Antibodies against the VRT101 laminin epitope correlate with human SLE disease activity and can be removed by extracorporeal immunoabsorption


Objective. We have previously shown that murine pathogenic lupus autoantibodies bind to VRT101, a 21-mer peptide located at the globular part of the laminin-α chain. In this study, we evaluated whether VRT101 also serves as a target for human lupus antibodies, upholding the hypothesis that VRT101 may serve as a potential target in the therapy of lupus.

Methods. Anti-VRT101 and anti-dsDNA reactivity were measured in the serum of lupus patients and compared with that of healthy individuals and patients with other rheumatic disorders. Statistical correlations between disease activity measured by the SLEDAI-2k scale and compatible serum anti-VRT101 and anti-dsDNA levels were defined. A VRT101-coupled sepharose column was assessed for its efficacy in removing serum anti-VRT101 antibody and its safety in extracorporeal apheresis in sheep.

Results. Anti-VRT101 and anti-dsDNA antibodies were significantly higher in SLE patients compared with patients with other rheumatic conditions. A high degree of correlation was detected between anti-VRT101 levels and the SLEDAI-2k activity in patients with SLE. Immunoabsorption of lupus patients’ sera on the VRT101–sepharose column removed most of the anti-VRT101 antibodies. The column was found to transfer effectively 3l of normal sheep plasma without significant removal of non-specific antibodies or other proteins.

Conclusions. Anti-VRT101 antibodies are abundantly detected in the serum of patients with SLE and correlate with disease activity. Specific removal of serum anti-VRT101 by extracorporeal plasmapheresis with specific immunoabsorption on the VRT101-coupled sepharose columns may serve as a new therapeutic tool for specific immunoadsorption of pathogenic antibodies in SLE patients.

Key words: SLE, Lupus nephritis, Laminin, Anti-DNA antibodies, Autoantibodies.

Introduction

Renal involvement in systemic lupus erythematosus (SLE) is a major determinant that affects prognosis and outcome [1]. Many pathogenic pathways have been implicated in the induction of glomerulonephritis in SLE; injury caused by deposition of DNA–anti-DNA immune complexes is commonly mentioned [2, 3]. Immune complex formation and deposition in renal glomeruli, blood vessels, skin, choroid plexus and other tissues probably initiate a virulent inflammatory process [2].

However, it is more commonly appreciated that the pathogenic process in SLE cannot be solely ascribed to the passive deposition of immune complexes or to DNA antibody binding. In spite of the fact that anti-DNA antibodies in SLE are important disease markers it seems that only a minority of them is nephritogenic [4]. Hylkema et al. [5] showed that Farr assay binding of lupus sera was reduced by 80% by prior extensive purification of anti-DNA antibodies with nucleosomes and histones.

Several other intracellular candidate molecules have been proposed as triggers of the renal inflammatory process exerted by binding of autoantibodies. α-Actinin, an actin-binding protein localized in glomerular podocytes and mesangium, is a possible target recently mentioned [6, 7]. Another molecule is α-enolase, a glycolytic enzyme, that has been proposed to be a major target of nephritogenic anti-DNA and non-anti-DNA antibodies [8].

An another mechanism that may lead to tissue damage in lupus is the cross reactivity of lupus autoantibodies with extracellular matrix (ECM) antigens. We have recently shown that one of the major antigens to which murine lupus autoantibodies bind, is the 1-laminin VRT101 component, abundantly found in the mesangial ECM [9, 10]. The titers of either anti-ECM or anti-laminin urinary antibodies in SLE patients were also found to correlate with SLE disease activity [10].

We have recently reported that the binding of lupus autoantibodies to ECM could be inhibited in vitro by laminin peptides. The laminin epitope recognized by the lupus antibodies was found to be a 21-mer peptide, designated VRT101, which is located at the globular part of the laminin-α1 chain [9].

In the current study, we extended our previous investigation of anti-laminin antibodies to the pathogenesis of human lupus nephritis. The VRT101 peptide was not only found to avidly bind human monoclonal anti-DNA antibodies but also to be highly reactive with sera antibodies of SLE patients. Furthermore, immunoadsorption of lupus sera via the VRT101-coupled column abolished most of the VRT101 activity without significant reduction of the total immunoglobulin levels. Specific immunoadsorption on the VRT101-coupled sepharose column may serve as a future therapeutic option for lupus patients.

Materials and methods

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]. The VRT101 peptide was prepared by standard solid-phase 9-fluorenlymethoxycarbonyl chemistry and analysed and purified by reverse-phase HPLC and tested by atom-bombardment mass spectrometry. The sequences of the various peptides were discussed previously [9].
Patients serum samples

Blood samples were collected from 95 SLE, 39 antiphospholipid syndrome (APS), 59 systemic sclerosis, 20 primary biliary cirrhosis, 20 myasthenia gravis patients and 157 healthy controls.

Informed consent was obtained from all enrollees according to the protocol that was approved by the institute's ethical committee.

Following blood coagulation the samples were centrifuged at a rate of 2500 r.p.m. and the sera were separated from the blood cells, aliquoted and stored at −70°C until use.

The SLE clinical disease activity was assessed by using the SLE disease activity scale-2000 (SLEDAI-2000) [14].

SLE patients monoclonal anti-DNA antibodies

Four human monoclonal IgG anti-DNA antibodies (mAbs), RH14, B3, D5 and DIL-6 were generated by using the human-human hybridoma technique from SLE patients and kindly provided by D. Isenberg (University College, London, UK) [15–17].

ELISA test for anti-VRT 101 levels

Polystyrene 96-well plates (Immunol II, Dynatech Laboratories, USA) were coated overnight at 4°C with 100 µl/well of 5 µg/ml of different laminin peptides, VRT101 in DDW, or R28, R30, R37, R18, R27, R26 and R35, in carbonate coating buffer (pH 9.6). Wells were washed three times with PBS-0.05% Tween-20 and blocked with 300 µl/well of 1% BSA in PBS for 60 min at room temperature. The plates were washed six times and subsequently incubated with either mAbs at a concentration of 5 µg/ml or with serum samples of the mentioned subjects diluted 1:100 with PBS-1% BSA-0.01% Tween-20 for 60 min at room temperature. The plates were rinsed six times with PBS-0.05% Tween-20 and incubated with alkaline phosphatase-conjugated anti-mouse or anti-human IgG Abs (Sigma) (1:2000 in PBS-1% BSA), for 1 h followed by rewashing and addition of 1 mg/ml N-[methyl-3H]imidazolephosphate (pNPP, Sigma), in 0.1 M glycine buffer (pH 9.6). Optical density (OD) was measured at 405 nm by an ELISA reader (Microwell System; Organon Teknika Turnhout, Belgium).

ELISA test for anti-dsDNA levels

Polystyrene plates (Immunol II, Dynatech Laboratories, USA) were coated overnight with 100 µl/PBS-poly-l-lysine (100 µg/ml) washed three times with distilled water and incubated with 100 µg/ml DNA (100 µg/ml) overnight. Wells were washed three times with PBS-0.05% Tween-20 and blocked with 300 µl/well of 1% BSA in PBS for 60 min at room temperature. The plates were subsequently incubated with the serum samples of the mentioned subjects (1:100 in PBS-1% BSA-0.01% Tween-20) for 60 min at room temperature, washed and incubated with alkaline phosphatase-conjugated anti-human IgG Abs and developed with pNPP as described previously.

Filter assay for anti-dsDNA levels

Serum antibody binding to dsDNA was also measured by using the Millipore (Bedford, MA, USA) filter assay [18]. Briefly, 5 µl of serum was diluted in 0.1 ml 0.2 M borate-saline buffer, pH 8.0. 14C-labelled DNA (10 µl, 2800 c.p.m) 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 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 less than 8%.

Specific immunoadsorption of antibodies on VRT101–sepharose columns (Lupusorb)

The VRT101 peptide (1 mg/ml) was conjugated to high performance N-hydroxysuccinimide-activated sepharose beads (Pharmacia, cat. #17-0717-01) according to the manufacturer’s instructions. The columns were prepared in GMP protocols by the Fresenius Hemocare Inc. (Seattle, WA, USA). Briefly, the VRT101 peptide was coupled by its mixture with the sepharose beads at 4 day overnight in 1 mM HCl buffer solution, afterwards the columns were blocked by 0.1 M of HCl–Tris (pH 8). The VRT101-coupled sepharose was washed twice by using glycine acetate Tris buffer 0.15M (pH 8.0) followed by HCl–Tris buffer 0.1M (pH 8), and this procedure was repeated six times. Latter measurements demonstrated that the coupling efficiency was 97%. The VRT101 column was termed the Lupusorb column.

Plasma samples were obtained from highly active SLE patients and were passed without further dilution through the VRT101 peptide–sepharose column, at a rate of 0.4 ml/1 ml sepharose/min, simulating the rate of the in vivo apheresis. The column was then eluted with PBS–HCl 0.01 M (pH 2.4) and the eluted samples neutralized with Tris–EDTA 2 M (pH 12). Binding of the serum before and after adsorption as well as the eluted fraction to VRT101 was measured by ELISA.

Extracorporeal apheresis using the Lupusorb column in sheep

A 50 ml Lupusorb column was connected to a Phresenius plasmapheresis machine and was pre-washed with 500 ml PBS. Sheep were anaesthesized, and connected via the jugular and leg veins to the inlet and outlet of the pheresis machine. Three litres of heparnized plasma were run at a rate of 20 ml/min for two and a half hours. At the end of the procedure the column was washed with 500 ml of PBS and a 5 ml sample from the column was eluted as previously described. The eluate was evaluated for the presence of sheep albumin and immunoglobulins.

Results

Binding of human lupus monoclonal Abs to laminin peptides

The laminin-α1 C-terminal VRT101 peptide was previously shown to bind to antibodies in the sera of lupus mice [9]. To analyse the interaction of pathogenic human lupus antibodies with the laminin molecule we tested the binding of anti-DNA mAbs generated from lupus patients, to peptides that compose the C-terminal of the laminin-α1 chain as well as to the R18 peptide, derived from the N-terminal of the molecule.

DIL-6, a human anti-DNA mAb, which was generated from a patient with active lupus nephritis, reacted selectively and intensely with the VRT101 peptide whereas the B3 mAb originating from a patient with SLE with arthritis had intermediate activity. The human lupus mAb’s D5 and RH 14 had negligible reactivity. None of these mAbs bound to other laminin peptides (Fig. 1).

Binding of sera to the VRT 101 peptide and to dsDNA

In order to assess whether the anti-VRT101 antibodies are specific to SLE patients we analysed the VRT101 binding capacity of 95 patients with active SLE by ELISA and compared their results with those measured in 157 healthy controls.

Sera derived from SLE patients had significantly higher titres of antibodies against the VRT101 peptide compared with healthy donors (Fig. 2). The average binding of SLE patients sera to VRT101 was 1.18 ± 0.06 (OD 405 nm; mean ± s.e) compared with 0.36 ± 0.03 in the healthy group (P = 0.0001). Anti-VRT101 antibodies were significantly higher in SLE patients compared with patients with the APS (P < 0.001). Patients with other
autoimmune disorders as well as normal healthy controls had significantly lower anti-VRT101 antibody binding compared with the SLE patients \((P < 0.0001)\). When the cut-off level for anti-VRT101 serum antibody was defined as the mean plus 2 s.d.s of the normal control levels, 57% of the SLE patients compared with 6% of the controls were found to be positive.

Serum activity level against anti-VRT101 was assessed in eight patients at different periods of their disease, at intervals lasting from 4 months to 2 years, when the degree of activity of their disease varied (Table 1). The SLE disease activity was quantified by the SLEDAI-2000 scale, based on the clinical records of the patients. By using a linear mixed model we assessed the degree by which anti-VRT101 predicts lupus activity levels. Such a correlation was indeed detected and it reached \(F(1,27.84) = 32.24\), leading to a partial correlation of 0.828 \((P < 0.001)\). An illustrative individual case is shown in Fig. 3; consecutive serum titres of anti-VRT101 samples of a single patient with lupus nephritis highly correlated with the disease activity throughout her disease flare and response to cyclophosphamide. Thus, the clinical activity correlation coefficient with the anti-VRT101 antibody was \(r = 0.96\), \((P < 0.0001)\) while corresponding levels of anti-DNA antibodies reached only \(r = 0.3\), \((P > 0.05)\). In this patient, whenever a flare was recorded, kidney involvement significantly contributed to the disease severity and to the SLEDAI-2000 scoring.

**Immunoadsorption of lupus patients’ sera on the VRT101–sepharose column**

The VRT101-conjugated sepharose was shown to be highly selective and reduced the anti-VRT101 serum binding more than 85% in the serum of four SLE patients (Fig. 4). This reduction is equivalent to a decrease in the anti-VRT101 antibody concentration of more than 95% according to the titration curve of serum anti-VRT101 binding (data not shown).

By using glycine acetate Tris buffer followed by HCl–Tris buffer we eluted the anti- VRT101 antibodies from the VRT101 sepharose column and found reconstitution of most of the anti-VRT101 antibody activity in the serum by ELISA.

### Table 1. Sequential analysis of anti-VRT101 titres of eight SLE patients during different phases of their disease

<table>
<thead>
<tr>
<th>No. of Clinical Events</th>
<th>SLEDAI-2000</th>
<th>Anti-VRT101 (OD 405 nm)</th>
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Disease activity is expressed by the SLEDAI-2000 scoring system. SLEDAI-2000, systemic lupus erythematosus activity index 2000; OD, optical density.
actually take part in the mediation of disease. Several studies in
been cast on whether anti-DNA antibodies and binding to DNA
expression and severity of lupus nephritis; however, doubt has
play a major role in its pathogenesis.

It is quite clear that the pathogenesis of lupus nephritis is complex
and multifactorial and cannot be entirely ascribed to the humoral
arm of the immune system; however it seems that autoantibodies
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expression and severity of lupus nephritis; however, doubt has
been cast on whether anti-DNA antibodies and binding to DNA
actually take part in the mediation of disease. Several studies in

About 54% of the anti-DNA antibodies activity was also removed
by the VRT101 column indicating that about half of the
anti-DNA antibodies activity cross-reacts with this laminin
peptide (Fig. 5).

Apheresis of sheep serum via the VRT101–sepharose column
To test the specificity and safety of the laminin-coupled column in
extracorporeal plasmapheresis, we have performed monthly
plasmapheresis in three sheep for two consecutive months.

Three litres of heparinized sheep plasma were easily passed on
a 50 ml VRT101 column at a rate of 20 ml/min. Apheresis was
performed once a month for 2 months in three sheep. Analysis of
the proteins eluted from the column revealed only immunoglobu-
lins and did not reveal any albumin, and the amount of
immunoglobulin absorbed on the column represented 0.015% of
the total globulin amount transferred on the column. Evaluation
of blood counts and liver functions also did not show significant
changes after apheresis (data not shown).

Discussion
It is quite clear that the pathogenesis of lupus nephritis is complex
and multifactorial and cannot be entirely ascribed to the humoral
arm of the immune system; however it seems that autoantibodies
play a major role in its pathogenesis.

It is widely known that anti-DNA titres are related to the
expression and severity of lupus nephritis; however, doubt has
been cast on whether anti-DNA antibodies and binding to DNA
actually take part in the mediation of disease. Several studies in

lupus animal models have shown that induction of nephritis
may be dissected from the coexistence of anti-DNA antibodies;
Christensen et al. [19] have shown that Toll-like receptor-
9-deficient MRL lupus mice lack anti-DNA antibodies but still
develop glomerulonephritis. Autoantibodies with diverse affin-
ities, to intracellular antigens other than DNA have also been
suggested to participate in the pathogenesis of lupus such as
vimentin, α-actinin [20, 21].

Recently, we have shown that one of the major components to
which lupus autoantibodies are directed to is the 1-laminin
component of the ECM [10]. Laminin has a major role in the
structure of the ECM of the glomerulus. Previous studies had
shown that the laminin molecule is essential for the proper
arrangement of a single cell epithelium layer adjacent to the
glomerular basement membrane and for the formation of the
convoluting structure of the glomerular capillaries [22].

Kootstra et al. [23] demonstrated that in the normal glomer-
ulus, laminin epitopes are present only in the mesangial matrix.
However, in pathological conditions such as chronic graft-vs-host
disease in mice, a murine model for lupus nephritis, laminin
molecules have also been detected in immune deposits within the
subepithelium and in cases of glomerulosclerosis also in the
subendothelium.

In a recent study, we showed that many lupus mAb anti-DNA
bind to the laminin peptide VRT101 [9]. Moreover, sera
originating from MRL/lpr/lpr and (NZB/NZW)F1 lupus-prone mice
intensely reacted with this peptide as their disease progressed.
Studying congenic strains carrying lupus susceptibility genes
(Sle1–Sle3) from the lupus-prone NZM2410 mouse on the
C57BL/6 background has indicated that anti-laminin antibodies
induce tissue damage independent of anti-DNA activity. Mice
carrying either one or two of the three lupus susceptibility genes
had high anti-DNA antibody titres, low anti-VRT101 reactivity
and minimal renal disease; however, mice carrying all three
susceptibility genes also developed along with high anti-DNA,
severe lupus nephritis accompanied by high anti-VRT101
antibodies. Interestingly, only monoclonal anti-DNA antibodies
that cross-reacted with the VRT101 epitope exerted glomerulone-
phritis in a the non-autoimmune RAG1(−/−) (B6.129.RAG1)–
deficient mouse model. Most importantly, treatment of MRL/lpr/
lpr mice with the VRT101 peptide prevented the development of
nephritis and extended animal longevity similar to therapy with
high daily doses of betamethasone [9]. These data imply that the
conjunction of anti-VRT101 to anti-DNA activity is highly
significant in the pathogenesis of the disease.

In the current study, we showed that human monoclonal
and polyclonal anti-DNA antibodies derived from SLE patients
cross-react with the VRT101 peptide. Significantly higher titres of
anti-VRT101 antibodies were found in SLE and APS patients
compared with healthy normal controls whereas patients with
systemic sclerosis, primary biliary cirrhosis and myasthenia gravis
had similar low binding levels. Anti-VRT101 serum activity was
also found to correlate with overall disease activity determined by
the SLEDAI-2000 index.

Adsorption of lupus sera to a VRT101-conjugated sepharose
gel column has demonstrated that while anti-VRT101 activity was
almost completely abolished, anti-DNA activity was only partially
affected. Some of the anti-VRT101 immunoadsorbed antibodies
eluted from the column also cross-reacted with DNA.

This concept naturally leads to an inevitable clinical application
advocating the removal of these pathological antibodies from the
peripheral blood. Several forms of immunoadsorptions have been
developed for autoimmune diseases. Basically, this modality can
be classified according to the type of antibody removal, non-
selective or selective. For instance, the Prosorba silica-based
system utilizes the non-selective binding of the Staphylococcus
aureus protein A to the Fc portion of the immunoglobulins in
order to remove them from the blood. This therapeutic modality
has been approved by the Food and Drug Administration for the
treatment of refractory rheumatoid arthritis and for resistant idiopathic thrombocytopenic purpura [24].

Selective immunoadsorption has been experimentally implemented in myasthenia gravis patients using a peptide originating from the human α2-subunit of the acetylcholine receptor. A study encompassing 22 randomly selected patients showed significant reduction of blocking antibodies with concomitant clinical improvement in more than half of these patients [25]. Another interesting experimental model in which selective removal of pathogenic antibodies was tried is the dilated-type cardiomyopathy. Autoantibodies against the β1-adrenergic receptor are present in 80% of the patients with idiopathic dilated cardiomyopathy and have been implicated in the pathogenesis of this disorder [26].

In SLE, reduction of anti-DNA levels has been achieved by removal of autoantibodies from the peripheral blood using the LJP394 molecule. This molecule is a selective B lymphocyte immunomodulator that consists of four double-stranded 20-mer oligodeoxynucleotides attached to an inert scaffold composed of a triethylene glycol core [27]. The administration of LJP394 to mice and humans resulted in reduction of serum dsDNA antibodies and dsDNA antibody-producing cells, probably by its binding to circulating antibodies and subsequent clearance of immune complexes [28, 29]. Interestingly, in most patients, treatment with LJP394 induced a significant reduction in anti-DNA levels and decreased the rate of renal flare, yet this agent was not shown to be effective in actual treatment of lupus nephritis. These results imply that non-anti-DNA antibodies may probably have a more significant role than previously acknowledged in the pathogenesis of the lupus nephritis [30].

As the VRT101 has been found to serve as an important target for lupus pathogenic antibodies, and has been shown to suppress nephritis in lupus mice we have prepared a VRT101-sepharose column in GMP conditions and tested its ability to remove anti-VRT101 antibodies. As can be seen in Fig. 5, one passage of lupus plasma on the column removed most of the anti-VRT101 antibodies. Fifty milliliter VRT101-sepharose columns were also used successfully for extracorporeal plasmapheresis in sheep and did not remove non-specifically significant amounts of other immunoglobulins.

In conclusion, anti-laminin antibodies may have a prominent role in the induction of lupus nephritis; although some of these antibodies cross-react with DNA, we have shown by immunoadsorption that the affinity of these antibodies to a laminin-derived peptide designated VRT101 is distinct and independent. These results set a profound basis for a clinical trial assessing the efficacy of extracorporeal-specific immunoadsorption of anti-laminin antibodies in patients with lupus nephritis.

Rheumatology key messages

- Patients with active SLE and particularly those with lupus nephritis have high titres of an anti-laminin peptide that correlate with disease activity.
- About half of the anti-VRT101 antibodies have no anti-DNA activity.

Y.N. is the chief scientist of Vertomedical Ltd.

References


