Significance of anti-nuclear and anti-extracellular matrix autoantibodies for albuminuria in murine lupus nephritis; a longitudinal study on plasma and glomerular eluates in MRL/l mice

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SUMMARY

The relationship between autoantibody reactivities and nephritis in systemic lupus erythematosus (SLE) is unclear. We studied MRL/l mice which developed a considerable albuminuria (either mice with short (< 1 week) or heavy and prolonged (3 weeks) albuminuria) and compared them with non-albuminuric age-matched controls, with young (12 weeks old) non-albuminuric mice and with mice which were followed for 36 weeks and did not develop albuminuria. In a longitudinal prospective study on plasma samples we correlated a variety of anti-nuclear reactivities and reactivities against extracellular matrix (ECM) components, with the onset of albuminuria. We found that at the onset of albuminuria, anti-DNA was higher while anti-nucleosome and anti-H2A/H2B-DNA subnucleosome reactivities were lower compared with age-matched non-albuminuric mice. We also studied glomerular eluates of these mice in ELISA and in indirect immunofluorescence (IF). In the eluates we found with IF that anti-glomerular basement membrane (GBM)-tubular basement membrane (TBM) antibodies were already present in 12-week-old non-albuminuric mice. These eluates showed no anti-nuclear antibodies. In eluates of albuminuric mice more immunoglobulin was deposited, and anti-ECM, anti-DNA and anti-nucleosome reactivities were higher than in eluates of age-matched non-albuminuric mice. The deposition of anti-nucleosome antibodies preceded the deposition of anti-DNA antibodies since they were deposited to a greater extent in mice with a short albuminuria. We conclude that anti-GBM-TBM antibodies are the first autoantibodies that deposit in glomeruli of MRL/l mice at an early age. The onset of albuminuria is associated with additional deposition of both anti-ECM and anti-nuclear (anti-nucleosome and anti-DNA) antibodies, but the difference with non-albuminuric mice seems to be more quantitative than qualitative.

Keywords lupus   nucleosome   autoantibodies and extracellular matrix

INTRODUCTION

Nephritis is one of the most serious manifestations of systemic lupus erythematosus (SLE), occurring in about 50% of these patients [1]. In renal biopsies granular deposits of immunoglobulin and complement are found in the glomeruli, suggesting an immune complex pathogenesis [2]. The causal relationship between auto-antibody formation and nephritis has intrigued many investigators, and a variety of autoantibody specificities have been assigned a pathogenic role in the development of lupus nephritis [3]. In this respect, especially antibodies towards nuclear antigens and glomerular basement membrane (GBM) components have been mentioned. With regard to anti-nuclear antibodies an important

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role has been assigned to anti-dsDNA antibodies [4–6], but also to anti-histone antibodies [7–9]. More recently, anti-nucleosome reactivity has been identified in SLE, apart from anti-DNA and anti-histone reactivities. Burlingame *et al.* [10] described how anti-nucleosome, or more precisely antibodies against the H2A/H2B-DNA subnucleosome complex, preceded the formation of anti-histone and anti-dsDNA antibodies in the lupus mouse strains MRL/l and BXSB. Lately, it was found that the prevalence of anti-nucleosome and anti-H2A/H2B-DNA subnucleosome reactivity is higher than that of anti-dsDNA and anti-histone reactivity [11,12]. In addition, we [13] and others [14,15] have identified MoAbs derived from lupus mice which were reactive only with the intact nucleosome and not with its components, DNA or histones. A correlation between both plasma anti-H2A/H2B-DNA sub-nucleosome and anti-nucleosome reactivity with nephritis has been described [11], and recently we showed that anti-nucleosome

antibodies complexed to nucleosomal antigens are able to bind to the GBM after renal perfusion in rats [13].

Antibodies directed against extracellular matrix (ECM) components of the GBM have also been proposed as candidates for the initiation of glomerular disease in lupus. In lupus sera, reactivity against heparan sulphate proteoglycan, laminin, collagen type IV, fibronectin and entactin has been described [16]. In another experimental model for lupus nephritis, the chronic graft-*versus*-host (GVH) model, a role for anti-basement membrane and anti-tubular brush border antibodies has been proposed [17–19]. Enrichment of anti-laminin reactivity in renal eluates relative to serum has been described in MRL/l and GVH mice [18,20]. Lately, Bernstein *et al.* developed an ELISA using the intact glomerulus as substrate and used it to show enrichment of anti-glomerular reactivity in renal eluates [21], in different murine lupus strains.

The aim of the present study was to analyse the relationship between plasma and glomerular antibody reactivities and nephritis in MRL/l mice. Although the MRL/l mouse strain is congenic, there is a marked variability in onset and course of the disease. We exploited this variability and selected mice developing considerable albuminuria within a short period of time (animals were divided into those with albuminuria for either 1 or for 3 weeks) and compared them with age-matched non-albuminuric mice and with mice surviving 36 weeks without developing albuminuria. Until now, most studies have focused on changes over time irrespective of clinical signs of nephritis, or have only studied animals with overt signs of nephritis without comparison with age-matched, non-albuminuric mice. By taking non-albuminuric MRL/l mice as a control group, we argued that we could perhaps identify antibody specificities in plasma or in glomerular eluates which are positively or negatively associated with albuminuria. To this end we tested plasma and eluates for antibody reactivities against various nuclear antigens and ECM components in both ELISA and immunofluorescence (IF). To check for non-specific trapping of immunoglobulin in the glomerulus we tested both plasma pools and glomerular eluates for antibody reactivity towards a non-relevant antigen, dinitrophenol (DNP).

MATERIALS AND METHODS

Animals

MRL/l (MRL, *lpr/lpr*) and MRL/n (MRL, +/+) mice were bred in the animal facilities of the University of Nijmegen, from stock originally obtained via the Scripps Clinic and Research Foundation (La Jolla, CA), from the Jackson Laboratory (Bar Harbor, ME).

Experimental approach

Plasma study. Plasma samples were collected at 6, 9, 12, 16, 20, 24, 28, 32 and 36 weeks of age ($n = 53$, 36 males and 17 females, at 6 weeks). Blood was collected under ether anaesthesia from the retro-orbital plexus, in EDTA vials. After centrifugation, plasma was collected and stored at $-20°C$. Albuminuria was screened every week with Albustix (Boehringer Mannheim, Mannheim, Germany). When albuminuria was more than +, urine was collected the next day in a metabolic cage for 18 h, while the animals had free access to water. Albuminuria was quantified in all urine samples by a radial immunodiffusion technique, as described [22,23]. It was found that albuminuria as assessed with Albustix

correlated very well with albuminuria determined in the immunodiffusion assay. Importantly, no false-negative and only few false-positive results were obtained (data not shown). Since every positive result obtained by Albustix screening was checked in the immunodiffusion assay, all false-positive assessments were identified.

Of the 53 mice at entry, 25 did not develop albuminuria (Albustix < +) throughout the study period. Of the 25 non-albuminuric mice, 13 died before the age of 36 weeks, and therefore 12 mice reached the age of 36 weeks. In these mice, urinary albumin excretion was measured at 36 weeks. The median albuminuria in this group was 60 μg/18 h (range 45–143 μg/18 h). The plasma samples of these mice were analysed.

Albuminuria developed in 28 mice. The median age of onset of albuminuria was 21 weeks (range 12–26 weeks). Of these 28 mice, nine had an albuminuria < 1000 μg/18 h and in 19 mice albuminuria was > 1000 μg/18 h (median 2800 μg/18 h, range 1010–28 500 μg/18 h). Since we wanted to study mice which developed a considerable (> 1000 μg/18 h) albuminuria within a short period of time, the plasma samples of 17 out of these 19 mice (two mice died in the metabolic cage, before the final blood sample could be taken) were used in this study and the results were compared with the non-albuminuric mice which reached the age of 36 weeks and remained negative on Albustix screening.

Elution study. In a different study, four groups of mice ($n = 10$ per group) were selected based on age and magnitude of albuminuria, and were screened every week with Albustix: group 1, 12-week-old mice, without albuminuria (Albustix ⩽ +); group 2, 18–24-week-old mice without albuminuria (Albustix ⩽ +); group 3, 18–24-week-old mice with short duration of albuminuria (urine albumin > 1000 μg/18 h, albuminuria period < 7 days); group 4, 18–24-week-old mice which had albuminuria (urine albumin > 1000 μg/18 h) on three subsequent occasions (albuminuria period at least 14 days, but not more than 21 days). After assigning a mouse to one of the four study groups, under anaesthesia (50 mg/kg sodium pentobarbital, Narcovet; Apharmo, Arnhem, The Netherlands), kidneys were flushed with PBS, perfused with Fe_3O_4 (BDH, Poole, UK) 13·0 mg/ml, and stored at $-80°C$. Perfusion with Fe_3O_4 was done in order to isolate glomeruli as described previously [24]. Glomerular eluates were pooled per group since individual samples did not allow analysis of antibody reactivity because of the low yield. For a proper comparison of antibody reactivities from the pooled glomerular eluates with plasma, plasma samples from all mice were collected just before renal Fe_3O_4 perfusion and also pooled per group.

Elution of antibodies from isolated glomeruli

Kidneys perfused with Fe_3O_4 were pooled ($n = 20$ per group), homogenized, and glomeruli were isolated by a magnet as described before [24]. The material thus obtained consisted of > 95% glomeruli. A 10-μl sample was taken from the glomerular suspension and the number of glomeruli counted in a haemocytometer. Antibodies were eluted from the glomeruli by acid elution (0·12 M glycine–HCl pH 2·8). In previous experiments performed in our laboratory, we found that after this elution additional procedures with 0·12 M glycine–NaOH pH 12 or with 3 M KSCN did not lead to further elution of immunoglobulin [20]. Elution was carried out under continuous shaking for 2 h at room temperature in the presence of protease inhibitors (1 mM PMSF, 0·05% w/v NaN_3, 20 mM EDTA and 100 KI units Trasylol). After

elution, the pH was immediately adjusted to 7·4 using 2 M Tris and the eluates were dialysed overnight at 4°C against PBS containing the above-mentioned protease inhibitors. The eluates were then concentrated to a final volume of 4 ml using an Amicon YM50 filter and stored at 4°C. All analyses on these eluates were performed within 48 h after the elution procedure.

Determination of immunoglobulin concentration
Plasma samples, eluates and plasma pools were measured in ELISA for immunoglobulin concentration. Nunc Maxisorb F96 immunoplates (Life Technologies Inc., Gaithersburg, MD) were coated overnight at 4°C with goat anti-mouse immunoglobulin (GM 17; CLB, Amsterdam, The Netherlands) 5 μg/ml, 100 μl/well. Plates were washed three times with distilled water and three times with PBS, containing 0·05% (v/v) Tween 20 (PBS–T). Samples were diluted in PBS–T containing 0·2% gelatin and incubated for 1 h at 37°C, 100 μl/well. Plates were washed again and incubated with peroxidase-labelled goat anti-mouse immunoglobulin (GM 17-HRP) diluted 1:1500, for 1 h at room temperature, 100 μl/well. The plates were washed again and developed with 3,5,3′,5′-tetramethylbenzidine (Merck), 100 μg/ml in 0·11 M sodium acetate pH 5·5 containing 0·003% H_2O_2. By adding 2 M H_2SO_4, 100 μl/well, colour development was stopped after 15 min and optical density (OD) at 450 nm was measured.

Autoantigen reactivities of plasma samples, plasma pools and eluates
Anti-dsDNA, anti-histone, anti-nucleosome, anti-H2A/H2B-DNA subnucleosome, anti-laminin, anti-collagen IV, anti-entactin and anti-DNP reactivities were assessed in ELISAs. In addition, eluates and plasma pools were tested in indirect IF on MRL/n (MRL, +/+) kidney sections.

Anti-dsDNA ELISA/anti-histone ELISA/anti-nucleosome ELISA. The anti-dsDNA, anti-histone and anti-nucleosome ELISAs were performed as described previously [13,25].

Anti-H2A/H2B-DNA subnucleosome ELISA. H2A/H2B-DNA subnucleosome was prepared as described [26]. Next, Nunc immunoplates were coated with the H2A/H2B-DNA subnucleosome complex 2·5 μg/ml in 0·15 M NaCl, 0·015 M trisodium citrate pH 7·0 for 1 h at room temperature. Further procedures were identical to the anti-nucleosome ELISA.

Anti-laminin ELISA. Mouse EHS Laminin from Engelbreth–Holm–Swarm (EHS) tumour (Life Technologies) was coated onto Greiner plates, 1 μg in 100 μl per well, overnight at room temperature. After washing the plates five times with PBS–T, plates were blocked with 10 mM Tris/HCl pH 7·4 containing 5% (w/v) bovine serum albumin (BSA), 150 μl/well, for 1 h at 37°C. Next, samples were diluted in PBS containing 1% (w/v) BSA, 100 μl per well for 1 h at 37°C. Plates were washed again five times and incubated for 1 h with peroxidase-labelled rat MoAb anti-mouse immunoglobulin (CLB-RM-19; CLB) diluted 1:1000 in PBS, 100 μl per well. The plates were washed again and developed with 3,5,3′,5′-tetramethylbenzidine (Merck), 100 μg/ ml in 0·11 M sodium acetate pH 5·5 containing 0·003% H_2O_2. By adding 2 M H_2SO_4, 100 μl/well, colour development was stopped after 15 min and OD at 450 nm was measured.

Anti-collagen IV ELISA. Mouse collagen type IV (Sigma) was coated onto Greiner plates, 1 μg in 100 μl per well, overnight at room temperature. Further procedures were identical to the anti-laminin ELISA.

Anti-entactin ELISA. Entactin (Upstate Biotechnology Inc.,

Waltham, MA) was coated onto Greiner plates, 1 μg in 100 μl per well, overnight at room temperature. Further procedures were identical to the anti-laminin ELISA.

Anti-DNP ELISA. DNP coupled to BSA (Calbiochem, La Jolla, CA) was coated to Nunc immunoplates, 1 μg in 100 μl per well, overnight at room temperature. After washing, the plates were blocked with 1% (w/v) gelatin in PBS for 1 h at 37°C. Further procedures were identical to the anti-laminin ELISA.

The titre in each ELISA was defined as the reciprocal of the dilution giving an OD of 1·0.

Immunohistology
Immunofluorescence was performed on 2-μm cryostat sections from all kidneys to study deposition of mouse immunoglobulin. Sections were incubated with F(ab)$_2$ FITC-labelled sheep anti-mouse IgG (Organon Teknika-Cappel N.V., Turnhout, Belgium). The intensity of staining in the capillary loops and mesangium was scored semiquantitatively on a 0–4 + scale (described previously [23]).

For indirect immunofluorescence, 2-μm cryostat kidney sections from 6-week-old MRL/n mice were incubated with the eluates or the plasma pools in two-fold dilution steps for 30 min. Subsequently, the sections were incubated with F(ab)$_2$ FITC-labelled sheep anti-mouse IgG (Organon Teknika-Cappel N.V.), diluted 1:750 in PBS 1% (w/v) BSA for 1 h at 37°C. The titre was defined as the reciprocal of the highest dilution still giving positive staining.

Statistical analysis
Plasma reactivities were analysed in two different ways. First, longitudinal plasma antibody reactivities of albuminuric and non-albuminuric mice were compared with a distribution free method for curve analysis as described by Koziol *et al.* [27]. Second, the plasma antibody reactivities at the onset of albuminuria were compared with the median of non-albuminuric mice at the same age in a sign test. Statistical analysis on the immunofluorescence score was performed using the Mann–Whitney U-test. In all tests $P < 0.05$ was considered significant.

RESULTS

Longitudinal plasma study
When plasma antbody reactivities over time from mice developing albuminuria were compared with those of non-albuminuric mice, there was a marked similarity between the two groups (Fig. 1a–d). Antibody reactivities to collagen IV, laminin and entactin were found from week 15–18 onwards. Immunoglobulin concentration, anti-DNA, anti-nucleosome and anti-H2A/H2B-DNA subnucleosome reactivities rose until week 24 and remained at this level thereafter in the non-albuminuric mice. Although the anti-DNA reactivity was some-what higher and the immunoglobulin concentration, anti-nucleosome and anti-H2A/H2B-DNA subnucleosome reactiv-ities were slightly lower in albuminuric mice, these differences were not statistically significant (Koziol test). A different kinetic pattern was seen for anti-histone antibody formation. In most mice (82%) anti-histone reactivity was very low until week 24, and increased thereafter.

When autoantibody reactivities at the onset of albuminuria were compared with the median at that age in the non-albuminuric group, anti-DNA reactivity was significantly higher in the
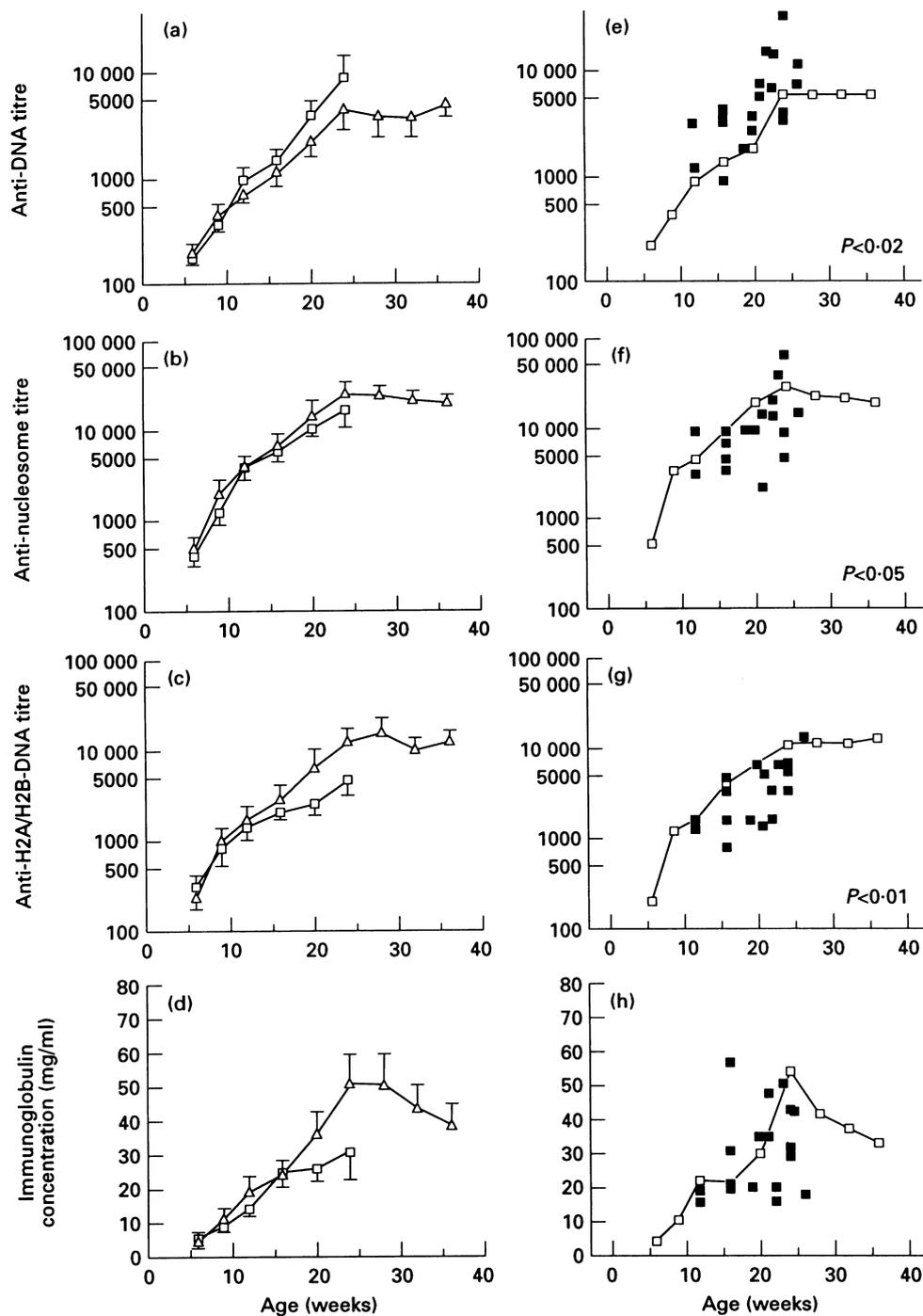
Fig. 1. Autoantigen reactivities in longitudinally obtained plasma samples of albuminuric (□) and non-albuminuric (△) mice (means and s.e.m. are given). (a) Anti-DNA, (b) anti-nucleosome, (c) anti-H2A/H2B-DNA subnucleosome, (d) immunoglobulin concentration. Autoantibody reactivity at the onset of albuminuria (■) of all albuminuric mice studied, compared with the median at that age in non-albuminuric controls (□). (e) Anti-DNA, (f) anti-nucleosome, (g) anti-H2A/H2B-DNA subnucleosome, (h) immunoglobulin concentration.

albuminuric group ($P < 0.02$, sign test, Fig. 1e). In an identical analysis, anti-nucleosome and anti-H2A/H2B-DNA subnucleosome titres were significantly lower in the albuminuric group ($P < 0.05$ and $P < 0.01$, respectively, Fig. 1f,g). Since anti-DNA antibodies are also measured in the anti-nucleosome and the anti-H2A/H2B-DNA subnucleosome ELISA (because DNA is a part of both antigens) [13,28], the depression of true anti-

nucleosome antibodies in albuminuric mice might even be more pronounced.

These differences (higher anti-DNA, lower anti-nucleosome and anti-H2A/H2B-DNA subnucleosome at the onset of albuminuria) are not a mere reflection of the total immunoglobulin levels, since these were not different at the onset of albuminuria between the groups (Fig. 1h). For anti-histone antibodies and for the
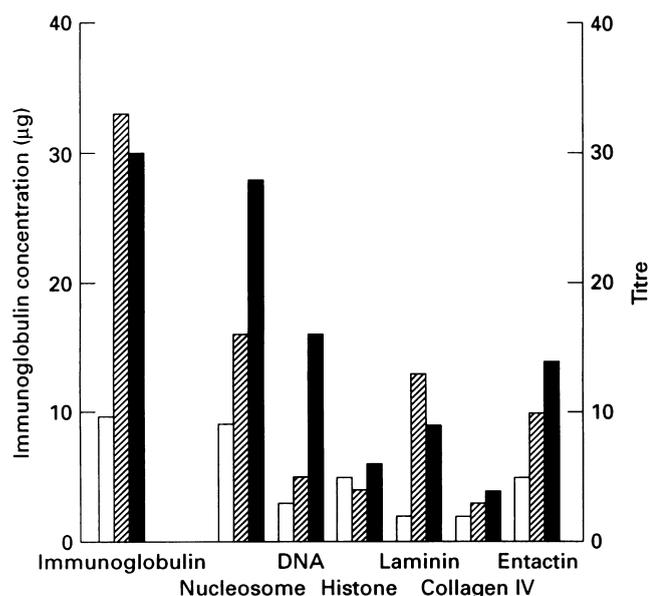
Fig. 2. Immunoglobulin concentration and titres towards the various autoantigens in glomerular eluates of the age-matched (18–24 weeks) albuminuric and non-albuminuric MRL/l mice tested. (Titres are not adjusted for the quantity of immunoglobulin.) □, Non-albuminuric mice (group 2); ▨, short albuminuria < 7 days (group 3); ■, prolonged albuminuria 14–21 days (group 4).

antibodies against ECM components, no differences at the onset of albuminuria were observed.

Elution study

The number of glomeruli obtained in the four groups studied was about 205 000 glomeruli per group, i.e. a yield of 10 250 glomeruli per kidney. The total amount of immunoglobulin eluted ranged from 2·4 μg in young (12-week-old) non-albuminuric mice to 33 μg in albuminuric mice, i.e. 0·12 and 1·65 μg immunoglobulin eluted per kidney, respectively. The highest amount of immunoglobulin was eluted from glomeruli of albuminuric mice (groups 3 and 4, Fig. 2). This is in line with our previous finding concerning glomerular IgG deposition in these mice [23]. With direct IF we observed that glomerular deposition of mouse immunoglobulin in non-albuminuric mice was predominantly restricted to the mesangial areas. In albuminuric mice immunoglobulin was located both in the mesangium and, to a significant extent, along the GBM. The degree of mesangial immunoglobulin (IF score 2·8 ± 0·4) and GBM immunoglobulin (IF score 2·6 ± 0·4) deposition in albuminuric mice was significantly greater ($P < 0.05$) than that in non-albuminuric mice (mesangial immunoglobulin, IF score 2·0 ± 0·3; and GBM immunoglobulin, IF score 1·2 ± 0·6)

In indirect immunofluorescence, glomerular eluates obtained from young non-albuminuric mice (group 1) showed binding to the glomerular and tubular basement membranes, whereas no nuclear binding was observed (Fig. 3a). Glomerular eluates of the other groups of older age-matched mice (groups 2–4) showed binding to the glomerular and tubular basement membranes and, in addition, to nuclei (Fig. 3b). In the plasma pools of all groups, nuclear binding at high titre was observed, whereas no binding to glomerular and/or tubular basement membrane was found (data not shown).

In ELISA, antibody reactivities towards all nuclear antigens and ECM components were found in eluates of the age-matched 18–24-week-old groups (groups 2–4, Fig. 2). Eluate reactivities in young mice of 12 weeks of age (group 1) were negative or close to background levels in all ELISAs. No detectable anti-DNP reactivity was found in any eluate, whereas in the plasma pools anti-DNP titres were high and comparable to anti-DNA and anti-nucleosome titres (data not shown). These high anti-DNP titres in plasma are found in many murine lupus strains [29]. In albuminuric mice (groups 3 and 4) approx. three times more immunoglobulin was eluted compared with age-matched non-albuminuric controls
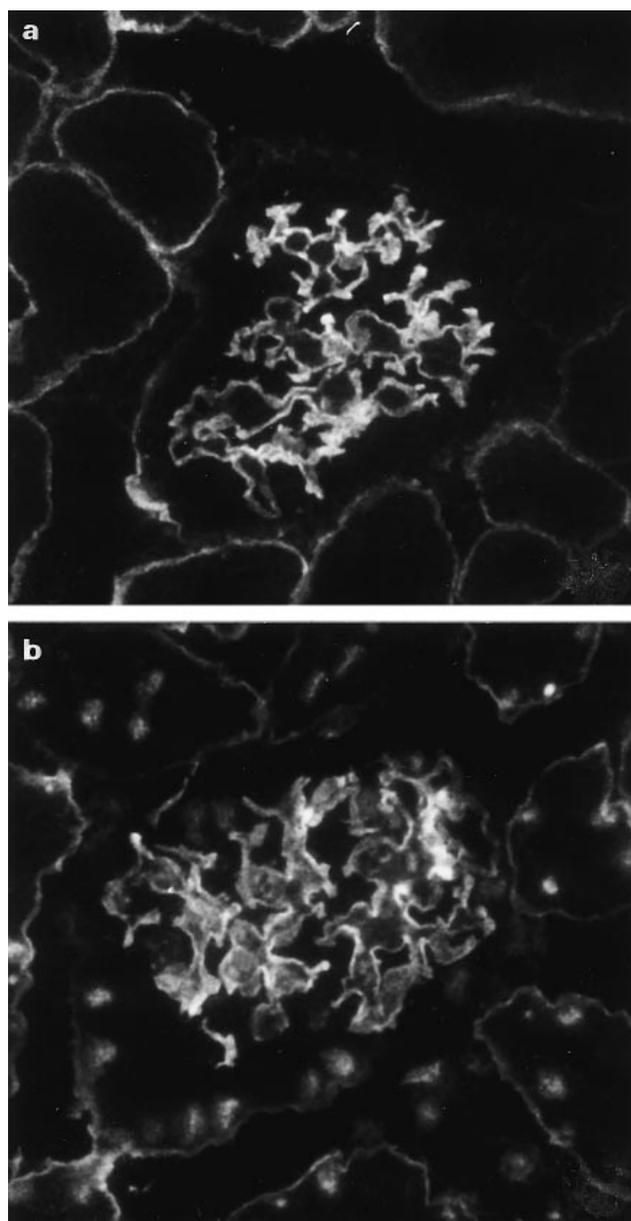
Fig. 3. Indirect immunofluorescence of glomerular eluates on MRL/*n* kidney sections. Note that with the eluate (0·4 μg/ml) of young non-albuminuric mice, only the glomerular basement menbrane (GBM) and the tubular basement membrane (TBM) stain, whereas the nuclei are negative (a). When glomerular eluates (0·4 μg/ml) of the 18–24-week-old groups are evaluated nuclei, GBM and TBM are stained (b).

Table 1. Enrichment factors (eluate reactivity per mg immunoglobulin/plasma reactivity per mg immunoglobulin) for reactivities towards different nuclear antigens and extracellular matrix (ECM) components in albuminuric mice (groups 4 and 5) and age-matched non-albuminuric controls (group 3)

	Group 3	Group 4	Group 5
Age (weeks)	18–24	18–24	18–24
Albuminuria	—	Short*	Long†
Nucleosome	5	6	7
DNA	4	7	9
Histone	35	14	27
Laminin	100	68	32
Collagen IV	133	16	36
Entactin	83	22	64

*Duration of albuminuria < 7 days.
†Duration of albuminuria 14–21 days.

(group 2), and anti-nuclear (except for anti-histone) and anti-ECM component titres were higher (Fig. 2).

Factors of enrichment for eluates compared with plasma (i.e. eluate reactivity per mg immunoglobulin/plasma reactivity per mg immunoglobulin) for all age-matched groups of mice were calculated. For all parameters tested, except for anti-DNP, enrichment in eluates was found (Table 1).

DISCUSSION

For the study of the relationship between antibody specificities and lupus nephritis, two approaches are possible. First, plasma antibody reactivities can be correlated with the onset of renal disease or a renal exacerbation. A disadvantage of this approach is that antibodies with high affinity for glomerular antigens will deposit in the kidney and will not be found in the circulation. Second, antibodies can be eluted from diseased glomeruli. In this way the nephritogenic antibodies deposited in the glomerulus are obtained. We adopted both approaches and studied the association between albuminuria and the reactivities in longitudinal plasma samples and with antibodies eluted from isolated glomeruli.

In the longitudinal plasma study there was a striking similarity between plasma antibody reactivities of mice developing albuminuria and mice which did not. Only when reactivities were compared at the moment albuminuria developed were higher anti-DNA and lower anti-nucleosome reactivities found. The finding that the absolute anti-DNA titre is higher at the onset of albuminuria is in line with many serological studies describing a relationship between serum anti-DNA levels and nephritis in both human and murine lupus [4–6]. Until now, there have been two studies, both in humans, correlating anti-nucleosome and/or anti-H2A/H2B-DNA subnucleosome reactivity to renal disease. In one study there is a correlation between both parameters and renal disease [11]; in the other, anti-nucleosome reactivity was not correlated with this disease manifestation [12]. Two features of the deposition of anti-nucleosome antibodies are noteworthy. First, the titre is already high in animals with short-lasting albuminuria, while the titre of deposited anti-DNA antibodies increases substantially in animals with longer lasting albuminuria. This suggests a different time course of deposition between the two specificities, i.e. anti-nucleosome deposition precedes that of anti-DNA. Second, glomerular deposition of anti-nucleosome antibodies in albuminuric mice is associated with lower plasma titres than non-albuminuric controls (Fig. 1f). This may be due to enhanced glomerular deposition of these antibodies. In contrast, the higher eluate anti-DNA titre, which is most pronounced in animals with prolonged albuminuria, is found in conjunction with higher plasma titres (Fig. 1e). This difference between anti-DNA and anti-nucleosome titres in plasma *versus* eluate suggests that from the anti-DNA antibodies only a subgroup deposits in the glomerulus, whereas for anti-nucleosome antibodies such a selective deposition does not seem to occur. A decrease of plasma titres might be due to an enhanced glomerular deposition.

Anti-histone antibodies were mainly seen later in life in mice not developing albuminuria. This pattern of anti-histone reactivity is in line with results found by Burlingame *et al.* [10]. These authors also found anti-histone reactivity later in the disease after the development of anti-chromatin (nucleosome) and anti-DNA antibodies. In the eluates, antibodies to histones are not different between albuminuric (groups 3 and 4, Fig. 2) and age-matched non-albuminuric mice (group 2, Fig. 2). It is questionable therefore whether these anti-histone antibodies are important for the development of albuminuria. This observation is in line with the clinical experience that in drug-induced lupus, renal disease is rare, despite high serum anti-histone reactivity but in the absence of anti-DNA titres.

Deposition of anti-GBM-tubular basement membrane (TBM) antibodies starts at an earlier stage than anti-nuclear antibodies, for in IF glomerular eluates of young mice show exclusively GBM/TBM staining, i.e. anti-ECM reactivity without anti-nuclear staining. These findings are in line with results found in another mouse lupus model, the GVH disease, in which glomerular eluates 4 weeks after induction of the disease contain only anti-ECM reactivity, while after 8 weeks anti-nuclear reactivity could also be detected [18]. Interestingly, when the eluates of the young non-albuminuric animals in our study were tested in ELISA on the ECM components laminin, collagen IV and entactin, these reactivities were negative or close to background levels, suggesting that other antibody specificities at this age are responsible for the GBM-TBM binding in IF. Therefore, apparently autoantibodies are formed with a high affinity for the GBM/TBM, since they are not found in the circulation, and are directed against an as yet undefined epitope within the GBM/TBM. However, the deposition of these anti-GBM/TBM antibodies alone does not lead to albuminuria.

Reactivity to collagen IV and laminin (together with other ECM proteins) has been described in MRL/l sera later in life from weeks 17–20 onwards [16]. We obtained similar results, since in MRL/l plasmas we found antibody reactivity to collagen IV, laminin and entactin from weeks 15–18 onwards, while from the age of 18–24 weeks these antibody specificities could be detected in the eluates.

These reactivities, especially anti-laminin, had the highest titres in albuminuric mice. Anti-laminin reactivity has been described before in glomerular eluates of albuminuric MRL/l mice [20].

Factors of enrichment for eluates compared with plasma (i.e. eluate reactivity per mg immunoglobulin/plasma reactivity per mg immunoglobulin) for all age-matched groups of mice were calculated. Both anti-nuclear and anti-ECM antibodies are enriched in

glomerular eluates of both albuminuric and non-albuminuric mice. Since plasma anti-ECM titres are much lower than anti-DNA and anti-nucleosome titres, the enrichment for anti-ECM antibodies is more pronounced than enrichment for anti-nuclear specificities. Eluate enrichment for anti-DNA [20,30] or anti-histone [9] antibodies has been described before in murine lupus.

In this study we provide evidence that deposition of autoantibodies directed against nucleosomes, DNA and ECM proteins is correlated with the development of albuminuria in lupus nephritis. Furthermore, anti-GBM-TBM antibodies deposit early in the disease (12 weeks) before any deposition of anti-nuclear autoantibodies, although this deposition is not sufficient to cause albuminuria. Our study does not permit any conclusion about their contribution to the development of albuminuria later in life. These antibodies are directed against an as yet undefined GBM/TBM epitope. Deposition of anti-DNA and anti-nucleosome occurs later in the disease. Since in the eluate higher titres of anti-DNA, anti-nucleosome and anti-ECM component reactivity are seen in albuminuric mice in conjunction with a higher amount of immunoglobulin deposition, these data suggest that the differences between albuminuric and non-albuminuric mice are more quantitative than qualitative.

ACKNOWLEDGMENTS

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