A Nephritogenic Peptide Induces Intermolecular Epitope Spreading on Collagen IV in Experimental Autoimmune Glomerulonephritis

Lanlin Chen,* Thomas Hellmark,† Vadim Pedchenko,‡ Billy G. Hudson,‡ Charles D. Pusey,§ Jay W. Fox,* and W. Kline Bolton*†

*Department of Medicine, Division of Nephrology, University of Virginia Health System, Charlottesville, Virginia; †Department of Nephrology, Lund University Hospital, Lund, Sweden; ‡Department of Medicine, Division of Nephrology, Vanderbilt University School of Medicine, Medical Center, Nashville, Tennessee; and §Department of Medicine Renal Section, Imperial College, London, Hammersmith Hospital, London, United Kingdom

This group previously identified a peptide p13 of α3(IV)NC1 domain of type IV collagen that induces experimental autoimmune glomerulonephritis (EAG) in rats with generation of antibodies to sites on α3(IV)NC1 external to the peptide as a result of intramolecular epitope spreading. It was hypothesized that intermolecular epitope spreading to other collagen IV chains also was induced. Rats were immunized with nephritogenic peptide that was derived from the amino terminal part of rat α3(IV)NC1 domain, and serum and kidney eluate were examined for antibodies to both native and recombinant NC1 domains of collagen IV. Peptide induced EAG with proteinuria and decreased renal function and glomerular basement membrane IgG deposits. Sera from these rats were examined by ELISA, which revealed reactivity not only to immunizing peptide but also to human and rat α3(IV)NC1 and to human α4(IV)NC1 domains. Kidney eluate that was depleted of α3(IV)NC1 antibodies still reacted to α4(IV)NC1, and α3(IV)NC1 column-bound antibody reacted with α3(IV)NC1. There was minimal reactivity to other collagen chains. Eluate that was adsorbed to NC1 hexamer from rat glomerular basement membrane lost all reactivity to glomerular constituents, and the eluted antibodies reacted to α3(IV)NC1 and α4(IV)NC1 domains. These studies show that a T cell epitope of α3(IV)NC1 induces EAG, intramolecular epitope spreading along α3(IV)NC1, and intermolecular epitope spreading to α4(IV)NC1 domain with minimal or no reactivity to other collagen chains or glomerular constituents. This is the first demonstration in EAG of intermolecular epitope spreading and identification of the spread epitopes.


Experimental autoimmune glomerulonephritis (EAG) in rats is a model of autoimmune Goodpasture’s disease in man and can be induced by crude glomerular basement membranes (GBM), purified α3(IV) noncollagenous (NC1) domains, and recombinant α3(IV)NC1 proteins (1–5). An amino terminal peptide of the rat α3(IV)NC1 induces EAG with antibodies deposited on the GBM in half of immunized animals (6,7). The peptide is a pure T cell epitope and does not induce cross-reactive antibody between the peptide and GBM (7,8). Nonetheless, in rats with GBM deposits, antibodies that are eluted from the kidneys react with GBM constituents, which occurs via epitope spreading (7–9).

Few studies have been performed on epitope spreading in glomerulonephritis. Evidence of determinant spreading was provided by Wu et al. (8), who demonstrated a T cell peptide–induced GBM antibody that recognized GBM antigens outside the immunizing epitope, although the identity of the determinants involved in the spreading was not delineated. We also showed that epitope spreading can be induced by a T cell epitope and that the nephritogenic antibody recognizes rat and human NC1 domains and, specifically, α3(IV)NC1 (7). To study epitope spreading further, it is necessary to identify the major epitopes that are involved in disease induction. Thereafter, mechanisms of epitope spreading and possible disease amplification, as described in other models, can be examined.

The purpose of these studies was to determine whether epitope spreading from the nephritogenic α3(IV)NC1 peptide extended to other collagen IV chains in the GBM (intermolecular spreading). Delineation of the involved protein epitopes and the pattern of epitope spread are important to understanding the pathogenesis and the progression of the autoimmune process. Other models of epitope spreading have demonstrated that interference within the cascade of organized spreading can have a modulatory effect on the autoimmune process (10–13). We show for the first time that intermolecular spread to α4(IV)NC1 domain occurs, that intramolecular and intermolecular spreading is limited to α3 and α4 among the six chains of collagen IV, and that other kidney antigens do not seem to be involved.
Materials and Methods

Preparation of Antigens and Antibodies

Recombinant human α1, α2, α4, α5, and α6(IV)NC1 domains were prepared as described previously in detail (5). Recombinant human α3(IV)NC1 likewise was prepared as described previously (3,14). Rat NC1 domain hexamers were isolated from kidney by column chromatography (15). Recombinant rat α3(IV)NC1 was prepared as originally described (2). In text and figures, α1 through α6 refers to recombinant α1 through α6(IV)NC1 domains. Column-purified rat NC1 is referred to as “rat NC1.” All of the α3(IV)NC1 constructs that were used in the studies have been shown to induce EAG in rats and were appropriately immunoblot positive (2,3,5). MAb 17 to α3(IV)NC1 and polyclonal antibody to the terminal 36AA of α3(IV)NC1 were used (3,4). Kidney-bound and circulating antibodies were obtained by acid elution and serial bleeds (7).

Immunosorption columns of rat α3(IV)NC1 domain and rat NC1 hexamers were prepared using cyanogen bromide−activated Sepharose 4B (Sigma-Aldrich, St. Louis, MO) (7). Column-bound and -unbound fractions were examined by ELISA assays as described previously (3,4,16). Electrophoresis and immunoblotting under reducing and non-reducing conditions were performed (3,4).

Experimental Animals, Immunizations, and Sample Preparation

Female WKY rats that were 4 to 6 wk of age were obtained from Harlan (Indianapolis, IN). The protocol was approved by the Animal Care and Use Committee and adhered to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were immunized with 300 μg of p13 peptide (SQTTANPSCPEGT), corresponding to amino acids 14 to 26 from rat α3(IV)NC1 domain, in PBS and complete Freund’s adjuvant (7). Rats were bled weekly (50 to 100 μl) for ELISA and killed at 9 wk with harvest of their kidneys for immunofluorescence and histologic studies and glomerular elutions (1,7). Kidneys with IgG on the GBM were eluted by the glycine method as described previously (7).

Statistical Analyses

Data are expressed as mean ± SEM. Statistical differences between groups were evaluated by the t test (7).

Results

Rats selected for this study had antibody bound to their GBM by immunofluorescence analysis. A previous study showed that rats that lack GBM-bound IgG have no circulating antibody to GBM or antibody activity from eluates to GBM by ELISA or by indirect immunofluorescence (6,7). All rats had abnormal proteinuria and proliferative glomerulonephritis (1,3,7).

Serum Antibody Studies

Previous studies showed that antibody specific to the p13 immunogen did not recognize GBM antigens (7,8). In these studies, immunization with p13 peptide induced a robust antibody response to p13 in the serum of immunized rats as well as lower levels of antibody to rat and human α3(IV)NC1 domain (Figure 1A). A significant increase in serum antibody to α3(IV)NC1 and α4(IV)NC1 domains was evident by 4 wk after immunization with p13 peptide (Figure 1B). Responses to α3(IV)NC1 peaked at 6 to 7 wk as previously reported (17).

Antibody to α4(IV)NC1 was more blunted but was statistically significant by week 4 and diminished to nonsignificant levels by week 7.

Kidney Eluate Antibody Studies

Antibodies that were eluted from kidneys of nephritic rats bound to rat and human α3(IV)NC1 and rat NC1 GBM hexamer by ELISA (Figure 2A). After immunosorption of eluate on immobilized rat α3(IV)NC1, the unbound fraction did not bind to either rat or human α3(IV)NC1, demonstrating complete adsorption of any cross-reactive antibodies between human and rat α3(IV) species. This fraction, however, continued to react with rat NC1 domain. The column-adherent anti-rat α3(IV)NC1 reacted strongly to rat as well as human α3(IV)NC1. Because native GBM NC1 hexamer is composed of all of the chains of type IV collagen, except α6(IV)NC1, individual recombinant α(IV)NC1 domains were tested in ELISA versus total kidney elute and fractions bound and unbound to rat α3(IV)NC1 column. As shown in Figure 2B, kidney eluate demonstrated low levels of binding to α2, α3, and α6(IV)NC1 with high levels versus α4(IV)NC1, even though serum antibody to α4(IV)NC1 was low (Figure 1). Unbound flow-through from the rat α3(IV)NC1 immunosorbent column continued to show reactivity to α4(IV)NC1 but was much diminished versus other recombinant chains. Antibody that was eluted from the
rat H9251 3(IV)NC1 immunosorbent column demonstrated strong reactivity to H9251 3(IV)NC1, as expected, with minimal reactivity with other chains. High homology and antibody cross-reactivity have been shown for the various chains of a3 to a4(IV)NC1 (5,18). Kidney eluate that was reactive with rat a3(IV)NC1, rat NC1, and human a3(IV)NC1 and a4(IV)NC1 lost all reactivity after passage over the rat NC1 immunosorbent column (Figure 2C), whereas the antibody that was eluted from the column continued to recognize those proteins. Antibodies that were eluted from nephritic kidneys recognized mainly conformational epitopes on a3 and a4(IV)NC1 domains (Figure 2D), as demonstrated by abolition of immunoblot reactivity under reducing conditions.

**Binding of Kidney Eluate and Fractions to Normal Rat and Human Kidney**

MAb and polyclonal antibodies that are specific to rat and human a3(IV)NC1 and a4(IV)NC1, respectively, co-localize in rats to the GBM and all tubular basement membranes (TBM) (19–22). By contrast, in the human kidney, a3(IV)NC1 and a4(IV)NC1 co-localize to the GBM and a very limited number of distal TBM (20,23,24). By indirect immunofluorescence, kidney eluate stained rat and human GBM and TBM (Figure 3, A and E) in the characteristic patterns described for rat and human a3(IV)NC1 and a4(IV)NC1 chain distribution, respectively. Rat a3(IV)NC1 column-unbound flow-through (Figure 3, B and F), reactive in ELISA versus a4(IV)NC1 but not a3(IV)NC1, stained rat and human kidney, respectively, in the pattern described for a3/a4(IV)NC1. Eluted rat a3(IV)NC1 column-bound antibody, strongly positive for a3(IV)NC1 but not other NC1 domains, fixed to rat and human kidney in the characteristic a3(IV)NC1 pattern (Figure 3, C and G). Background tubular cytoplasmic staining was present for all rat indirect immunofluorescence, including normal rat serum controls (Figure 3D). Rat NC1 column-unbound kidney eluate was negative versus both human and rat kidney sections, consistent with the ELISA (Figure 2C).

**Discussion**

Epitope spreading from a determinant on one molecule to other determinants on the same molecule (intramolecular spread) and to other molecules (intermolecular spread) has been described in many models of autoimmunity and in human autoimmune diseases (25–33). This spread follows a hierarchical, predictable pattern and consists of T cell, B cell, and T/B cell spread (12,34). It is accepted that spread of autoimmunity is

\[ a3(IV)NC1 \]

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consequent to tissue damage and \( T_{H1} \)-cytokine–mediated damage and recruitment of additional T cells, resulting in amplification of the injurious autoimmune process (35,36). Interruption of the patterned spread or induction of a regulatory \( T_{H2} \) cytokine milieu downregulates disease expression (11,12). Although many antibodies may be induced during epitope spreading, these may have no evident ability to induce or augment disease. We do not know whether the antibodies to \( \alpha4(IV)NC1 \) are injurious in EAG. Human \( \alpha4(IV)NC1 \) recombinant protein induces EAG, but it is not known whether this is \( \alpha \) T cell–mediated damage, antibody injury, or both. These studies were not directed toward examining a pathogenic role of antibody to \( \alpha4(IV)NC1 \), only to identifying other constituents of GBM that are involved in epitope spreading in EAG to provide a basis for additional studies. Future studies will be needed to examine any pathogenic role of these antibodies. Conceivably, they could be involved in exposing additional cryptic epitopes as part of the amplification process. Recent studies in the NOD diabetic mouse model demonstrated the pivotal importance of B cells in antigen presentation, essential for \( T_{H1} \)-mediated epitope spreading with augmented tissue damage, but also for \( T_{H2} \)-mediated spreading with marked downregulation of disease expression (37). We do not know the mechanisms of downregulation of antibody to \( \alpha3(IV)NC1 \) in EAG previously described or for downregulation of anti-\( \alpha4(IV)NC1 \) shown here. Studies are in progress to understand this observation further.

Studies of epitope spreading in pathogenesis and intervention require first the identification of the epitopes involved. The purpose of our studies was to identify further the epitopes that are involved in spreading in the EAG rat model. Our results demonstrate for the first time that only collagen (IV) chains are involved in antibody epitope spreading in EAG. The antibody formation could develop only \( \alpha \) via epitope spreading, rather than cross-reactive antibodies, because the rats were immunized only with the 13 AA p13, which elicits antibody to itself but not to GBM. Moreover, epitope spreading is restricted to NC1 domains of collagen because adsorption of kidney eluate by rat NC1 completely removed anti-kidney antibody activity. The ELISA studies further showed that the spread was predominantly, if not only, to the \( \alpha3(IV) \) and \( \alpha4(IV) \) NC1 domains. Although there was slight antibody reactivity to other chains, this likely occurred by cross-reactivity because the NC1 domains are highly homologous and antibody cross-reactivity has been noted by others (5,18). The only significant antibody response in our studies was to the \( \alpha3 \) and \( \alpha4(IV) \) NC1 domains. This is consistent with our own work as well as that of others, which showed that native basement membrane that contained \( \alpha1 \) and \( \alpha2(IV)NC1 \) did not induce disease in this model; neither do \( \alpha1, \alpha2, \alpha5, \) and \( \alpha6 \) recombinant NC1 domains (5,38). However, both peptides and recombinant \( \alpha3 \) and \( \alpha4(IV) \) NC1 domains induce EAG in rats (3,6,7,19,39). Dissection of NC1 spread domains for \( \alpha3 \) and \( \alpha4 \) is complicated by their colocalization in the kidney (19–24). Although the patterns are different in humans and rats, the distribution of each is the same within their respective kidney. However, the column immunosorption studies clearly showed that \( \alpha3(IV) \) NC1 column-unbound fraction that lacked antibody reactivity to \( \alpha3(IV) \) NC1 by ELISA still strongly stained kidney in the characteristic \( \alpha3/\alpha4(IV) \) NC1 pattern, and the \( \alpha3(IV) \) NC1-specific antibody, which lacked reactivity with \( \alpha4(IV) \) NC1, also stained in the characteristic pattern, providing strong evidence that epitope spreading from the T cell peptide occurred to both \( \alpha3(IV) \) NC1 and \( \alpha4(IV) \) NC1 but not other NC1 domains. Although other, non-NC1 proteins could be involved, the abrogation of anti-kidney reactivity by the rat GBM NC1 hexamer affinity column and the ability to induce EAG with only \( \alpha3(IV) \) NC1 and \( \alpha4(IV) \) NC1 suggest that they are the dominant, if not the only, glomerular proteins involved.

The studies reported pertain to the antibody spread epitopes in EAG and not the T cell epitopes. However, their identification now allows assessment of these two NC1 domains for other T cell epitopes involved. Elaboration of the specific and limited B and T cell epitopes should provide a framework for future studies to define the chronological sequence and hierarchical dominance of the nephritogenic antigens. Their identification also should provide a better understanding of patho-
genic mechanisms and interventions that might be used to prevent, ameliorate, or treat EAG in rats. Oral GBM and nasal recombinant rat α3(IV)NC1 both prevent development of EAG in this model (40,41). Identification of specific peptides that are involved with regulatory T cell recruitment could allow targeted specific intervention. Information that is derived from the rat model should provide valuable insight into the pathogenesis of Goodpasture’s disease in humans and hopefully illuminate pathways for therapeutic intervention.

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