Autoantibodies to components of chromatin, which include double-stranded DNA (dsDNA), histones and nucleosomes, are central in the pathogenesis of lupus nephritis. How anti-chromatin autoantibodies exert their nephritogenic activity, however, is controversial. One model assumes that autoantibodies initiate inflammation when they cross-react with intrinsic glomerular structures such as components of membranes, matrices or exposed nonchromatin ligands released from cells. Another model suggests glomerular deposition of autoantibodies in complex with chromatin, thereby inducing classic immune complex-mediated tissue damage. Recent data suggest acquired error of renal chromatin degradation due to the loss of renal DNaseI enzyme activity is an important contributing factor to the development of lupus nephritis in lupus-prone (NZBxNZW)F1 mice and in patients with lupus nephritis. Down-regulation of DNaseI expression results in reduced chromatin fragmentation and in deposition of extracellular chromatin-IgG complexes in glomerular basement membranes in individuals who produce IgG anti-chromatin autoantibodies. The main focus of the present review is to discuss whether exposed chromatin fragments in glomeruli are targeted by potentially nephritogenic anti-dsDNA autoantibodies or if the nephritogenic activity of these autoantibodies is explained by cross-reaction with intrinsic glomerular constituents or if both models coexist in diseased kidneys. In addition, the role of silencing of the renal DNaseI gene and the biological consequences of reduced chromatin fragmentation in nephritic kidneys are discussed.

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endonuclease in the kidney (25), which is accompanied by a reduced chromatin fragmentation capacity in the nephritic kidneys (26).

**THE PARADOX OF ANTI-dsDNA ANTIBODIES: ARE THEY REALLY PATHOGENIC?**

Not all individuals with anti-dsDNA antibodies in their circulation develop nephritis, although anti-dsDNA antibodies are believed to be directly involved in the nephritic process (16). One model proposes that only those anti-dsDNA antibodies that cross-react with intrinsic glomerular antigens induce lupus nephritis, which could explain why not all patients with anti-dsDNA antibodies develop the disease. Another model states that the nephritogenic potential of anti-dsDNA/anti-chromatin antibodies is exerted because the antibodies target extracellular chromatin fragments in glomeruli (15). This result would explain that anti-dsDNA antibodies are pathogenic only in situations where chromatin is exposed in glomeruli.

Cross-reacting anti-dsDNA antibodies may react with, for example, α-actinin (27,28), extracellular matrix components (9,11,29), cell surface structures (30,31) and entactin (32). Until now, no results from prospective multicenter studies have been published that analyze in an unbiased way the impact of the described cross-reactive antibodies in the development of lupus nephritis. In one prospective clinical study in patients with lupus nephritis, a relationship was found between anti-dsDNA/anti-chromatin antibodies and renal parameters, which, for example, was not observed for anti-α-actinin antibodies (33). Lack of impact of anti-α-actinin antibodies on nephritis was also demonstrated in the (NZBxNZW)F1 (BW) mouse model for lupus nephritis, since antibodies eluted from diseased kidneys hardly bound α-actinin, whereas a large fraction of the antibodies bound dsDNA, histones and nucleosomes (10). Whether an anti-dsDNA/anti-chromatin antibody initiates and executes a pathogenic activity might therefore be determined by the availability of chromatin fragments within glomeruli and not by the potential cross-reactivity of these antibodies. In the absence of extracellular chromatin, the anti-dsDNA antibodies remain non-pathogenic epiphenomena, aside from their diagnostic potential for SLE.

**WHAT IS THE EVIDENCE THAT CROSS-REACTION OF ANTI-dsDNA ANTIBODIES WITH INTRINSIC RENAL ANTIGENS IS A SINE QUA NON IN THE PROCESS OF LUPUS NEPHRITIS?**

The cross-reaction model to explain the nephritogenic potential of anti-dsDNA antibodies shows some caveats. Dual specificity per se does not identify which one of the cross-reactive renal ligands actually are bound by these antibodies in vivo. Even proving that antibodies eluted from nephritic kidneys possess dual specificity, for example, by demonstrating that eluted anti-dsDNA antibodies cross-react with renal nonchromatin target structures such as α-actinin (27,28), laminin (29) or entactin (32), does not reveal which of the target structures these antibodies actually bind in vivo.

To determine the actual in vivo target structures for anti-dsDNA antibodies, different high-resolution analytical assays such as colocalization immune electron microscopy (IEM) techniques can be used (34,35). For example, colocalization IEM has the potential to reveal whether in vivo-bound IgG colocalizes or not with antibodies to dsDNA, histones and other chromatin-associated proteins added to the kidney sections in vitro. If a glomerular target antigen is a nonchromatin structure, but an intrinsic target structure, binding of anti-dsDNA/chromatin antibodies in vivo should be traced to the extracellular matrix, including the glomerular basement membrane (GBM), and not to deposited chromatin present as electron dense structures (EDSs) in the GBM (35). When we traced IgG bound in vivo to GBM-associated EDSs, we observed IgG binding confined to EDSs (35), which argue against in vivo binding of antibodies to, for example, laminin (29) or entactin (32). In addition, we could not demonstrate the presence of laminin and α-actinin within EDSs in murine (10,35) and human lupus nephritis (34). Thus, there seems to be no direct evidence that cross-reaction of anti-dsDNA antibodies with intrinsic renal antigens is important in the process of lupus nephritis.

**WHAT IS THE PROBABILITY THAT THE IMMUNE SYSTEM CONTINUOUSLY PRODUCES CROSS-REACTING ANTI-dsDNA ANTIBODIES?**

If anti-dsDNA antibodies are so central in the pathogenesis of lupus nephritis as we believe, and if cross-reaction of the antibodies with renal structures is a prerequisite for the pathogenic potential of the antibodies as others believe, then the immune system must be stimulated in a way that preserves the dual specificity of the antibodies over time.

Generation of antibody specificity is a result of several stochastic processes from random heavy-chain V-D-J and light-chain V-J gene segment recombination (combinatorial diversity), non-templated nucleotide insertion (functional diversity), to heavy- and light-chain association and to somatic hypermutation (36,37) (for a scholar overview, see relevant chapters by Murphy et al. [38]). These stochastic processes do not permit discrimination between self and nonself antigens, and generation of antibodies with specificity for dsDNA is therefore not a rare event (39–43). These anti-dsDNA antibodies are often observed to be cross-reacting or even polyspecific antibodies (44–48).

Many of the studies on the potential nephritogenic effect of cross-reactive antibody populations are performed by using monoclonal antibodies. In contrast to the dynamic affinity maturation as a consequence of continuous somatic mutations of antibodies during a humoral immune response (49,50), the specificity and affinity of monoclonal antibodies are frozen in time and therefore mostly retain their (dual) specificity that was present at the time of hybridoma cell genera-
tion. It may be argued whether the use of cross-reactive monoclonal anti-dsDNA antibodies to study their pathogenic potential is relevant to understand how anti-dsDNA antibodies bind in lupus nephritis. The biological meaning of somatic hypermutation is to increase affinity for the immunogen (in the case of SLE, components of chromatin) and to limit poly-specificity. Because in lupus nephritis both sets of possible target structures (that is, intrinsic glomerular structures and DNA/chromatin components) are present in the glomerulus (15,35), the following three considerations must be taken into account to clarify the role of cross-reactivity in lupus nephritis: (i) Is a given cross-reaction between DNA and a glomerular antigen retained during affinity maturation of the immune response? (ii) If cross-reaction is significant, and a given cross-reactive non-DNA glomerular antigen is supposed to bind the antibody, then are the affinities for both the DNA and the non-DNA antigen different? Theoretically, the affinity should be higher for the cross-reactive antigen than for DNA if binding to the cross-reactive antigen is the interaction that mediates the development of nephritis. (iii) Robust assays must be used to clarify the chemical nature and localization of glomerular structures targeted in vivo by nephritogenic anti-dsDNA antibodies. This step can be performed by electron microscopy and coherent studies of cross-reactive antibodies in sera and in renal eluates of diseased individuals.

ARE ANTI-dsDNA ANTIBODIES REQUIRED FOR THE DEVELOPMENT OF LUPUS NEPHRITIS?

Not all patients with lupus nephritis have detectable levels of anti-dsDNA antibodies in their circulation, indicating that lupus nephritis is not always linked to the presence of these antibodies. This has been experimentally demonstrated in murine models of lupus nephritis, where in fact the activity of T cells accounted for the kidney injury (51,52). Consistent with this latter observation, the production of anti-chromatin autoantibodies is not absolutely required for the development of lupus nephritis (53). Thus, the pathogenesis of lupus nephritis includes elements of antibody-independent processes.

Such observations may explain the results of the Lupus Nephritis Assessment with Rituximab (LUNAR) study (that is, an anti-B-cell therapy) (54). Although rituximab reduced CD19+ B cells and resulted in a relative improvement of serum levels of complement (C3 and C4) and reduced anti-dsDNA antibody levels, renal response rates were similar in the treatment and placebo groups. Thus, the nephritic process remained largely unaffected during the treatment period. This result may theoretically be explained in two ways: either reminiscent levels of anti-dsDNA antibodies were sufficient to maintain nephritis, or infiltrating autoimmune (possibly cytotoxic) T cells in the kidney may have been involved in a way that maintained the nephritic process, since T cells are not affected by rituximab. However, from the LUNAR study design, the role of T cells remains elusive, since renal biopsies were not analyzed at the end point. Nevertheless, although anti-dsDNA antibodies may not always be required in progressive lupus nephritis, most data so far indicate that anti-dsDNA antibodies are inflicted in the disease severity (8,55–57).

A CENTRAL ROLE FOR CHROMATIN IN THE DEVELOPMENT OF LUPUS NEPHRITIS

Our data in BW mice support the concept that the pathogenesis of murine lupus nephritis is characterized by two distinct stages. Late forms of membra-noproliferative lupus nephritis evolve as a consequence of early mesangial lupus nephritis (24,58). In the next sections, we will outline the molecular and transcriptional mechanisms accounting for this two-stepped nephritic process.

BASIC OBSERVATIONS IN EARLY MURINE MESANGIAL LUPUS NEPHRITIS

In several studies, we showed that chromatin fragments serve as central targets for nephritogenic anti-dsDNA/anti-nucleosome antibodies in all stages of lupus nephritis (35,59,60). The early phase of lupus nephritis correlated with deposition of chromatin fragment–IgG complexes in the mesangial matrix. By high-resolution analytical approaches, such as IEM, colocalization IEM and colocalization terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) IEM, IgG was never observed to bind in vivo directly to the GBM or to the mesangial matrix. But IgG did bind to TUNEL-positive EDSs associated with glomerular matrices and membranes (34,35) (Figure 1). This result was demonstrated in murine lupus nephritis (35) and was verified in patients with lupus nephritis (34).

The link between production of anti-dsDNA antibodies and chromatin deposits in the mesangium indicates that the antibodies were directly responsible for this process (24), since we never observed chromatin in glomerular matrices and membranes in anti-dsDNA antibody-negative BW mice (22,24).

PROGRESSION OF MURINE LUPUS NEPHRITIS: A POSSIBLE ROLE FOR RENAL DNASEI

At a certain time point, clinically silent mesangial nephritis progresses into end-stage organ disease. This point was characterized by the deposition of chromatin fragment–IgG complexes in the GBM and by severe proteinuria. The progression was strongly associated with a loss of renal DNaseI gene expression at both the transcriptional and the translational levels and with a severe loss of renal DNaseI enzyme activity as well. Silencing of the renal DNaseI gene was demonstrated by Western blot, zymography, immunohistochemistry, immunofluorescence, quantitative polymerase chain reaction and unpublished Affymetrix microarray analyses (22–24,58). Because DNaseI is required for chromatin breakdown during apoptosis as well as necrosis (61,62), loss of this enzyme activity may lead to accumulation of apoptotic chromatin fragments in glomeruli. In line with this result, local accumulation of chromatin fragment–anti-dsDNA anti-
body complexes in GBM may be the core factor that imposes progressive renal inflammation in SLE (23,35,63).

The role of reduced DNaseI in the pathogenesis of SLE has been discussed for decades. This discussion led to the opinion that DNaseI could be a promising drug for substitutive therapy to prevent autoimmunity (64–66). Studies aimed at analyzing this option were performed in both BW mice (64,66) and human patients (65). However, this idea fell into oblivion for years because the treatment largely failed. Injection of DNaseI in prenephritic BW mice delayed, but did not prevent, the disease in one study (64). In another study, administration of DNaseI in BW mice did not affect development or progression of lupus nephritis (66). Similarly, intravenous or subcutaneous administration of recombinant human DNaseI in patients with lupus nephritis class III–V did not affect kidney function or activity of the disease (65). These studies demonstrated indirectly that serum DNaseI had little or no influence on degradation of apoptotic chromatin in the kidney (61).

Treatment failure may thus indicate that DNaseI in the extracellular compartments has a poor impact on chromatin deposited in the GBM.

Data from our laboratory as well as from other laboratories provide the basis for the following conclusions: (i) DNaseI is strongly expressed in healthy kidneys (67) (see also gene expression profiles from the BioGPS portal at http://biogps.org) and account for 80% of total renal endonuclease activity (25); (ii) DNaseI expression is selectively reduced compared with other endonucleases expressed in nephritic kidneys (23); (iii) DNaseI expression is furthermore selectively silenced in affected kidneys and not in other organs and tissues that express DNaseI (23); (iv) silencing of the renal DNaseI gene is the result of an active regulation of gene expression and not caused by cell death, since other renal genes analyzed so far are not silenced but are expressed at normal levels (23,58); and (v) there is a highly significant inverse correlation between loss of DNaseI enzyme activity and progression of lupus nephritis, and with deposition of chromatin fragments in the GBM, while with normal or subnormal renal DNaseI gene expression, no deposits in GBM were observed (24,58). These conclusions do not, however, clarify if extracellular chromatin deposition secondary to loss of renal DNaseI is a direct or indirect consequence of local or systemic tissue damage and if the formation of anti-dsDNA antibodies in complex with chromatin is a secondary event that participates in development of nephritis.

What the data demonstrate, however, is that chromatin–IgG complexes are involved in progression of lupus nephritis.
Thus, silencing of renal DNaseI expression (68) may have immediate and harmful consequences linked to activation of the complement system by chromatin–IgG complexes (79). In addition, accumulated apoptotic chromatin fragments that are enriched in apoptosis-induced chromatin modifications (80–82) may affect cells of the innate immune system by triggering TLR7–9 and the Clec4e receptor. The apoptosis-induced chromatin modifications present in accumulated chromatin may be involved in sustained systemic autoimmune responses against chromatin (80–82).

A principal component analysis (PCA) biplot of gene expression data from murine (Figure 2A) and human (Figure 2B) lupus nephritis demonstrated the importance of renal DNAseI gene shutdown for progression of the inflammatory organ disease. The data are taken from murine and human parameters published by Thiyagarajan et al. (68). These PCA biplots aim to display variances of the parameters. The angles between the various biplot axes (shown as arrows) indicate the correlations between the variables (shown as arrows). The angles between the arrows tell whether they are clustered and indicate that they correlate with each other. The different arrows point at the variables. The figure, therefore, shows which variables are clustered to indicate that they appear together. Similarly, the position of the samples of individual mice (shown as the signs 1, 2 and 3 for group 1–3 mice) relative to the arrows, indicates which variable(s) have had the largest effect on disease progression. The result of the biplots demonstrates that groups emerging from this analysis perfectly correlated with the groups of BW mice, as defined by Licht et al. (20), defined as prenephritic BW mice (group 1), BW mice with deposits of EDSs in the mesangial matrix (group 2) or with deposits in the GBM (group 3). In (B), a similar biplot was generated for the human data, where the three patients with low DNAseI expression levels are included. The most striking observation in these biplots is that the DNAseI vector points away from the individuals with severe lupus nephritis (to the left in the biplots), demonstrating that loss of DNAseI correlates inversely with disease progression, whereas MMP-2, MMP-9, TLRs and EDSs associated with GBM are clustered and points at the most severely diseased murine and human individuals, in harmony with statistical analyses demonstrated by Thiyagarajan et al. (68). This figure is modified from Thiyagarajan et al. (68).
One possibility is a direct effect of early inflammation and secretion of proinflammatory cytokines in the context of early mesangial nephritis (studies in progress). By inspection of the DnaseI gene organization in the University of California, Santa Cruz, genome browser (http://genome.ucsc.edu/index.html), we found an overlap of 59 nucleotides in the annotated transcript with a transcript from the convergently transcribed tumor necrosis factor receptor–associated protein 1 (Trap1) gene in the 3′-untranslated regions. This gene organization is peculiar and is likely to preclude coexpression of the two genes. Given the fact that transcription proceeds well beyond the 3′ end of the mature transcript (83,84), the overlap between the transcripts of the two genes is substantial, and recent data demonstrate that it is unlikely that the two genes can be transcribed simultaneously. This was directly demonstrated by Hobson et al. (85) when they analyzed
transcription of convergent gene pairs. They used a combination of biochemical and genetic approaches and demonstrated that polymerases transcribing opposite and overlapping DNA strands cannot bypass each other. On the contrary, RNA polymerase II (RNAPII) molecules stop, and most importantly, do not dissociate from the DNA strands on head-to-head collision (85). This result suggests that opposing polymerases represent insurmountable blockades for each other (85). Finally, RNAPIIinduces chromatin modification, such as specific histone methylation and deacetylation, through the Set2/Rpd3S pathway (86,87). Such modifications will result in low to no expression of both genes.

The mutual suppression of one gene by expression of the other is called transcriptional interference (88). Recently, we showed that transcription of the DNasel gene is inversely affected by Trap1 gene expression (89). More detailed analyses on regulation of DNasel gene expression and the role of transcriptional interference with Trap1 gene expression are currently performed in our laboratory, involving studies of other regulators of DNasel gene transcription such as miRNA (manuscript in preparation, K D Horvei, D Thiyagarajan, S Fismen, OP Rekvig, SD Johansen, H Nielsen) and the effect of DNA methylation.

ORIGIN OF EXPOSED GLOMERULAR CHROMATIN

The processes that lead to exposure of renal chromatin are still an unresolved. Accelerated apoptosis or impaired clearance of apoptotic cells is thought to be involved (90–92). However, despite intensive investigations during the last decade, existing data on problems linked to disturbed apoptosis or clearance of apoptotic cells are not conclusive. Recent data expand our insight into these processes, since it was demonstrated that a loss-of-function variant in DNaseIL3 causes a familial form of SLE with lupus nephritis (93). DNaseIL3 is an apoptotic endonuclease predominantly expressed in macrophages (94) and dendritic cells (see http://biogps.org) and therefore links an inactive apoptotic executor to a possible cause of lupus nephritis.

The cellular origin of chromatin fragments exposed in glomeruli in the context of lupus nephritis is difficult to assess. Accumulation of chromatin fragments in the GBM correlates in time with the loss of expression of the most important renal endonuclease (that is, DNasel), and this result suggests a renal origin of the chromatin fragments. The fact that chromatin found in the GBM in human lupus nephritis can contain the polyomavirus BK large T antigen (95,96) also suggests a renal origin, since the BK virus possesses a selective tropism for tubular cells (97).

Another possibility is that that chromatin fragments reach glomeruli through the circulation, eventually in complex with IgG (40,63,98). This result explains the systemic character of tissue damage in SLE. Comparative studies of components of immune complexes and their localization in skin and glomerulus membranes and matrices demonstrated that they have similar structure (99,100). However, deposition of immune complexes in glomeruli did not predict deposition in skin. Examination of DNasel and MMP expression in the skin in MRL-lpr/lpr mice demonstrated a stable activity of DNasel and an increase in MMP-2 and MMP-9 enzyme activities during disease progression (100). These results indicate that circulating immune complexes can impose SLE manifestation in different organs, while progression of lupus nephritis may be organ-restricted because of the secondary mechanisms linked to silencing of the renal DNasel gene.

CONCLUSION

There is comprehensive and coherent support for a model of lupus nephritis in which extracellular chromatin plays a direct role as a target structure for anti-dsDNA/anti-chromatin antibodies. Thus, a notable change in thinking entailed by recent studies is that chromatin fragments exposed in glomeruli are released from dying renal cells and that these fragments are not appropriately degraded during the programmed cell death process because of an acquired loss of the dominant renal endonuclease DNasel. In this situation, accumulated chromatin fragments may be targeted by potentially nephritogenic anti-chromatin antibodies. In this sense, interaction of the antibodies and the chromatin targets is a homologous interaction. Therefore, the core nature of both murine and human lupus nephritis may point at exposure of chromatin in glomeruli and its complex formation with IgG as key events in disease pathogenesis and disease progression.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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