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Treatment with a Laminin-Derived Peptide Suppresses Lupus Nephritis[1]

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The role of DNA as the target for pathogenic lupus autoantibodies in systemic lupus erythematosus is equivocal and renal damage may be due to cross-reactivity of lupus Abs with glomerular components. We have previously shown that lupus autoantibodies bind to the laminin component of the extracellular matrix. In the present work, we have analyzed the fine specificity of the interaction of pathogenic murine lupus autoantibodies with this molecule and the effect of inhibiting their binding to laminin during the course of the disease. We have found that pathogenic murine lupus autoantibodies react with a 21-mer peptide located in the globular part of the α-chain of laminin. Immunization of young lupus-prone mice with this peptide accelerated renal disease. Analysis of transgenic, congenic, and RAG-1$^{-/-}$ mice confirmed the importance of this epitope in the pathogenesis of lupus renal disease. We have synthesized a panel of peptides that cross-react with the anti-laminin Abs and have found that the binding of lupus autoantibodies to the extracellular matrix could be inhibited in vitro by some of these competitive peptides. Treatment of MRL/*lpr/lpr* mice with these peptides prevented Ab deposition in the kidneys, ameliorated renal disease, and prolonged survival of the peptide-treated mice. We suggest that laminin components can serve as the target for lupus Abs. The interaction with these Ags can explain both the tissue distribution and the immunopathological findings in lupus. Moreover, inhibition of autoantibody binding to the extracellular matrix can lead to suppression of disease. *The Journal of Immunology,* **2005, 175: 5516–5523.**

C ertain similarities in the pathology of tissue damage between systemic lupus erythematosus (SLE)[3] and immune complex-mediated, chronic experimental serum sickness led investigators to postulate that both diseases share the same pathogenetic mechanism, i.e., the formation and deposition of immune complexes in renal glomeruli, blood vessels, skin, and the choroid plexus of the brain. It was further postulated that autoantibodies present in the sera of SLE patients and in experimental animal models interact with DNA and the resulting immune complexes are deposited in the various tissues and initiate the inflammatory process. Alternatively anti-DNA Abs may bind to the kidney via nucleosomal particles. The most "problematic" element in the suggested mechanism of immune complex disease has been the unsubstantiated role of the DNA Ag for the following reasons: 1) most patients with active SLE, like normal subjects, have little free DNA in the circulation; 2) DNA-anti-DNA complexes are infrequent in circulating blood and represent a very small portion of the immune complexes in SLE; 3) free DNA and preformed DNA-anti-DNA immune complexes, injected into mice, are removed to the liver very rapidly; 4) There is little evidence for the presence of DNA Ag in

kidney eluates of SLE patients or lupus-prone mice. Consequently, various aspects of the classic, serum sickness-like model have been modified to suggest that the mechanism of renal and tissue damage in SLE is due to the binding of anti-DNA Abs to glomerular components in the absence of DNA by way of cross-reaction (1–3).

One of the Ags that was recently suggested as being involved in the immune injury to renal tissue is α-actinin, which is predominantly localized in the podocyte foot processes and mesangial cells (4, 5). Mostoslavsky et al. (5) reported that pathogenic (but not nonpathogenic) monoclonal anti-DNA Abs bind kidney tissue via this intracellular protein. Another group corroborated these results showing the binding of pathogenic murine anti-DNA directly to α-actinin (6). Despite these bindings, many researchers find it difficult to explain how the myriad of pathogenic manifestations of lupus Abs result from an interaction with an intracellular Ag such as α-actinin.

We have shown previously that one of the major components to which lupus Abs bind is the α1 laminin component of the extracellular matrix (ECM). The titers of either anti-ECM or anti-laminin urinary Abs correlated with SLE disease activity (7). Analysis of the α1 laminin component revealed that the lupus Abs bind to the E3 C-terminal constituent of the α1 laminin chain (data not shown). These findings were also corroborated by Kootstra et al. (8), who demonstrated, in an experimental murine model of lupus nephritis, that in the normal glomeruli laminin is present only in the mesangial matrix, whereas after disease induction its expression expands to the subepithelium, along the glomerular basement membrane to the subendothelium and finally to the periphery of end-stage sclerotic lesions.

In the present study, we have extended our investigation on the pathogenic capacity of anti-laminin peptide Abs in the induction of glomerulonephritis (GN) in different models of lupus mice and, more importantly, we have underlined the therapeutic potential of these peptides.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; ECM, extracellular matrix; GN, glomerulonephritis.

Materials and Methods

Mice

MRL/*lpr/lpr*, (NZB/NZW)F$_1$, and BALB/c mice were purchased from The Jackson Laboratory. Congenic lupus mice carrying lupus susceptibility genes (*Sle1-Sle3*) from the lupus-prone NZM2410 mouse on the C57BL/6 background were also studied (9, 10). These mice were divided according to the following expression of the lupus-prone genes: B6.NZMc1 (locus *Sle1*), B6.NZMc4 (locus *Sle2*), B6.NZMc7 (locus *Sle3*), B6.NZMc1/c7 (*Sle1+Sle3*), and B6.NZM c1/c4/c7 (*Sle1+Sle2+Sle3*). Mouse serum samples were obtained from 7- to 9-mo-old congenic mice. The animal studies have been reviewed and approved by the Ethics Committee and the Authority for Animal Facilities at the Hebrew University of Jerusalem.

Synthetic laminin peptides

Synthetic peptides were kindly provided by Schuger et al. (11). In brief, the peptides were derived from various domains of the α1 laminin molecule (12, 13). Four additional peptides with selected amino acids substitutions based on the composition of the VRT101 peptide were prepared by standard solid-phase 9-fluorenylmethoxycarbonyl chemistry and were analyzed and purified by reverse-phase HPLC and tested by fast atom-bombardment mass spectrometry. The sequences of the various peptides are presented in Table I.

Monoclonal Abs

The anti-DNA mAbs C72, ID9, J38, 11C4, and J64 of the IgG2a isotype and A52 and 3F7 of the IgG2b isotype originating from the NZB × NZW lupus mouse were prepared as previously described (5, 14).

Thirteen anti-laminin mAbs were used in this study. The primary mAb was the H50 (IgG2b) anti-laminin derived from an MRL/*lpr/lpr* mouse. All other anti-laminin mAbs (66, A46, B8E, 61, 129, A10C, A10D, 68, 104, 87, 131, E23) were derived from B6 mice, transgenic for a μ H chain comprising the H50 variable region (15). All of these mAbs carried the same H50 IgM H chain in conjunction with different light chains. The E23 Ab, which also expresses the H50 H chain and has low anti-laminin activity, served as a control.

Direct binding of Abs to laminin peptides (ELISA)

Polystyrene 96-well plates (Nunc) were coated overnight at 4°C with 50 μl/well of 10 μg/ml different laminin peptides, VRT101, R28, R30, R37, R18, R27, R26 and R35, in carbonate-coating buffer (pH 9.6) or with 10 μg/ml DNA (Sigma-Aldrich) in PBS (pH 7.4). Wells were washed three times with PBS-0.05% Tween 20 and blocked with 300 μl/well of 1% BSA in PBS for 30 min at room temperature. The plates were subsequently incubated with various concentrations (0.3–5 μg/ml) of the mAbs or with serum samples, (1/100–1/25,600) in PBS-1% BSA for 60 min at room temperature and then rinsed as before and reincubated with alkaline phosphatase-conjugated anti-mouse IgG and IgM Abs (Sigma-Aldrich) for 1 h, followed by 1 mg/ml *p*-nitrophenylphosphate (Sigma-Aldrich), in 0.5 M NaHCO$_3$, 2 mM MgCl$_2$ (pH 9.5). OD was measured at 405 nm by an ELISA reader (Microwell System; Organon Teknika Turnhout).

Inhibition of anti-DNA mAb binding to VRT101 by laminin peptides

Inhibition assays were performed by incubation of the mAb C72 (5 μg/ml) with increasing concentrations (0.1–100 μg/ml) of dsDNA, VRT101, VRT102, VRT104, VRT105, and VRT107 for 60 min before testing its binding to VRT101 by ELISA.

Binding of mAbs and mouse sera to dsDNA

Ab binding to dsDNA was also measured by using the Millipore filter assay (16). Briefly, 5 μl of serum was diluted in 0.1 ml of 0.2 M borate-saline buffer (pH 8.0). ^{14}C-Labeled DNA (10 μl, 2800 cpm) was added to 100 μl of the diluted serum or to 2.5 μg of mAb. The mixture was incubated for 30 min at 37°C and for an additional 60 min at 4°C. The mixture was filtered under reduced pressure using 0.45-μm nitrocellulose filters (Millipore). The filters were washed twice with 3-ml aliquots of borate buffer, dried at room temperature for at least 16 h, and counted with toluene-based scintillation fluid in a beta scintillation counter. Results are expressed as the mean of duplicate samples. The samples differed from the mean value by <8%.

In vivo pathological effect of anti-DNA Abs on mouse kidneys

The in vivo glomerular pathogenicity of the anti-DNA mAbs was tested by using the nonautoimmune RAG-1$^{-/-}$ (B6,129.RAG1)-deficient mouse model (The Jackson Laboratory) (5, 17). Eight-week-old mice (five mice per group) were injected with 10^7 cells of various anti-DNA hybridoma cell lines. Blood samples were taken every 4 days for measurement of IgG and anti-DNA activity. Proteinuria was measured by dipsticks (Albustix; Bayer Diagnostics), and glomerular pathology was evaluated in all mice by light microscopy and immunofluorescence.

Immunization of young MRL/lpr/lpr mice with VRT101

To test whether immunization with VRT101 can cause acceleration of renal diseases, we have immunized young MRL/*lpr/lpr* mice with VRT101 and evaluated their anti-VRT level and renal disease at a relatively young age when anti-VRT levels are low and renal disease is normally minimal. Eight-week-old MRL/*lpr/lpr* mice were immunized s.c. with either VRT101 in PBS, 500 μg weekly, or with PBS (20 mice/group). At the age of 3 mo, mice were bled and anti-DNA and anti-VRT101 levels were measured. Four mice in each group were sacrificed and their kidneys were evaluated by light microscopy.

Treatment of MRL/lpr/lpr mice with the laminin peptides

Eight-week-old MRL/*lpr/lpr* mice (11–15 mice/group) were treated i.p. with laminin peptides (VRT101, VRT102, and R28) or with 100 μg of betamethasone (~3 mg/kg body weight) for periods of 180 or 260 days. Control mice received PBS. Mouse weight and survival were registered at 3, 4.5, 6, and 9 mo. Proteinuria was measured by dipsticks at 4 and 6 mo and anti-DNA and anti-VRT101 Abs at 5 mo of age. At the age of 5–6 mo, 5–10 mice were sacrificed to assess renal pathology.

Evaluation of renal pathology

Renal pathology was assessed in three mouse strains: 1) RAG-1-deficient mice inoculated with anti-DNA-producing hybridoma cell lines; 2) congenic lupus mice carrying the lupus susceptibility genes (*Sle1-Sle3*) from the lupus-prone NZM2410 mouse on the C57BL/6 background; and 3) MRL/*lpr/lpr* mice treated with i.p. injections of laminin peptides.

Mouse kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. For localization studies, cryosections of the kidneys were stained with FITC-conjugated goat anti-mouse Igs and counterstained with 0.1% Evans blue, then inspected by a Zeiss fluorescence microscope. Sections were also inspected by light microscopy (H&E) and graded as I–IV according to the World Health Organization classification of lupus nephritis. GN was graded as 0, or +1 to +3 according to Ig deposition as assessed by immunofluorescence. The renal pathology was evaluated by a renal pathologist "blinded" to the samples identity.

Results

Binding of monoclonal and serum polyclonal lupus Abs to laminin peptides

C-terminal peptides of laminin α1 chain have been shown to confer laminin-important biological activities such as promotion of cell adhesion and binding sites for cellular adhesion molecules (12, 13). To analyze the interaction of pathogenic lupus Abs with the laminin molecule, we tested the binding of anti-DNA Abs to peptides that compose the C-terminal of the laminin α-chain as well as to the R18 peptide derived from the N-terminal of the molecule

Table I. *Sequence of peptides within the α1 chain of laminin*[a]

Name	Sequence
R18 (TG-1)	RPVRHAQCRVCDGNSTNPRERH
R37 (GD-1)	KATPMLKMRTSFHGCIK
R26 (GD-3)	KNLEISRSTFDLLRNSYGVRK
R28 (GD-4)	DGKWHTVKTEYIKRKAF
R35 (GD-5)	TSLRKALLHAPTGSYSDGQ
R30 (GD-6)	KQNCLSSRASFRGCVRNLRLSR
VRT101 (R38)	KEGYKVRLDLNITLEFRTTSK
VRT102	KEGYKVRLDLNTTLEFRTTSK
VRT104	AEGYAVALDLNITLEFATTSA
VRT105	KEGYKVELDLNITLEFETTSK
VRT107	KEGYKVRLDLNITLEFETTSK

[a] Synthetic peptides (R18–38) were prepared from various domains of the α1 chain of the laminin molecule (12, 13). Four additional peptides (VRT102–107) represent substitutions in the VRT101 peptide sequence.
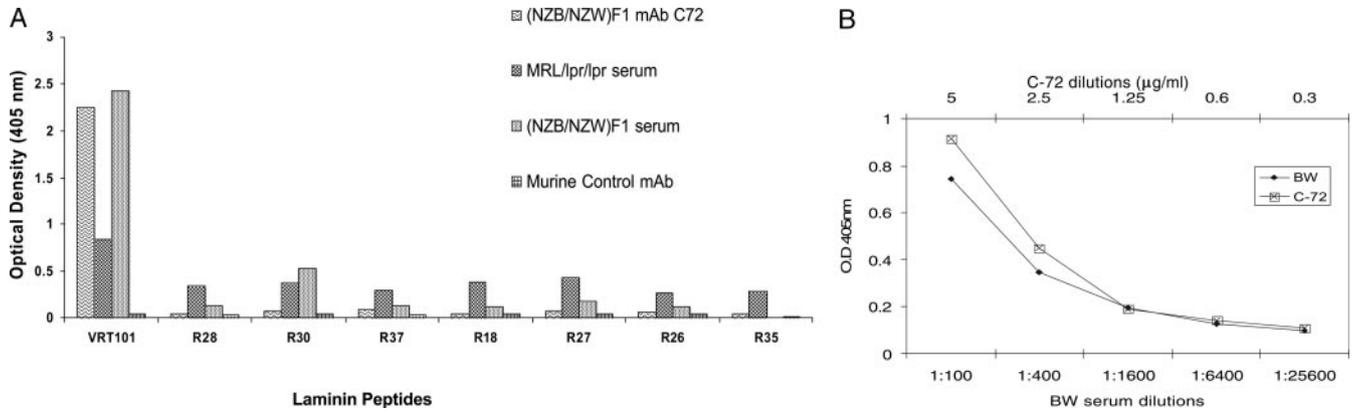
FIGURE 1. *A,* Binding of mouse lupus Abs to laminin peptides. Mouse mAbs (anti-DNA C72 and a nonrelated control; 5 μg/ml) and single serum samples from 6-mo-old (NZB $\times$ NZW)F$_1$ and 4-mo-old MRL/*lpr/lpr* female mice (1/100) were tested for binding to laminin peptides by ELISA. Results are the mean of duplicates measured by OD at 405 nm. *B,* Binding avidity of mAb C72 and (NZB $\times$ NZW)F$_1$ serum to VRT101. C72 (0.3–5 μg/ml) and serum from an 8-mo-old (NZB $\times$ NZW)F$_1$ mouse (1/100–1/25,600) were tested for binding to VRT101 by ELISA. The results represent the mean of duplicates measured by OD at 405 nm.

(Table I). An unrelated mouse mAb served as control. The (NZB $\times$ NZW)F$_1$-derived mAb C72 cross-reacted exclusively with the VRT101 peptide, which is located at the C-terminal portion of the laminin α1 chain. Serum derived from a 6-mo-old (NZB $\times$ NZW)F$_1$ and a 4-mo-old MRL/*lpr/lpr* female mouse avidly reacted with the VRT101 peptide, but exhibited a lower degree of reactivity with the other laminin peptides (Fig. 1*A*). The binding avidity of C72 (0.3–5 μg/ml) and (NZB $\times$ NZW)F$_1$ serum (1/100–1/25,600) to VRT101 was tested by ELISA (Fig. 1*B*).

Binding of sera from lupus-prone mice to VRT101 peptide

To assess the association of disease activity and anti-VRT101 serum titers in murine lupus models, we analyzed the anti-VRT101-binding capacity in sera of 10 female MRL/*lpr/lpr* (2- and 6-mo old) and 10 female (NZB $\times$ NZW)F$_1$ (2- and 8-mo old) mice. Each age group included sera of five mice. Sera from eight 6-mo-old BALB/c mice served as negative controls.

A consistent elevation in the binding to VRT101 was noticed in the two murine lupus models over time (Fig. 2). The average binding of MRL/*lpr/lpr* sera to VRT101 increased over time from an OD (at 405 nm) of 0.42 $\pm$ 0.13 (mean $\pm$ SE) at the age of 2 mo to 1.01 $\pm$ 0.14 by the age of 6 mo. Similarly, in (NZB $\times$ NZW)F$_1$ mice, the binding to VRT101 increased from 0.19 $\pm$ 0.02 at the age of 2 mo to 1.08 $\pm$ 0.27 by the age of 8 mo. The degree of binding to VRT101 in the BALB/c mice was significantly lower.

Anti-DNA and anti-VRT101 reactivity in the sera of congenic lupus mice

Three congenic strains carrying lupus susceptibility genes (*Sle1-Sle3*) from the lupus-prone NZM2410 mouse on the C57BL/6 background were characterized for their component phenotypes. B6.NZMc1 (locus *Sle1*) mediates the loss of tolerance to nuclear Ags, B6.NZMc4 (locus *Sle2*) lowers the activation threshold of B cells, and B6.NZMc7 (locus *Sle3*) mediates a dysregulation of CD4$^+$ T cells. Morel et al. (9, 10) have created a collection of bi- and tricongenic strains with these intervals and assessed the auto-immune phenotypes they elicit in various combinations. Dicongenic and tricongenic strains were produced by intercrossing the strains. It was reported that the B6-bicongenic mouse strain B6.NZMc1/c7 (*Sle1*+*Sle3*) developed high titers of autoantibodies, yet with only moderate GN, whereas the coexpression of B6.NZMc1/c4/c7 (*Sle1* + *Sle2* + *Sle3*) as a B6-triple congenic mouse resulted in severe systemic autoimmunity and fully penetrative, fatal GN (18).

As shown in Table II, the c1(*Sle1*) and c7(*Sle3*) monogenic strains had a similar degree of either anti-DNA or anti-VRT101 serum reactivity, whereas the c4(*Sle2*) strain had a lower anti-DNA titer. The serum of the hybrids of two loci *Sle1/Sle3* recorded enhanced anti-DNA and anti-VRT101 reactivity, compared with the single locus strains and with BALB/c mice ($p < 0.02$). The hybrid mouse carrying all three gene loci (*Sle1/Sle2/Sle3*) that developed severe GN had a significantly higher serum anti-VRT101 activity compared with each of the other strains ($p < 0.05$) and a higher anti-DNA titer compared with the *Sle3* strain ($p < 0.01$). In contrast, the titer of the anti-DNA Abs in the sera of the triple gene hybrid strain was similar to that of the double congenic strain (Table II).

Binding of pathogenic anti-DNA mAbs to VRT101

We found that all pathogenic anti-DNA mAbs recognized peptide VRT101 whereas the less or non-pathogenic Abs bound this peptide to a lesser extent (Table III). The (NZB $\times$ NZW)F$_1$ anti-DNA mAbs, A52, C72, 3F7, and 1D9 produced a similar pattern of intense linear deposition along the glomerular capillary wall, in all of the mice of each group, whereas 11C4 induced mostly mesan-gial deposition with little or no structural changes observed by microscopy (Table III). The J38 and J64 mAbs did not induce any
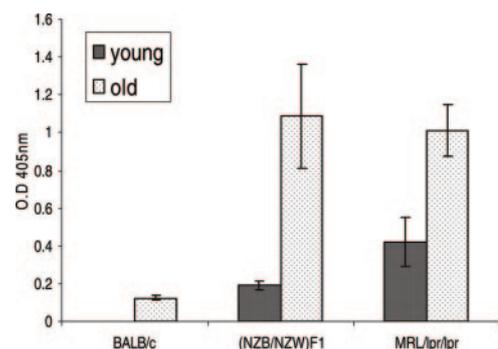

FIGURE 2. Binding of sera from two lupus mouse models to VRT101. MRL/*lpr/lpr* sera (2- and 6-mo old; 1/100) and (NZB $\times$ NZW)F$_1$ sera (2- and 8-mo old; 1/100) were tested for binding to VRT101 peptide by ELISA. Each age group included sera from five mice. Sera from 6-mo-old BALB/c mice (eight mice per group) served as control. Results are the mean $\pm$ SE measured by OD at 405 nm. $p < 0.02$ in both models comparing older to younger mice or to BALB/c mice.

Table II. *Anti-DNA and anti-VRT101 serum levels and renal pathology in congenic lupus mice[a]*

Mice	Anti-VRT101 (OD 405 nm)	Anti-DNA (cpm)	Renal GN
c1 (*Sle1*) (*n* = 8)	0.28 ± 0.04^b	1328 ± 167^b	−
c4 (*Sle2*) (*n* = 8)	0.32 ± 0.1	713 ± 104	−
c7 (*Sle3*) (*n* = 8)	0.24 ± 0.02	1482 ± 103	−
c1/c7 (*Sle1/Sle3*) (*n* = 6)	$0.4 \pm 0.1*$	$2540 \pm 167*$	+1
c1/c4/c7 (*Sle1/Sle2/Sle3*) (*n* = 10)	$0.87 \pm 0.14**$	$2711 \pm 167*$	+3
BALB/c (*n* = 8)	0.13 ± 0.04	73 ± 21	ND

[a] Sera from congenic lupus mice (7–9 mo old; 1/100), carrying lupus-susceptibility genes, were tested for binding to VRT101 and DNA and their kidney pathology was expressed as follows: +3, severe diffuse GN; +2, moderate diffuse GN; +1, mild GN; +/−, borderline GN; and ND, not done.
[b] Mean $\pm$ SD.
*, $p < 0.02$ compared to BALB/c, c1, c4, and c7 strains.
**, $p < 0.05$ compared to BALB/c, c1, c4, c7, and c1/c7 strains.

pathological effects. All of these mAbs bound dsDNA with a similar avidity but the mAbs differed in their VRT101 binding. A52, C72, 3F7, and 1D9 bound VRT101 with a higher avidity then J38, whereas 11C4 and J64 did not react with the VRT101 laminin epitope. It seems therefore that the pathogenic activity of these anti-DNA Abs is associated with the recognition of laminin determinants rather than with their DNA reactivity.

Binding of anti-laminin mAbs to DNA and VRT101

Thirteen anti-laminin mAbs were tested for binding to DNA and VRT101. All of them, i.e., H50, 66, A46, B8E, 61, 129, A10C, A10D, 68, 104, 87, 131, and E23, bound the VRT101 peptide and some bound to DNA to various degrees (Fig. 3). The results demonstrated a diverse relationship between anti-DNA and anti-laminin activity. Several Abs exhibited a clear predilection for laminin with lower anti-DNA activity (H50, 66, A46, B8E, 61, 129, 104, 87, and E23) and others showed a higher avidity for DNA (A10C, A10D, 68, and 131). Two of these mAbs (H50, A10C) were previously tested for pathogenicity in non-lupus mice and were found to form glomerular and basement membrane deposits, after the respective hybridoma cells had been injected into the peritoneum of histocompatible CB6F1/J mice (15). Of these, H50 was highly reactive with the VRT101 but had minimal anti-dsDNA activity and A10C reacted with both Ags, indicating that VRT101, rather than DNA binding, was associated with renal pathology induced by both Abs.

Table III. *VRT101 peptide binding and renal pathogenicity of anti-DNA mAbs[a]*

mAbs	Anti-DNA Abs (cpm)	Anti-VRT101 Abs (OD 405 nm)	Pathogenicity[b]
A52	1752	1.31	+3
C72	1637	1.25	+3
3F7	1400	1.4	+3
1D9	1784	1.08	+2
J38	1600	0.52	−
11C4	1800	0.03	$+/-^c$
J64	2038	0.08	−

[a] Cells (10^6) of different anti-DNA-producing hybridoma lines (originating from (NZB × NZW) F$_1$ mice) were inoculated in 1 to 8-wk-old RAG-1-deficient mice (six mice per group). Sera were tested for DNA (by RIA) and VRT101 (by ELISA) binding and kidney pathology was evaluated as detailed in *Materials and Methods*. Results are the mean of duplicates (ELISA) and triplicates (RIA).
[b] Pathogenicity was evaluated in all five mice of each group. All of the pathogenic Abs that bound the kidneys showed a similar pattern of pathology and were also found to induce proteinuria in these mice. The pathogenic Abs produced an intense linear deposition along the glomerular capillary wall. The kidney pathology was expressed as follows: +3, severe diffuse GN; +2, moderate diffuse GN; +1, mild GN; and +/−, borderline GN.
[c] 1IC4 Ab induced mostly mesangial deposition with little or no structural changes observed by light microscopy.

Inhibition of the anti-DNA mAb C72 binding to VRT101 by laminin peptides

To test the specificity of the binding of C72 mAb to VRT101 peptide, we performed competition assays with DNA and with various concentrations of several laminin peptides (Fig. 4). C72 titer used for inhibition assays was 5 μg/ml (Fig. 1B). DNA, VRT102, VRT101, and VRT107 showed a 50% inhibition at concentrations of 3.3, 9.1, 30.2, and 57.5 μg/ml, respectively. VRT102 differs from VRT101 by a single amino acid (Table I) and has the same net charge of +2, whereas VRT107 also differs by a single amino acid but has a neutral net charge. VRT104 and VRT105 had no inhibitory effect on the binding of the C72 mAb to VRT101 (Fig. 4). These peptides differ from VRT101 by two amino acids and have a negative charge of −3 and −2, respectively. These results indicate that both structure and charge play a role in the cross-reactivity of these peptides.

Immunization of MRL/lpr/lpr mice with the VRT101 peptide

Immunization of MRL/*lpr/lpr* mice with s.c. injections of VRT101 induced high levels of anti-VRT101 and accelerated the development of proliferative GN. At 3 mo of age, anti-VRT101 levels increased from 0.26 ± 0.02 in the PBS-treated mice to 1.6 ± 0.23 (mean $\pm$ SE) in the VRT-immunized mice. No change in anti-DNA levels was seen. Two of four VRT-immunized mice developed diffuse proliferative GN, whereas all of the PBS-treated mice had mild mesangial changes only.

Treatment of MRL/lpr/lpr mice with laminin-derived peptides

Since both VRT101 and VRT102 peptides inhibited the binding of lupus Abs to the laminin peptide in vitro, we have tested their
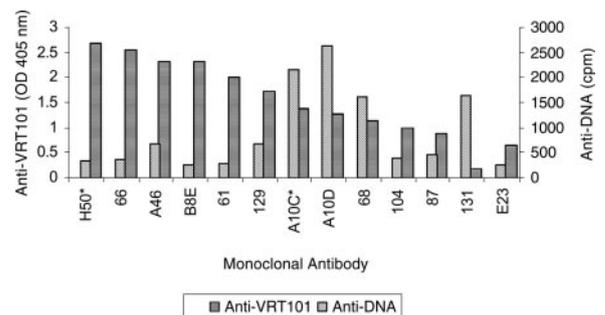

FIGURE 3. Binding of anti-laminin mAbs to DNA and to VRT101 peptide. Thirteen mAbs (5 μg/ml) were tested for binding to DNA (by RIA) and to VRT101 (by ELISA). Results are the mean of duplicates (ELISA) and triplicates (RIA) and are expressed by OD (405 nm) for VRT101 binding and by cpm for DNA binding. *, Only H50 and A10C Abs were previously tested for pathogenicity and have been shown to cause renal disease (15).

FIGURE 4. Competitive inhibition of mAb C72 binding to VRT101. C72 (5 μg/ml) was preincubated for 1 h with several laminin peptides or dsDNA (0.1–100 μg/ml) and then tested for binding to VRT101 by ELISA. Results are the mean of duplicates measured by OD at 405 nm.
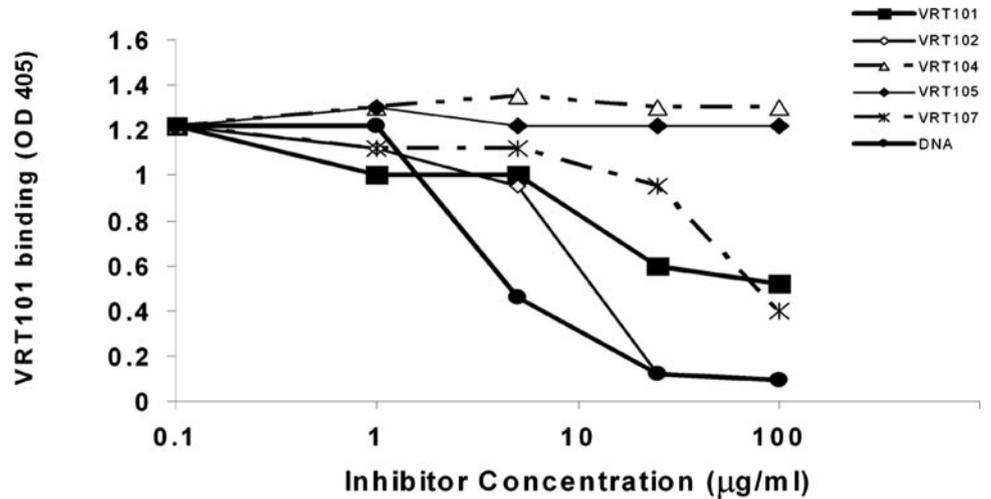
therapeutic effect on the course of murine lupus. Initial studies showed that treatment of RAG-1$^{-/-}$ mice that had been inoculated with 10^7 mAb C72 hybridoma cells, with 5 weekly s.c. injections of 50 μg of VRT101 for 2 wk, resulted in a reduced kidney immune complex deposition, from moderate deposition in the

PBS-treated mice to mild or no deposition in the peptide-treated mice. Based on these results and on the finding that VRT102 was the best laminin peptide inhibitor in vitro (Fig. 4), we have tested its therapeutic effect in vivo compared with the laminin peptide R28. We treated MRL/*lpr/lpr* mice with three weekly injections of
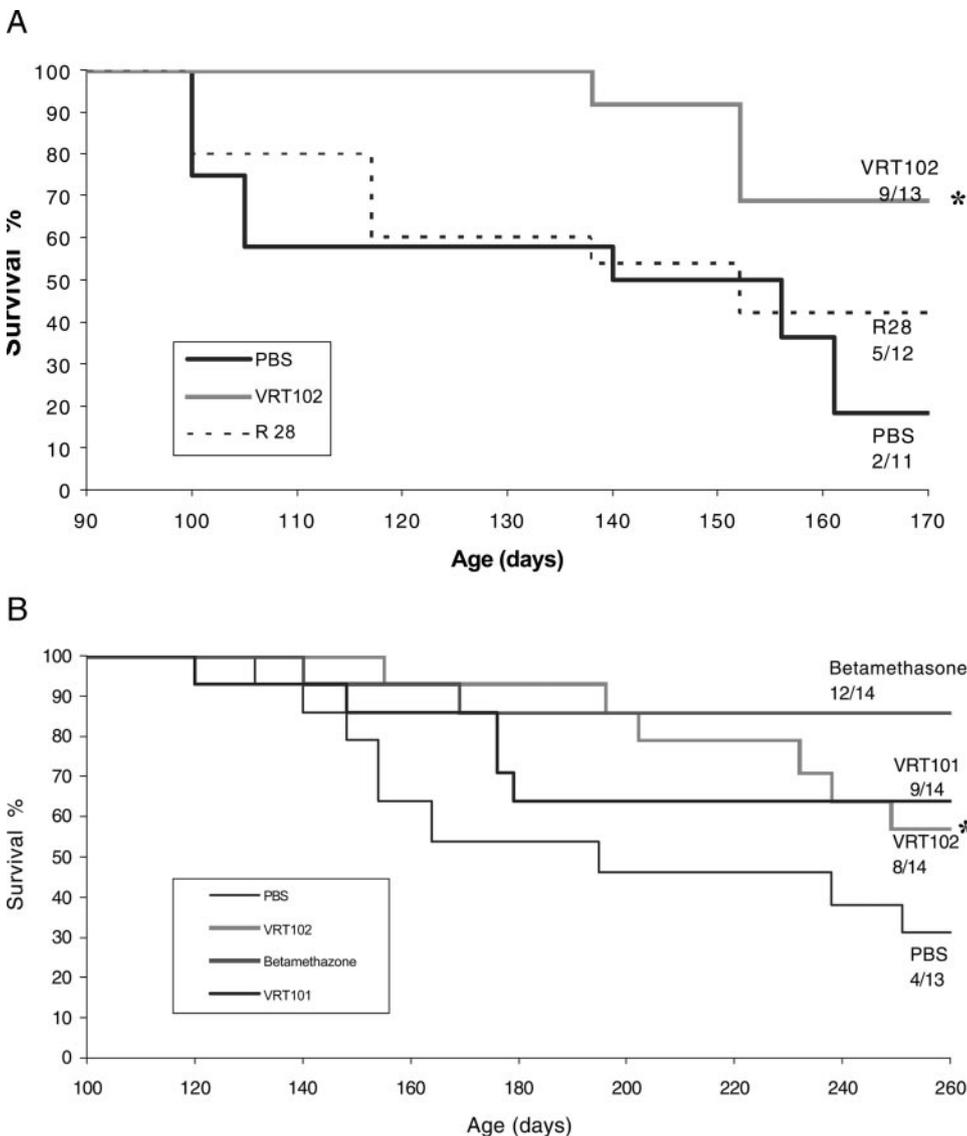

FIGURE 5. *A,* Survival rates of MRL/*lpr/lpr* mice treated with VRT102, R28, and PBS. Mice were injected i.p. with 50 μg of peptide, three times weekly, for 180 days. Control mice received PBS. The rates are expressed as percentage of treated mice. The numbers in parentheses express surviving mice/treated mice at the end of the experiment. *, $p < 0.01$ comparing survival of VRT102- to PBS-treated mice by Wilcoxon-Gehan test implemented as StatXact6. *B,* Survival rates of MRL/*lpr/lpr* mice treated with VRT101, VRT102, betamethasone, and PBS. Mice were injected i.p. with 80 μg of peptide or with 100 μg of betamethasone, five times weekly, for 260 days. Control mice received PBS. The rates are expressed as percentage of treated mice. The numbers in parentheses express surviving mice/treated mice at the end of the experiment. *, $p < 0.05$ comparing survival of VRT102- to PBS-treated mice by Wilcoxon-Gehan test implemented as StatXact6.

50 μg of VRT102, R28, or PBS for 180 days. We found that VRT102, but not the R28 peptide (which is not included in the E3 fragment of the C-terminal globular region of the laminin α1 chain) prolonged the survival of MRL/*lpr/lpr* mice significantly (Fig. 5*A*). We then added to the therapeutic regimen VRT101, which was characterized by better solubility features.

We found that a maximal effect of laminin-peptide administration was obtained by injection of 80 μg of either VRT101 or VRT102 peptide five times weekly for 260 days (Fig. 5*B*). Only 30% of the PBS-treated mice survived 9 mo, whereas ~60% of the VRT-treated mice and 85% of the betamethasone-treated mice survived that time. Peptide therapy also significantly prevented the accumulation of ascitic fluid and edema (demonstrated by body weight) and the average weight (±SE) of each group was as follows; PBS-treated mice, 46.2 ± 0.52 g; VRT101-treated mice, 43.8 ± 0.42 g; VRT102-treated mice, 44.3 ± 0.35 g; and betamethasone-treated mice, 38.0 ± 0.06 g ($p < 0.01$ in peptide-treated compared with PBS-treated mice). The peptide treatment also progressively reduced the protein levels in the urine of the treated mice. At 4 mo of age, 6% of the PBS-treated mice already had urine protein levels that were ≧500 mg/dl, whereas none of the peptide- or steroid-treated mice showed similar levels. At 6 mo of age, 43% of the PBS-treated mice already had these urine protein levels, whereas only 25% of the peptide-treated and 8% of the steroid-treated mice had similar levels. However, there was no change in anti-VRT101/102 or anti-DNA Ab levels in the sera of the peptide- or steroid-treated compared with PBS-treated mice. Similarly, the peptide treatment had no effect on lymphadenopathy or T cell infiltrates in the spleen and inguinal lymph nodes of peptide-treated mice. Furthermore, T cells from PBS- or VRT101-treated mice did not specifically proliferate in response to VRT101 before or after the treatment.

We used the VRT101/102-based therapeutic model to assess the degree of renal disease, i.e., GN and of Ig deposition in the glomeruli of the treated mice; When animals were treated with 50 μg of peptide three times weekly, we detected milder kidney manifestations in the mice treated with VRT102 than in those treated with the laminin peptide R28 or with PBS; of nine mice treated with VRT102, six had mesangial proliferative GN (class II), one had focal proliferative GN (class III), and two had diffuse proliferative GN (class IV), whereas of six mice treated with R28, five had GN class IV and only a single mouse was categorized with class II or III GN. All of the five mice that were treated with PBS had class IV GN.

When animals were treated five times weekly with 80 μg of peptides, we found that all of the VRT101-treated mice had class II GN (Fig. 6*B*). Of four mice treated with betamethasone, one had class I, two had class II, and one had class III GN (data not shown), and two of the PBS-treated mice had class II, one had class III, and one had class IV GN (Fig. 6*A*). Four of seven mice treated with VRT101 had no deposition (Fig. 6*D*), one had mild deposition, and two had moderate deposition of Igs in the glomeruli whereas all of the PBS-treated mice had extensive deposition of Igs (Fig. 6*C* and Table IV).

Discussion

It is well acknowledged that anti-DNA titers are linked to the expression and severity of lupus nephritis; however, whether immunological pathogenicity is entirely ascribed to these Abs remains questioned. In addition to their capacity to bind to nucleosomal components, it has been postulated that anti-DNA Abs may also mediate renal injury by cross-reacting with non-nucleosomal kidney Ags. Mostoslavsky et al. (5) observed that anti-DNA mAbs that bind to kidney glomeruli cross-react with the intracellular protein α-actinin. We and others have previously shown that the serum and urine of lupus mice with active nephritis contain Abs that bind to the laminin component of the ECM (7, 19). Analysis of various fragments of the molecule revealed that the Abs bind to the E3 C-terminal component of the α1 chain of the laminin molecule. Interestingly, Abs to this laminin molecule were shown to correlate with disease activity (7).

In the present work, we have tested the interaction of lupus polyclonal and mAbs with a set of laminin peptides comprising the E3 fragment of the C-terminal globular region of laminin α1 chain,

FIGURE 6. Kidney sections of 5-mo-old MRL/*lpr/lpr* mice treated with PBS or VRT101 peptide. *A,* Diffuse and global endocapillary proliferation with extensive crescent formation and interstitial inflammatory infiltrates in PBS-treated mice (H&E staining; original magnification, ×200). *B,* Mild mesangial cell hyperplasia in VRT101-treated mice (H&E staining; original magnification, ×200). *C,* Fluorescent staining of a glomerulus with massive mesangial and some pseudolinear distribution of immune deposits in PBS-treated mice (immunofluorescence for IgG; original magnification, ×200). *D,* Absence of immune deposits in VRT101-treated mice (immunofluorescence for IgG; original magnification, ×200).

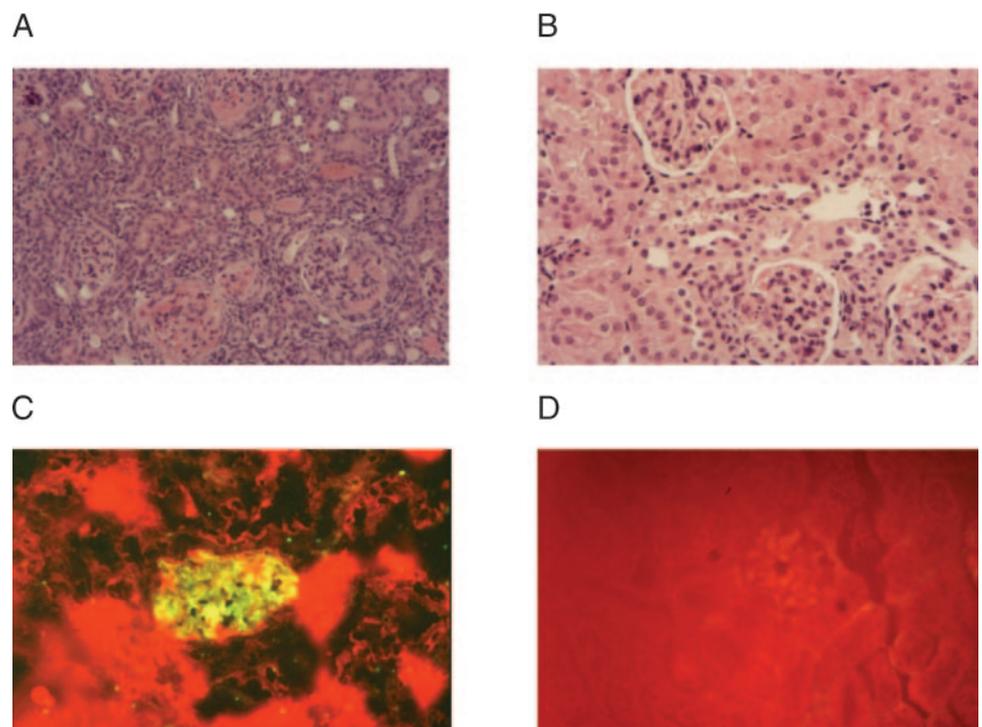Table IV. *Ig deposition and renal disease severity to VRT101-treated MRL/lpr/lpr mice*[a]

Treatment	Renal GN	Deposition of Igs		
		IgA	IgG	IgM
PBS	II	−	+1	+2
PBS	IV	+3	+2	+2
PBS	II	+1	−	+2
PBS	III	+1	+2	+2
VRT101	II	−	−	−
VRT101	II	−	−	−
VRT101	II	−	+2	+2
VRT101	II	−	−	−
VRT101	II	−	−	−
VRT101	II	−	−	+1
VRT101	II	−	−	+2

[a] MRL/*lpr/lpr* mice were injected i.p. with 80 μg of VRT101 peptide or with PBS, five times weekly, for 260 days. Renal pathology and immune complex deposition were evaluated as described in *Materials and Methods*. Deposition of Igs: +1, mild; +2, moderate; and +3, severe. Renal GN: class II, mesangial proliferative GN; class III, focal proliferative GN; and class IV, diffuse proliferative GN.

which is involved in the functional roles of the laminin molecule (20). The VRT101 peptide was found to serve as an important antigenic determinant. Abs to this peptide were detected in the sera of MRL/*lpr/lpr* and (NZB × NZW)F$_1$ mice but were not detected in the sera of non-lupus mice. Furthermore, the pathogenic anti-DNA mAb C72 cross-reacted with this peptide. The level of anti-VRT101 Abs correlated with disease activity; as the disease severity of the MRL/*lpr/lpr* and (NZB × NZW)F$_1$ mice progressed over time, the titer of anti-laminin peptide VRT101 Abs rose in parallel.

Although these findings show that VRT101 binding is associated with disease activity, they could not differentiate between the role of VRT101 binding to that of DNA binding because anti-DNA levels also increase with progressive disease in the spontaneous murine models of lupus. Therefore, we chose to study the correlation of anti-VRT101 and renal disease by using SLE-congenic mice, in which a dichotomy between anti-DNA levels and renal pathology has been found. Wakeland and colleagues (9, 10, 18) have developed congenic mouse strains that possess different SLE-susceptibility gene loci based on the NZM2410 mouse model. Mice carrying a single locus (*c1*, *c4*, or *c7*) develop hypergamma-globulinemia and anti-DNA Abs but not kidney disease. Mice carrying all three susceptibility gene loci (*c1xc4xc7*) develop a severe form of renal lupus, whereas mice carrying only two loci (*c1xc7*) have a much milder disease. We have found that the (*c1xc4xc7*) mice with severe nephritis had significantly higher titers of the anti-laminin peptide VRT101 Abs than (*c1xc7*) mice and anti-DNA Ab titers were similar in the two strains. Congenic mice with no renal disease with or without anti-DNA Abs did not exhibit anti-VRT101 binding. These results indicate that renal disease in these congenic mice is associated with VRT101 binding rather than with their DNA binding.

These findings were supported by the observed acceleration of renal disease in young MRL/*lpr/lpr* mice immunized with VRT101, inducing high levels of anti-VRT101 Abs with no change in the anti-DNA levels.

These results are in agreement with the study of Liang et al. (21) who have analyzed the reactivity to nucleosomes, ssDNA, dsDNA, and glomerular substrate in a large panel of antinuclear and non-nuclear binding mAbs obtained from NZM2410 lupus mice. A large fraction of mAbs demonstrated polyreactivity to DNA, histones, and glomerular Ags that was dependent on DNase I-sensitive nuclear antigenic bridges. Of great interest was the finding that

glomerular-binding anti-dsDNA Abs exerted the most severe renal disease, the nonglomerular-binding anti-dsDNA Abs were modestly pathogenic, and the anti-nucleosome Abs were clearly nonpathogenic.

To test the pathogenicity of the anti-DNA/anti-laminin mAbs, we have used the RAG-1-deficient mice. Eilat and coworkers (5, 22) were able to use this model for assessing tissue damage produced by anti-DNA autoantibodies. RAG-1$^{-/-}$ mice do not produce B and T cells due to a targeted mutation in a gene that is essential for recombination of Ig and TCR genes (17). Several IgG2a and IgG2b hybridoma cells from various anti-DNA clones that were injected into RAG-1$^{-/-}$ mice produced nephritis and proteinuria with deposition of Abs in the kidney. Interestingly, we have found that all pathogenic anti-DNA mAbs cross-reacted intensively with the VRT101 peptide, whereas anti-DNA mAbs that did not bind VRT101 or bound it with low avidity did not deposit in the kidneys and did not produce significant renal damage (Table III). Although a subpopulation of anti-DNA Abs recognizes ECM elements like the laminin molecule, the renal pathogenicity in lupus cannot be solely attributed to the binding of anti-DNA Abs. We further addressed this issue by analyzing a group of monoclonal anti-laminin Abs. We have shown that some of these Abs reacted with both DNA and VRT101, whereas others reacted only with VRT101. Two of these anti-VRT101 Abs, H50 and A10C, were previously tested for pathogenicity and shown to induce renal damage (15). Only one of these Abs, i.e., A10C, cross-reacted with DNA whereas H50 bound only to VRT101, indicating that in these Abs it is the VRT101, rather than the DNA binding, that is associated with renal pathogenicity.

The induction of kidney disease with monoclonal anti-VRT101 Abs in these two murine-induced lupus models indicate that these Abs are indeed nephritogenic.

Waters et al. (23) have demonstrated that breaking tolerance to dsDNA, nucleosomes, and other nuclear Ags is not required to induce GN in the lupus-prone mouse NZM2328. In this strain, two loci were identified; *Cgnz1* is linked to chronic GN with severe proteinuria and early mortality in females, whereas locus *Adnz1* is linked to anti-nuclear Ag and anti-dsDNA Ab production. By generating congenic strains in C57L/J mice, a non-lupus-prone strain, they succeeded to generate female mice that had chronic GN and severe proteinuria without circulating anti-nuclear, anti-dsDNA, and anti-nucleosome Abs. Immune complexes were detected in the diseased kidneys yet eluates from these kidneys did not contain anti-nuclear, anti-dsDNA, and anti-nucleosome Abs, indicating the presence of non-anti-dsDNA nephritogenic Abs. Recently, Christensen et al. (24) have shown that TLR-9-deficient MRL mice lack anti-DNA Abs but still develop GN. These results corroborate the finding that anti-dsDNA production is not obligatory to the induction of lupus GN; furthermore, the emergence of these two basic manifestations of SLE is probably controlled by different genetic loci.

Most promising is the fact that the laminin peptides provided beneficial therapeutic effects. Treating MRL/*lpr/lpr* mice with five injections weekly of the VRT peptides decreased the weight gain caused by accumulating ascitic fluid and reduced the level of proteinuria. Moreover, this therapeutic modality suppressed renal disease and markedly improved the longevity in these mice.

Treatment of MRL/*lpr/lpr* mice with the VRT101 peptide induced a reduction in Ig deposition in the kidneys without a significant effect on the anti-DNA or anti-VRT101/2 Ab levels in their serum. This therapeutic effect was better with daily injections, compared with a once weekly treatment (data not shown). Moreover, preliminary results in RAG-1$^{-/-}$ mice, inoculated with the anti-DNA/anti-VRT101 C72 hybridoma, show that peptide treatment inhibited the

deposition of the mAb C72 in their kidneys. These results suggest that the effect of the VRT101 on renal disease is obtained by in vivo inhibition of the deposition of the pathogenic Abs in the kidneys.

The α1 laminin molecule is essential for many biological processes and is a basic component of the ECM, appearing in the normal kidney within the mesangium but in lupus kidneys it propagates into extramesangial sites (8). This finding may clarify the pathogenic role that anti-laminin Abs may have in lupus nephritis in which early mesangial involvement develops into panglomerular diffuse proliferative GN. The VRT101 laminin peptide promotes cell adhesion (13) and it is possible that inhibiting this activity may also play a role in the pathogenesis of lupus renal disease. Interestingly, Peterson et al. (25) have demonstrated by a cDNA microarray analysis of glomerular specimens originating from patients with SLE an increased production and up-regulation of genes encoding ECM components.

In another study, we have detected high titers of anti-VRT101 Abs exclusively in the serum of lupus patients but not in the serum of patients with other autoimmune diseases or in the serum of normal controls (H. Amital, M. Heilweil, and Y. Naparstek, manuscript in preparation), indicating that this laminin epitope plays a major role in the human disease as well.

On the basis of these results, we suggest that lupus nephritis is probably mediated by the deleterious action of anti-laminin Abs. Furthermore, we have demonstrated that continuous daily administration of laminin peptides to lupus-prone mice has a protective effect that increases survival.

Acknowledgments

Disclosures

Y. Naparstek is the Chief Scientific Officer of Verto Medical.

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