100 dimers through different binding sites located on the antibody variable regions, in a mechanism of “multireactivity,” as reviewed recently (32). We have reported previously that the double reactivity of several rheumatoid factors for IgG and histones was related to distinct binding sites (33, 34). Topographic mapping of these sites was performed by using the whole histones and histone peptides in inhibition experiments and reinforced by using murine monoclonal anti-idiotype antibodies reacting with distinct idiotopes on the rheumatoid factors.

Third, an explanation for the specificity of monoclonal antibodies examined in this study is that mAbs are directed against a topographic determinant constituted by a segment of DNA associated with an epitope normally found in the (H3-H4)$_2$ tetramer region near the surface of the octamer core of the nucleosome. This hypothesis referring to a mechanism called “dual reactivity” (32) is supported by the finding that the antibodies have a very high $K_a$ value for nucleosome (around $2.7 \times 10^{10}$ m$^{-1}$) and lower $K_a$ values for dsDNA and 83–100 dimers. It is probably because the initial affinity particularly high with the nucleosome that reaction with parts of the original epitope is still detectable both in ELISA and in the BIAcore.

In order to reinforce our assumption, we have analyzed our results with regards to the crystal structure of the histone octamer that has been resolved at 3.1 Å (35–37). A close examination of the chicken octamer structure showed that the 83–100 domain of H3 is only partially surface-oriented. Residues 83–87 define the boundaries of the path of the polypeptide as it emerges to and “dives away” from the surface of the octamer. Residues 88–100 appear to be buried in octamer and not available for surface-probing by ligand molecules, and the two H3 residues at sites 96 are not close to each other. In the nucleosome model built from x-ray crystallographic data of the chicken octamer (35–37), the 83–87 residues are predicted to be located in a DNA binding area and would be under the path of the double helix. Thus we have difficulty in explaining how the mAbs can cross-react with mononucleosomes in view of the current nucleosome models. We can argue, however, that the crystal structure was obviously obtained in a chemical environment very different from the reaction conditions used with antibodies and involved the complete octamer and not the nucleosome assembly. Furthermore, it has to be pointed out that the crystal structure of the histone octamer was solved from chicken erythrocyte and not from mammal nuclear material. In birds and fishes, the cysteine residue 96 (found in human, bovine, and murine H3, for example) is replaced by a serine residue. As we have shown that the 83–100 dimer, but not the 83–100 monomer, was recognized by the four mAbs tested in this work, it might be concluded that in mammal octamer, cysteine residue 96 plays an important role and that this region assembles in a slightly different shape compared with chicken. In this regard, it is interesting to note that cysteine 96 has been reported to be more reactive to sulfhydryl reagents, and for this it has been suggested that it might be located close to the octamer surface (38, 39).

Several authors have recently pointed out the fundamental role of anti-nucleosome antibodies in the pathogenesis of lupus. It is probable that many studies based on the use of purified nuclear proteins or DNA tested separately have obscured an important part of the antibody reactivity underestimating specificity for nucleosomes (10). It is possible that it is precisely because such subsets of antibodies are able to interact with the complete nucleosome structure and with individual components of this structure that they have a pathogenic role, in particular in lupus nephritis (1). In view of our present knowledge of the nucleosome structure, our results may further suggest that nucleosomes in an abnormal conformation have triggered the production of these cross-reactive antibodies.

This study presents the first detailed kinetic analysis of the interactions between several antibodies and apparently distinct antigenic determinants. A more definitive picture should be obtained by combining the present results and informations derived from the sequence of variable regions of these antibodies (30). All these data allow us to better understand the structural basis of autoantibody reactivity. Finally, this analysis shows that perhaps a large number of autoantibodies defined as anti-dsDNA antibodies on the basis of the Farr assay (re-
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