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# Immunodominant epitopes of α3(IV)NC1 induce autoimmune glomerulonephritis in rats

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**Immunodominant epitopes of α3(IV)NC1 induce autoimmune glomerulonephritis in rats.**

*Background.* The major Goodpasture antibody binding epitopes have been localized to the amino-terminal third of the noncollagenous domain (NC1) of the α3 chain of type IV collagen [α3(IV)NC1]. The present study determined whether the same epitopes induce glomerulonephritis in rats.

*Methods.* We immunized Wistar Kyoto (WKY) rats with human α3(IV)/α1(IV)NC1 chimeric proteins or full-length recombinant α3(IV)NC1 ($\alpha3_{732}$). Chimeric protein constructs were thirds of α3(IV)NC1 (CP333) replaced by corresponding sequences of homologous nonreactive α1(IV)NC1 (CP111). All chimeric proteins contained 30 amino acids of type X collagen at the amino terminus except $\alpha3_{732}$. Two other constructs, T195 EA (EA) and T194 EB (EB), were entirely α1(IV)NC1, except for antibody-immunodominant amino acids from the first and second thirds of α3(IV)NC1.

*Results.* Construct immunized animals developed specific antibody responses to recombinant proteins and native human, bovine and rat NC1. CP311 immunized rats, as well as $\alpha3_{732}$ rats, had glomerular IgG, fibrin, and glomerulonephritis with proteinuria by 3 weeks. CP331 produced more severe disease, comparable to positive controls. CP111 produced no disease. EA, but not EB, induced severe glomerulonephritis. Half-dose each of EA plus EB induced disease identical to full-dose EA alone.

*Conclusion.* The amino third of α3(IV)NC1 which contains the major epitope for Goodpasture antibody binding, also induces glomerulonephritis in rats. The middle third of α3(IV)NC1 does not induce glomerulonephritis but appears to enhance disease with the amino terminal third. Finally, the presence of the collagen X leader sequence appears to convey greater nephritogenicity. These studies suggest that not only the nephritogenic epitope itself, but flanking sequences and the conformational context of the nephritogenic epitope may influence its ability to cause glomerulonephritis.

Goodpasture syndrome is an autoimmune disease characterized by rapidly progressive glomerulonephritis, lung hemorrhage, and linear deposits of IgG along the glomerular basement membrane (GBM) [1]. The disease is mediated by autoantibodies to type IV collagen in the GBM and lung alveoli [1]. The primary target for these antibodies has been identified as the noncollagenous domain (NC1) of the α3 chain of col IV [α3(IV)NC1] [2–4]. The α3 chain, one of six genetically distinct α chains (α1 to α6) [4], is present only in certain specialized organ basement membranes, including kidney and lung [5].

All of the α chains of collagen IV have similar structures: a short noncollagenous amino terminal domain, 7S; a long central triple helical collagenous domain; and a carboxyl terminal NC1 domain [4]. The NC1 domains of all α chains show high degrees of amino acid sequence homology [6–8]. In addition, there is a highly conserved pattern of 12 cysteine residues that form six intrachain disulfide bonds in NC1. These disulfide bonds enable the NC1 domain to be properly folded into a three-dimensional globular structure [9]. Denaturation of NC1 by reducing the disulfide bonds causes loss of reactivity to autoantibodies, suggesting that the Goodpasture epitope is conformational. This requirement for intact tertiary structure for Goodpasture antibody binding is well recognized [10, 11].

Over the past two decades, extensive efforts have focused on identifying the Goodpasture epitope. Work using linear synthetic peptides as well as using recombinant proteins expressed in bacterial systems have been fraught with problems and limitations in characterizing these conformational epitopes [12–15]. Subsequent work from several groups using recombinant α3(IV)NC1 expressed in mammalian cells to preserve the three-dimensional structure of the Goodpasture epitope have all demonstrated the critical role of the amino terminal portion of α3(IV)NC1 for Goodpasture epitope binding [16–21]. Recent studies mapping the Goodpasture epitope have used chimeric molecules of human α3(IV)NC1 and α1(IV)NC1. With this approach, parts of the α3(IV)NC1 were replaced by the corresponding sequences of homologous but nonreactive α1(IV)NC1 [16, 19]. Using this strategy, Hellmark, et al [16] and Borza et al [21] have

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further defined the amino terminal third of α3(IV)NC1 as the predominant binding site for Goodpasture antibodies. Hellmark et al [16] showed that all of the sera from 77 Goodpasture patients bind to this region, and demonstrated correlation with progression of Goodpasture disease [16]. Further mutational analyses have demonstrated a limited number of critical amino acids in the amino terminal end of α3(IV)NC1 relative to Goodpasture sera binding [20–23].

Previous studies have been performed in vitro to map antibody binding with various epitopes. None have demonstrated that these epitopes not only bind Goodpasture autoantibody, but also are capable of inducing disease. The goal of the present study was to determine whether the α3(IV)NC1 domain, particularly the amino terminal portion of this domain (i.e., the antibody reactive epitopes [16]) also contains epitopes causing nephritis. As this cannot be studied in humans, we have utilized a model of experimental autoimmune glomerulonephritis (EAG) in rats. This model, induced by native or recombinant GBM antigens, has been shown to result in severe proliferative glomerulonephritis with crescents, hematuria, proteinuria, and uremia; therefore, it mimics the organ-specific form of human Goodpasture syndrome [24–26]. Similar models have been described in rabbits [27] and mice [28, 29]. In this study, the potential capacity of different portions of the α3(IV)NC1 domain to induce EAG in rats was determined by using mammalian recombinant α3(IV)NC1 and α3(IV)NC1/α1(IV)NC1 chimeric proteins as immunogens. The results demonstrated that the α3(IV)NC1 domain, and specifically the amino terminal portion of α3(IV)NC1, induced EAG in rats. Furthermore, the presence of the middle portion together with the amino terminal portion of α3NC1 enhanced EAG production relative to other constructs. The results suggest the conformational context of the nephritogenic epitope influences its ability to cause EAG, and that specific amino acids not only influence autoantibody binding, but disease induction as well. Finally, adjacent nonnephritogenic regions of the construct seem to influence the degree of EAG induced by the nephritogenic epitope.

## METHODS

### Experimental animals

Hypersusceptible normal male Wistar Kyoto (WKY) rats, weighing 200 g, were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals were maintained in standard conditions in the Department of Comparative Medicine for several days of acclimatization before experiments.

### Antibodies

Human autoantibodies were from serum of patients with Goodpasture syndrome. Monoclonal antibodies specifically against the α3(IV)NC1 domain (Mab17)[30], and against 6× histidine (6× his) epitope (anti-his.G) (Qiagen, Valencia, CA, USA) were used. Rat sera were from animals immunized with recombinant proteins and native GBM. Horseradish peroxidase (HRP)-conjugated antibodies and fluorescein isothiocyonate (FITC)-conjugated goat antirat IgG and fibrinogen were purchased from ICN/Cappel (Costa Mesa, CA, USA).

### Preparation of bovine GBM

Bovine kidneys from a local abattoir were frozen at −70°C until used. Glomeruli were isolated from homogenized cortical tissue by differential sieving as previously described [25, 31]. To prepare GBM for immunization, whole glomeruli were sonicated on ice for 30 seconds to obtain disrupted GBM. Portions of GBM were then digested with type VII collagenase (Sigma Chemical Co., St. Louis, MO, USA), and the resultant solubilized GBM (csGBM) was lyophilized and used as immunizing antigen.

### Recombinant human α3(IV)NC1 and chimeric α 3/α1(IV)NC1

The other immunizing antigens used in this study were recombinant human α3(IV)NC1 and its chimeras [16]. These included one full-length α3(IV)NC1 domain ($\alpha 3_{732}$) and three different α3 /α1 chimeric NC1 domains. The strategy and method of constructing the α3/α1 chimeric proteins has been described previously in detail [16]. Briefly, chimeric proteins were constructed by replacing thirds of the α3NC1 domain with the corresponding sequence of the human α1(IV)NC1 domain using polymerase chain reaction (PCR) methods. The different chimeric proteins were obtained and named according to the origin of the NC1 domain. The chimeric proteins used in this study were as following: CP111 is the recombinant α1(IV)NC1 domain with four added restriction sites (*Bam*HI, *Hin*dII, *Xba*I, and *Not*I), which divided α1(IV)NC1 into three regions (Fig. 1A); CP311 contains one-third amino terminal portion of the α3(IV)NC1 with the rest of the α1(IV)NC1 domain; CP331 consists of two-thirds amino terminal portion of α3(IV)NC1 domain and one-third carboxyl terminal portion of the α1(IV)NC1 domain. All of the chimeric cDNAs were inserted into the expression vector, pCEP4-BM40-HisEK, which is based on the pCEP4 vector (Invitrogen, Carlsbad, CA, USA), and sequenced by ABI310 automated sequencer (Perkin-Elmer, Boston, MA, USA). The constructs were expressed in HEK-293 cells. The secreted proteins contained a BM40 signal peptide followed by a 6× histidine (6 his) tag and a 30 amino acid length of type X collagen and the NC1 domain from collagen IV (Fig. 1A). All of the constructs were tested for translation of protein with the correct molecular weight (~31 kD), using an in vitro system (Promega, Madison, WI, USA) with $S^{35}$-labeled

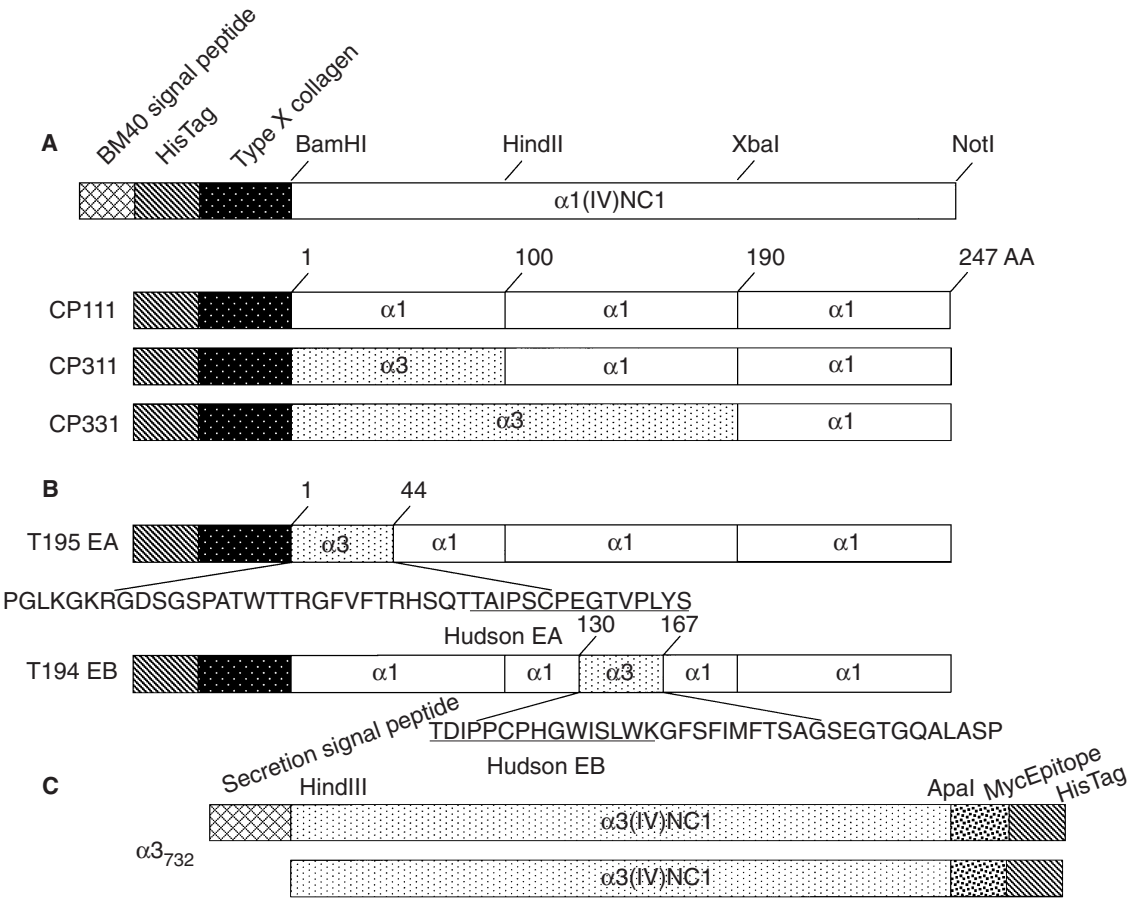


**Fig. 1. Schematic representation of cloning strategy for constructing human α3/α1 chimeric proteins [16, 23] and full-length human α3(IV)NC1 domain.** (*A*) The chimeric constructs consist of a 6× His tag, a type X collagen triple helix (30 amino acids) and an α1 type IV collagen NC1 domain cloned into the mammalian expression vectors (pCEP4-BM40-HisEK). Three different chimeric constructs, CP111, CP311, CP331, named according to the origin of the respective portions of the NC1 domain, are shown here. The *Bam*HI, *Hin*dII, *Xba*I, and *Not*I cleavage sites were introduced into the α1(IV)NC1 cDNA, and then homologous portions of the α3(IV)NC1 were exchanged. (*B*) Constructs EA and EB as described in text. Amino acids of α3(IV)NC1 on the backbone of α1(IV)NC1 with antibody-immunodominant sites underlined [20, 21]. (*C*) The full-length α3(IV)NC1 domain cloned into the expression vector (pSecTagA) was expressed as the fusion protein with a myc epitope and a 6× His tag at the carboxyl terminus ($\alpha3_{732}$). The amino acid sequence for the α3(IV)NC1 of the chimeric constructs at the type X collagen/type IV collagen junction is type X collagen/DPGLKG. The amino acid of $\alpha3_{732}$ at the amino terminus starts as SLGLKG. The signal peptides in (A) and (B) are cleaved off in the cells.

cysteine and T7 RNA polymerase. HEK-293 cells used for cDNA expression were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) (Gibco BRL, Rockville, MD, USA) in the presence the selection reagent (G-418) Gibco BRL). During harvesting, the cells were kept in FCS-free DMEM/F-12, and culture medium was collected and purified by ProBind resin (Invitrogen).

Cultures of CP131 and CP113 did not produce sufficient quantities of protein to allow isolation of the construct and immunization of rats. Therefore, new constructs were developed to encompass both of the antibody binding sites described by Hudson and Colleagues [20, 21]. To construct the two new chimeric proteins, we used the extension PCR technique [22] (Fig. 1B). Construct T195 EA (EA) consists entirely of α1(IV)NC1 domain except for 45 amino acids of α3(IV)NC1. The Hudson EA site is underlined. Likewise, T194 EB (EB) consists entirely of α1(IV)NC1 with 37 amino acids of α3(IV)NC1 containing the Hudson EB site, underlined. Both constructs contain the BM40 signal peptide followed by a 6× his tag + EK site and a short length of type X collagen as described above.

$\alpha3_{732}$ is the full-length α3(IV)NC1 domain, without the collagen X leader (Fig. 1C). The original cDNA encoding the α3(IV)NC1 domain was a gift from A. Michael/M. Kleppel. The 5′ end was extended by PCR amplification to include the region coding the junction between the triple helix and the NC1 domain (GLKGKRGDS...). The construct was then subcloned into a mammalian expression vector (pSecTagA) (Invitrogen) and expressed in the HEK-293 cell system. The secreted protein was fused with a myc epitope and 6× his tag at the carboxyl terminus. The cells were cultured and harvested as described above except zeocin (Invitrogen) was used as the selection reagent. The proteins from culture medium

were purified by affinity chromatography using Ni-NTA agarose columns (Qiagen). The protein was sequenced by Edman degradation method and by mass spectrometry [abstract; Bolton WK, et al, *J Am Soc Nephrol* 10:543A, 1999], and the expected size is ~29 kD. All the recombinant proteins were quantified by bicinchoninic acid (BCA) protein assay and by measuring the absorbance at 280 nm.

### Immunizations

The immunizing antigens were suspended in 0.1 mol/L acetic acid and emulsified with an equal amount of complete Freund's adjuvant (CFA $H_{37}Ra$) (Sigma Chemical Co) and given as a single injection in the left hind footpad and subcutaneously. Animals were divided into groups to receive immunization with various recombinant proteins and native GBM. The amount of protein injected into each animal (100 μg/rat) was kept constant for all groups. Group I rats were immunized with CP111 ($N = 6$), group II rats with CP311 ($N = 6$), group III rats with CP331 ($N = 6$), group IV rats with $\alpha 3_{732}$ ($N = 6$), group V rats with bovine csGBM ($N = 9$), group VI rats with EA ($N = 6$), group VII with EB ($N = 6$), and group VIII with 50 μg EA plus 50 μg EB ($N = 5$) (EA + EB). Control animals ($N = 3$) received phosphate buffered saline (PBS) emulsified in an equal volume of CFA.

### Urine analysis, serum creatinine, and urea nitrogen assays

Animals were placed in metabolic cages and 24-hour urine samples were collected weekly following immunization for a total of 7 weeks. Total urinary protein (TUP) was determined using 3% sulfosalicylic acid with bovine serum albumin (BSA) as a standard [25]. The upper limit of normal for 24-hour TUP in rats is 10 mg. Urine was also examined for hematuria (0 to 3+) using reagent strips (Multistix 10 SG) (Bayer Corporation, Elkhart, IN, USA). To measure serum creatinine and urea nitrogen, blood samples obtained via the tail vein from each rat every 2 weeks following immunization were examined using diagnostic kits from Sigma Chemical Co. for creatinine (Procedure No. 555) and urea nitrogen (Procedure No. 640).

### Immunofluorescence studies and histologic examination

Kidney tissue was obtained at death from rats that died of uremia, or at sacrifice, 8 to 12 weeks after immunization. Tissue was snap-frozen in isopentane (2-methylbutane) (Fisher, Newark, DE, USA) on dry ice. For direct immunofluorescence staining of rat IgG and fibrinogen, 4 μm cryostat sections of kidney were stained with FITC-conjugated goat antirat IgG and fibrinogen. The intensity of immune deposits was semiquantitatively graded from 0 to 4+. For light microscopy, kidney tissue was fixed in 10% buffered formalin, dehydrated in alcohol, and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) and examined. Histologic scores were assigned in a masked fashion, using a 4-point scale [32].

### Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12.5% gels, under nonreducing conditions. For immunoblotting, the proteins separated on SDS-PAGE were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blotted membranes were blocked with 5% dry milk in 0.1% PBS-Tween 20 and washed with PBS-Tween 20. The membranes were then incubated for 2 hours with primary antibodies diluted in PBS-Tween 20. Upon incubation, the membranes were washed thoroughly with PBS-Tween 20, followed by 1-hour incubation with HRP-conjugated secondary antibodies. After washing four to five times with PBS-Tween 20, the immunoreactive proteins were identified using chemiluminescence (Pierce, Rockford, IL, USA).

### Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Immulon II) (Dynatech, Chantilly, VA, USA) were coated with 200 ng of antigen in 200 μL of coating buffer. The plates were incubated overnight at room temperature. After washing three to four times with PBS-Tween 20, the plates were blocked with 1.5% BSA/PBS-Tween 20 for 1 hour at 37°C. The plates were washed again with PBS-Tween 20 and incubated with the various rat sera diluted in 1.5%BSA/PBS-Tween 20 for 2 hours at 37°C. The plates were washed again and incubated 1 hour at 37°C with the HRP conjugated secondary antibody (antirat IgG) at 1:5000 dilution in PBS-Tween 20. The color was developed using the ABTS substrate (2,2′azino-di-(3-ethylbenzthiozoline sulfonic acid)) (Sigma Chemical Co.). The absorbance for each well was read at 405 nm using Multiscan ELISA plate reader (MTX Lab Systems, Flow Laboratories, McLean, VA, USA). All assays were run in duplicate.

### Statistical analyses

Data are expressed as the mean ± SE. Statistical differences between groups were evaluated by the Student *t* test and analysis of variance (AVOVA) [31] using the S-Plus program(Insightful Corporation, Seattle, WA, USA). The presence or absence of the 6 his tag and the collagen X leader protein, both different from native csGBM, could influence immune responses [33]. Therefore, comparisons of the first with the second part of α3(IV)NC1 were made only for the nephritogenic constructs containing the same structure (i.e., thirds of α3(IV)NC1 and α1(IV)NC1, collagen X, and the 6 his tag).

## RESULTS

### Recombinant α3(IV)NC1 and chimeric α3/α1(IV)NC1 domain

The immunizing antigens used in this study included a full-length human recombinant α3(IV)NC1 domain ($\alpha3_{732}$), chimeric α3/α1(IV)NC1 constructs (CP111, CP311, CP331, EA, and EB), as well as native bovine GBM. All of the recombinant proteins were secreted into the culture medium as the fusion proteins containing the 6× his tag [16], and purified by affinity chromatography on $Ni^{+}$ column. The proteins were quantified and examined by Western blot before injecting equal quantities into animals. As shown in Figure 2, all the recombinant proteins were recognized by the anti-histidine antibody (anti-His.G), with CP111, CP311, CP331, EA, and EB at the predicted molecular weight of approximately 31 kD, and $\alpha3_{732}$ of 29 kD. Mab17, a monoclonal antibody specific for α3(IV)NC1, recognized $\alpha3_{732}$ as well as CP331, which contains the two thirds amino terminal portion of α3(IV)NC1 (Fig. 2A) [30]. Mab17 did not recognize CP311, which contains the one third most proximal amino terminal portion of α3(IV)NC1, nor EA or EB. The Mab 17 antibody epitope is known to be conformational and requires both amino acids 17–31 (first third) and 127–141 (second third) of α3(IV)NC1 to bind [20]. Mab17 also recognized native GBM, but not CP111, as expected. In addition, serum from a patient with Goodpasture disease (Fig. 2B and C) reacted with all the recombinant proteins except CP111 and EB. Reactivity of the Goodpasture serum with CP311, CP331 and $\alpha3_{732}$ was markedly reduced or abrogated after reduction of the antigen demonstrating reactivity with nonlinear, conformational epitopes.

### Reactivity of rat serum with recombinant and native NC1 domain

Sera from animals were analyzed by ELISA for antibodies against various constructs, as well as native NC1 domains from human, bovine, and rat GBM (Fig. 3). The extent of response correlated well with the type of construct used for immunization. Rats immunized with CP111, an α1NC1 construct, showed a strong antibody response against α3/α1(IV)NC1 chimeras with a higher absorbance for CP311 than CP331, but no reactivity for $\alpha3_{732}$. This reflects a greater number of B-cell epitopes

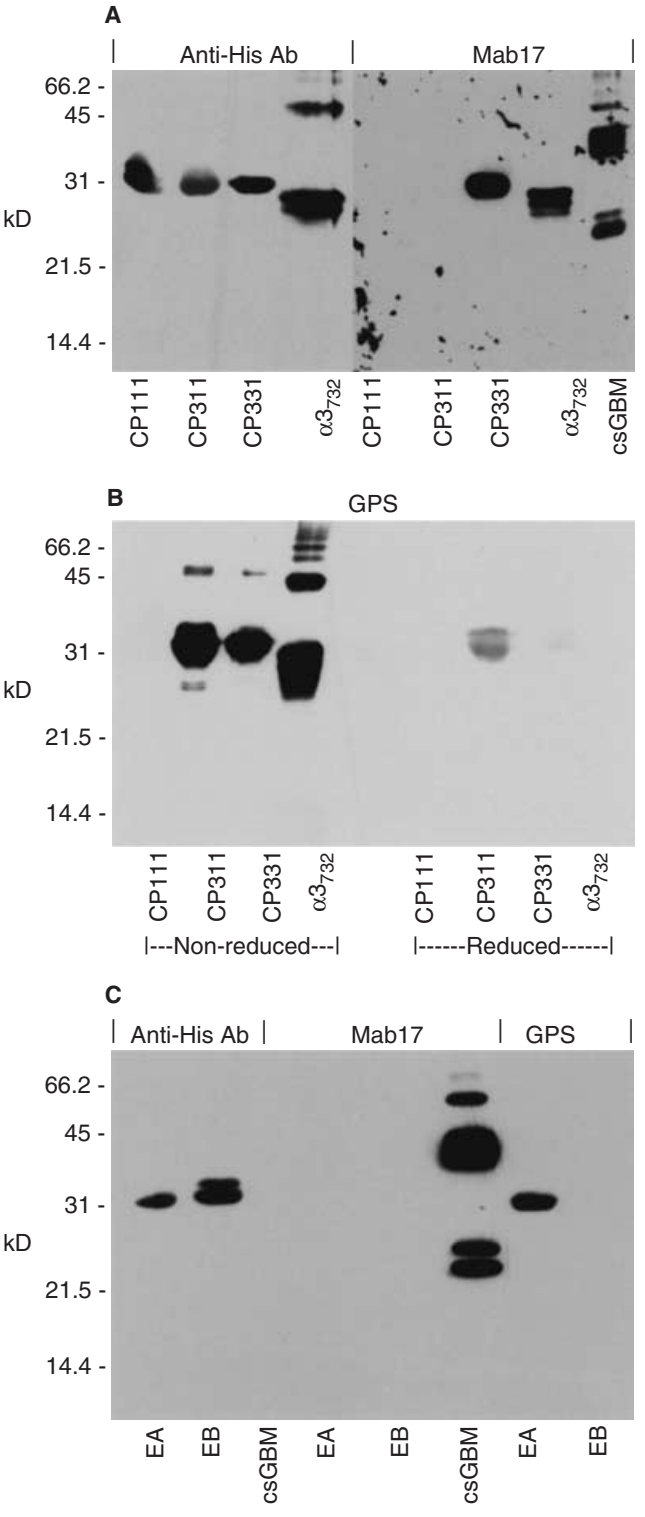


**Fig. 2. Immunoblotting of different constructs with anti-His antibody (lanes 1 to 4), and Mab 17 (lanes 5 to 9), and with Goodpasture serum from a patient under nonreduced and reduced conditions.** Mab17 is specific for the conformational structure formed by approximation of sites in the amino third of α3(IV)NC1 (amino acids 17–31) with the middle third of α3(IV)NC1 (amino acids 127–141)[20]. (*A*) Lanes 1 and 5 contain CP111, lanes 2 and 6 contain CP311, lanes 3 and 7 contain CP331, lanes 4 and 8 contain $\alpha3_{732}$, and lane 9 contains native glomerular basement membrane (GBM) (bovine). Lanes 1 to 4 were blotted with anti-His antibody and lanes 5 to 9 with Mab 17. The upper band in lane 9 is the native α3(IV)NC1 dimer. (*B*) Lanes 1 and 6 contain CP111, lanes 2 and 7 contain CP311, lanes 3 and 8 contain CP331, and lanes 4 and 9 contain $\alpha3_{732}$. Lane 5 is empty. Lanes 1 to 4 were blotted under nonreduced and lanes 6 to 9 under reduced conditions. (*C*) Lanes 1, 4, and 7 contain EA, lanes 2, 5, and 8 contain EB, lanes 3 and 6 contain csGBM. Lanes 1 to 3 were blotted with anti-His, lanes 4 to 6 with Mab 17, and lanes 7 and 8 with Goodpasture serum. Blotted under nonreduced conditions.

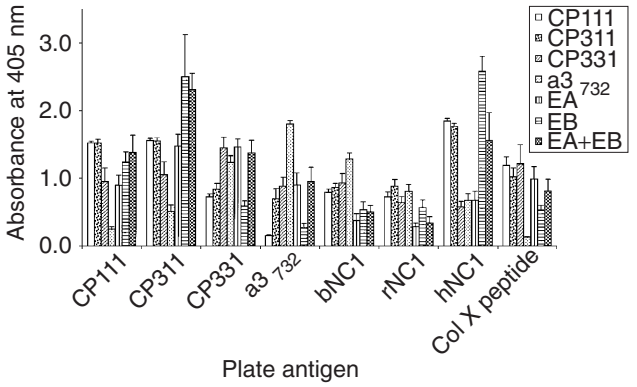


**Fig. 3. The specific reactivity of sera from construct-immunized rats toward various recombinant proteins and native NC1 domain was determined by direct enzyme-linked immunosorbent assay (ELISA).** Sera from the 6-week bleed were used at 1:500 dilution. The serum antibodies bind specifically to the recombinant as well as the native NC1 domains purified from bovine (bNC1), rat (rNC1), and human (hNC1) glomerular basement membrane (GBM). NC1 domains were prepared by multistage column chromatography, as described previously in detail [61, 62]. Chimeric construct-immunized rats developed antibody to type X collagen (col X), but not those immunized with $a3_{732}$. Mean ± SEM.

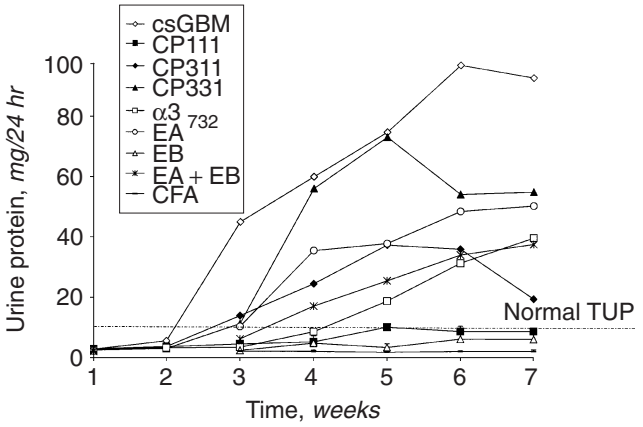


**Fig. 4. Time course of proteinuria of rats immunized with different constructs and native glomerular basement membrane (GBM).** Total urine protein (TUP) of each animal in each immunization group was determined weekly. Results shown represent the mean TUP of each group. Normal TUP < 10 mg. The degree of proteinuria for CP111 and EB were not different from complete Freund's adjuvant (CFA) rats. All other constructs resulted in proteinuria significantly greater than CFA, CP111, and EB, $P < 0.05$. TUP was not different between csGBM and CP331 rats. TUP for csGBM rats was significantly greater than all other proteinuric groups ($P < 0.05$ to $P < 0.0004$). CP331 induced greater proteinuria than $\alpha3_{732}$ ($P < 0.02$).

for anti α1NC1 antibodies on CP311 than on CP331, and even less on $\alpha3_{732}$. It also reflects the antibodies to collagen X on chimeric proteins, not present on $\alpha3_{732}$. By contrast, rats immunized with $\alpha3_{732}$, an α3NC1 construct, showed a similar but reversed antibody response, with highest response against CP331, moderate against CP311 and minimal against CP111. Animals immunized with α3/α1(IV)NC1 chimeras (CP311 and CP331) showed antibody responses to $\alpha3_{732}$, α3 chimeric proteins, and α1 chimeric proteins to a similar extent. Thus, each recombinant antigen elicited the greatest antibody response to itself, as expected. The response to other constructs reflected the composition of the construct and plate antigen. Animals immunized with larger segments of α1 constructs had higher reactivity vs. constructs with greater percent α1 in the construct, and an inverse reactivity with $\alpha3_{732}$. In addition, all animals demonstrated a robust response to native NC1 domains from human, bovine, and rat GBM which contain both α3NC1 and α1NC1. The greater amount of antibody binding in sera from CP111 and CP311 immunized rats for human NC1 could be due to the higher composition of α1(IV)NC1 in the preparation. All rats immunized with chimeric proteins, which contain collagen X, developed a strong antibody response against collagen X. Rats immunized with $\alpha3_{732,}$ lacking collegen X, showed no reactivity. Finally, sera from rats immunized with constructs containing EA and EB, consisting mostly of α1 chain plus collagen X and histidine, had broad spectrum reactivity to antigens containing either α1 or α3 moieties, as would be expected. Negative-control rats immunized with CFA alone did not exhibit any antibody response against any recombinant or native protein (data not shown).

## Nephritogenicity

*Proteinuria.* As shown in Figure 4, animals immunized with CP311, CP331, $\alpha3_{732,}$ and native GBM all developed proteinuria beginning 3 to 4 weeks following immunization, which increased with time throughout the course of the study. However, there were differences in the time of onset and severity of proteinuria among the different construct recipient rats, despite the presence of antibody with similar binding against native bovine and rat GBM. CP331 was the most potent chimeric protein construct for inducing rat EAG. All six rats receiving CP331 had heavy proteinuria at 4 weeks following injection, reaching a mean level of 73 ± 36 mg/24 hours at 5 weeks, similar to protein excretion levels of rats given native GBM. By contrast, only two of six rats given CP311 and one rat given $\alpha3_{732}$ had proteinuria at 4 weeks. The development of proteinuria among CP331 recipient rats was so rapid and severe, that by the time of sacrifice (8 weeks), only two of six CP331 rats survived, the others dying of uremia. A slower and less pronounced degree of proteinuria was seen in animals immunized with CP311 and $\alpha3_{732}$. At 7 weeks following injection, five of six rats given CP311 and four of six rats given $\alpha3_{732}$ developed proteinuria. Rats immunized with EA developed heavy proteinuria with the same time course and severity of CP311, while only one of six EB immunized rats had mild proteinuria. EA and EB rats immunized with half-dose antigen, nonethe-less had proteinuria comparable to CP311 and EA full-dose rats. Among six rats given CP111, only one rat had mild proteinuria (24 mg/24 hours), which started at

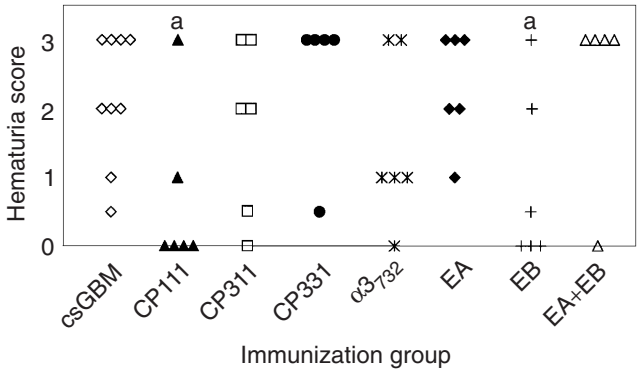


**Fig. 5. Hematuria of rats immunized with various constructs and native glomerular basement membrane (GBM).** Urine was examined weekly for hematuria. Results shown represent the hematuria of individual rats in each immunization group at week 5 following injection. [a]$P < 0.05$ vs. csGBM. Complete Freund's adjuvant (CFA) rats did not develop hematuria.

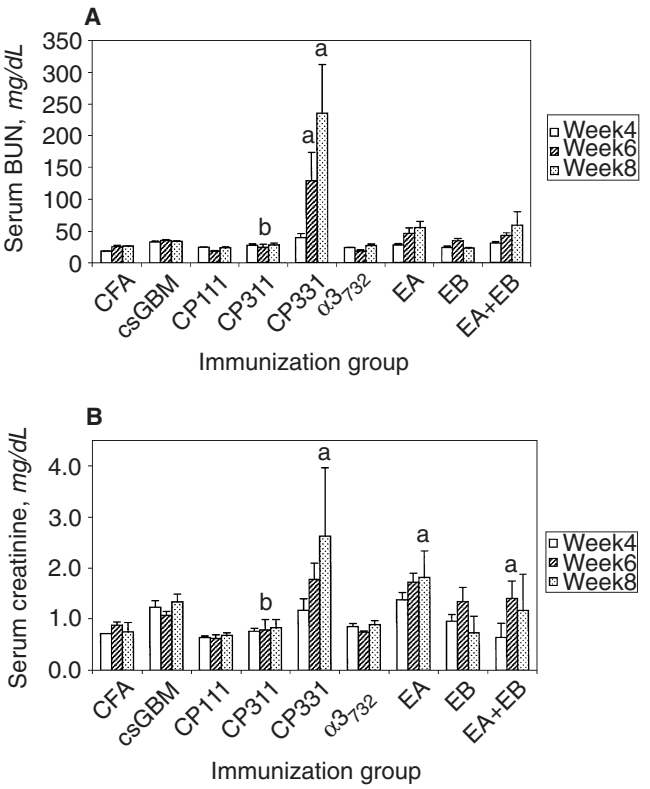


**Fig. 6. Levels of serum urea nitrogen (*A*) and creatinine (*B*) in rats immunized with various recombinant proteins and native glomerular basement membrane (GBM).** [a]$P < 0.05$ vs. complete Freund's adjuvant (CFA) controls; [b]$P < 0.05$ vs. CP331.

week 5 and continued at the same level throughout the rest of study. Negative-control animals given CFA alone did not develop any abnormal proteinuria. Differences in protein excretion with time (Fig. 4) reflect a combination of factors in this model including a peak of proteinuria at weeks 4 to 6, death of the most proteinuric rats, and diminished proteinuria with time as we and others have observed [26].

*Hematuria.* The urines of all animals were tested for hematuria. The results (Fig. 5) correlated well with the occurrence of proteinuria. At 5 weeks following injection, while one CP331-immunized rat had died of uremia, all CP331 rats except one had heavy hematuria, similar to rats immunized with native GBM. Only two CP311 and two $\alpha 3_{732}$ recipient rats had heavy hematuria at 5 weeks. By 7 weeks, all CP331 recipient rats had severe hematuria, as did five CP311 recipient rats and four $\alpha 3_{732}$ recipient rats. Hematuria in EA and EA + EB rats was comparable to other nephritic rats. Two EB-immunized rats had hematuria and a third EB rat had trace hematuria. Rats immunized with CP111, however, showed minimal hematuria throughout the course of study. One animal had light hematuria without deposits by fluorescence and no glomerulonephritis by light microscopy. One rat had trace fluorescent deposits of IgG and fibrin and mild proteinuria and glomerulonephritis.

*Serum urea nitrogen and creatinine.* The serum urea nitrogen and creatinine were also determined in animals injected with various recombinant proteins (Fig. 6). Rats given CP 331 showed a progressive increase in serum urea nitrogen beginning by 4 weeks following immunization which increased to 235 mg/dL at the time of sacrifice (8 weeks). CP311, CP111, and $\alpha 3_{732}$ recipient rats did not develop significant elevations in serum urea nitrogen. CP331 immunized rats also had an increase in the serum creatinine from 0.5 mg/dL to 2.6 mg/dL, while no significant abnormality was observed in CP311, CP111, and $\alpha 3_{732}$-immunized rats. EA and EA + EB immunized rats had increases in creatinine compared to CFA and CP111 rats. EB rats had no increase in blood urea nitrogen or creatinine. Consistent with our previous findings [25], rats immunized with native bovine csGBM showed a mild increase of serum creatinine from 0.6 mg/dL to 1.1 mg/dL at time of sacrifice, although no significant change for serum urea nitrogen levels was observed. With additional time, csGBM immunized rats routinely develop elevated blood urea nitrogen and creatinine, and die of kidney failure.

## Histologic findings

*Direct immunofluorescence for IgG and fibrinogen.* Rat IgG was deposited in a linear pattern along the GBM and to a lesser extent along the tubular basement membrane (TBM) in all rats except those immunized with CP111 (Fig. 7). However, the intensity of IgG staining on the GBM in animals immunized with recombinant proteins was significantly less than that seen in animals immunized with native GBM, despite the presence of intense fibrinogen deposition.

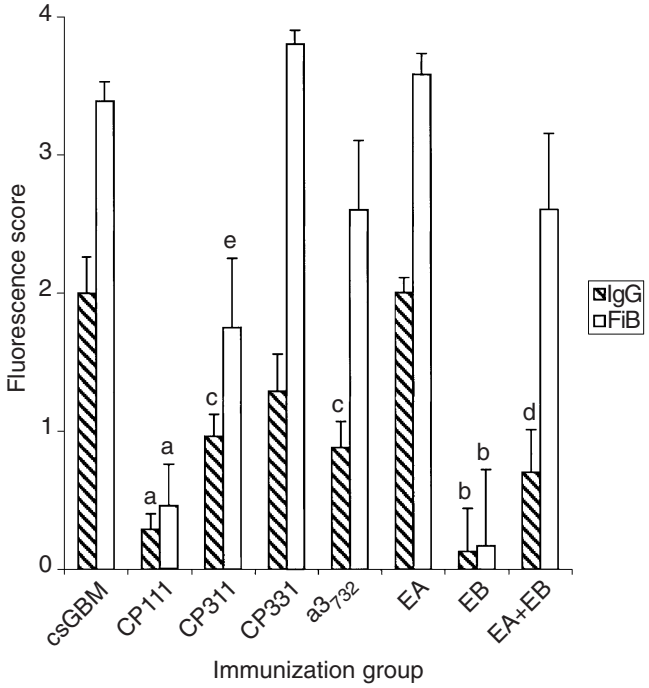


**Fig. 7. Histologic scoring by immunofluorescence in rats immunized with recombinant proteins and native glomerular basement membrane (GBM).** Kidney sections were stained for rat IgG and fibrinogen. Mean ± SEM. [a]$P < 0.0005$ vs. csGBM; [b]$P < 0.001$ vs. CSGBM; [c]$P < 0.01$ vs. csGBM; [d]$P < 0.02$ vs. csGBM; [e]$P < 0.005$ vs. CP331.

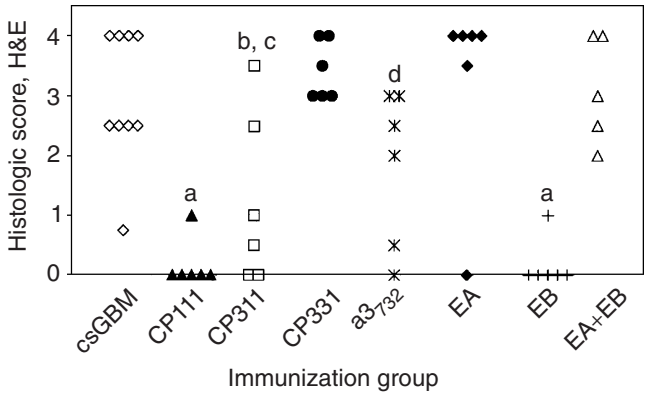


**Fig. 8. Histologic scores by hematoxylin and eosin for rats immunized with various constructs.** [a]$P < 0.001$ vs. csGBM; [b]$P < 0.03$ vs. csGBM; [c]$P < 0.01$ vs. CP331; [d]$P < 0.02$ vs. CP331.

CP331 immunized rats had intense fibrinogen deposits within the glomeruli in a pattern and distribution comparable to that seen in animals immunized with native GBM (Fig. 7). CP311- and $\alpha 3_{732}$-immunized rats showed less intensity of fibrinogen deposits compared to CP331 recipient rats. EA-immunized rats had IgG and fibrinogen deposits comparable to csGBM, while only one EB-immunized rat had trace to one plus deposits of IgG and fibrinogen. EA + EB-immunized rats had minimal IgG deposits, but fibrinogen comparable to csGBM.

Among six CP111-immunized rats, only one rat with mild proteinuria and hematuria showed trace linear IgG staining along the GBM with minimal fibrinogen deposits. The other five rats showed neither IgG binding nor deposits of fibrinogen within glomeruli. Negative-control animals given CFA alone showed no antibody binding (data not shown).

*Light microscopy.* Analysis of histologic damage by light microscopy (Fig. 8) revealed severe glomerulonephritis in CP331 immunized rats, comparable to that seen in native GBM immunized rats and described in detail previously [25, 32]. Examples of histology are provided in Figure 9. The degree of damage in two rats reached the highest score 4, and the remaining four rats showed scores of 3 to 3.5. This was associated with intense interstitial fibrosis and tubular atrophy, and interstitial mononuclear cell infiltrates. Modest glomerular abnormalities were seen in half of CP311- and $\alpha 3_{732}$-immunized rats (range of score 2 to 3). Five of six EA-immunized rats had glomerulonephritis comparable or worse than csGBM and comparable to CP331. EA + EB rats had severe glomerulonephritis similar to csGBM. One of six EB rats had very mild glomerulonephritis. The CP111-recipient animals had normal glomeruli except the one with hematuria and proteinuria, which had focal areas of interstitial fibrosis. Animals given CFA alone showed completely normal glomeruli.

## DISCUSSION

Goodpasture syndrome consists of glomerulonephritis with pulmonary hemorrhage associated with circulating and tissue-bound antibodies to basement membranes. Studies in animals immunized with GBM have demonstrated the disease can be transferred by antibody alone [28, 34, 35]. However, the disease is T-cell dependent for its induction and can be transferred by sensitized T cells [28, 31, 36–40]. Animals immunized with GBM develop a broad spectrum of antibodies to various NC1 domains. Serum from patients with Goodpasture syndrome also have a broad spectrum of antibodies against various components of the GBM with most directed to collagen IV [14, 41–43]. However, the majority of antibody reactivity is to α3(IV)NC1 domain [41, 42]. Further evidence for the role of α3NC1 in disease production has been provided by the production of glomerulonephritis in animals immunized with monomers and dimers of $\alpha_3$ and recombinant α3(IV)NC1 [27, 44, 45]. While the immunodominance demonstrated by Goodpasture syndrome sera binding to the amino terminal portion of α3 would suggest that this region contains the epitope responsible for induction of the disease [16, 18–23], some investigators have demonstrated immunoreactive regions in other portions of α3, including amino acids 127–141 and the carboxy terminal 36 amino acid [18, 20, 21]. The presence of circulating

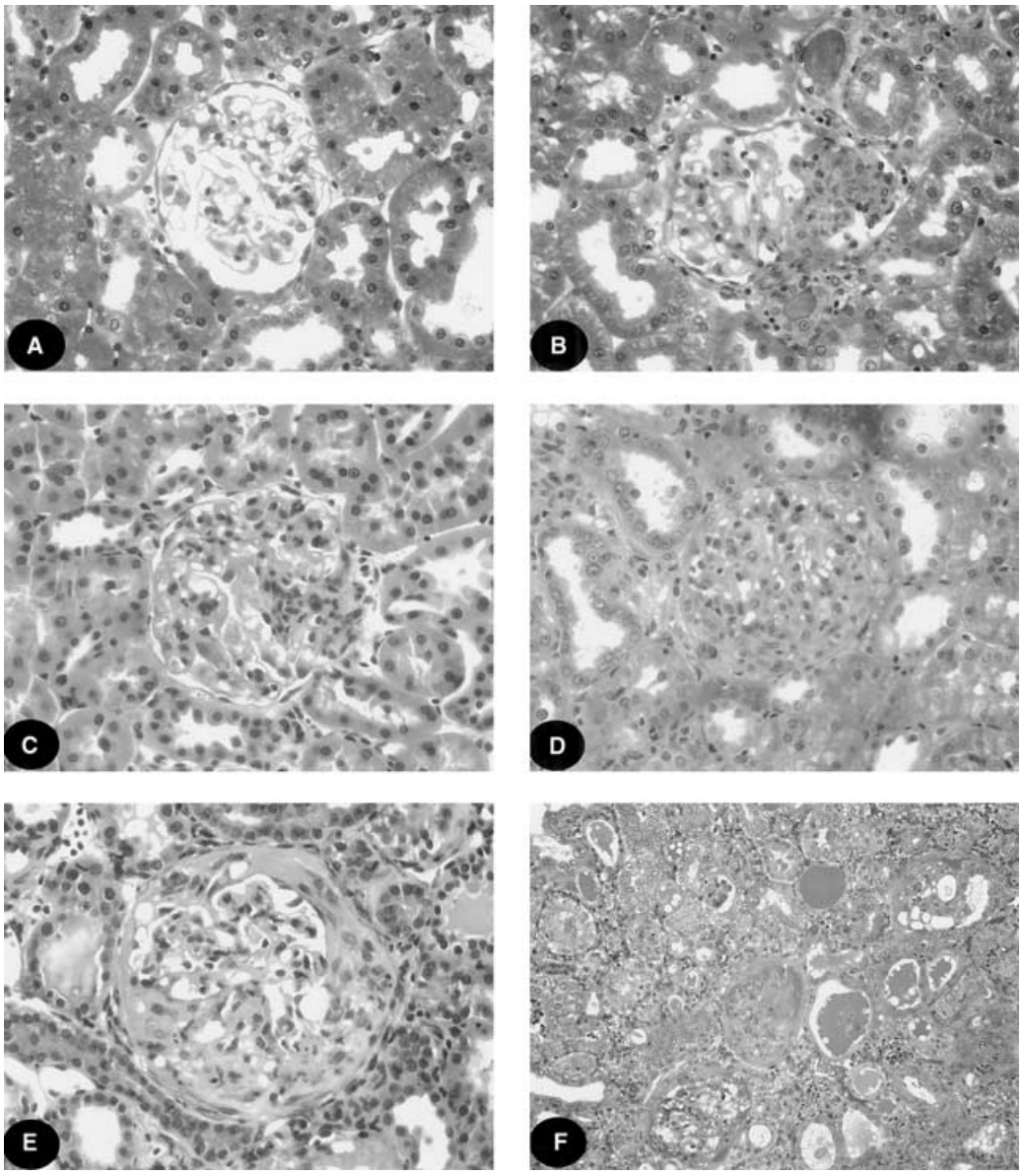


**Fig. 9. Illustrative examples of histologic grading scores from animals immunized with recombinant proteins.** (*A*) Section from an animal immunized with CP111 showing normal glomerular architecture, absence of tubular abnormalities, identical to normal rats (hematoxylin and eosin ×4000. (*B*) Section from an animal immunized with EB, the only animal of this group to show histologic abnormalities, illustrating a 1+ lesion with segmental tuftal proliferation but no other abnormalities (hematoxylin and eosin ×400). (*C*) Animal immunized with recombinant protein m732 illustrating a 2+ lesion in a glomerulus with hypercellularity decreased capillary lumens (hematoxylin and eosin ×400). (*D*) CP331 immunized animal showing 3+ glomerular proliferation with tubular dilatation, marked decrease in capillary lumens and Bowman's space, and early interstitial infiltration (hematoxylin and eosin ×400). (*E*) Section from a rat immunized with EA illustrating a 4+ lesion with marked glomerular compression with a fibrocellular crescent, and tubular dilatation with casts (hematoxylin and eosin ×400). (*F*) Low power view of an animal immunized with CP331 illustrating 4+ lesions with glomerular obsolescence, glomerular fibrosis and crescents, tubular dilatation with marked cast formation, and interstitial cellular infiltration (hematoxylin and eosin ×200). Detailed histologic descriptions on a chronologic basis and grading are provided in [25, 32].

antibodies may not portend pathogenic consequences as demonstrated in multiple animal models of autoimmune organ-specific disease transferable by activated T cells [39, 46–50]. In addition, epitope spreading whereby the initiating pathogenic epitope can result in the recruitment of additional epitope-specific T and B cells may result in antibodies and T-cell proliferation as an epiphenomenon of the process. This may or may not contribute to tissue damage [51, 52]. This suggests caution in extrapolating data from in vitro antibody binding studies to pathogenic events occurring in vivo. Since it has previously been shown that the nephritogenic epitope in the experimental analogue of Goodpasture syndrome, EAG in rats, resides in α3(IV)NC1, we have investigated whether the in vitro antibody binding information from Goodpasture syndrome sera and NC1 domains correlates with disease

induction in this model. Identification of both the target epitopes involved in initiation of EAG and the epitopes responsible for induction of disease is important to better understand the pathogenesis of the process and provide a framework for the development of therapeutic strategies in humans.

The construction of chimeric proteins allows examination of the role of site-specific conformational epitopes in the experimental model, which are not available using synthetic polypeptides. Indeed, studies by a number of investigators suggest that conformational epitopes requiring interaction of amino acids at great lengths from each other in the α3(IV)NC1 domain are necessary for antibody binding and presumably for disease induction [20–23]. However, in vitro antibody binding does not provide proof of the ability of epitopes to induce disease. For these reasons, we are investigating epitopes that may be involved in disease induction to determine if these are the same epitopes recognized by antibodies from patients with Goodpasture disease and to identify the specific epitope or epitopes involved in the pathogenesis of EAG.

The chimeric proteins used in the current study have been shown to bind the serum of patients with Goodpasture syndrome [16, 22]. The constructs were designed to consist of thirds of the α3(IV)NC1 domain with various thirds of α1(IV)NC1 domain. Whole-chain α3(IV)NC1 has been shown to bind Goodpasture syndrome and to induce EAG, while α1(IV)NC1 does not cause EAG nor bind Goodpasture syndrome serum [16, 45, 53]. We have previously shown that constructs containing the amino terminal third of α3 had the highest level of binding and worse prognosis than for patients demonstrating other antibody activities [16]. The results of the current experiments also demonstrated the necessity for the amino terminal portion of α3 for induction of disease in animals. Rats immunized with the chimeric backbone of α1(IV)NC1 domain had no glomerulonephritis while those immunized with whole α3(IV)NC1 domain or chimeric protein constructs consisting of the first third or the first plus second third of $\alpha_3$ developed EAG. We had hypothesized that the amino terminal third would contain the nephritogenic epitope based on antibody-binding studies in vitro. Indeed, CP311 did induce EAG. However, the disease was less florid with less proteinuria and slower onset than that seen with CP331 or native GBM despite identical amounts of antigen and conditions of immunization. The degree of intramolecular interaction of the immunogen in our studies is unknown, as the antigen is emulsified in CFA and then injected. Most of the chimeric protein constructs appeared to be monomeric, as opposed to csGBM (Fig. 2) and monomers induce less disease than dimers [44]. However, the amount of native α3(IV)NC1 monomer/dimer in csGBM, 3% [54] is far less than in the recombinant proteins, implying much greater propensity of native antigen to induce disease. CP331, flanked by the collagen X leader protein and the middle portion of α3(IV)NC1, induced a more florid disease than CP311 or $\alpha 3_{732}$ and was comparable to that induced by native csGBM (Figs. 4 and 6 to 8). Since CP311 would only contain the first of two epitopes described by Netzer et al [20] and Borza et al [21] and CP331 would contain the first and second epitope described by that group, it might be postulated that the two epitopes together are necessary for full-disease expression. However, $\alpha 3_{732}$ containing the entire α3(IV)NC1 domain but lacking the collagen X leader protein actually produced slightly less severe disease and was slower in onset than either CP311 or CP331. This could be consequent to the factors noted above. It could also suggest that the collagen X leader protein may have had some role in the severity of disease production after immunization or that the folding of the molecule is differently affected by collagen X or the neighboring construct. Studies with EA and EB constructs confirmed the pivotal importance of the amino terminal third of α3(IV)NC1 for disease induction. EA induced florid disease, similar to full-length native csGBM and constructs containing the full amino terminal third of α3(IV)NC1. EB alone, containing the second antibody immunodominant region, induced essentially no disease. Yet half dose of EA + EB induced full-spectrum disease. The degree of severity of glomerulonephritis in this model is dose related (personal observation [24, 55, 56]). Thus, comparable disease of half-dose EA plus half-dose EB further suggests an augmenting effect of the second third of the molecule on disease induction, even though the EA and EB sites were on different chains. Future studies will need to address a possible augmenting role of the collagen X leader protein.

The antibodies that developed after immunization with the various constructs are consistent with a robust response to the collagen X leader protein in CP311, CP331, CP111, EA, and EB-immunized animals. Since we did not have chimeric proteins of these constructs lacking the collagen X leader protein, we were unable to distinguish the antibody specificities for the collagen X compared to the remainder of the chimeric protein. Rats immunized with $\alpha 3_{732}$ did not respond to collagen X or CP111, and animals immunized with chimeric protein containing the amino third or amino two thirds of α3 responded to $\alpha 3_{372}$ lacking collagen X, while CP111 did not. These findings are consistent with a species of $\alpha 3_{372}$, CP311, and CP331 antibodies specific for the α3(IV)NC1 domain, without significant anti-α1NC1 cross-reactivity, as shown by Sado et al [45]. As demonstrated here, and by other studies, immunization with α1NC1 induces circulating antibody to α1NC1, but not deposition on the GBM, despite its high content of α1(NC1) [4, 45, 53, 54, 57–59].

The histologic findings in the present study are of interest. Native csGBM antigen immunization was associated

with intense deposits of IgG on the GBM and formation of crescents with a marked interstitial infiltrate, fibrosis, and scarring as previously described [25]. Much lesser amounts of GBM-bound IgG were noted with CP311, CP331, and $\alpha 3_{732}$. While this might be presumed to be on the basis of their monomeric conformation, since NC1 monomers induce less disease than dimers [44], the histologic and clinical consequences of immunization with these constructs suggest that other factors may also be involved. As shown in Figures 4 to 8, despite their monomeric conformation, there was considerable difference in the ability of these three constructs to induce EAG. This suggests that factors other than failure to assume a dimeric conformation were involved in the variance in disease induction.

The differences in the amount of GBM-bound IgG may result from several possibilities. It is known from both humans and experimental animals that anti-GBM antibodies can be formed, deposited on the GBM, be clearly detectable by immunofluorescence, and yet cause no disease [25, 56, 60]. Presumably the epitope specificities for the antibody are important in disease induction. This is suggested by studies transferring disease with antibodies derived from urine, serum, and monoclonal antibodies that induce florid glomerulonephritis after fixation to the GBM [28, 34, 35]. It is also possible that epitope spreading occurs with release of GBM antigens and the formation of secondary antibodies, which then deposit in the GBM, increasing the overall amount of IgG present in the GBM seen in csGBM-immunized rats [51, 52]. This, however, seems unlikely since rats immunized with CP331 had disease comparable to that seen in csGBM animals but markedly less GBM-bound IgG. On the other hand, since it is known that EAG is T-cell dependent [28, 31, 36–40], one could postulate that there are several or multiple T-cell epitopes that may be responsible for the overall phenotypic expression of the disease either alone or in combination with antibody, and that the repertoire of T cells activated by the various constructs influence the phenotypic expression. Evidence to support this is indirectly suggested by the studies in which two discrete sites of α3(IV)NC1 domain, amino acids 17–31 and 127–141, appear to be involved in in vitro studies of binding to Goodpasture sera [20, 21]. The first site, amino acids 17–31, would be contained in our chimeric protein constructs CP311 and EA, whereas both sites would be contained in CP331. It is further supported by the description of multiple T-cell epitopes in this model and humans [32]. In addition, the type of epitope-initiated disease may play a role in the degree to which recruitment of nonspecific T cells and macrophages may occur, with subsequent augmentation of injury. Further evidence of a potential T-cell–dependent antibody independent mechanism is provided by studies of Wu et al [33, 39]. They immunized rats with linear recombinant bacterial α3(NC1), which induced EAG without GBM-bound antibody but with circulating antibody to immunogen and EAG transfer with cells.

Multiple antibody binding sites of α3(IV)NC1 have been identified by various investigators [32], with two immunodominant antibody binding sites [16, 20–22]. Our studies confirm our postulate that the immunodominant antibody binding sites on α3NC1 domain are also capable of inducing EAG in our model. However, only the amino terminal site containing Hudson EA, induces glomerulonephritis, not the second, EB site. Our studies also suggest that other portions of the molecule than the amino terminal third may be involved in disease induction or modification of disease expression. It raises the possibility that more than one epitope may be involved and that flanking regions related to the nephritogenic epitope influence disease expression. Additional studies will be necessary to further clarify these possibilities and to further define the nephritogenic epitopes involved in EAG production. Refinement in the delineation of the nephritogenic epitopes provides an infrastructure whereby peptide specific immunotherapy directed to T-cell epitopes could eventually become part of the therapeutic armamentarium for treatment of patients with Goodpasture disease. The current studies narrow the spectrum of potential disease responsible epitopes and provide yet further support for the concordance of the experimental animal model to the analogous disease in humans.

## NOTE ADDED IN PROOF

After we reported the observations that are the basis for the current manuscript [abstract; Bolton WK, et al, *J Am Soc Nephrol* 11:472A, 2000], Borza et al reported in abstract form confirmation that the EA amino terminal portion of α3(IV)NC1 induced disease while the second portion, EB did not, and that the EA + EB combination produced more severe disease [abstract; Borza D-B, et al, *J Am Soc Nephrol* 13:171A, 2002]. They also reported that other segments α3(IV)NC1 did not induce disease, but did not report antibody responses nor possible modulating effects of other portions of the molecule.

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