Nephritogenic Potential of Anti-DNA Antibodies against Necrotic Nucleosomes

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Systemic lupus erythematosus (SLE) is a disorder linked to loss of immune tolerance to self-antigens and the production of a variety of autoantibodies.1 Antibodies directed against nuclear constituents are clinically important. Subpopulations of antinuclear antibodies, especially those binding double-stranded DNA (dsDNA), may initiate lupus nephritis2 and represent one of the classification criteria for SLE.3 Not all anti-dsDNA antibodies, however, are involved in the evolution of lupus nephritis. This suggests that only select anti-dsDNA antibodies initiate cellular or molecular mechanisms making them nephritogenic. In this review, we examine the mechanism by which anti-dsDNA antibodies acquire their nephritogenic effects.

Anti-dsDNA antibodies were discovered in 1957.4–6 They were assumed to be nephritogenic because glomerular in vivo–bound antibodies possess specificity for dsDNA and nucleosomes,7–9 and DNA binds glomerular collagens with high affinity.10,11 Today, this notion is still controversial. Although there is broad agreement that anti-dsDNA antibodies have nephritogenic potential, there is no consensus on how they exhibit their pathogenicity. Recent data confirm a hypothesis that chromatin fragments play a central role in lupus nephritis. Why chromatin fragments are released into the renal extracellular milieu is not entirely clear. Increased apoptotic activity, deficient clearance of apoptotic chromatin, reduced fragmentation of secondary necrotic chromatin, and increased release of chromatin as a result of the cytotoxic effect of autoimmune CD8+ T cells all have been advanced as possible mechanisms.

WHAT MAKES AN ANTI-DSDNA ANTIBODY NEPHRITIC?

Some patients with lupus produce antibodies against dsDNA and nucleosomes but do not develop lupus nephritis; therefore, one must assume there are distinct pathways—or at least a selection principle—that determine the pathogenic effect of induced antibodies. Two mechanisms have received attention over the years. Antibodies may cross-react with native glomerular structures and induce inflammation.

ABSTRACT

Systemic lupus erythematosus is an inflammatory autoimmune syndrome of unknown cause. Kidney disease is a central and serious complication in this syndrome. Deposition of chromatin-containing immune complexes within glomerular membranes is considered a key event in the pathogenesis of lupus nephritis. One set of autoantibodies that participate in these complexes is directed against components of chromatin, particularly against double-stranded DNA (dsDNA). Matzinger’s danger model implicates chromatin fragments as both inducers and glomerular targets for nephritogenic anti-dsDNA and anti-nucleosome antibodies. In context of this model, apoptosis, secondary necrosis, and exposure of chromatin fragments may causally trigger autoimmunity and subsequent lupus nephritis. The exposure of glomerular basement membrane–associated extracellular chromatin depends on an observed acquired downregulation of renal DNase1 transcription and loss of nuclease activity preceding development of severe nephritis; this downregulation would result in reduced fragmentation and clearance of chromatin fragments. These fragments bind glomerular basement membrane structures with high affinity. In addition, exposed chromatin fragments contain structures that stimulate the innate immune system through Toll-like receptors and the adaptive immune system to produce affinity-maturated pathogenic anti-chromatin and anti-dsDNA antibodies that are central to the development of lupus nephritis. J Am Soc Nephrol 20: 696–704, 2009. doi: 10.1681/ASN.2008010112
Alternatively, the nephritogenic effect of anti-dsDNA antibodies depend on binding chromatin fragments associated with glomerular basement membranes (GBMs) with antibodies remaining nonpathogenic in the absence of exposed chromatin. Whether antibody avidity is essential for antibody binding in vivo is controversial.

**BINDING OF ANTI-dsDNA ANTIBODIES IN GLOMERULI DOES NOT REQUIRE HIGH INTRINSIC AFFINITY**

A significant proportion of B cells have the potential to recognize and respond to immunogenic DNA.\(^2,12–15\) The strength of binding between an antibody and a single epitope is called affinity, whereas involvement of multiple binding sites by a single antibody yields functional affinity or avidity. DNA is regarded as immunogenic when complexed with DNA-binding peptides of a nonself\(^{16–23}\) or self origin\(^{24,25}\) (for review, see reference\(^2\)). DNA–peptide complexes stimulate DNA-specific B cells and peptide-specific T cells analogous to the hapten carrier model for induction of anti-hapten antibodies. A consequence of sustained stimulation with dsDNA–peptide complexes is affinity maturation of the induced anti-dsDNA antibodies.\(^{14,26–30}\) It is assumed that high avidity of anti-dsDNA antibodies contributes to pathogenicity\(^7,9,31–33\); however, little information is available to confirm this statement.\(^8,34\)

We have analyzed intrinsic affinity of circulating and glomerular in vivo–bound anti-dsDNA antibodies in individual nephritic (NZB×NZW)F1 mice. Affinity is higher in antibodies eluted from kidneys as compared with circulating antibodies; however, affinity of antibodies in renal eluates from different nephritic (NZB×NZW)F1 mice with severe proteinuria varies considerably, from low affinity to very high.\(^35\) These data suggest that intrinsic antibody affinity is not a key nephritogenic parameter.

**ANTI-dsDNA ANTIBODY SPECIFICITY FOR CHROMATIN FRAGMENTS IS A PATHOGENIC FACTOR**

Dual specificity of antibodies for dsDNA and naturally exposed glomerular constituents represents the most logical explanation for why some but not all anti-dsDNA antibodies are pathogenic.\(^36–40\) Some serum anti-DNA antibodies recognize inherent non-DNA/non-nucleosomal renal antigens,\(^36,39–43\) but dual specificity does not identify which of the cross-reactive renal ligands actually binds these antibodies in vivo. Even proving that antibodies eluted from nephritic kidneys possess dual specificity (e.g., eluted anti-dsDNA antibodies that cross-react with α-actinin\(^39,42\) or with laminin\(^40\) does not permit any inferences as to which of the structures bound these antibodies in vivo; however, such observations may explain why some but not all anti-dsDNA antibody-positive patients develop lupus nephritis; only patients producing cross-reacting subpopulations of anti-dsDNA antibodies
would do so. This explanation has never been substantiated by evidence.

An alternative model suggests that availability of chromatin fragments in the circulation or in glomeruli is required for anti-chromatin antibodies to exert full pathogenic effect.\(^{44-47}\) In recent studies, electron-dense structures (EDS) associated with GBM and the mesangial matrix constitute the main target for \textit{in situ}–bound antibodies in both murine\(^{48}\) and human lupus nephritis (Figure 1).\(^{49}\) Binding of antibodies to other glomerular structures was not observed.\(^{48-50}\)

In subsequent studies, \textit{in vivo} glomerular target structures for IgG antibodies were analyzed by immune electron microscopy (IEM) and by co-localization IEM, an EM-based form of two-dimensional confocal microscopy.\(^{48,50}\) IEM analyses demonstrate that antibody deposits are confined to EDS in glomerular capillary membranes and the mesangial matrix. \textit{In vivo}–bound autoantibodies co-localize perfectly with experimental chromatin–bound autoantibodies in both murine\(^{48}\) and human lupus nephritis (Figure 1).\(^{49}\) Binding of antibodies to other glomerular structures was not observed.\(^{48-50}\)

A compilation of results of experiments addressed to determine the nature of EDS associated with GBMs in nephritic kidneys\(^{a}\) (Table 1). These data correspond with evidence that antibodies eluted from nephritic (NZB×NZW)F1 kidneys possess specificity for nucleosomes and DNA;\(^{46}\) however, these results are not conclusive, because the experimental anti-chromatin antibodies used in the co-localization IEM assay may have been cross-reactive.\(^{39,42,43,53-61}\) Therefore, we developed a co-localization terminal deoxynucleotidyl transferase dUTP nicked-end labeling (TUNEL) IEM assay.\(^{50,62}\) In this assay, nicked endogenous extracellular DNA was localized by terminal deoxynucleotidyl transferase–mediated introduction of biotinylated nucleotides and autoantibodies by IEM.\(^{50,62}\) The result of this assay demonstrated that nicked DNA consistently co-localizes with \textit{in vivo}–bound autoantibodies in glomerular membrane–associated EDS (Figure 2). This result is in harmony with the fact that chromatin fragments bind GBM and mesangial matrix with high affinity, as demonstrated by surface plasmon resonance.\(^{62}\) These observations remain unambiguous, consistent, and coherent with results from murine\(^{47,48}\) and human\(^{50}\) lupus nephritis (Table 1).

Although our knowledge of the composition of EDS is not exhaustive, these studies clearly demonstrate that chromatin particles are a major component of these structures, although other, yet-unidentified moieties may be present. Considering the dominant autoimmune specificity for chromatin among autoantibodies eluted from nephritic kidneys,\(^{49}\) the appearance of chromatin in glomerular deposits is a striking feature of lupus nephritis. Whether chromatin fragments bind GBM as part of preformed immune complexes or whether such complexes form \textit{in situ} after chromatin is deposited has not been determined. The nature of GBM-associated EDS in the context of lupus nephritis is most probably distinct from subepithelial deposits, as seen in Heymann nephritis, for example, in which the major target for autoantibodies is megalin and the receptor-associated protein of podocyte origin.\(^{63,64}\)

**GENERATION OF NECROTIC CHROMATIN IS A KEY EVENT IN THE GENESIS OF LUPUS NEPHRITIS**

Apoptosis is a well-orchestrated, controlled process in which cell death guar-

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Table 1. Summary of results of experiments addressed to determine the nature of EDS associated with GBMs in nephritic kidneys\(^{a}\)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Healthy Glomeruli</th>
<th>Nephritic Glomeruli (NZB×NZW)F1</th>
<th>Present in Membranes outside EDS</th>
<th>Human Lupus Nephritis</th>
<th>References</th>
<th>Interpretation of EDS Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Not detected</td>
<td>Dense pattern confined to EDS</td>
<td>Negative</td>
<td>Dense pattern in EDS</td>
<td>48–50</td>
<td>Presence of DNA, histones, and transcription factors indicate accumulation of nucleosomes in EDS associated with membranes</td>
</tr>
<tr>
<td>Histones</td>
<td>Not detected</td>
<td>Dense pattern confined to EDS</td>
<td>Negative</td>
<td>Dense pattern in EDS</td>
<td>48–50</td>
<td></td>
</tr>
<tr>
<td>Transcription factors(^{b})</td>
<td>Not detected</td>
<td>Presence confined to EDS</td>
<td>Negative</td>
<td>Present in EDS</td>
<td>48–50</td>
<td></td>
</tr>
<tr>
<td>Laminin</td>
<td>Normal membrane constituent</td>
<td>Surrounding EDS</td>
<td>Normal pattern</td>
<td>Not analyzed</td>
<td>48</td>
<td>Laminin and collagen are not part of EDS</td>
</tr>
<tr>
<td>Collagen</td>
<td>Normal membrane constituent</td>
<td>Surrounding EDS</td>
<td>Not analyzed</td>
<td>Not analyzed</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>(\alpha)-Actinin</td>
<td>In podocytes and mesangial cells</td>
<td>Present in membranes, interstitium, and EDS</td>
<td>Random pattern in the extracellular space</td>
<td>Not analyzed</td>
<td>49</td>
<td>(\alpha)-Actinin is extracellular in nephritic glomeruli</td>
</tr>
</tbody>
</table>

\(^{a}\)Reprinted from reference\(^{62}\), with permission.

\(^{b}\)For demonstration of presence of transcription factors in EDS, antibodies to TATA-box–binding protein were used, because these are constitutively bound to nucleosomes.
guarantee a safe, fast, and noninflammatory clearance of the dead cells while maintaining immunologic tolerance to cellular antigens. If clearance of apoptotic cells is reduced, then this may explain the observed increase in the number of dead cells in glomeruli, and programmed cell death may transform into a state of disorganized cell fragmentation, possibly providing “danger” signals. This process may have an immense impact on loss of immune tolerance and inflammation related to lupus nephritis (Figure 3).

The activation of nucleosome-specific T cell populations in vivo is more concordant with Matzinger’s danger paradigm than with Medzhitov’s and Janeway’s distinction of infectious nonself versus noninfectious self (see reference for discussion). Matzinger’s danger model states that the immune system does not care whether infectious nonself or noninfectious self provides the danger signals. In this sense, self may be as harmful as nonself structures.

When apoptotic cells are not cleared in vivo, they deviate from a well-organized apoptotic process and enter an energy-independent state of disorganized cell fragmentation instigated by non-apoptotic proteolytic enzymes and nucleases. The apoptotic process is thus transformed into secondary necrosis that results in an altered self-form of chromatin unmasked from apoptotic blebs and able to provide the same danger signals as infectious nonself structures. Consequently, chromatin fragments released from apoptotic blebs are consumed by immature dendritic cells and bind to Toll-like receptor 7 (TLR7) or TLR9 through exposed RNA structures or CpG motifs, respectively. Signaling through TLR7 or TLR9 induces dendritic cell maturation, up-regulation of co-stimulatory molecules, and processing and presentation of antigenic peptides derived from chromatin. Such activated dendritic cells prime nucleosome-specific or Sm/RNP-specific T-helper cells that provide help to DNA or RNP-specific B cells to be transformed into antibody-producing plasma cells (Figure 3).

Because TLR7 and TLR9 are confined to the endosomal compartment, their activity depends on the internalization of their respective ligands by other receptors recognizing chromatin constituents. Mammalian DNA binds to TLR9 through CpG motifs, and peptides derived from chromatin may be processed and presented on the cell surface within MHC class II molecules. This scenario may be sufficient to activate innate and adaptive immune systems to produce anti-dsDNA or anti-nucleosome antibodies (Figure 3).

Another pathway that directs chromatin into macrophages and permits interaction with TLRs has been described. Means et al. demonstrated a novel functional interaction between Fc receptors and TLRs. This interaction follows a pathway in which CD32 (FcγRIIa) transfers anti-dsDNA antibody-dsDNA-containing immune complexes to TLR9 in lysosomes. This interaction induces plasmacytoid dendritic cell activation and IFN-α production. Thus, complexes of chromatin fragments and anti-dsDNA antibodies may contribute to the pathogenesis of SLE. High levels of IFN-α play a role in loss of tolerance to autoantigens in SLE, because IFN-α drives maturation of monocytes into dendritic cells. The ability of these dendritic cells to internalize, process, and present secondary necrotic chromatin and activate self-rec
Figure 3. The role of necrotic chromatin fragments in activating the innate and adaptive immune system and as target structures for induced anti-chromatin autoantibodies. (1) Apoptotic cells are not cleared, and chromatin is released, exposed, but not fragmented as a result of nuclease (DNase1) deficiency. (2) This results in deposition of chromatin fragments in tissue. (3) Immature dendritic cells are activated by exposed chromatin structures such as RNA or CpG motifs. They then process and present chromatin-derived peptides in context of MHC class II molecules, upregulate co-stimulatory molecules, and secrete cytokines and ILs to activate relevant T helper cells. (4) T cells specific for chromatin-derived peptides recirculate and interact with dsDNA-specific B cells binding circulating nucleosomes and presenting relevant peptides for T cells committed to respond to such peptides. This results in plasma cell transformation and secretion of anti-chromatin antibodies. (5) These recognize and bind membrane-associated chromatin fragments and initiate inflammation. Lupus nephritis may be the end result. (6) This would be consistent with the danger model of Matzinger to explain how necrotic chromatin activates autoimmune B cells and T cells and also with the pathogenic potential for the induced antibodies. They are pathogenic only in context of exposed target structures.

Theoretically, glomerular cells can be killed by cytotoxic T cells. Functional, autoimmune CD8+ T cells with potential to kill autologous cells have been detected in lupus. In addition, autoimmune B cells promote activation of autoimmune CD4+ and CD8+ T cells in lupus-prone MRL-lpr/lpr mice. Activated CD8+ T cells have also been detected in nephritic kidneys from human SLE. CD8+ T cell–mediated intraglomerular killing of cells could cause a release of chromatin and formation of complexes with anti-chromatin antibodies in vivo.

There is experimental precedence for this model. De novo expression of polyomavirus large T antigen in a binary tet-off regulated T antigen transgenic mouse model resulted in activation of CD8+ and CD4+ T cells and in sustained production of antibodies to dsDNA, nucleosomes, and T antigen. In T antigen–expressing mice, EDS were observed in GBMs. These EDS bound experimental antibodies to T antigen, dsDNA, histones, and transcription factors as demonstrated by co-localization IEM. This demonstrates that they contain chromatin fragments in complex with T antigen. In addition, these observations indicate that chromatin fragments have been released from T antigen–expressing cells secondary to CD8+ T cell–mediated killing of cells that express that T antigen. IEM analyses shows that autoantibodies bound strictly to these EDS. This tet-regulated T antigen–transgenic model may be used to explain how chromatin fragments are released and how

split up GBMs by enzymatic degradation. Although formal evidence is missing, this process may facilitate immune complex deposition and progression of glomerular disease. Recent data from our group consistently demonstrate increased MMP activity within glomeruli of nephritic but not prenephritic (NZB × NZW)F1 mice.
they serve as target structures for nephritogenic antibodies.

**IMPACT OF ACQUIRED RENAL DNASE1 DEFICIENCY ON CHROMATIN EXPOSURE IN GLOMERULI**

Reduced clearance of apoptotic cell debris plays a causal role in necrotic transformation of apoptotic chromatin and in deposition of chromatin among glomeruli,45,65,66,92 yet, there is no apparent explanation for the reduced clearance of chromatin.45,46,93 Recently, we questioned whether reduced fragmentation of apoptotic chromatin could account for reduced clearance of these structures. In initial experiments, we observed that nucleosomal DNA fragmentation in campthothecin-induced apoptotic cells in freshly isolated kidneys from nephritogenic (NZB×NZW)F1 mice is markedly reduced compared with the effective fragmentation in similarly induced apoptotic cells in kidneys from nonautoimmune mice.94 The secreted endonuclease DNase1, produced in tubular and glomerular cells, is responsible for more than 80% of total nuclease activity in the kidney,95 and reduced DNA fragmentation correlated with reduced levels of DNase1 mRNA and a near-absent DNase1 activity in nephritic kidneys.94 Loss of DNase1 activity is not observed in kidneys from prenephritic (NZB×NZW)F1 or age-matched nonautoimmune mice. Notably, there is no reduction of DNase1 mRNA or enzyme activity in skin of the same nephritic autoimmune mouse. This indicates there is not a systemic loss of DNase1 activity in these mice (Hedberg et al., manuscript submitted). By real-time PCR analyses of DNase I, DNase II, endonuclease G, DNA fragmentation factor subunit, and cell death–inducing DNA fragmentation factor subunit–like effector B, only the expression of DNase I mRNA is dramatically reduced in the kidneys of proteinuric (NZB×NZW)F1 mice.94

Importantly, we observed a chronological relationship among reduced enzyme activity of renal DNase1, deposition of chromatin fragments in GBM, and development of proteinuria. This indicates a direct link between reduced fragmentation of extracellular chromatin, deposition of chromatin fragments in GBM, and pathogenicity of anti-chromatin autoantibodies. Experimental deletion of the DNase1 gene causes lupus-like disease and progression to renal failure in mice on selected genetic backgrounds.96 A nonsense mutation in the DNase1 gene has also been reported in two patients with SLE.97 In light of these reports, acquired DNase1 deficiency in lupus-prone mice provides further evidence of a central role for DNase1 in SLE pathogenesis and the development of renal manifestations. Whether loss of DNase1 activity is a characteristic phenomenon in other lupus-prone mouse strains and in human lupus nephritis is under investigation.

**IMPACT OF NECROTIC CHROMATIN ON TLR9**

Recent data from TLR9-deficient MRL/MpJpr/lpr mice may be inconsistent with the concept that chromatin exposure induces pathogenic autoimmunity. In the absence of TLR9, antibodies to dsDNA, as detected by the Crithidia luciliae immunofluorescent test, are reduced, autoimmune disease is exacerbated, and lymphocytes and dendritic cells are more activated.98 In agreement with previous reports, TLR9 regulates anti-dsDNA antibodies in autoimmune mice99; however, the differences between wild-type MRL/MpJpr/lpr and TLR9-deficient MRL/MpJpr/lpr mice with respect to levels of anti-nucleosome and anti-dsDNA antibodies are relative and not absolute. Nephritis in TLR9-deficient mice is more severe than in wild-type mice, although the differences are not statistically significant.98 TLR7-deficient MRL/MpJpr/lpr mice have less serious kidney disease, produce significantly less anti-Sm antibodies, yet the production of anti-dsDNA antibodies is unaffected. These observations reveal contradictory inflammatory and regulatory roles for TLR7 and TLR9, despite that TLR7 and TLR9 have similar tissue expression and signaling pathways.100 These results suggest a requirement for TLR9 in the initiation of anti-dsDNA antibodies in vivo.98–100 but nephritis in MRL/MpJpr/lpr mice is relatively controlled by TLR7 as is the production of antibodies against RNA protein–containing antigens.

Important information about specificity of antibodies bound in glomeruli of TLR7- and TLR9-deficient mice is lacking in these studies. We have noticed that in (NZB×NZW)F1 mice, autoantibodies bind only to extracellular membrane–associated necrotic chromatin.48,50 Antibodies eluted from such kidneys are specific for nucleosomes and dsDNA.59 Specificity for Sm/RNP or other autoantigens was not analyzed. It is not unlikely, however, that such antibodies also bind in glomeruli, because apoptotic and necrotic chromatin contain Sm/RNP complexes.101 If tissue-exposed chromatin presents dsRNA or CpG to TLR7 and/or TLR9, respectively, then this may induce autoimmunity to RNP and chromatin complexes. If chromatin is not fragmented and cleared appropriately, then it may subsequently become a target for induced autoantibodies. In that sense, the fine molecular specificity of nephritogenic antibodies binding chromatin is not the critical parameter but rather their ability to bind necrotic chromatin associated with membranes in glomeruli.

Molecular specificity of pathogenic autoimmunity may be controlled by TLRs,100 but exposure of chromatin structures may be the event that allows the autoantibodies to bind and become pathogenic. Even in TLR9-deficient mice, anti-chromatin antibodies appear. Yu et al.102 observed in a murine model of SLE with hyperreactive B cell activation, mediated by mutant phospholipase Cγ2, that TLR9 deficiency did not preclude spontaneous anti-DNA autoantibody formation and development of nephritis.

**CONCLUSIONS**

Considering the data discussed here, central initial events in lupus nephritis...
may include loss of renal nuclease activity. Reduced renal DNase1 activity is observed only in context of severe membranoproliferative lupus nephritis, whereas the activity is normal in all pre-nephritic and age-matched control mice. The initial causal event in lupus nephritis may be an unusual exposure to large chromatin fragments. Necrotic chromatin fragments, not anti-chromatin antibodies, may represent a key element in understanding the basis for the development of nephritis as a central organ manifestation in lupus. Release of chromatin fragments and their subsequent binding to GBM may be the events that render potentially nephritogenic anti-dsDNA (or anti-chromatin) antibodies nephritic. Without this partner, they are nonpathogenic but remain in the body as sleeping wolves waiting for their partners to appear.

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DISCLOSURES

None.

REFERENCES


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