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Nephritogenic Lupus Antibodies Recognize Glomerular Basement Membrane-Associated Chromatin Fragments Released from Apoptotic Intraglomerular Cells

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Antibodies to dsDNA represent a classification criterion for systemic lupus erythematosus. Subpopulations of these antibodies are involved in lupus nephritis. No known marker separates nephritogenic from non-nephritogenic anti-dsDNA antibodies. It is not clear whether specificity for glomerular target antigens or intrinsic antibody-affinity for dsDNA or nucleosomes is a critical parameter. Furthermore, it is still controversial whether glomerular target antigen(s) is constituted by nucleosomes or by non-nucleosomal glomerular structures. Previously, we have demonstrated that antibodies eluted from murine nephritic kidneys recognize nucleosomes, but not other glomerular antigens. In this study, we determined the structures that bind nephritogenic autoantibodies *in vivo* by transmission electron microscopy, immune electron microscopy, and colocalization immune electron microscopy using experimental antibodies to dsDNA, to histones and transcription factors, or to laminin. The data obtained are consistent and point at glomerular basement membrane-associated nucleosomes as target structures for the nephritogenic autoantibodies. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling or caspase-3 assays demonstrate that lupus nephritis is linked to intraglomerular cell apoptosis. The data suggest that nucleosomes are released by apoptosis and associate with glomerulus basement membranes, which may then be targeted by pathogenic anti-nucleosome antibodies. Thus, apoptotic nucleosomes may represent both inducer and target structures for nephritogenic autoantibodies in systemic lupus erythematosus. ***(Am J Pathol 2006, 168:1779–1792; DOI: 10.2353/ajpath.2006.051329)***

Anti-double-stranded DNA (anti-dsDNA) antibodies are diagnostically and pathophysiologically central in systemic lupus erythematosus (SLE)[1–4] and represent a formal SLE classification criterion (American College of Rheumatology criterion no. 10,[1]). Clinically, glomerulonephritis is one of the most serious manifestations of SLE.[5–7] In this particular clinical context, anti-dsDNA antibodies are a proven central pathogenic factor.[3,8] There is, however, no firm and objective distinction that separates nonpathogenic from pathogenic anti-dsDNA antibodies ([2,3,5–7,9]). Antibody characteristics that may determine pathogenic potential may be intrinsic affinity and specificity for structures unique for nucleosomes or for cross-reacting non-DNA/nucleosomal planted or inherent kidney antigens.[3,10–15]

The following problems and models are contemporarily under discussion. Do pathogenic anti-dsDNA antibodies bind nucleosomal structures, presumably released from apoptotic cells,[9,11,16] or do they cross-react with non-nucleosomal planted antigens like α-actinin[13,17–19]? Antibodies recognizing intrinsic glomerular structures, like components of glomerular basement membranes (GBMs; an abbreviation used here for both mesangial and capillary membranes) or tubular basement membranes, including laminin, collagen IV, or entactin,[20–24] or cellular membranes[12,25,26] have all been eluted from nephritic kidneys, indicating pathogenic impact of this rather diverse repertoire of autoantibodies.

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Morphological changes in lupus nephritis, including prominent accumulation of electron-dense deposits (EDDs) associated with GBMs and correlation between EDDs and the clinical courses of lupus nephritis, were reported about 30 years ago.[27–29] These deposits are believed to constitute immune complexes, where the target antigens may contain chromatin constituents, as discussed by Berden et al.[8,9]

In agreement with such observations, we have recently demonstrated that antibodies eluted from nephritic (NZBxNZW)F1 (B/W) mice recognize eukaryotic nucleosomes. Among these antibodies, a significant subpopulation cross-reacted with dsDNA and histone H1.[30] By immune electron microscopy (IEM), we observed that *in vivo*-bound antibodies are confined to EDDs associated with GBMs. Others have eluted similar anti-nucleosomal antibodies from lupus nephritis kidneys (see, eg, Refs. [23,24,31]). In harmony with such observations, Kramers et al[32] have demonstrated that autoantibodies complexed with nucleosomes can bind GBMs, whereas purified, noncomplexed autoantibodies do not. Because heparan sulfate in GBMs may be a potential target for nucleosomes in glomeruli,[33] the binding of nucleosome-complexed autoantibodies in GBMs may be explained by interaction of nucleosomes with GBMs, as discussed by Berden et al.[9] Although antibody specificity for nucleosomes has not been correlated with the locus of binding in, eg, GBMs, it is tempting to speculate that GBM-associated EDDs may reflect deposition of nucleosomes complexed with autoantibodies.

We defined two aims for the present study: to determine the nature and potential origin of the antigens binding the antibodies *in situ* and to determine the exact intraglomerular localization of antibody deposits. To shed new light on these problems, we have here analyzed murine nephritic glomeruli by transmission electron microscopy (TEM), IEM, and high-resolution colocalization IEM, in addition to confocal microscopy. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL) and caspase-3 assays for apotosis demonstrate that glomerular cells of nephritic mice are apoptotic, and apoptotic chromatin fragments (or nucleosomes) are associated particularly with the GBMs. Data from TEM, IEM, and colocalization IEM demonstrate that EDDs are deposited in GBMs of nephritic mice. These EDDs are not inherent parts of GBMs of nephritic kidneys but represent externalized chromatin particles because they bind experimental antibodies to histones, dsDNA, and transcription factors, all of which colocalize with *in situ*-bound autoantibodies. Both autoantibodies and experimental antibodies to chromatin constituents bound strictly to these ectopic chromatin structures. No antibody interaction was seen with regular inherent basement membrane structures. Thus, the present data demonstrate that nephritogenic autoantibodies in nephritic B/W mice bind ectopic apoptotic nucleosomes associated with GBMs and not other constituents of the glomerulus.

Materials and Methods

Mice

BALB/c, NZB, and NZW mice were purchased from Harlan (Oxon, United Kingdom). Hybrid lupus-prone B/W mice were generated by crossing the NZB and NZW mouse strains. Treatment and care of the animals were in accordance with the guidelines of Norwegian Ethical and Welfare Board for Research Animals. The study was approved by the Institutional Review Board.

Antigens

Calf thymus dsDNA and chicken α-actinin were purchased from Sigma (St. Louis, MO). Nucleosomes were prepared from BALB/c spleen cells and characterized as described previously,[34] except that we digested shared chromatin by micrococcal nuclease for 60 minutes instead of 30 minutes. The purified nucleosomes were shown to be of similar size and composition as described previously[34] and consisted of DNA from 200 up to several thousand base pairs, all five histone classes, and nonhistone proteins.

Monoclonal and Oligoclonal Antibodies

The murine nephritogenic anti-dsDNA monoclonal antibody (mAb) 163p77[14] and the human anti-dsDNA mAb 33C9[35] were kindly provided by Dr. Marion (Memphis, TN) and Dr. Winkler (Erlangen, Germany), respectively. Rabbit oligoclonal or murine monoclonal anti-histone H1 (H1), anti-histone H3 (H3), anti-α-actinin, and anti-TATA box-binding protein (TBP) antibodies as well as the GBM-specific rabbit anti-collagen IV (fine specificity for type IV α-isoform of mouse, rat, and human collagen) and anti-laminin-β2 antibodies were all from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-laminin antibodies were from Sigma (St. Louis, MO); and the monoclonal anti-polyomavirus T-antigen (T-ag) antibody Ab-2 was obtained from Calbiochem (San Diego, CA). Peroxidase-conjugated anti-mouse, -human, and -rabbit IgG antibodies used in enzyme-linked immunosorbent assays (ELISAs) were from Sigma.

Serial Serum Samples and Determination of Proteinuria

Sera from individual B/W mice were obtained at the age of 11, 17, 21, 30, and 33 weeks. The sera were stored at −20°C until used. Proteinuria was determined by stix from Bayer Diagnostics (Bridgend, United Kingdom): 0–1+, ≤0.3 g/L, regarded as physiological proteinuria; 2+, ≤1 g/L; 3+, ≤3 g/L; and 4+, ≥20 g/L.

ELISA

For the detection of serum antibodies, ELISA was performed exactly as described previously,[10,36,37] using microtiter plates (Nunc MaxiSorp; Nunc, Copenhagen, Den-

mark) coated with nucleosomes (10 μg/ml as DNA), calf thymus dsDNA (10 μg/ml), or α-actinin (10 μg/ml). All antigens except nucleosomes, which were diluted in water, were diluted in phosphate-buffered saline (PBS), pH 7.2. Blocking solutions were not used in these ELISAs, because they may contain nucleosomes, as demonstrated by Dieker et al.[38] Cut-off levels for each ELISA were determined as described in detail elsewhere,[30,37] and $OD_{490} \leq 0.2$ was regarded negative. Appropriate negative and positive control antibodies were included in each ELISA for intra-assay validation of the cut-off levels.[30]

Examination of Kidneys for Morphological Changes and Autoantibody Binding

Kidneys from the B/W or BALB/c mice were subjected to morphological and histopathological examination by light microscopy, confocal microscopy, TEM, IEM, and finally by colocalization IEM. One kidney from each mouse was fixed in depolymerized paraformaldehyde for TEM and IEM analyses. The other kidney was divided into two parts, one was embedded using Tissue-Tek (Chemiteknik AS, Oslo, Norway) and frozen at −70°C for confocal microscopy, whereas the second part was fixed in formalin and paraffin-embedded.

Light Microscopy

Histopathology assessment was done on 4-μm sections of paraffin-embedded, formalin-fixed kidneys stained with hematoxylin and eosin. Sections were evaluated by two pathologists (E.M. and L.J.) without knowing the condition of the mice.

Confocal Microscopy

Cryosections (4 μm) of kidneys of nephritic B/W mice were incubated with rabbit IgG anti-collagen IV or anti-laminin-β2 antibodies for 30 minutes. Subsequently, the slides were washed with PBS and incubated with goat anti-mouse IgG antibodies conjugated with Alexa Fluor 488 to detect deposits of IgG autoantibodies and with goat anti-rabbit IgG antibodies conjugated with Alexa Fluor 546 (Molecular Probes, Inc., Eugene, OR) to test whether the autoantibodies colocalize with anti-laminin-β2/anti-collagen IV antibodies. Images were collected using a Zeiss Axiovert 200 microscope (Carl Zeiss, Heidelberg, Germany) equipped with an LSM5 10 confocal module and processed using Adobe Photoshop.

TEM, IEM, and Colocalization IEM

TEM was performed to analyze for morphological changes related to nephritis development in the B/W mice, exactly as described recently.[30]

For IEM, ultra-thin cryosections of kidneys from BALB/c or nephritic B/W mice were prepared and processed as described previously.[30,39] *In vivo*-bound glomerular autoantibodies were detected by incubating the sections with rabbit anti-mouse IgG (RaM IgG) antibodies (ICN/Chappel, Aurora, OH) followed by protein A conjugated with 5-nm gold particles (PAG-5 nm) (University of Utrecht, The Netherlands). This also represents step 1 in the colocalization IEM (see below).

The colocaliztion IEM developed in this study is based on similar double-labeling assays described before.[40] Step 1 (IEM, see above) was first performed to trace *in vivo*-bound autoantibodies, followed by incubation of the sections with experimental murine mAbs directed against the chromatin constituents dsDNA, H1 and H3, and TBP or with rabbit antibodies against laminin, a major constituent of GBMs (step 2). The sections incubated with the murine mAbs were subsequently incubated with RaM IgG followed by PAG-10 nm to visualize the presence of the antibodies added in step 2. Binding of rabbit antibodies to laminin was directly detected by PAG-10 nm. By this approach, the structures recognized by the autoantibodies could be determined by eventual colocalization with the added experimental structure-specific antibodies. In separate control experiments, rabbit anti-H1/H3/TBP or a human anti-dsDNA antibody (33C9[35]) was used instead of murin monoclonal antibodies, because rabbit and human IgG antibodies bind PAG conjugates directly, and addition of RaM IgG could be omitted. By this technique, monospecific staining of GBM structures by the experimental antibodies could be performed without concomitant staining of *in vivo*-deposited autoantibodies. Specificity and validity of double-labeling of autoantibodies and experimental antibodies for colocalization was verified using control antibodies (anti-T-antigen antibody Ab-2) or PBS instead of experimental antibodies in step 2. Micrographs were taken at ×50,000 to 70,000 total magnification using a JEM-1010 transmission electron microscope (Jeol, Tokyo, Japan).

Apoptosis Assays

Intraglomerular apoptosis was evaluated by a TUNEL assay using a fluorescence-based *In situ* Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany). The assay protocol was as recommended by the manufacturer, applied to 4-μm sections of paraffin-embedded kidneys of B/W mice at different ages and of 25-week-old BALB/c mice. TUNEL-positive control sections, included in each experiment, were generated by digestion of BALB/c kidney sections with 3000 U/ml micrococcal nuclease for 10 minutes. Samples were analyzed using the fluorescent Zeiss Axiovert 200 microscope.

Presence of apoptotic cells was also determined by caspase-3 activity. Paraffin-embedded kidney sections were first boiled in citrate buffer, pH 6, and incubated overnight at 4°C with a polyclonal rabbit anti-caspase-3 antibody (R&D Systems, Abingdon, United Kingdom). The sections were subsequently washed with PBS and incubated with secondary biotinylated anti-rabbit IgG antibody and

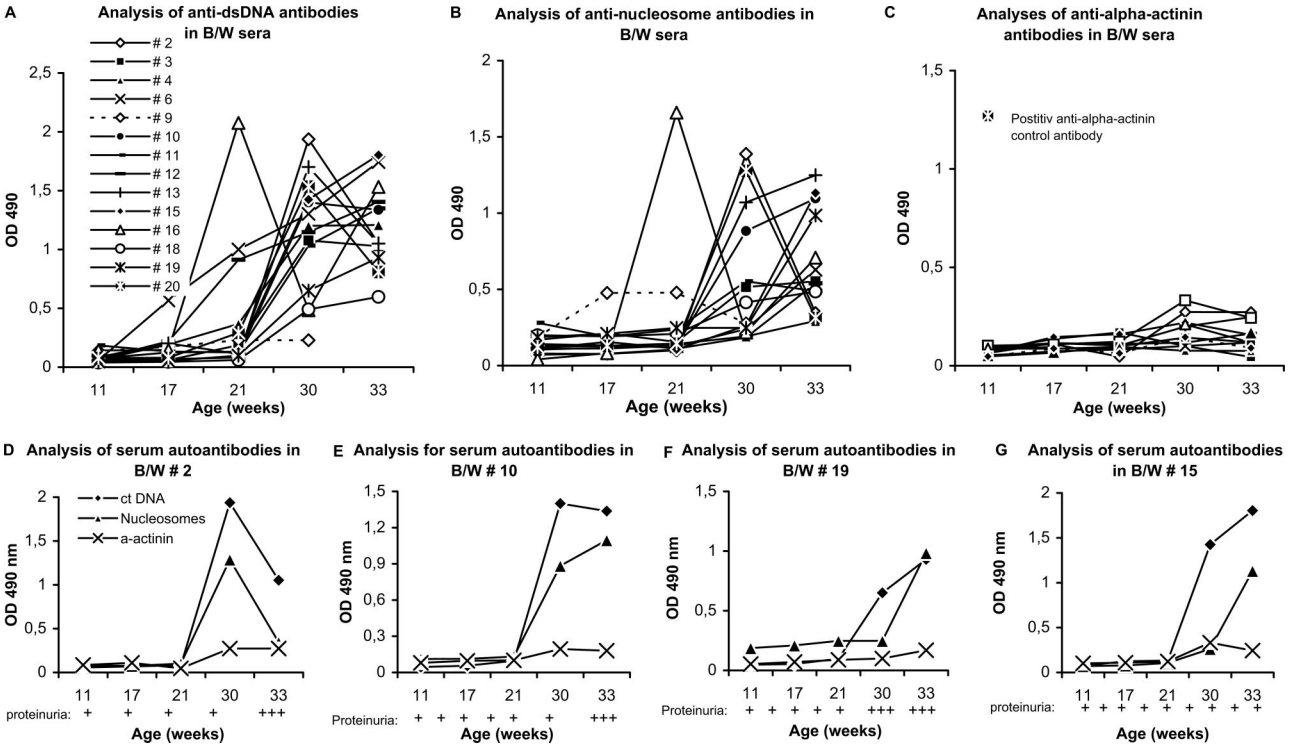


Figure 1. Autoantibody profiles determined over 33 weeks in 14 (NZBxNZW)F1 mice. Antibodies were determined at regular intervals against dsDNA (**A**), nucleosomes (**B**), and α-actinin (**C**). Four antibody patterns were detected before or at the time proteinuria was developing, decreasing (**D**), stable (**E**), or increasing (**F**) antibody levels. In one mouse, the antibodies increased, still without proteinuria (B/W15; **G**). The symbols given for individual mice in **A** are representative for the symbols in **B** and **C**, whereas the symbols in **D** are representative for those in **E–G**.

streptavidin-horseradish peroxidase conjugate using cell and tissue staining kit horseradish peroxidase-3,3′-diaminobenzidine (DAB) (R&D Systems).

Results

Analyses of Serum Antibodies to dsDNA, Nucleosomes, and α-Actinin and Their Relationship with Development of Proteinuria in Lupus-Prone B/W Mice

To determine the temporal relationship between anti-dsDNA or anti-nucleosome antibody production and proteinuria and to analyze whether anti-α-actinin antibodies are linked to nephritis development, 14 lupus-prone B/W mice were selected for follow-up studies over a 33-week observation period. The mice were analyzed for production of antibodies to dsDNA, nucleosomes, or α-actinin at regular intervals, and proteinuria was determined weekly. All mice produced significant amount of antibodies to dsDNA (Figure 1A) and anti-nucleosome antibodies (Figure 1B), whereas only three of the mice produced antibodies to α-actinin above cut-off levels (Figure 1C). Anti-dsDNA and anti-nucleosome antibodies were detected within the same time span, as serum antibodies to dsDNA and nucleosomes appeared on average at 25 and 26 weeks of age, respectively, and approximately 4 weeks before development of proteinuria (Table 1). Four principally different antibody profiles were observed concomitant with or before development of proteinuria: an abrupt decrease of serum antibody levels (Figure 1D); a plateau (Figure 1E); steadily increasing levels (Figure 1F); or increase in serum levels (still) without development of proteinuria (Figure 1G). The anti-dsDNA and anti-nucleosome antibodies tended to follow each other with similar profiles (Figure 1, A–G).

Morphological Studies of Nephritic B/W Kidneys

Included in these studies are kidneys from BALB/c mice, one 10-week-old non-nephritic, antibody-negative B/W mouse (B/W52), or three anti-dsDNA/nucleosome antibody-positive B/W mice—one without proteinuria (B/W15) and two with proteinuria (B/W2 and B/W16; Table 1).

Hematoxylin and eosin-stained kidney sections from BALB/c or the young B/W52 did not demonstrate glomerular pathology (Figure 2, A and B). Similar sections from B/W15 were characterized by moderate mesangial proliferation but no clearly identifiable extracellular chromatin particles (Figure 2C). Corresponding sections from B/W2 and B/W16 demonstrated moderate mesangial matrix proliferation (Figure 2, D and E, for B/W2 and B/W16, respectively) and development of wire-loop lesions and intraglomerular cell death with extracellular chromatin particles (for examples, see areas indicated by circles in Figure 2, D and E).

Table 1. Antibodies to dsDNA, Nucleosomes, and α -Actinin and Their Relationship to Proteinuria in B/W Mice Studied over 33 Weeks

B/W mouse no.	Age (weeks) at production of			Age at proteinuria*	Antibody pattern prior to proteinuria	Pattern example (see Figure 1)†
	Anti-dsDNA	Anti-nuc	Anti-α-actinin			
2	30	30	30	2 (+3)	Decreasing	D
3	30	30	nd‡	32 (+3)	Stable	E
4	21	33	nd	29 (+3)	Stable	E
6	17	30	nd	26 (+3)	Increasing	F
9	21	17	nd	24 (+3)	Low antibody levels	–
10	30	30	nd	32 (+3)	Stable	E
11	30	21	nd	32 (+3)	Stable	E
12	17	33	33	32 (+2)	Increasing	F
13	30	30	nd	32 (+3)	Stable	E
15	30	30	30	(+1)	No proteinuria over 33 weeks	G
16	21	21	nd	33 (+4)	Decreasing	D
18	30	21	nd	32 (+3)	Increasing	F
19	30	17	nd	17 (+1)	Increasing	F
20	21	30	nd	32 (+2)	Decreasing	D
Mean (±SD)	25.6 (±5.4)	26.6 (±5.8)	Not determined	30.5 (±2.8)		

*Proteinuria was determined by stix from Bayer Diagnostics (Bridgend, United Kingdom): 0–1+, ≤0.3 g/l; 2+, ≤1 g/l; 3+, ≤3 g/l; 4+, ≥20 g/l.
†The antibody profiles, as determined by ELISA using 1/100 serum dilution on serial serum samples, possessed four principally different profiles linked to development of proteinuria, as demonstrated in Figure 1, D–G.
‡nd, not detected.

Analyses of Apoptosis in B/W Glomeruli by TUNEL and Caspase-3 Assays

To investigate whether intraglomerular cell death is caused by apoptosis, the TUNEL assay was performed on kidney sections of BALB/c and of B/W mice nos. 52, 15, 2, and 16. The BALB/c and the B/W52 glomeruli were TUNEL assay negative (Figure 2, F and G), whereas glomeruli of B/W15 and B/W2 were strongly positive with a granular staining of the mesangium and also along capillary basement membranes (Figure 2, H and I, for B/W15 and B/W2, respectively). Similarly, the TUNEL assay applied to glomeruli of the B/W16 resulted in staining particularly localized to the mesangium and capillary basement membranes (Figure 2J).

The caspase-3 assay, as an alternative assay detecting early apoptosis, was negative for BALB/c and the young B/W52 glomerular cells (Figure 2, K and L, for BALB/c and B/W52, respectively). In glomeruli of B/W15, B/W2, and B/W16, the caspase-3 assay was positive for a large proportion of mesangial cells (Figure 2, M, N, and O, respectively). Thus, results of the two different apoptosis assays are consistent and demonstrate strong tendency for apoptosis of glomerular cells in nephritic but not in young non-nephritic B/W mice nor in BALB/c mice. Interestingly, glomeruli of B/W15 mice, which produce significant amounts of anti-dsDNA and anti-nucleosome antibodies and have GBM-associated EDDs (see below; Figure 6A) while still lacking proteinuria, were positive for both apoptosis assays (Figure 2, H and M).

These data strongly suggest that nucleosomes and larger chromatin particles in context of lupus nephritis may be released from intraglomerular apoptotic cells and distributed to the mesangium and to the GBMs as potential target antigens for antibodies to dsDNA/nucleosomes.

In Vivo-*Bound GBM-Associated IgG Autoantibodies Recognize Structures Distinct from GBMs, as Determined by Colocalization IEM*

A detailed investigation of the nature and exact localization of the target antigens for autoantibodies deposited in glomeruli was performed using confocal and colocalization IEM. Kidney specimens of the nephritic B/W mice were selected for these analyses based on observations that demonstrated mesangial proliferation and intraglomerular cell death (Figure 2 for B/W15, B/W2, and B/W16). In the following, typical data are presented for B/W16. By TEM, accumulation of EDDs was observed along GBMs (Figure 3A, arrows), and *in vivo* binding of autoantibodies, visualized by 5-nm gold, was strictly restricted to EDDs (Figure 3B). By confocal microscopy, IgG autoantibodies (Figure 3C) and antibodies to collagen IV (Figure 3D) were partly in confocus (Figure 3E, yellow color). Similarly, autoantibodies (Figure 3F) and anti-laminin-β2 antibodies (Figure 3G) also colocalized topographically and were partly in confocus (Figure 3H). IEM for detection of IgG autoantibodies demonstrated that these bound strictly to EDDs associated with GBMs (Figure 3I), whereas rabbit anti-laminin antibodies bound regular GBM structures distinctly different from EDDs (Figure 3J). Double-labeling of these sections with IgG autoantibodies visualized by 5-nm gold and with anti-

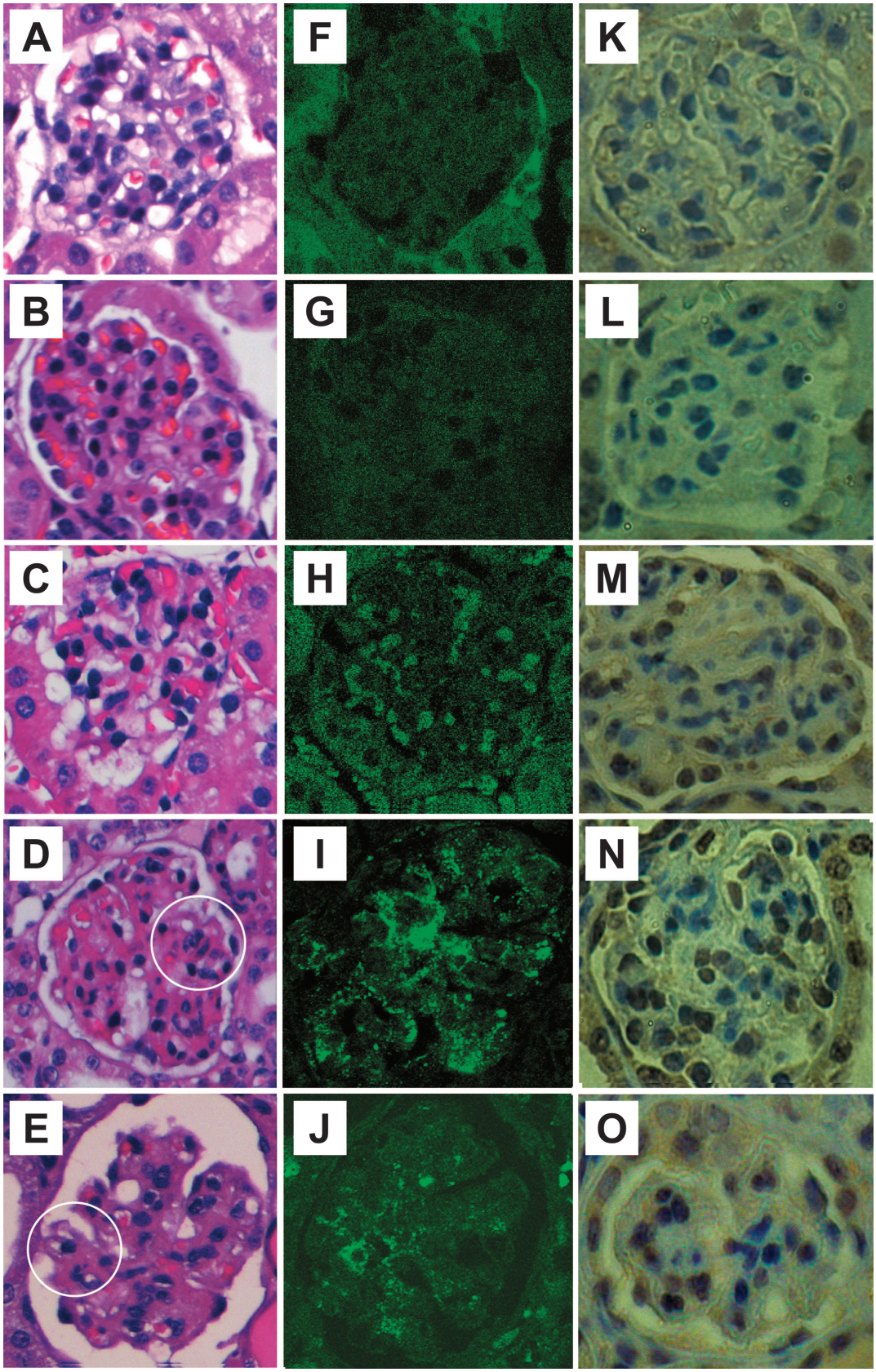


Figure 2. Morphological and apoptosis studies of murine glomeruli. Glomeruli of BALB/c (**A**, **F**, and **K**), non-nephritic young B/W52 (**B**, **G**, and **L**), anti-dsDNA-positive, nonproteinuric B/W15 (**C**, **H**, and **M**), nephritic B/W2 (**D**, **I**, and **N**), or the nephritic B/W16 (**E**, **J**, and **O**) were analyzed for morphological changes by light microscopy (**A–E**). Intraglomerular apoptosis was analyzed by TUNEL assay (**F–J**) or by caspase-3 assay (**K–O**). **K–O:** Caspase-3-positive cells are brown; caspase-3-negative ones are blue. **Circles** in **D** and **E** indicate areas with wire-loop lesions and extracellular chromatin fragments.

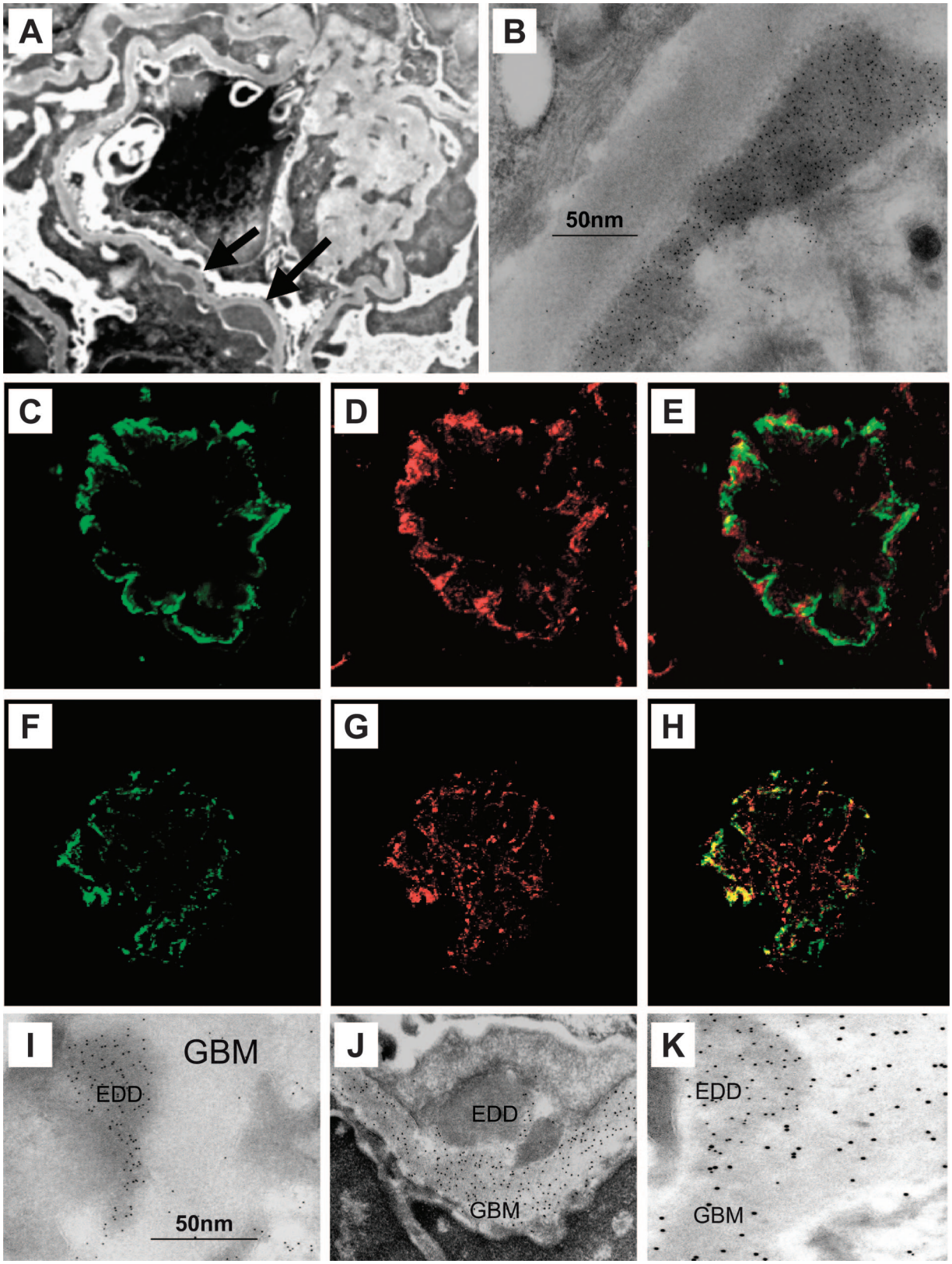


Figure 3. Morphological analyses of nephritic glomeruli of B/W16 by TEM and IEM and by confocal microscopy and colocalization IEM. Morphology is analyzed by TEM (**A**) and localization of autoantibody deposits by IEM (**B**). EDDs in **A** are indicated by **arrows**. **B:** Autoantibody deposits (shown as 5-nm gold) are confined to these structures. Confocal microscopy (**C–H**) demonstrates that autoantibody deposits (displayed in green; **C**) and bound anti-collagen IV antibodies (red; **D**) colocalize topographically and are in certain areas in confocus (yellow; **E**). **F–H:** Autoantibody deposits (green; **F**) and bound anti-laminin-β2 antibodies (red; **G**) are partly in confocus (yellow; **H**). By IEM analyses (**I** and **J**), autoantibodies are displayed by 5-nm gold particles (**I**) and anti-laminin antibodies by 10-nm gold particles (**J**). Colocalization IEM analyses (**K**) demonstrate autoantibodies (5-nm gold) and anti-laminin-β2 antibodies (10-nm gold) in EDDs and GBMs, respectively.

laminin antibodies by 10-nm gold demonstrates that autoantibodies and test antibodies like those to laminin are in juxtaposition but bind to different structures of nephritic GBMs (Figure 3K). Similar results are obtained using glomerular section from other nephritic B/W mice (data not shown). Thus, although confocal microscopy indicates that autoantibodies and test antibodies to laminin partially are in confocus, results from colocalization IEM demonstrate that the anti-laminin antibody binds in the GBMs, whereas the *in vivo*-bound autoantibodies recognize EDDs associated with GBMs. Furthermore, similar structures complexed with antibodies are seen not only in association with GBMs but also in the mesangium (data not shown).

GBM-Associated Electron-Dense Deposits Recognized by Autoantibodies in Vivo *Constitute Chromatin Particles as Determined by Two-Step High-Resolution Colocalization IEM*

Data given above for B/W16 demonstrate that IgG autoantibody binding *in vivo* is confined to GBM-associated EDDs. To characterize the nature of EDDs recognized by these autoantibodies, we developed a high-resolution colocalization IEM technique. TEM applied to glomeruli of a BALB/c mouse revealed that GBMs and the mesangium were largely normal, with normal podocyte foot processes (Figure 4A). Colocalization IEM was performed by first adding RaM IgG/PAG-5 nm (step 1), followed by incubation of the sections with the negative control antibody Ab-2 and RaM IgG/PAG-10 nm (step 2). No significant deposits of 5- or 10-nm gold could be traced in GBMs, indicating the absence of *in vivo*-bound antibodies (Figure 4B). This result demonstrates that RaM IgG-PAG-5 nm/RaM IgG-PAG-10 nm do not bind GBMs nonspecifically. If an anti-dsDNA mAb was added instead of Ab-2 in step 2, still no GBM-associated antibody deposits could be detected (Figure 4, C and D), whereas nuclear deposits of the anti-dsDNA mAb could be observed, as visualized by nuclear binding of 10-nm gold (Figure 4, C and E). In subsequent experiments, this technique was applied to kidneys of anti-dsDNA antibody-positive (B/W2, B/W15, and B/W16) or antibody-negative (B/W52) mice with (B/W2 and B/W16) or without (B/W15) proteinuria. Glomeruli of B/W52 were devoid of EDDs associated with GBMs, and IEM revealed no antibody deposits in GBMs (data not shown).

In Figure 5, TEM and colocalization IEM data are demonstrated for the nephritogenic B/W2 mouse, having proteinuria and anti-dsDNA/anti-nucleosome antibody production (Figure 1, A and B; Table 1). Glomeruli were characterized by confluent podocyte foot processes and subepithelial accumulation of EDDs along GBMs (Figure 5A, arrows). Autoantibody binding *in vivo* was strictly localized to EDDs as demonstrated by IEM after staining with RaM IgG, PAG-5 nm (Figure 5B, enlarged in C). To test for unspecific binding, these sections were incubated with an irrelevant IgG antibody (anti-T-antigen mAb Ab-2) followed by RaM IgG, PAG-10 nm. This resulted in 5-nm but not 10-nm gold staining of the EDDs (Figure 5, D and E), thus demonstrating that IgG antibodies used in step 2 do not bind nonspecifically.

In subsequent experiments, Ab-2 in step 2 was substituted by anti-H1 (Figure 5F, enlarged in G), by anti-TBP (Figure 5H, enlarged in I), or by the 163p77 anti-dsDNA (Figure 5, J–M) mAbs. These experiments demonstrate that all experimental anti-chromatin antibodies (against H1/TBP/dsDNA) but not irrelevant control antibodies like the anti-T-ag antibody Ab-2 colocalize with *in vivo*-bound autoantibodies, as is demonstrated by simultaneous presence of 5- and 10-nm gold particles in EDDs. Importantly, both *in vivo*-bound autoantibodies and the experimental antibodies bound strictly to EDDs and not to regular GBM structures. In the experiment using anti-dsDNA mAb 163p77, this antibody bound the nuclei of the sections, as demonstrated by nuclear localization of 10-nm gold (Figure 5K, enlarged in L). In the GBM, however, the anti-dsDNA antibody colocalizes with autoantibody deposits restricted to EDDs, because 5- and 10-nm gold particles are simultaneously present in these EDDs (Figure 5K, enlarged in M) but not outside these structures.

Two further anti-dsDNA/anti-nucleosome antibody-positive B/W mice were selected for such studies, B/W15 and B/W16. Although the B/W15 mouse still after 33 weeks did not have measurable proteinuria (Table 1), the glomeruli were characterized by GBM-associated EDDs (Figure 6A, arrows) and by *in vivo* autoantibody binding exclusively restricted to these structures (Figure 6B, enlarged in C). By colocalization IEM, it is evident that these autoantibodies colocalized with the anti-dsDNA mAb 163p77 (Figures 6D and 5E) in a way similar to what was observed in glomeruli of B/W2.

Similar results were obtained in the anti-dsDNA and anti-nucleosome antibody-positive nephritic B/W16 mouse (Figure 1, A and B; Table 1). Confluent podocyte foot processes characterized the glomeruli, and subepithelial accumulation of EDDs was detected along GBMs (Figure 7A, arrows). By IEM, autoantibody deposits were shown to be confined to EDDs (Figure 7B). Colocalization IEM using the anti-dsDNA 163p77 mAb demonstrated, as for the former B/W mice, that the autoantibodies, reflected by 5-nm gold, and the experimental anti-dsDNA antibody, visualized by 10-nm gold, bound target antigens located within EDDs (Figure 7C, enlarged in D). As was demonstrated for B/W2, gold particles of 10-nm size, reflecting anti-dsDNA mAb 163p77, were bound in the nuclei (Figure 7C, enlarged in E).

Thus, in the anti-dsDNA antibody-positive B/W15 mouse without proteinuria and in proteinuric B/W mice, EDDs are associated with GBMs, and these EDDs contain target antigens for autoantibodies and experimental murine anti-chromatin antibodies, like those recognizing histones, transcription factor TBP, and dsDNA. This is further evident from the fact that rabbit antibodies to H1, H3, or TBP and the human monoclonal anti-dsDNA antibody 33C9[35] target the same electron-dense structures in these mice (data not shown).

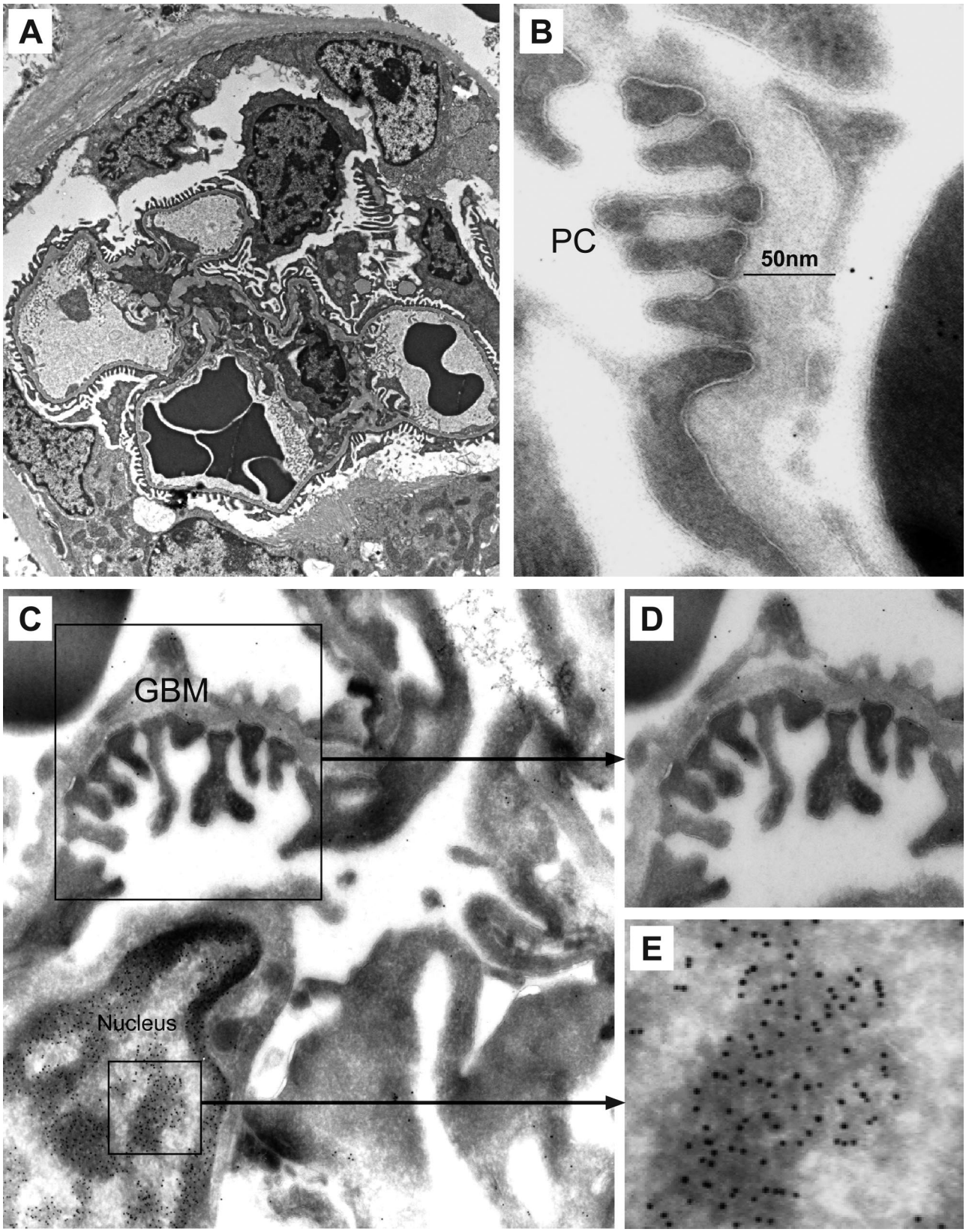
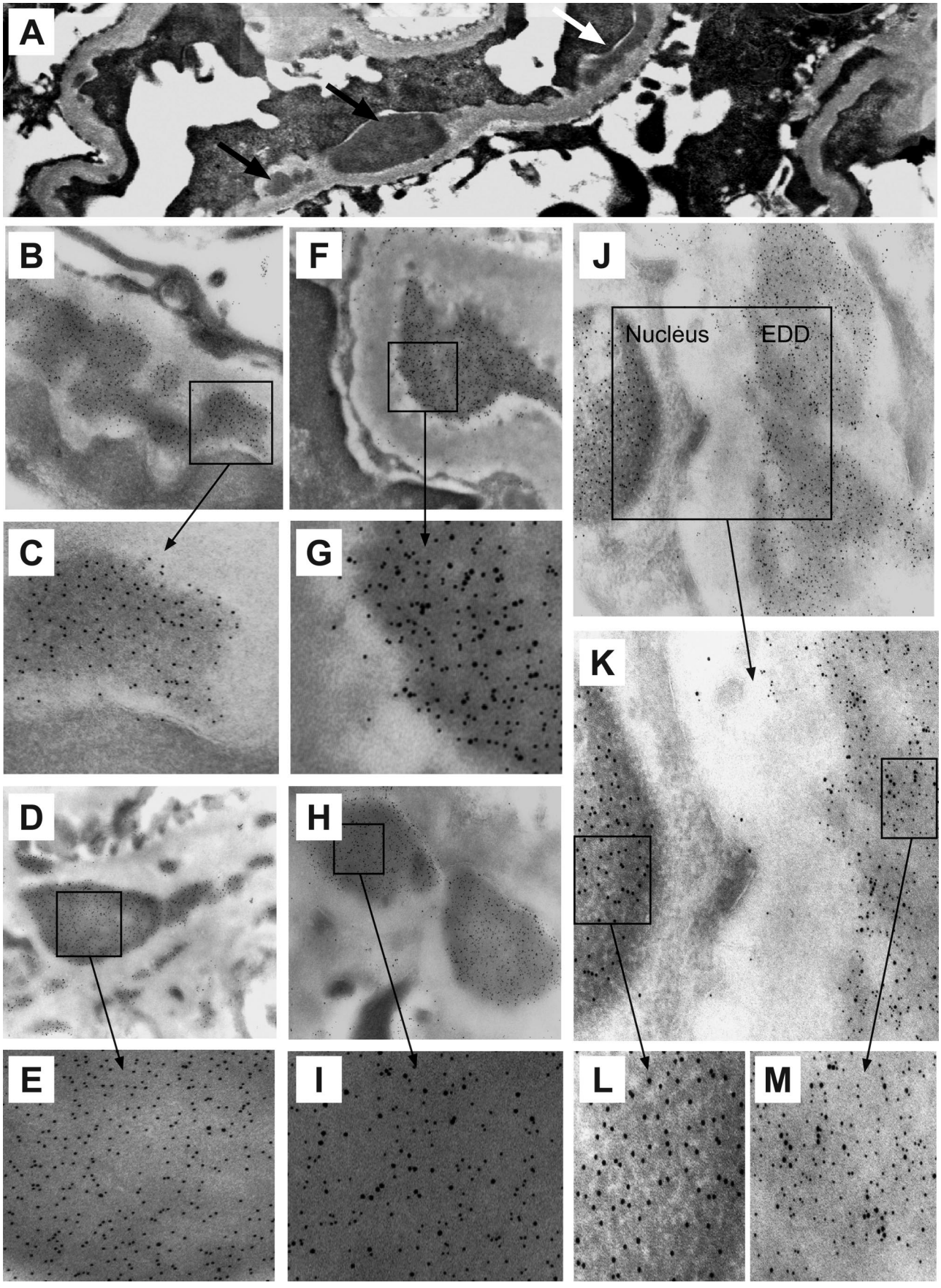


Figure 4. TEM, IEM, and two-step colocalization IEM of BALB/c glomeruli. **A:** TEM analysis was performed to assess glomerular morphology. **B:** Sections are incubated with RaM IgG/PAG-5 nm to search for *in vivo*-bound autoantibodies (step 1). In step 2, colocalization IEM (**B**) was performed with an irrelevant control antibody (anti-T-antigen mAb Ab-2). Absence of 10-nm gold demonstrates that IgG or PAG conjugates do not bind nonspecifically (**B**). Next, step 2 was performed with anti-dsDNA mAb 163p77 instead of Ab-2. GBM is not stained by 5- or 10-nm gold particles (**C**, enlarged in **D**), whereas the nuclei are stained by anti-dsDNA mAb, reflected by 10-nm gold particles (**C**, enlarged in **E**). For experimental details on colocaliztion IEM used in Figures 4 to 7, see Materials and Methods.


A
B
F
J
Nucleus
EDD
C
G
K
D
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M

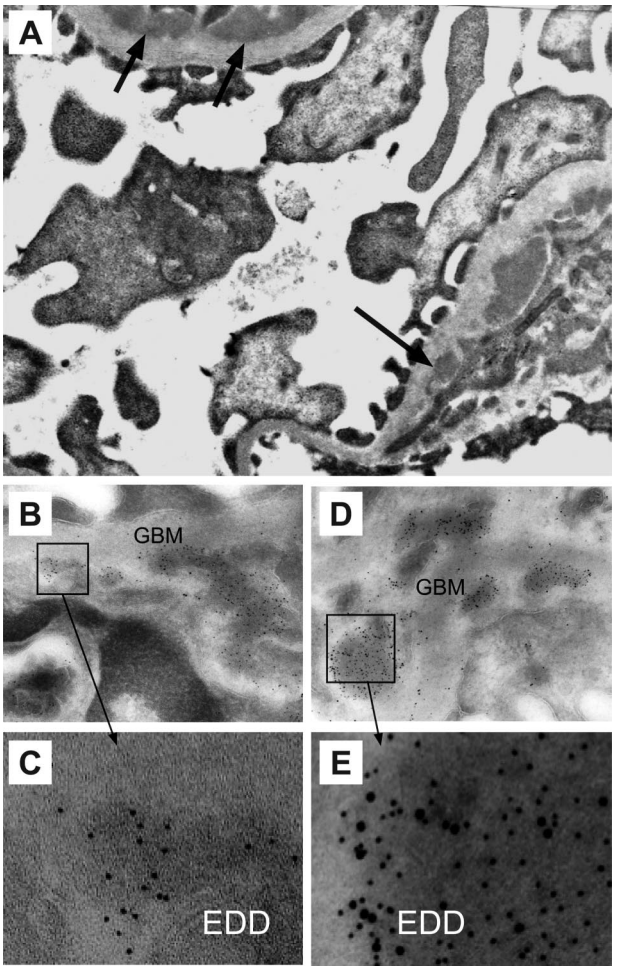

Figure 6. TEM, IEM, and two-step colocalization IEM of nonproteinuric B/W15 glomeruli. **A:** TEM analysis demonstrates partially confluent podocytes, and GBM-associated EDDs (**arrows**). By IEM (step 1), autoantibody deposits are traced by 5-nm gold (**B**, enlarged in **C**). Colocalization IEM (step 2) was performed using the anti-dsDNA mAb 163p77, and binding was traced by 10-nm gold (**D**). Autoantibodies and the anti-dsDNA mAb colocalize in the GBM-associated EDDs (5- and 10-nm gold in **D**, enlarged in **E**).

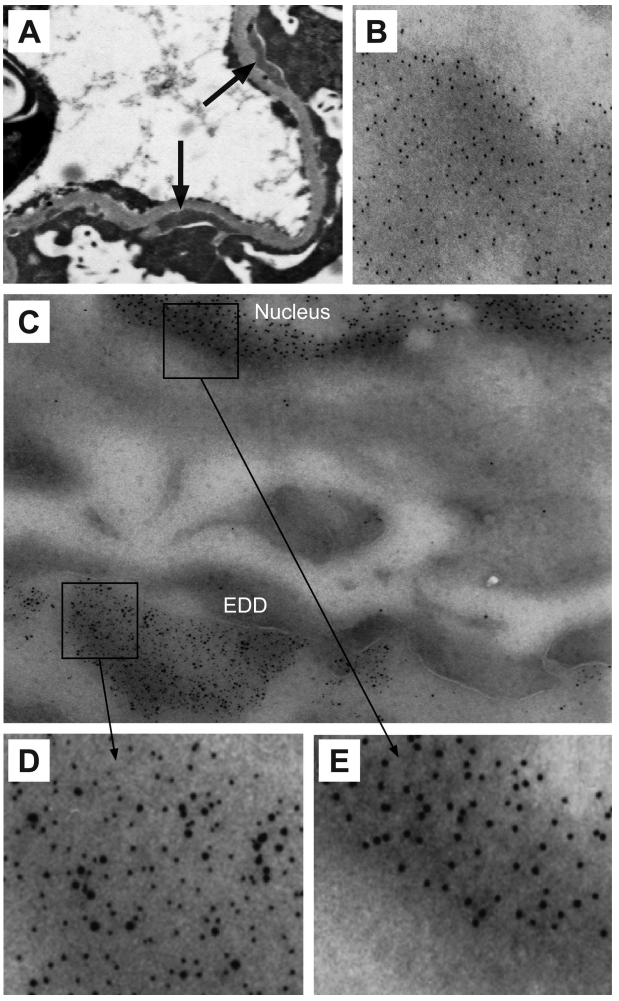

Figure 7. TEM, IEM, and two-step colocalization IEM of nephritic B/W16 glomeruli. **A:** TEM analysis demonstrates confluent podocytes and GBM-associated EDDs (**arrows**). **B:** IEM analysis (step 1) demonstrates autoantibodies in EDDs (5-nm gold). Colocalization IEM (step 2) was performed using the anti-dsDNA mAb 163p77. The anti-dsDNA mAb (10-nm gold) colocalizes with autoantibodies (**C**, enlarged in **D**). In the nucleus, the anti-dsDNA mAb is detected (**C**, enlarged in **E**).

Discussion

There is no agreement as to which antibody specificities are nephritogenic in SLE. Moreover, the critical event(s) accounting for initiation of lupus nephritis has never been determined. Development of distinct nephritogenic antibody specificities may be one important factor; release of antigens binding potentially nephritogenic antibodies may be another critical pre-requisite (for details, see the introduction). Irrespective of the complexity of potentially nephritogenic autoantibodies associated with SLE, a consensus has evolved that antibodies to dsDNA are a central pathogenic factor involved in development of lupus nephritis.[3,5,8,41] However, it has not been established whether there exists a nephritogenic anti-dsDNA antibody distinctly different from other anti-dsDNA antibodies. The divergent models to explain lupus nephritis may have evolved simply because we still lack conclusive data that provide definitive insight into the nephritic process(es). The purpose of this study was to identify structures that represent targets for *in vivo* glomerular-bound autoantibodies, and to characterize these structures by sets of experimental antibodies specific for candidate antigens using high-resolution colocalization IEM. Different apoptosis assays were used to search for origin of potential autoantibody-binding GBM-associated structures.

Initially, we observed different anti-dsDNA and anti-nucleosome antibody profiles before or concomitant with development of proteinuria. These profiles may give

Figure 5. TEM, IEM, and two-step colocalization IEM of nephritic B/W 2 glomeruli. **A:** TEM analyses revealed confluent podocytes and electron deposits in GBMs (**arrows**). By standard IEM (step 1), autoantibody deposits are traced by 5-nm gold (**B**, enlarged in **C**). Colocalization IEM (step 2) was performed using the negative control anti-T-ag antibody Ab-2 (**D**, enlarged in **E**) or anti-histone H1 (**F** and **G**), anti-TBP (**H** and **I**), or the anti-dsDNA 163p77 (**J–M**) monoclonal antibodies. Autoantibodies and the three chromatin-specific experimental antibodies (the three latter antibodies traced by 10-nm gold) are colocalized in EDDs (enlarged in **G**, **I**, and **M**, for anti-H3, anti-TBP, and anti-dsDNA antibodies, respectively). Anti-T antigen did not bind (absence of 10-nm gold in **D** and **E**).

some hints as to whether nonpathogenic antibodies transform into pathogenic ones by, eg, somatic mutations, or whether anti-dsDNA antibodies per se bind *in vivo* to, eg, nucleosomes at the time they are released from dying cells. If the latter explanation is correct, this would explain why intraglomerular apoptosis and subsequent antibody binding to ectopic nucleosomes results in decrease in serum antibody levels and precedes proteinuria. In some of the mice studied here, antibody levels did not decrease. This may be explained by a stronger antibody production in these mice, resulting in an antibody overproduction that may hide that antibodies disappear from the circulation. If, as an alternative explanation, somatic mutations of IgG variable region genes account for generation of "new" nephritogenic anti-dsDNA antibody subpopulations, then the precursor serum antibodies would not bind in glomeruli. Consequently the antibody levels would be stable or increasing and not drop, whereas the new, transformed antibodies would bind, and potentially be undetected in the serum. The present data are not consistent with this explanation. The different antibody profiles related to development of proteinuria among B/W mice may, nevertheless, make changing antibody levels difficult to use as predictor for onset of proteinuria (see, eg, Ref. [42]). Anti-α-actinin antibodies were detected in only 3 of the 14 B/W mice studied here. Thus, this antibody specificity may not be a critical factor for nephritis development, consistent with lack of this specificity in eluates from B/W nephritic kidneys.[30]

By IEM, we made two significant observations that relate to the nature of the target antigen for nephritogenic autoantibodies. First, EDDs were present in GBMs of nephritic mice but not in young non-nephritic mice, as originally described about three decades ago.[27–29] These EDDs were mostly localized subendothelially but also subepithelially, as exemplified in Figures 5A and 7A. Second, these EDDs represent unique target structures for *in vivo*-bound autoantibodies, because EDDs associated with GBMs were stained by rabbit anti-mouse IgG and PAG conjugates, whereas GBM was not. This implies that GBM itself did not bind antibodies *in vivo*. Also, these large-sized EDDs have recently been shown by Kalaaji et al[30] to possess an extracellular localization different from the fine-dispersed mesangial and GBM-associated distribution of externalized α-actinin in glomeruli of nephritic kidneys. Whether the electron-dense structures reflected proliferation of certain inherent GBM structures or entrapment in GBM of structures released to circulation or intraglomerularly has never been formally determined. The most adequate previous examination of the nature of these structures was performed by Van Bruggen et al.[43] By indirect immunofluorescence using antibodies to histones and nucleosomes, they found that such antibodies bound extracellular structures in glomeruli of lupus nephritis.[43] These results suggest that nucleosomes may have associated with, eg, GBMs or the mesangial matrix.

We approached this problem by applying sets of antibodies to colocalization IEM. These sets implied antibodies to chromatin constituents like dsDNA, histone H1, and H3 and to transcription factors like TBP, which is constitutively bound to chromatin,[37] in addition to the GBM-specific anti-laminin antibody. The results of these studies were surprisingly clear and consistent and demonstrated that autoantibodies bound electron-dense structures that were recognized by the experimental anti-chromatin antibodies. In addition, antibodies to dsDNA, histones, and TBP produced in different species as those described above also colocalized with EDD-associated autoantibodies (data not shown). Because antibodies eluted from nephritic B/W kidneys[30] and experimental antibodies generated in different species all recognize components of nucleosomes and stain EDDs, the nature of such EDDs is most likely reflecting released chromatin fragments complexed *in vivo* with autoantibodies. Similar mechanisms may be operational for binding of autoantibodies in other basement membranes, as indicated by Grootscholten et al.[44]

Early electron microscopy studies on lupus nephritic kidneys revealed the presence of EDDs associated with GBMs,[27] and correlation between EDDs and the clinical course of lupus nephritis has been reported.[27–29] These deposits have been assumed to contain chromatin constituents and could therefore represent target structures for pathogenic anti-dsDNA antibodies (Refs. [8] and [9] and references therein). The present results confirm such assumptions and demonstrate that GBM-associated EDDs may derive from apoptotic cells that release chromatin fragments to the extracellular space.

The present data demonstrating intraglomerular, extracellular chromatin are consistent with the results of apoptosis assays like caspase-3, as an early assay for apoptosis,[45,46] or the TUNEL assay.[47] The strong tendency for apoptosis of glomerular cells may explain how chromatin fragments are released locally and subsequently associated with the mesangial matrix and GBMs, as discussed by Dieker et al.[48] This is also indicated by results of studies in human nephritic kidneys.[49] By release from multiple apoptotic cells within glomeruli, sufficiently high local concentrations of nucleosomes may be reached to associate significantly with GBMs, where nephritogenic autoantibodies may bind and initiate nephritis. Thus, nucleosomes associated with GBMs may originate from intraglomerular cells and not necessarily from circulation. What is still an unsolved problem is why intraglomerular cells undergo apoptosis as a potential source for extracellular nucleosomes. One possibility may be initial binding of autoantibodies to mesangial cell membranes.[12,25,26] Another possibility is release of nucleosomes from cells killed by cytotoxic T cells. There is experimental precedence for this possibility. Functional, autoimmune CD8+ T cells have been detected in SLE with potential to kill autologous cells,[50] and autoimmune B cells have, at least in the autoimmune MRL strain, been shown to promote activation of autoimmune $CD4^+$ and CD8+ T cells.[51] Moreover, activated CD8+ T cells have been detected in glomeruli of lupus nephritic kidneys.[50] Such CD8+ T cells may be involved in intraglomerular killing of cells, and subsequent release of nucleosomes to the mesangium and GBMs. Externalized nucleosomes may by themselves exert a pathogenic function in early lupus nephritis, as described by Coritsidis et al.[52] Based on the present results, the critical unanswered questions are why nucleosomes associate

with GBM structures and whether the GBM-associated nucleosomes are apoptotic. We are currently approaching the answers to these questions by analyzing the affinity of eukaryotic nucleosomes for GBM structures by surface plasmon resonance. Furthermore, we are analyzing the size distribution of GBM-associated nucleosomal dsDNA to determine whether the nucleosomes really derive from apoptotic fragmentation of chromatin (experiments in progress).

Taken together, the present data are coherent with a process implying intraglomerular apoptosis and trapping of apoptotic nucleosomes in mesangium and particularly in GBMs. A consequence of this process is that nucleosomes become available as target antigens for anti-dsDNA and anti-nucleosome autoantibodies. This is consistent with the strict localization of *in vivo*-bound autoantibodies in the electron-dense structures in GBMs and with the recent observation that antibodies eluted from nephritic B/W kidneys recognize dsDNA and nucleosomes.[30] The present data argue against a model implying binding of nephritogenic autoantibodies to cross-reactive, non-nucleosomal antigens potentially available in the glomerulus. Although tentative, this interpretation creates a meaningful basis for experimental strategies aiming at describing a potential link between apoptosis and release of apoptotic nucleosomes to mesangium and GBMs.

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