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Chromatin Specificity of Anti-Double-Stranded DNA Antibodies and a Role for Arg Residues in the Third Complementarity-Determining Region of the Heavy Chain

Amanda M. Guth, Xianghua Zhang, Diana Smith, Thiago Detanico, and Lawrence J. Wysocki

A spontaneous, autoreactive autoantibody called SN5–18 (IgG2b, κ) binds to a complex of H2A/H2B/dsDNA in chromatin, but erroneously appears to bind dsDNA when the Ab is used in a form that is not highly purified. Because of this finding, we evaluated the antigenic specificity of a prototypic anti-dsDNA Ab, 3H9/Vκ4, now used widely in transgenic studies of tolerance and autoimmunity. We found that the purified mAb 3H9/Vκ4 binds chromatin and specifically a complex of H2A/H2B/dsDNA, but not dsDNA in solid phase or in solution. When used in the form of culture supernatant or as a standard protein G preparation, mAb 3H9/Vκ4 appears to bind dsDNA, apparently due to nuclear proteins in the preparation that assemble on target DNA. Because of the reported role of VκCDR3 Arg residues in dsDNA binding and the near identity of the SN5–18 sequence to other dsDNA-specific Ab, we tested the contributions of two VκCDR3 Arg residues in SN5–18 to chromatin specificity. We found that both these Arg residues at positions 104 and 106 were required for detectable chromatin binding. These results indicate a role for VκCDR3 Arg residues in chromatin specificity of lupus-derived autoantibodies. Further, they provide an explanation for a possible discrepancy in the form of tolerance observed in different anti-DNA Ig transgene models. The Journal of Immunology, 2003, 171: 6260–6266.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the synthesis and secretion of autoantibodies that are frequently directed against nuclear Ag such as dsDNA, chromatin, and ribonucleoprotein components (1, 2). These autoantibodies contribute to disease in part by forming immune complexes that may deposit in the glomeruli of kidneys, often precipitating kidney failure and death (1, 2). Mouse models of lupus, such as the MRL/Mp-lpr/lpr strain, and F1 hybrids between either NZB and SWR or NZB and NZW strains have been exploited in studies to address the pathological mechanism of autoantibody production. A considerable body of evidence obtained from these animal models supports the idea that lupus autoantibodies are products of T cell-dependent immune responses. As such, lupus autoantibodies often bind autoantigens with significant avidity. They are products of oligoclonal B cell responses and class switch recombination, and their V region structures are frequently modified by somatic hypermutation (3–9).

Postulated mechanisms that lead to a break in tolerance and escape of autoreactive B cells are heavily dependent upon accurate knowledge of the antigenic specificities of lupus autoantibodies. In one study it was suggested that the precursor B cell to an autoreactive lineage (clone) initially expressed a receptor with specificity for ssDNA (9, 10). This was later supported by a mutagenesis analysis (10). Somatic hypermutation apparently created the anti-dsDNA specificity of the widely studied 3H9/Vκ4 member of this clone. This is an important conclusion because ssDNA specificity is common among nonpathological Ab, while dsDNA specificity is considered pathological and a hallmark of lupus. Other studies also have implied that positively charged amino acids, particularly Arg residues in VκCDR3, are important and sometimes essential for conferring dsDNA specificity (3, 4, 11–20).

The importance of accurately defining antigenic specificity is illustrated by the paradox that lupus autoantibodies often appear to bind multiple and seemingly disparate antigenic structures (3, 21–30). For example, Bloom et al. (3) reported one such Ab with affinity for both dsDNA and La, a common nuclear protein autoantigen. Similarly, we found that culture supernatants of a lupus-derived hybridoma (7) contained autoantibodies that appeared to bind individually to dsDNA and histone H2A/H2B complexes. Highly purified preparations of this Ab, however, bound only to a complex of H2A/H2B and dsDNA (31). Nuclear exudates in the culture supernatant apparently were able to provide components that complement the immobilized Ag (histones or dsDNA) that were the intended targets in our immunoassays. It is noteworthy that our Ab (SN5–18) is nearly identical in sequence to both the 3H9/Vκ4 member of this transgenic model and Dna4 (17).

The finding that specificity can be obscured in binding assays that involve unpurified autoantibody preparations prompted us to more closely examine the prototypic anti-dsDNA lupus Ab, 3H9/Vκ4, which is currently the subject of widespread studies of tolerance and autoimmunity. Conclusions of tolerance studies in transgenic mouse models that use the mAb 3H9 VκH gene have been inconsistent, even though B cells in the various models ostensibly bind the same autoantigen, dsDNA. We found that highly purified mAb 3H9/Vκ4 binds chromatin, specifically H2A/H2B/dsDNA complexes, but not dsDNA. Because our autoreactive Ab with specificity for histone/dsDNA complexes contained...
V$\gamma$CDR3 Arg residues, which have been associated with specificity for dsDNA, we also examined the role of V$\gamma$CDR3 Arg residues in chromatin specificity. We found that two V$\gamma$CDR3 Arg residues in the unmutated precursor to the SN5–18 autoantibody are required for binding to the histone/DNA complex.

Materials and Methods

Antibodies

SN5–18R (IgG2b, κ) is a germline-reverted version of a chromatin-binding Ab that was previously described (7, 31). We reverted three natural somatic mutations (one silent) in the heavy chain gene encoding the original Ab to their known germline sequence to produce the Ab, SN5–18R. These alterations affected neither its affinity nor its specificity compared with the original Ab (31). We generated additional mutants lacking the V$\gamma$CDR3 Arg residues as described below. The 3H9/V$\kappa$4 hybridoma (IgG2b, κ) was provided by Dr. A. Marshak-Rothstein (Boston University, Boston, MA) (5). We confirmed the identity of its rearranged V$\kappa$4–J$\kappa$1 gene by sequencing an amplified product of genomic DNA from the hybridoma. H241 (IgG2b, κ) is an anti-dsDNA Ab and was a gift from Dr. D. Stollar (Tufts University, Boston, MA). Control Ab include mAb 36–71 (IgG1, κ), mAb 36–65 (IgG1, κ) and JRB (IgG2b, κ). All three bind the hapten p-azophenylsulfate.

Production and purification of Abs

Ab were purified as previously described (31). Either ascites or culture supernatant was passed through a protein G column, and Ab was eluted with a 0.5 M NaCl/0.1 M glycine, pH 2.5, solution and dialyzed against PBS. Eluate from this column has been termed protein G purified. The dialyzed material was subjected to microcentrifugation to remove any precipitate, and the supernatant was incubated with DNase I (1 μg/ml; Worthington Biochemical, Lakewood, NJ) for 90 mm at 37°C in the presence of 2 mM MgCl$_2$. After DNase treatment, the Ab was passed through a Sepharose column conjugated with affinity-purified goat anti-mouse Ig (heavy and light chain specific; Zymed, San Francisco, CA). Before elution, the column was washed extensively with 1 M NaCl in PBS) to disrupt immune complexes by dissociating histones associated with DNA. Bound Ab was eluted with the glycine solution described above. The eluate was dialyzed against PBS and is referred to as 2x purified in the text. Ab purity was assessed by SDS-PAGE. To ensure that equivalent concentrations of Ab in the different preparations were used in binding assays, fine adjustments in Ab concentration were made after quantification in a europium-based immunoassay (32). Briefly, 96-well microtiter plates (Costar 3539; Corning, NY) were coated overnight with 10 μg/ml of rat anti-mouse IgG2b (clone R12-3; BD Pharmingen, San Diego, CA). Coated plates were washed with PBS and blocked with a PBS solution containing BSA (2 mg/ml) and gelatin (1 μg/ml). Ab were added at various dilutions, followed by 0.5 μg/ml biotin-labeled rat anti-mouse κ and finally 50 ng/ml streptavidin-conjugated Eu$^{3+}$ (PerkinElmer/Wallac, Turku, Finland). Bound Eu$^{3+}$ was chelated and detected with a time-resolved fluorometer (VICTOR2; Wallac) using an excitation wavelength of 340 nm and an emission wavelength of 615 nm. Ab concentrations were then normalized and used in subsequent binding assays.

Binding assays

To test for dsDNA specificity, 96-well microtiter plates (Falcon 3912; BD Labware, Franklin Lakes, NJ) were coated with poly-λ-lysine (2–10 μg/ml; Sigma-Aldrich, St. Louis, MO) overnight at room temperature. For some of the assays, mouse dsDNA was first treated with S1 nuclease using 1.5 U of S1 nuclease/μg of dsDNA in S1 nuclease buffer (0.2 M NaCl, 1 mM ZnSO$_4$, 0.5% glycerol, and 0.05M NaAc, pH 4.5) and then added to the plate. However, it was determined that S1 nuclease-treated mouse dsDNA and autosomal (one silent) the heavy DNA-containing bacteria. Plasmid was isolated and cut with EcoRI (New England Biolabs, Beverly, MA). The DNA fragments were then ligated into an IgG2b vector that contains a bacterial gene for guanine phosphoribosyltransferase conferring resistance to mycophenolic acid and a BALB/c IgG2b constant region gene with the intronic H chain leader sequence (35). This new vector was then transformed into DH5α bacteria. Plasmid was isolated and cut with EcoRI and sequenced using the primers in the J H 3(5’–GAT TCC GTG CAG ATA TTC-3’) and V$\kappa$4(5’–GCT TGC AGT TGT CCC TAC TG-3’) sequence are underlined). Single mutants at either Arg codon were generated with a similar primer containing only one GCT change. The newly mutated DNA was boiled and annealed to a single-stranded plasmid containing the SN5–18R V$\kappa$4 gene. This was used to transform Ecoli DH5α to produce site-directed homologous recombination into DH5α bacteria. Plasmid was isolated and cut with EcoRI and sequenced using the primers in the J H 3(5’–GAT TCC GTG CAG ATA TTC-3’) and V$\kappa$4(5’–GCT TGC AGT TGT CCC TAC TG-3’) sequence are underlined). Single mutants at either Arg codon were generated with a similar primer containing only one GCT change. The newly mutated DNA was boiled and annealed to a single-stranded plasmid containing the SN5–18R V$\kappa$4 gene. This was used to transform Ecoli DH5α to produce site-directed homologous recombination into DH5α bacteria.

Results

Extent of purification determines the specificity of an anti-nuclear Ab

SN5–18 is an anti-chromatin Ab derived from a spontaneously autoimmune (NZB × SWR) F1, female mouse in the advanced stages of SLE (7). It exhibits all the hallmarks of a product of T cell-dependent immunity. It binds chromatin with high avidity, it is derived from a B cell belonging to a large clone, it is the product of class switch recombination (IγG2b), and its V region is modified by somatic mutations. The results of initial specificity tests suggested that the mAb SN5–18 in the form of hybridoma culture supernatants bound to a complex of histones H2A and H2B. This result was misleading because it was later found that purified mAb SN5–18 bound to a complex of histones H2A/H2B and dsDNA, but not to the protein or DNA components alone (31). This same phenomenon and specificity pattern were observed for mAb SN5–18R, which represents the precursor to the SN5–18 lineage, mAb SN5–18R was generated by reverting the somatic mutations in mAb SN5–18 to their known germline counterpart (7). Both Ab
were purified in a procedure that involved sequential use of affinity columns, first protein G and then goat anti-mouse Ig, and steps to dissociate histones from DNA and to digest DNA (see Materials and Methods). We refer to Ab prepared in this manner as 2× purified. Like hybridoma supernatants, mAb SN5–18R purified only with protein G (termed protein G purified) still demonstrated apparent binding to histones alone or dsDNA alone (Fig. 1). This same phenomenon was seen with mAb SN5–18 (data not shown). Protein G purification proved to be insufficient to eliminate contaminants in culture supernatants that obscured the true binding specificity of this anti-chromatin Ab. In contrast, the 2× purified preparation of mAb SN5–18 bound chromatin, but not dsDNA.

Purified mAb 3H9/Vk4 binds chromatin, but not dsDNA alone

Because our Ab appeared to bind dsDNA when it was either supernatant derived or protein G purified, but not when it was 2× purified, we thought it was important to similarly test the specificity of the prototypic anti-dsDNA Ab, 3H9/Vk4 (9). This Ab was derived from an autoimmune MRL-lpr/lpr mouse and has served as a foundation for studies of tolerance and autoimmunity to a natural lupus autoantigen, dsDNA. To this end we tested mAb 3H9/Vk4 for potential activity against dsDNA. Three forms of the mAb were compared: a culture supernatant, a protein G-purified preparation, and a 2× purified preparation. As shown in Fig. 2A, mAb 3H9/Vk4 in the culture supernatant appeared to bind dsDNA. Upon protein G purification, this binding was still evident, although somewhat reduced. Upon 2× purification, however, there was no detectable binding to dsDNA. In contrast, 2× purified mAb 3H9/Vk4 bound strongly to chromatin (Fig. 2B). As a positive control, we subjected another well-characterized anti-dsDNA Ab, H241 (37), to our purification scheme and found that 2× purified mAb H241 retained activity toward dsDNA (Fig. 2C).

To determine whether mAb 3H9/Vk4 could bind dsDNA in solution, we performed a competition assay in which microtiter plates were coated with chromatin, followed by the addition of either 2× purified mAb 3H9/Vk4 or 2× purified mAb H241 in addition to varying concentrations of soluble dsDNA. As shown in Fig. 3A, only mAb H241 was competed for binding to chromatin by soluble dsDNA. On the basis of these results, it appears that mAb 3H9/Vk4 binds chromatin, but not pure dsDNA. Impure preparations, on the other hand, appear to bind dsDNA, apparently due to contaminants derived from hybridoma culture supernatants.

SP2/0 supernatant reconstitutes apparent activity of mAb 3H9/Vk4 against dsDNA

There are reports that cell culture supernatants sometimes contain histones and dsDNA released from dying cells (38–40). To directly test the idea that components in hybridoma culture supernatants reconstituted the antigenic target of mAb 3H9/Vk4 on wells coated with dsDNA, we performed the following experiment. Various quantities of culture supernatant from SP2/0 cells were mixed with 2× purified mAb 3H9/Vk4, and the mixtures were incubated in wells coated with dsDNA (Fig. 3B). This addition of supernatant reconstituted the apparent dsDNA binding by 2× purified mAb 3H9/Vk4, and the level of binding increased as the percentage of SP2/0 supernatant was increased. This result suggested that there were constituents in the culture supernatant that associated with either mAb 3H9/Vk4 or immobilized dsDNA.

Histones reconstitute apparent activity of mAb 3H9/Vk4 against dsDNA

We noticed that protein G preparations of mAb 3H9/Vk4 and mAb SN5–18R contained low m.w. contaminants that ran coincidentally with histones upon SDS-PAGE under reducing conditions (data not shown). Moreover, a considerable precipitate was always present upon dialysis of the eluate from the protein G column. We routinely removed this precipitate from the protein G preparation by centrifugation before using the Ab.

To directly test the idea that histones nucleate immobilized dsDNA to create an antigenic target for mAb 3H9/Vk4, we tested a 2× purified preparation for binding to microtiter trays coated with dsDNA alone, total histones alone, or a mixture of dsDNA and histones (C), which were preincubated and then coated to plates. Binding was detected with an 125I-labeled rat anti-mouse κ Ab. Error bars indicate SDs.

**FIGURE 1.** Apparent specificities of three preparations of the anti-nuclear autoantibody, SN5–18R. Binding of mAb SN5–18R (IgG2b) at various points in the purification scheme to plastic-immobilized dsDNA (A), total calf histones (B; Worthington), and dsDNA plus total calf histones (C), which were preincubated and then coated to plates. Binding was detected with an 125I-labeled rat anti-mouse κ Ab. Error bars indicate SDs.

**FIGURE 2.** Apparent specificities of three preparations of anti-nuclear mAb 3H9. A. Binding of mAb 3H9/Vk4 at various points in the purification scheme to immobilized dsDNA. B. Binding of purified mAb 3H9/Vk4 to mouse thymic chromatin. C. Binding of mAb H241 at various points in the purification scheme to immobilized dsDNA. Error bars indicate SDs.
and histones that had been preincubated together before application to the microtiter plates (Fig. 4). 2× purified mAb 3H9/Vε4 bound to those plates coated with a mixture of histones and dsDNA, but not to those coated with dsDNA alone or histones alone. Strong binding was also seen when wells were first coated with dsDNA and then incubated with histones followed by a washing step. These results stand in sharp contrast to the behavior of mAb 3H9/Vε4 in culture supernatant and protein G-purified mAb 3H9/Vε4, both of which displayed apparent activity against dsDNA alone. These results support the idea that histones released from dying cells nucleate plastic-immobilized dsDNA to generate dsDNA alone. Binding was negligible for all histones and dsDNA combinations. Lower levels of binding were seen against some of the other histone/DNA combinations, in particular H2B/dsDNA. While this binding could be due to a specific interaction between mAb 3H9/Vε4 and H2B/dsDNA, it is possible that it merely reflects trace contamination of the H2B preparation by H2A because the individual histones are only ~90% pure.

2× purified 3H9 does not bind bacterial chromosome

In view of a report (41) that an engineered 3H9 scFv fragment bound to dsDNA, ssDNA, nucleosomes, and cardiolipin, we wondered whether this bacterially derived version of the Ab might contain contaminating chromatosomal proteins that substitute for histones. For this reason, we tested 2× purified mAb 3H9/Vε4 for binding to bacterial chromosome-coated plates. As shown in Fig. 5B, the positive control Ab, H241, bound strongly, while 3H9/Vε4 bound negligibly. On the basis of this result, it appears that bacterial chromosomes cannot provide protein components that associate with DNA to reconstitute the antigenic target of mAb 3H9/Vε4.

Mutation of CDR3 Arg residues in SN5–18R ablates chromatin binding

Previous studies of lupus-related autoantibodies have implicated the importance of VH/CDR3 Arg residues in dsDNA specificity (3, 4, 11–20). Specifically, VH/CDR3 Arg residues were shown to be critical for dsDNA binding by an Ab encoded by the mAb 3H9/ Vε4 heavy chain gene (10). We considered the possibility that VH/CDR3 Arg residues might be important for chromatin binding for several reasons. Purified mAb 3H9/Vε4 and mAb SN5–18R contained VH/CDR3 Arg residues, and both Ab bound chromatin, but not dsDNA. In addition, mAb SN5–18R was nearly identical to dsDNA coat then 3H9
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2× purified mAb 3H9/Vε4 was then tested for binding at various concentrations. Binding was detected with an 125I-labeled rat anti-mouse κ Ab. Error bars denote SDs.

Refrain specificity of 3H9/Vε4

To more accurately define the antigenic target of mAb 3H9/Vε4, we tested a 2× purified preparation against various histone/DNA combinations. To this end microtiter plates were coated with histones alone, dsDNA alone, combinations of histones, or combinations of histones and dsDNA (31 combinations in all). Strong binding activity was detected against H2A/H2B/dsDNA complex, a prototypical nuclear Ag recognized by other lupus-associated autoantibodies (Fig. 5A). Binding was negligible for all histones and combinations of histones without dsDNA. Lower levels of binding were seen against some of the other histone/DNA combinations, in particular H2B/dsDNA. While this binding could be due to a specific interaction between mAb 3H9/Vε4 and H2B/dsDNA, it is possible that it merely reflects trace contamination of the H2B preparation by H2A because the individual histones are only ~90% pure.

FIGURE 3. A. Failure of purified mAb 3H9/Vε4 to bind dsDNA in solution. 2× purified mAb 3H9/Vε4 or 2× purified mAb H241 (1 μg/ml) was added together with varying amounts of soluble dsDNA to plates coated with 10 μg/ml mouse thymic chromatin. Bound mAb was detected with a biotin-labeled rat anti-mouse Ab. Error bars indicate SDs.
B. Culture supernatants from SP2/0 cells and incubated in wells coated with 10 μg/ml dsDNA. These results support the idea that histones released from dying cells nucleate plastic-immobilized dsDNA to generate dsDNA alone. These results support the idea that histones released from dying cells nucleate plastic-immobilized dsDNA to generate dsDNA alone. Binding was negligible for all histones and dsDNA combinations (31 combinations in all). Strong binding was also seen when wells were first coated with dsDNA and then incubated with histones followed by a washing step. These results stand in sharp contrast to the behavior of mAb 3H9/Vε4 in culture supernatant and protein G-purified mAb 3H9/Vε4, both of which displayed apparent activity against dsDNA alone. These results support the idea that histones released from dying cells nucleate plastic-immobilized dsDNA to generate dsDNA alone.

FIGURE 4. Histones reconstitute apparent activity of mAb 3H9/Vε4 for dsDNA. Plates were coated with dsDNA, total calf histones (Worthington), or a mixture of both. In one case dsDNA was first applied to the plate, followed by a wash and a secondary coat with histones. 2× purified mAb 3H9/Vε4 was then tested for binding at various concentrations. Binding was detected with an 125I-labeled rat anti-mouse κ Ab. Error bars denote SDs.

Mutation of CDR3 Arg residues in SN5–18R ablates chromatin binding

Previous studies of lupus-related autoantibodies have implicated the importance of VH/CDR3 Arg residues in dsDNA specificity (3, 4, 11–20). Specifically, VH/CDR3 Arg residues were shown to be critical for dsDNA binding by an Ab encoded by the mAb 3H9/ Vε4 heavy chain gene (10). We considered the possibility that VH/CDR3 Arg residues might be important for chromatin binding for several reasons. Purified mAb 3H9/Vε4 and mAb SN5–18R contained VH/CDR3 Arg residues, and both Ab bound chromatin, but not dsDNA. In addition, mAb SN5–18R was nearly identical to other lupus-associated mAb that were reported to be specific for DNA (17, 18).

To explicitly determine whether Arg residues in VH/CDR3 are required for chromatin binding, we tested the role of Arg residues at positions 104 and 106 in the VH of mAb SN5–18R.
Mutagenesis was performed to mutate these Arg residues to Ser residues, either individually or together (Fig. 6A), and 2× purified Ab preparations were tested in binding assays. Heavy and light chain pairing was intact in the mutant Ab, as demonstrated in sandwich immunoassays (data not shown). Results of the binding assays performed revealed that either of the Arg to Ser conversions was sufficient to ablate binding to chromatin, indicating that both Arg residues were required for chromatin specificity (Fig. 6B).

**Discussion**

In this study we demonstrated that certain lupus-associated autoantibodies with apparent specificity for dsDNA actually bind chromatin, but not pure, uncomplexed DNA. The concept that autoantibodies sometimes have specificities for complexes of nuclear proteins and DNA is not new. The literature contains numerous reports indicating that such Ab arise in human SLE and in murine lupus-like disease (3, 21–29, 42). However, our analysis demonstrates the importance of using purified preparations of anti-nuclear Ab when assessing antigenic specificity. This led to the surprising finding that mAb 3H9/Vk4 falls into the chromatin-specific category of anti-nuclear Ab. For historical reasons, we purified the anti-nuclear Ab using two affinity columns sequentially and by treating preparations of Ab eluted from the first (protein G) column with DNase and high salt, which degrade DNA and extract histones that are carried along with the Ab. The importance of high salt in eliminating apparent cross-reactions of anti-DNA Ab was implicated in studies by Smeenk et al., (43), who demonstrated that the Farr assay was much more discriminating than conventional ELISA in revealing high affinity anti-DNA Ab associated with SLE. In addition, this group (44) and Subiza et al. (45) demonstrated that DNase treatment often reduced cross-reactions of anti-DNA Ab with a variety Ags, including histones. More recently, we have found that treating culture supernatants with DNase and then subjecting them only to an anti-Ig column, followed by a high salt wash before elution of the Ab, is usually sufficient to yield pure preparations that bind chromatin, but not uncomplexed, free dsDNA (data not shown).

The Ab we have examined, including mAb 3H9/Vk4, appear to bind chromatin and specifically to bind a complex of H2A/H2B and dsDNA. Our interpretations need to be qualified because the histone preparations are not absolutely pure (H2A and H2B are >90% pure, while H1, H3, and H4 are ~95% pure). It is conceivable that other proteins that might be present as trace contaminants are important for binding by mAb 3H9/Vk4. For example, non-histone factors are apparently necessary for assembly of histone proteins on DNA. However, we believe that it is unlikely that mAb 3H9/Vk4 is binding to nonhistone proteins alone, based on the fact that...
that the sum of binding of 2× purified mAb 3H9/Vk4 to H2A/dsDNA and to H2B/dsDNA does not equal the binding seen when H2A, H2B, and dsDNA are combined (Fig. 5). Thus, while we cannot exclude the possibility that another component participates directly or indirectly in the formation of the antigenic target, a critical element appears to be a complex of H2A/H2B/dsDNA. Moreover, our results are consistent with three possibilities regarding the antigenic determinants seen by mAb 3H9/Vk4 and mAb SN5–18: 1) that the Ab binds exclusively to protein that is organized by DNA, 2) that the Ab binds exclusively DNA that is contorted by protein, or 3) that the Ab binds both to protein and DNA determinants. Distinguishing conclusively between these alternatives may require an x-ray structure. We cannot say with certainty why purified 3H9 is highly specific for chromatin in our hands but cross-reactive with other Ags when tested as an engineered scFv (41). The cross-reactions observed are unlikely to be due to contaminating bacterial nucleoid material, because we found this to be nonantigenic in our assay. One possibility is that the protein A domain in the context of the FV portion alters charge or confirmation to render the protein more nonspecifically adhesive.

Because our SN5–18 R Ab closely resembles other Ab that were reported to bind DNA and that contained numerous VhCDR3 Arg residues, we tested the role of VhCDR3 Arg residues in the specificity of SN5–18 R. We found that both VhCDR3 Arg residues at positions 104 and 106 were essential. Although other investigators have speculated on a role for VH CDR3 Arg residues, we tested the role of VH CDR3 Arg residues in the specificity of 3H9 Ab (46), our data experimentally confirm this idea. It is conceivable that chromatin-specific Ab may make direct contacts with DNA in the context of chromatin, or perhaps with DNA-binding proteins that are modified, for example, by phosphorylation (47). Interestingly, some dsDNA-binding Ab do not contain numerous Arg residues in their VhCDR3; mAb H241 contains only one Arg. It is important to point out that while our data show that some anti-dsDNA Ab are actually chromatin specific, there are clear examples of mAb, such as H241, that bind free dsDNA (48–50). Also, we cannot say whether a mutant of 3H9, containing an arginine residue at codon 56 in the heavy chain, might bind free DNA or a more complex epitope (51).

Defining the antigenic specificities of lupus-associated Ab is important because specific Ab are key indicators of disease status. It has been reported that anti-dsDNA Ab appear with different kinetics than anti-chromatin Ab in lupus (52). Therefore, without knowing the true specificity of an Ab, it cannot be conclusively determined when during the course of autoimmune disease a particular Ab arose. Ab specificity is also important in studies addressing the origin of anti-nuclear Ab in systemic autoimmune disease. It has been speculated, for example, that some anti-dsDNA Ab are derived from precursors that originally bound ssDNA, and that somatic mutation and selection produced dsDNA binding descendants. This scenario, in fact, was suggested for the origin of the autoreactive 3H9/Vk4 C B cell (9).

Defining antigenic specificities of anti-nuclear Ab is also important to interpretations regarding self-tolerance in the B cell repertoire. Studies in transgenic mice expressing a 3H9/Vk4 C B cell receptor have revealed evidence for central tolerance and receptor editing in the bone marrow of nonautoimmune-prone mice (53). However, Mandik-Nayak et al. (54) obtained a seemingly different result for transgenic mice expressing the 3H9 heavy chain together with endogenous λ light chains, despite the fact that in both cases the transgenic B cells were assumed to bind dsDNA. These authors observed the presence of 3H9 heavy chain- and Aλ light chain-bearing B cells; however, these cells were excluded from the follicle, and no anti-dsDNA Ab was detected in the serum. While it is conceivable that different editing capabilities in the two populations of B cells could account for the alternative tolerance pathways (55), it is also possible that they have different antigenic specificities. In fact, Radic and Weigert (42) proposed that light chains play a role in determining the antigenic specificity of anti-nuclear Ab. Ab that truly bind pure dsDNA may encounter their Ag only sparsely in vivo or may bind it only weakly in the context of chromatin (46, 56, 57). Another possibility is that pure dsDNA is located in a different microenvironment than chromatin in vivo. Regardless of the reason, our results illustrate the importance of using highly purified Ab to define the antigenic specificities of anti-nuclear Ab.

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