Antigen specificity of anti-nuclear antibodies complexed to nucleosomes determines glomerular basement membrane binding in vivo

Monoclonal anti-nuclear antibodies which are complexed to nucleosomes are able to bind to the glomerular basement membrane (GBM) in vivo, whereas purified antibodies do not bind. The positively charged histone moieties in the nucleosome are responsible for the binding to anionic determinants in the GBM. We tested the hypothesis that the specificity of the autoantibodies complexed to the nucleosome influences the glomerular binding of the antibody-nucleosome complex. We induced the formation of these immune complexes in vivo, by intraperitoneal inoculation of hybridomas producing monoclonal anti-nuclear antibodies (four anti-histone, three anti-double stranded (ds)DNA and three anti-nucleosome antibodies) into nude BALB/c mice. In ascites and plasma from the mice inoculated with these hybridomas, nucleosome/autoantibody complexes were detected in comparable amounts. Immunofluorescence of kidney sections revealed that about 60% of the mice inoculated with anti-nucleosome or anti-dsDNA hybridomas had immunoglobulin deposits in the GBM, whereas only 15% of the mice with anti-histone hybridomas showed these deposits (p < 0.04). In the Matrigel®-ELISA (used as a GBM surrogate) ascites from anti-nucleosome or anti-DNA hybridomas displayed significantly higher titers (p < 0.002) than ascites from anti-histone hybridomas. In conclusion, nucleosome/immunoglobulin complexes comprising anti-nucleosome or anti-dsDNA auto-antibodies do bind more frequently to the GBM in vivo than nucleosome/immunoglobulin complexes containing anti-histone antibodies. It therefore appears that the specificity of the antibody bound to the nucleosome is a critical determinant for the nephritogenic potential of the nucleosome-autoantibody complex.

1 Introduction

Systemic lupus erythematosus (SLE) is a disease characterized by the presence of autoantibodies which react with nuclear antigens [1, 2]. In recent years it has become clear that the nucleosome is an important auto-antigen in SLE [3, 4]. Nucleosomes are not only important in the induction phase of lupus but also for the evolution of tissue lesions, especially glomerulonephritis. The observation that DNA circulates in SLE patients in the form of oligonucleosomes [5, 6] and that nucleosome-specific antibodies are formed in the majority of SLE patients [7–9] suggested that formation of nucleosome anti-nucleosome complexes might be an alternative explanation for the pathogenesis of lupus nephritis. Indeed, recently nucleosomes were identified for the first time in GBM deposits in human lupus nephritis [10]. In renal perfusion studies we could show that nucleosome/autoantibody complexes bind to the GBM via an interaction of cationic histone moieties within the nucleosome to anionic heparan sulfate (HS) [11, 12]. From these renal perfusion experiments we also learned that a relative decrease of DNA or a relative increase in histone content within the nucleosome could increase the capacity of the nucleosome/autoantibody complex to bind to HS in the GBM [12]. The presence of the N-terminal regions of the core histones carrying many positive charges seems to be a critical determinant for this binding since masking of these positive charges by heparin completely prevents GBM binding [13]. These N-terminal regions harbor the epitopes for histone-specific autoantibodies [14, 15]. We reasoned that binding of anti-histone antibodies to these N-terminal regions would decrease the capacity of the nucleosome-autoantibody complex to bind to the GBM, whereas DNA and nucleosome-specific autoantibodies would relatively spare these N-terminal regions. Therefore, we compared the glomerular binding of different nucleosome-, histone- and dsDNA-specific monoclonal antibodies complexed to nucleosomes after intraperitoneal inoculation in nude BALB/c mice. This procedure will induce nucleosome/Ig complex formation in vivo [16]. In purified non-complexed form, none of the tested antibodies binds to the GBM in vivo as assessed by renal perfusion studies.

2 Materials and methods

2.1 Animals

BALB/c nu/nu mice and Wistar rats were bred in the animal facilities of the University of Nijmegen.
2.2 Renal perfusion of purified anti-nuclear antibodies

Non-complexed anti-histone, anti-DNA and anti-nucleosome mAb were obtained by purification under high salt conditions preceded by a DNase treatment as described [12]. Renal perfusion studies in Wistar rats were performed as described previously [11, 12].

2.3 Isoelectric focussing (IEF) of purified anti-nuclear antibodies

IEF was carried out in a Phast system separation unit (pH range of the gels 3–9) according to the manufacturer’s instructions (Pharmacia, LKB, Uppsala, Sweden).

2.4 Inoculation with anti-nuclear hybridomas in nude BALB/c mice

From a panel of hybridomas producing monoclonal anti-nuclear antibodies, four anti-histone, three anti-dsDNA and three nucleosome-specific hybridomas were selected. mAb 2, 32 and 34 are anti-nucleosome-specific hybridomas directed against H2A-H2B/DNA (mAb 2 and 32) or H3-H4/DNA (mAb 34) [17] mAb 2 was obtained by fusion of spleen cells from a graft-versus-host mouse and mAb 32 and 34 from NZB/W mice. mAb 36, 53 and 56 are anti-DNA binding hybridomas obtained from NZB/W mice [18]. mAb KM2, LG2-1, LG2-2 and MRA12 are anti-histone hybridomas originating from MRL/lpr mice. The anti-histone hybridomas KM2, LG2-1 and LG2-2 are directed against determinants located in the basic N-terminal domain of the core histones (KM2 recognizes aa 1-20 of H2A and aa 1-29 of H4, LG2-1 recognizes aa 30-45 of H3 and LG2-2 recognizes aa 1-13 of H2B) [14]. MRA12 recognizes a conformational determinant of the H1 molecule requiring both the globular and C-terminal domains [19, 20]. All mAb are IgG2a except for mAb 2 and 53 which are IgG2b. An IgG2a anti-human CD7 monoclonal (WT1) served as control.

Seven-week-old BALB/c nu/nu mice received 0.5 ml sterile pristane (4,6,10,14-tetramethylpentadecane; Sigma, Poole, GB) 10 days prior to intraperitoneal (i.p.) injection of 3 x 10^6 hybridoma cells. As controls, six mice received hybridoma cells secreting anti-human CD7 and four mice pristane alone. When mice developed visible tumor growth and/or ascites, ascites and plasma were collected for determination of antibody levels, anti-nuclear reactivity and the amount of nucleosome/Ig complexes. Albuminuria was screened with Albustix (Boehringer Mannheim, Germany). Only those animals that developed both visible ascites (and tumors) and anti-nuclear antibody reactivity were subjected to further examination. Although the time required to develop ascites varied (albeit insignificantly), mice were always killed 3 to 5 days after the onset of visible ascites.

2.5 Determination of immunoglobulin concentration and antibody reactivities in ascites and plasma

The immunoglobulin (Ig) concentrations and the anti-histone, anti-dsDNA and anti-nucleosome reactivities were assessed by ELISA, as described previously [12].

2.6 Anti-Matrigel ELISA

Matrigel™ (Beckton Dickinson, Bedford, MA), a solubilized basement membrane preparation extracted from the mouse Engelbreth-Holm-Swarm (EHS) tumor, consisting of laminin, entactin, collagen IV and heparan sulfate proteoglycan, was used as a “GBM surrogate”. Matrigel was first treated with DNase (DNase grade II, Boehringer) to remove DNA contamination and was coated to Greiner plates (Greiner, Frickenhausen, Germany) at 1 μg in 100 μl PBS per well overnight at room temperature. After washing the plates five times with PBS containing 0.1 % Tween-20, plates were blocked with 10 mM Tris-HCl pH 7.4 containing 5 % (wt/vol) bovine serum albumin (BSA). 150 μl/well, for 1 h at 37°C. Next, samples were diluted in PBS containing 1 % (wt/vol) BSA, 100 μl per well for 1 h at 37°C. Plates were washed again five times and incubated with peroxidase-labeled rat anti-mouse mAb Ig (CLB-RM-19, CLB, Amsterdam, The Netherlands) diluted 1/1000 in 100 μl PBS/well. Further procedures were identical to the anti-nuclear ELISA as described [12]. Purified anti-dsDNA, anti-histone and anti-nucleosome antibodies did not bind to DNase-treated Matrigel, whereas antibodies specific for laminin, entactin, collagen IV, HS-glycosaminoglycan (GAG) or HSPG core protein showed high reactivity (data not shown).

2.7 Nucleosome/IgG complex assay

The nucleosome/IgG complex assay was performed as previously described [16]. Briefly, Protein A/G (Pharmacia)-coated plates were incubated with plasma or ascites samples (diluted 1/20 or 1/250 respectively). After three washes, nucleosome/IgG complexes were identified using DNA-specific probes (biotinylated nucleotides). In addition the nucleosome/IgG complex assay was performed using biotinylated anti-histone mAb KM2 instead of the biotinylated nucleotides, allowing identification of the nucleosome/IgG complexes via their histone part. Ascites samples were diluted 1/20 instead of 1/250 since the assay using biotinylated anti-histone mAb was less sensitive than the assay using biotinylated nucleotides. Samples containing only free (non-complexed) nucleosomes, histones and/or DNA were negative in both assays.

2.8 Characterization of DNA in plasma and ascites

DNA was extracted from plasma and ascites using phenol saturated with 0.1 M Tris (pH 8). For DNA quantification, a DNA assay, was performed [16, 21]. DNA concentrations were determined by reference to standard curves constructed from pBR 322 DNA. Electrophoresis of DNA was carried out in 1.5 % agarose gels in 40 mM Tris-acetate buffer pH 8 containing 1 mM EDTA. Gels were stained with ethidium bromide (1 μg/ml).

2.9 Characterization of nucleosome/Ig complexes

For identification of DNA and histones in the nucleosome/Ig complexes, plasma/ascites samples were mixed with protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) for 30 min. After centrifugation, the beads were
washed twice with PBS. DNA was isolated by phenol extraction. The presence of histones in the nucleosome/Ig complexes which were bound to the protein A-Sepharose beads was determined by SDS-PAGE (12%) analysis followed by double silver staining [12].

2.10 Immunohistology

Direct immunofluorescence (IF) for IgG and C3 deposits was carried out as previously described [22]. Both the amount and intensity of the staining in capillary loops and mesangium were scored. No staining at all was scored as 0, moderate staining was scored as 1 and strong staining was scored as 2.

2.11 Statistical analysis

Statistical analysis was performed using the Fisher exact test and the Mann Whitney U test. $p < 0.05$ was considered to be statistically significant. Spearman's correlation was used in linear regression analyses.

3 Results

3.1 Characteristics of purified monoclonal anti-nuclear antibodies

Purified non-complexed anti-histone (KM2, MRA12, LG2-2 and LG2-1), anti-DNA (36, 53 and 56) or anti-nucleosome (2, 32 and 34) antibodies of the hybridomas reacted strongly with histones, dsDNA or nucleosomes respectively. None of the purified antibodies bound to the GBM in vivo as assessed by renal perfusion studies. Isoelectric points of the purified anti-nuclear antibodies ranged between 5.8 and 7.4, for all mAb, except for mAb 34 which had a pI value of 7.0–8.0.

3.2 Ascites development and antibody reactivity

Thirteen (± three) days after the inoculation with hybridoma cells, the mice developed visible ascites with high Ig levels (1610 ± 1285 µg/ml). In control mice, inoculated with an anti-CD7 hybridoma, no reactivity against nuclear antigens was found in ascites samples (titer/mg Ig < 60). Ascites produced by the anti-histone hybridomas displayed high reactivity against histones (titer/mg Ig: 67180 ± 47161) and also against nucleosomes (titer/mg Ig: 20763 ± 9517). In the anti-DNA ascites, the anti-DNA and anti-nucleosome titers were respectively 37292 ± 19025 and 32591 ± 11634. Anti-nucleosome hybridomas induced predominantly anti-nucleosome reactivity (titer/mg Ig: 18276 ± 9116) but also low levels of anti-DNA (titer/mg Ig: 126 ± 18) and anti-histone (titer/mg Ig: 155 ± 50). Ig levels and anti-nuclear reactivities in plasma samples were comparable to those obtained for ascites (data not shown) indicating that there was transfer to the systemic circulation.
complexes were detected in sections of nude-mouse with undressed 79 ± 7.4% (respectively). Also with the red-mon system no
undressed (5) and TdT (6) DNA hybridization was observed in the DNA from sections containing undressed DNA, hybridized with protein Ag-sphere mouse.

In addition, sections containing control probes (negative control) and undressed DNA, hybridized with protein Ag-sphere mouse, showed high signals (negative control) and undressed DNA, hybridized with protein Ag-sphere mouse.

In the undressed/diagram assay in which we used dual

3.3 Quantitation of undressed IgG complexes

complexes in sections was revealed by ELISA.

Figure 2: Direct immunohistochemistry on biopsy sections of nude mouse with anti-human CD7 (A) and anti-DNA (B).

and anti-HL/Hyphiona hybrid (MRM-2) of the control hybrid.
mesangial deposits

Table 1. Immunofluorescence findings in kidney sections of mice inoculated with the anti-DNA (nos. 56, 36 and 53), anti-histone (KM2, LG2-1 and LG2-2), anti-nucleosome (nos. 32, 34 and 2) or control (anti-CD7) hybridomas. Incidence of positive staining (score ≥ 1) on immunofluorescence.

<table>
<thead>
<tr>
<th>Hybridomas</th>
<th>GBM deposits</th>
<th>Mesangial deposits</th>
<th>Nuclear staining</th>
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<tbody>
<tr>
<td>Anti-CD7</td>
<td>0/6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/6</td>
<td>0/6</td>
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<tr>
<td>Anti-histone</td>
<td>2/13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9/13</td>
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<tr>
<td>Anti-dsDNA</td>
<td>9/15</td>
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<td>Anti-nucleosome</td>
<td>8/13</td>
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<sup>a</sup> Number of mice with positive staining/total number of mice

<sup>b</sup> Anti-histone vs. anti-nucleosome hybridomas (p = 0.04); anti-histone vs. anti-dsDNA hybridomas (p = 0.02)

2B, lanes 3–5). No DNA was isolated from beads incubated with control ascites (Fig. 2B, lane 2). SDS-PAGE electrophoresis of the material eluted from the protein A Sepharose beads revealed the presence of Ig heavy and light chains and the four core histones, while H1 was not detectable. Only Ig heavy and light chains were found on protein A beads incubated with control ascites or MRA12 ascites (data not shown).

3.5 Glomerular deposition

The kidneys of the mice with nucleosome/Ig complexes in their ascites/plasma (i.e. all mice except those inoculated with the anti-H1 hybridoma MRA12) and of mice inoculated with the control hybridoma were examined by direct IF for Ig deposition. Different staining patterns were observed. Deposits along the glomerular capillary wall were observed in 8/13 mice that received anti-nucleosome and in 9/15 mice that received anti-DNA hybridomas. In contrast, only 2/13 mice that received anti-histone hybridomas had GBM-Ig deposits, which was significantly different (p ≤ 0.04). In mice that received anti-human CD7 hybridoma or pristane alone no deposits along the capillary walls and only scant mesangial deposits of IgG2a were observed (Fig. 3A).

An increased incidence and severity of deposits were observed in the mesangium of mice that received anti-nuclear hybridomas, as compared to the anti-CD7 bearing control (Table 1). Nuclear staining was observed in 9/13 mice injected with the anti-histone hybridomas (Fig. 3C), in 10/15 mice bearing anti-dsDNA and in 12/13 mice carrying anti-nucleosome hybridomas (Table 1). The nuclear staining was always observed in mice with positive GBM-Ig deposits (Fig. 3D) except for mAb 53 (Fig. 3B).

The pathogenic potential of the bound nucleosome/Ig complexes can be derived from the observation that C3 deposition was seen in some (16%) of the mice with complex deposition. Furthermore, mild proteinuria (300 mg/ml) developed only in mice inoculated with the anti-DNA (2/15) or anti-nucleosome (3/13) hybridomas.

3.6 Binding to extra cellular matrix (Matrigel-ELISA)

In a Matrigel-ELISA (used as a GBM surrogate), ascites of mice inoculated with anti-nucleosome or anti-DNA hybridomas displayed significantly higher titers than ascites of mice inoculated with anti-histone hybridomas (median titers: 20 and 60 versus 2; p values < 0.002).

4 Discussion

Large amounts of nucleosomes are released from cells undergoing apoptosis in hybridoma cell cultures [23] and in ascites from mice inoculated intraperitoneally with hybridomas [16]. If this release occurs in the presence of anti-nuclear antibodies one can envision that nucleosome-autoantibody complexes are formed, as recently described for anti-DNA hybridomas [16]. Our study confirms and extends these observations since in ascites of mice inoculated with anti-histone or anti-nucleosome hybridomas, nucleosome/Ig complexes are also present. The DNA in these complexes has characteristics of nucleosomal DNA released by apoptosis [5, 6]. Within the nucleosome/Ig complexes the four core histones are also present, but no H1, confirming previous observations in bovine serum [24] and hybridoma supernatants [12, 23].

The amount of nucleosome/Ig complexes, found in ascites or plasma, was equal and not influenced by the anti-nuclear specificity of the hybridoma product with the exception of anti-H1. In control mice inoculated with the anti-CD7 hybridoma an identical amount of nucleosomes was found, but no nucleosome/Ig complexes, excluding nonspecific generation of these complexes. Despite the fact that the amount of nucleosome/Ig complexes was not significantly different among mice inoculated with the various anti-nuclear specificities, we observed clear differences in the glomerular localization of these complexes: high for anti-DNA and anti-nucleosome, low for anti-histone hybridomas. This is in line with the suggestion that anti-histone auto-antibodies are less nephritogenic in lupus [25–28] than anti-DNA [29] or anti-nucleosome antibodies [8, 30]. We also found that the amount of anti-histone antibodies deposited in glomeruli was similar in age-matched albuminuric and non-albuminuric MRL/lpr lupus mice in contrast to anti-nucleosome and anti-DNA reactivities which were increased in albuminuric animals [31].
There was a correlation between the in vivo GBM binding and the in vitro Matrigel reactivity in ascites. The observed Matrigel reactivity and in vivo GBM-binding of these autoantibodies is due to complexation to nucleosomes since these same antibodies in purified non-complexed form bound neither to Matrigel nor to the GBM in vivo.

The methodologies exploited in this study have also been used by others to evaluate glomerular immune deposit formation of murine [32–34] or human [35] anti-DNA antibodies. In these studies, the antibody deposition in mesangium and GBM varied for the different antibodies used. Based on these observations, it was postulated that direct binding of anti-DNA antibodies to intrinsic glomerular antigens is the major mechanism for this immune deposit formation. However, for some of the mAb described, the formation of GBM deposits was only observed after i.p. hybridoma inoculation and not after i.v. injection of purified mAb [33, 35], which suggests that this glomerular binding is due to complex formation with nucleosomes [11, 16, 36].

In many of the mice inoculated with anti-nuclear hybridomas, nuclear localization of autoantibodies (in vivo ANA) was observed in the different tissue specimens. Recently, it was reported that this in vivo ANA is a feature of anti-nuclear antibodies complexed to nucleosomes since binding to the cell surface and transport into the cytoplasm only occurred if the antibodies were complexed to nucleosomes [37, 38]. The observed nuclear localization of our mAb was a fixation artifact, since if we used a paraformaldehyde fixation, instead of acetone, this nuclear staining did not occur [38].

In conclusion, this study shows that complexes formed between nucleosomes and anti-DNA or anti-nucleosome autoantibodies more frequently localize in the GBM than those formed between nucleosomes and anti-histone antibodies. In our view, this latter is due to the fact that these anti-histone antibodies bind to the positively charged N-terminal regions of the core histones. This reduces the capacity of these histone moieties to bind to HS-associated anionic sites within the GBM.

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5 References