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The α3 chain of type IV collagen induces autoimmune Goodpasture syndrome

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ABSTRACT **Human Goodpasture syndrome is a lethal form of autoimmune disease that is characterized by pulmonary hemorrhage and glomerulonephritis. The tissue injury is mediated by autoantibodies that bind to glomerular and alveolar basement membrane. The target autoantigen is α3(IV) collagen, one of six genetically distinct chains that comprise type IV collagen, and the epitope is sublocalized to the noncollagenous domain (NC1) of the α3 chain. The present study reports the unique capacity of α3(IV)NC1 dimer from bovine kidney to aberrantly engage the immune system of rabbits to respond to self, mimicking the organ-specific form of the human disease, whereas the other chains of type IV collagen are nonpathogenic. However, α3(IV)NC1 hexamer was nonpathogenic, suggesting the exposure of a pathogenic epitope upon dissociation of hexamer into dimers. Exposure of the pathogenic epitope by infection or organic solvents, events which are thought to precede Goodpasture syndrome, may be the principal factor in the etiology of the disease. The pathogenicity of α3(IV) collagen brings full circle a decade of research that has identified four novel chains (α3–α6) of type IV collagen.**

Goodpasture (GP) syndrome has attracted the interest of the biomedical community for 75 years because it is a lethal form of autoimmune injury that produces a triad of pulmonary hemorrhage, glomerulonephritis, and anti-glomerular basement membrane (anti-GBM) autoantibodies (1–5). These autoantibodies develop in genetically predisposed individuals as dictated by polymorphism of the major histocompatibility complex (6), and the antibodies by themselves passively transfer glomerulonephritis to naive animals (7). The target antigen in glomerular and alveolar basement membrane has been identified as α3(IV) collagen (8–10), one of six genetically distinct chains that comprise type IV collagen (3) (Fig. 1). The *COL4A3* gene encoding the GP autoantigen has also been cloned and localized to chromosome 2 bands q35–37 (15, 16). The GP epitope resides within the NC1 noncollagenous domain of the α3(IV) chain [α3(IV)NC1], is sublocalized to the last 36 aa of the carboxyl terminus, and is sequestered in the native hexamer configuration (11, 17) (Fig. 1).

In the present study, the potential capacity of α3(IV) collagen to induce GP syndrome in rabbits was determined by using the dimer and hexamer forms of the α3NC1 domain as immunogens. For comparison, the analogous forms of the NC1 domain of α1, α2, α4, and α5 were also studied. The findings revealed the unique capacity of the α3NC1 domain to elicit pathogenic autoantibodies that produced clinical features in naive rabbits, mimicking the organ-specific form of human GP syndrome.

MATERIALS AND METHODS

Chemicals and Reagents. General chemicals, reagents, and supplies were obtained from Sigma and Fisher Scientific. Anti-rabbit IgG conjugated to horseradish peroxidase was obtained from Dako, anti-rabbit IgG conjugated to alkaline phosphatase was obtained from Sigma and Fisher, and anti-rabbit IgG conjugated to fluorescein or lissamine rhodamine was obtained from Cappel; ELISA plates were obtained from Nunc, and New Zealand White rabbits were obtained from Myrtle's Rabbitry, Thompson Station, TN. Protein and creatinine assay kits were obtained from Pierce and Sigma, respectively.

General Methods. GBM and its constituents were prepared as described (8, 9, 13). The NC1 hexamer, D1 and D2 dimers, α1/α2NC1 monomers, α3NC1 monomers, and α4NC1 monomers were isolated as described (8, 9, 13). SDS/PAGE and immunoblotting and direct and inhibition ELISAs were performed as reported (11, 13, 17).

Immunization of Rabbits with Bovine Type IV Collagen Domains. The New Zealand White rabbits were divided into five groups based on immunization: group I (n = 3 rabbits), control; group II (n = 2), complete Freund's adjuvant (CFA); group III (n = 3), α1(IV)/α2(IV)NC1 dimers (D1) in CFA; group IV (n = 10), α3(IV)NC1-containing dimers (D2) in CFA; and group V (n = 3), NC1 hexamer (α1–α5NC1 domain) in CFA.

On day 1 (after the rabbits were acclimatized for a week to the new surroundings in the animal care room), preimmune serum and urine were collected from all animals. All rabbits were weighed and the ones weighing the most were placed in group IV. After all the preimmune arrangements were made, rabbits from groups III, IV, and V were immunized subcutaneously with their respective antigens. Group II received 500 μl of CFA mixed with 500 μl of phosphate-buffered saline (PBS). Group III received 300 μg of α1(IV)/α2(IV)NC1 dimers mixed with 500 μl of CFA. Group IV received α3(IV)NC1-containing dimers (300 μg) mixed with 500 μl of CFA. Group V received 500 μg of NC1 hexamer in 500 μl of CFA. Booster injections were given every 2 weeks with the same amount of antigen.

Body weight was determined every 2 weeks and a note was made of those animals losing weight rapidly. A 15-hr urine collection was made every week, using metabolic pans. Urinary protein was measured by the bicinchoninic acid method (18). The rabbits were bled every 2 weeks to check for serum creatinine levels (19) and circulating antibodies.

Postmortem Analysis and Tissue Collection. Upon the death of two animals in group IV, the remaining animals were euthanized 8 weeks after the initial immunization. All rabbits were given 0.75 ml of xylazine followed by 1.25 ml of ketamine after 5–10 min. After the rabbits were completely immobilized, with loss of corneal reflex, they were weighed and placed on the operation table for abdominal incision. Immediately upon entry into the animal's abdominal region,

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Abbreviations: CFA, complete Freund's adjuvant; GBM, glomerular basement membrane; GP, Goodpasture.

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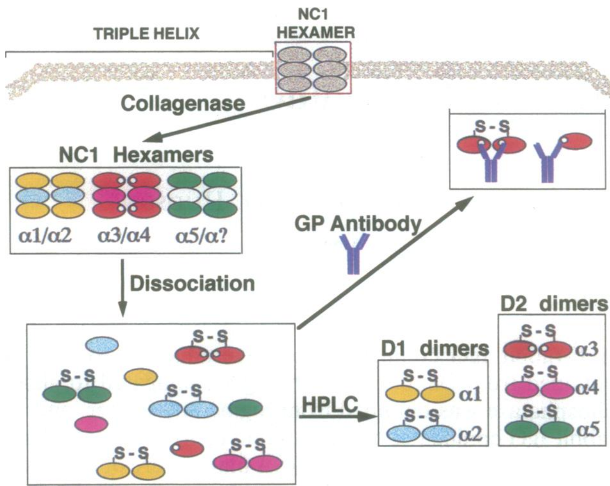

FIG. 1. Structure of GBM type IV collagen and NC1 immunogens. The monomeric (building block) unit of type IV collagen is a triple-helical molecule composed of three α chains. Each chain is characterized by a noncollagenous domain (NC1) of ≈230 aa at the carboxyl terminus, a long collagenous domain of about 1400 aa that contains several non-Gly-Xaa-Yaa interruptions, and a 7S domain at the amino terminus (3). The type IV collagen from GBM contains five α chains ($\alpha1$–$\alpha5$) (11) and it may contain the chain $\alpha6$ (12). Chains $\alpha1$–$\alpha5$ are assembled into at least three kinds of triple-helical molecules, with chain compositions of $(\alpha1)_2\alpha2$, $(\alpha3)_2\alpha4$, and $(\alpha5)_2\alpha$? (13, 14, 29) and which are further linked through NC1 domains to like kinds of triple-helical molecules. The triple-helical monomers are assembled into a suprastructure through end-to-end interactions [NC1–NC1 dimers, and 7S–7S tetramers (not shown)]. At the NC1 junction, the NC1 domains (monomers) of the six α chains form a NC1 hexamer. NC1 dimers are disulfide-crosslinked NC1 monomers of two α chains from adjoining triple-helical molecules. The NC1 hexamer can be excised from the suprastructure by collagenase digestion, yielding at least three kinds of hexamers from GBM that differ in the α chain origin of NC1 subunits. The GP epitope, located on the $\alpha3$NC1 domain, is sequestered within the NC1 hexamer, shown as white circles on the $\alpha3$NC1 domain (red) (11). Upon disruption of the conformation and tertiary or quaternary structure of the hexamer by protein denaturants, the epitope on $\alpha3$NC1 monomers and dimers is unmasked and becomes accessible for binding to GP antibody. In the present study three NC1 immunogens were used: the NC1 hexamer mixture, containing all five ($\alpha1$–$\alpha5$) NC1 domains; D1 dimers, containing $\alpha1/\alpha2$NC1 dimers; and D2 dimers, containing $\alpha3/\alpha4/\alpha5$NC1 dimers. The NC1 hexamer was prepared under conditions that maintain its native structure (13). In contrast, the D1 dimers and D2 dimers were prepared under conditions that disrupt the quaternary structure of the hexamer and the conformation of its subunits (13). D1 is composed exclusively of a mixture of $\alpha1$NC1 and $\alpha2$NC1 homodimers, and D2 is composed of a mixture of $\alpha3$NC1 homodimers (80%) along with $\alpha4$NC1 and $\alpha5$NC1 homodimers and $\alpha1/\alpha3$NC1 heterodimer (13). The color scheme is $\alpha1$, yellow; $\alpha2$, light blue; $\alpha3$, red; $\alpha4$, magenta; and $\alpha5$, green.

the left renal artery was clamped and the left kidney was excised, wrapped in aluminum foil, and quick-frozen in liquid nitrogen. The blood from the rabbits was collected from the heart (60 ml) and a catheter was placed in the aorta and flushed with saline to remove all the remaining blood from the renal circulation. The inferior vena cava was incised for the outflow. After the saline flush, the kidneys were perfusion-fixed with 4% paraformaldehyde until the kidneys and liver were thoroughly perfused. The fixed kidney was removed and with a sharp blade, vertical slices were made, and tissue was placed in a container of 4% paraformaldehyde for immunofluorescence and light microscopy. Pieces of lung and liver were similarly processed for microscopic studies.

Immunofluorescence Microscopy. To localize endogenous IgG, perfusion-fixed (4% paraformaldehyde) kidneys and lungs were quick-frozen, and frozen sections 5 and 6 μm thick, respectively, were cut from the renal cortex and lung. Endogenous bound IgG was identified with either fluorescein- or lissamine rhodamine-conjugated anti-rabbit IgG diluted to 1:100 with PBS. After the sections were washed thoroughly with PBS, they were coverslipped with glycerol/gelatin. The slides were viewed under a Nikon microscope fitted with UV epifluorescence lighting.

Elution of Kidney-Bound Antibodies. Cortex from quick-frozen and unfixed kidneys was sliced out and homogenized in 0.05 M Tris·HCl, pH 7.5/0.15 M NaCl with proteinase inhibitors (5 mM *N*-ethylmalemide, 5 mM benzamidine hydrochloride, 25 mM 6-aminohexanoic acid, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride). The homogenate was washed and centrifuged 10 times at 3000 × *g* for 20 min, until a clear solution was obtained. The insoluble material was eluted with cold 0.1 M glycine, pH 2.8/0.5 M NaCl and centrifuged. The supernatant was adjusted to neutral pH by addition of 1 M Tris. This sample was precipitated with ammonium sulfate (40% saturation) and the precipitate was dissolved in 2 ml of 0.05 M Tris·HCl, pH 7.5/0.05 M NaCl/0.02% NaN_3 and dialyzed twice against excess PBS.

RESULTS AND DISCUSSION

The potential capacity of the $\alpha3$(IV) collagen chain to induce experimental GP syndrome was determined by using the NC1 noncollagenous domain in dimer and hexamer forms as immunogens (Fig. 1). For comparison, the analogous forms of the NC1 domains of $\alpha1$, $\alpha2$, $\alpha4$, and $\alpha5$ chains of type IV collagen were also studied. Three NC1 immunogens (NC1 hexamer, D1 dimer, and D2 dimer) were used (Fig. 1). Hexamers were studied because the GP epitope is sequestered within the NC1 hexamer on $\alpha3$NC1 monomers and dimers (Fig. 1). NC1 dimers were studied because the GP antibodies have a higher affinity for $\alpha3$NC1 dimers than monomers (13) and because NC1 dimers from each of the five known ($\alpha1$–$\alpha5$) chains have been isolated and characterized. Heterologous bovine NC1 domains were used because the multiple NC1 components of only this species have been characterized thus far (13).

The immunogens were administered subcutaneously to New Zealand White rabbits. Ten of 10 rabbits immunized

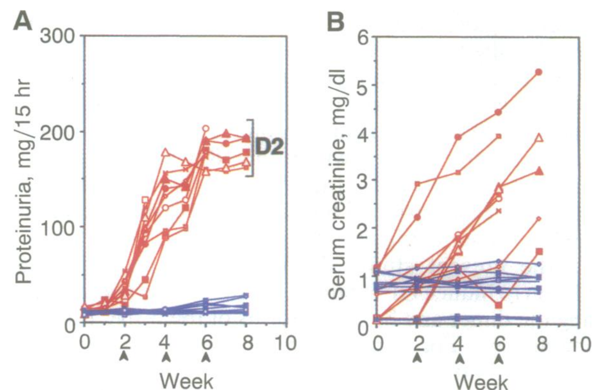

FIG. 2. Effect of NC1 immunogens on renal function of rabbits. Three rabbits were immunized with NC1 hexamers (blue), 3 with D1 dimers (blue), 10 with D2 dimers (red), 2 with CFA alone (blue), and 3 with no treatment (blue). At intervals urinary protein (*A*) and serum creatinine (*B*) were measured. The renal function of rabbits immunized with D2 dimers was greatly diminished (15- to 19-fold increase in proteinuria and 3- to 5-fold increase in serum creatinine), whereas the renal function of all other rabbits was unchanged. At the end of the study, rabbits were sacrificed, their kidney and lung tissues were examined by light microscopy, and the specificity of circulating and tissue-bound antibodies was determined. Arrowheads below the abscissa indicate time points of booster immunizations. The different symbols represent individual animals in each group.

with D2 dimers developed prominent proteinuria (indicating damage of the renal glomerulus) and elevated serum creatinine levels (indicating diminished renal function) (Fig. 2). In contrast, rabbits immunized with NC1 hexamers, D1 dimers, or CFA alone developed neither proteinuria nor elevated serum creatinine levels (Fig. 2). These results suggest that D2 dimers are pathogenic, whereas D1 dimers and NC1 hexamers are nonpathogenic.

The affected rabbits immunized with D2 dimers exhibited renal and pulmonary pathology that was similar to that observed in humans with GP syndrome. In the renal glomeruli, endogenous immunoglobulin was bound in a linear fashion to the GBM (compare Fig. 3 *B* and *C* with Fig. 3*A*). Glomeruli from the affected rabbits did not develop necrotizing glomerulonephritis with crescent cells; however, inflammatory cells were found in most glomeruli (data not shown). Crescentic glomerulonephritis is more characteristic of subacute glomerular injury, as observed in GP syndrome, whereas the present study focused on the developmental phase of the disease. The pulmonary lesion in rabbits was also characterized by endogenous immunoglobulin bound focally to alveolar basement membrane (Fig. 3 *E* as compared with *D*) and focal petechial hemorrhages with interstitial pneumonitis (Fig. 3 *G* as compared with *F*). The animals exhibited labored respiration as the disease progressed, and when two D2-immunized rabbits died, the study was terminated.

The specificity of autoantibodies from affected rabbits immunized with D2 dimers was determined. The circulating antibodies were found to bind to the immunogens (D2 dimers), as well as to D1 dimers (Fig. 4*A*), indicating crossreactivity among the various NC1 dimers. The crossreactivity probably relates to the high degree of homology (50–70% sequence identity) among the NC1 domains of the five α chains of type IV collagen (17). In contrast, the kidney- and lung-bound antibodies bound to the immunogen (D2 dimers)

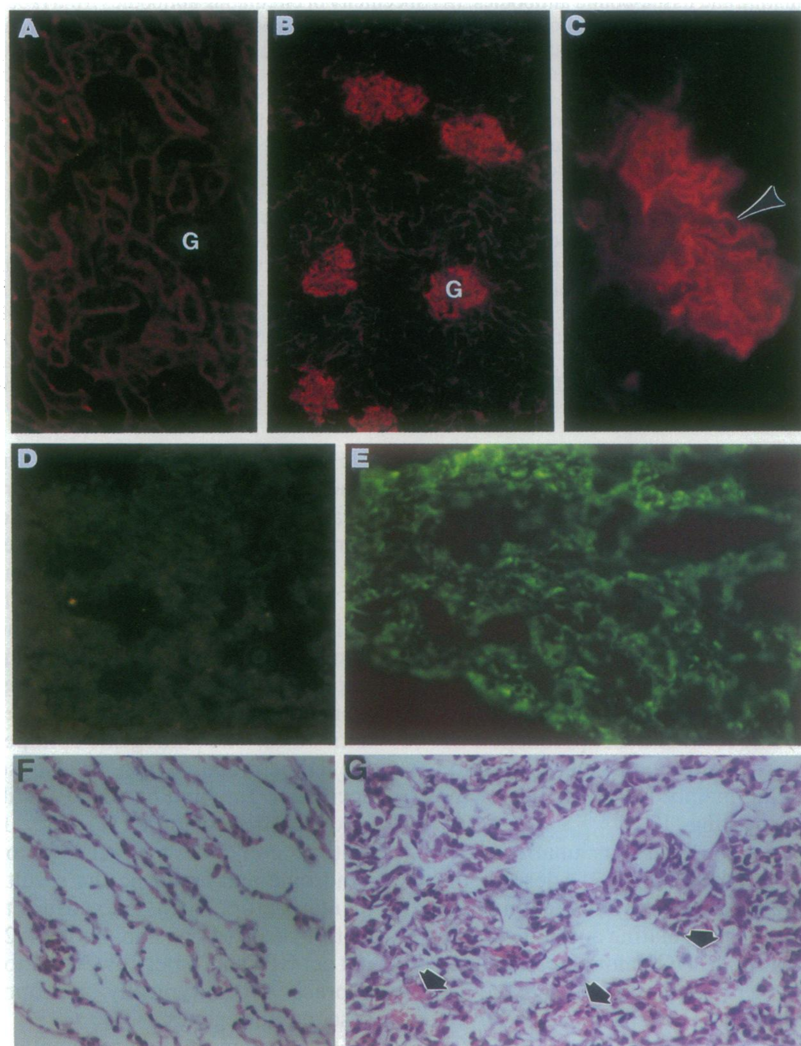

FIG. 3. Light microscopy of renal and pulmonary tissues. The presence of endogenously bound IgG was determined by fluorescence microscopy of renal tissue (*A–C*) incubated with donkey anti-rabbit IgG (labeled with lissamine-rhodamine) and pulmonary tissue (*D* and *E*) incubated with goat anti-rabbit IgG (labeled with fluorescein). Rabbits immunized with $\alpha1/\alpha2$NC1 dimers showed only background fluorescence in renal tissue (*A*) and pulmonary tissue (*D*). In contrast, rabbits immunized with $\alpha3/\alpha5$NC1 dimers exhibited intense fluorescence (red) in renal tissue, localized to the glomerular capillary loops (*B*) and characterized by linear staining along the GBM (*C*, higher magnification) and intense fluorescence (green) in pulmonary tissue, indicating the presence of endogenously bound IgG in both kinds of tissue. Renal and pulmonary tissues of rabbits immunized with $\alpha1/\alpha5$NC1 hexamers or CFA or sham-immunized did not exhibit endogenously bound IgG (data not shown). Hematoxylin/eosin staining of pulmonary tissue showed that rabbits immunized with $\alpha1/\alpha2$NC1 dimers (*F*) exhibited normal morphology, whereas pulmonary tissue of rabbits immunized with $\alpha3/\alpha5$NC1 dimers (*G*) exhibited some interstitial pneumonitis (arrowheads) with focal interstitial petechial hemorrhage with some extravasation of RBC into the alveolar spaces (arrows). For the analyses, kidneys were quick-frozen in liquid nitrogen and cryoprotected. Lungs were fixed by perfusion with 4% paraformaldehyde and then sectioned (6 μM). G denotes glomerulus and the arrowhead shows linear binding of the autoantibody to the glomerular loop.

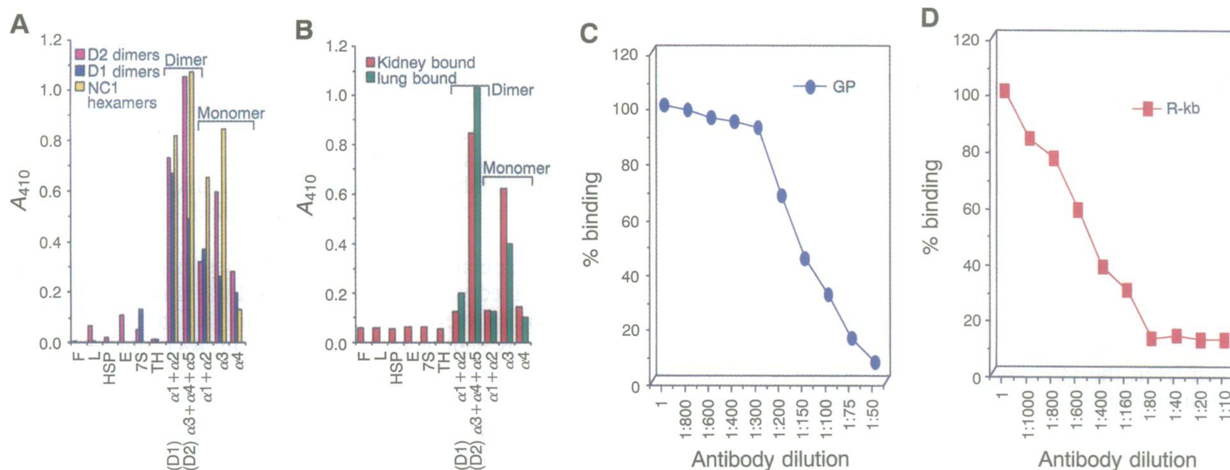

FIG. 4. Specificity of circulating and tissue-bound antibodies. (*A*) Specificity of circulating antibodies from rabbits immunized with D1 dimers (blue), D2 dimers (magenta), and NC1 hexamers (yellow) toward basement membrane components and collagen IV domains was determined by ELISA measurements, using components as previously described (13). The dilution of antibodies used was 1:50. Circulating antibodies from control rabbits without any immunization and rabbits immunized with CFA did not bind any of the basement membrane components or collagen IV domains (data not shown). (*B*) Specificity of kidney-bound (red) and lung-bound (green) antibodies from the affected rabbits immunized with $\alpha3/\alpha5$NC1 dimers was determined by ELISA measurements. The kidney-bound and lung-bound antibodies were eluted from the tissues by established procedures. The tissue-bound-antibody dilution used was 1:100. F, fibronectin; L, laminin; HSP, heparan sulfate proteoglycan; 7S, 7S domain of collagen IV; TH, triple-helical domain of collagen IV; $\alpha_1 + \alpha_2$, $\alpha1/\alpha2$NC1–(D1) dimers; $\alpha3 + \alpha4 + \alpha5$, $\alpha3/\alpha4/\alpha5$NC1 (D2) dimers; $\alpha1 + \alpha2$, $\alpha1/\alpha2$NC1 monomer; $\alpha3$, $\alpha3$NC1 monomer; $\alpha4$, $\alpha4$NC1 monomer. (*C*) Serological identity between the human GP antibodies and the kidney-bound autoantibodies from the affected rabbits (R-kb antibodies) was determined by inhibition ELISA. The ELISA plates were coated with 100 ng of D2 dimers and incubated overnight at room temperature. The plates were washed thoroughly and blocked with 2% bovine serum albumin for 30 min at 37°C. In the first experiment, the plates were incubated with various dilutions of GP serum for 1 hr at 37°C. Upon washing, the plates were incubated with 1:50 dilution of R-kb antibodies for the 1-hr duration at 37°C. Upon washing again, the plates were incubated with anti-rabbit IgG conjugated to alkaline phosphatase. The absorbance was read at 410 nm, which gave a value of 0.858 for 100% R-kb binding with no GP binding. The control rabbit serum at a dilution of 1:10 did not bind to $\alpha3$(IV)NC1 dimers (data not shown). (*D*) The order of antibodies was reversed and anti-human IgG antibody conjugated to alkaline phosphatase was used (red). The absorbance was read at 410 nm, which gave a value of 1.834 for 100% GP binding with no R-kb binding. The control human serum at a dilution of 1:50 did not bind to the $\alpha3$(IV)NC1 dimers (data not shown), as previously reported (13).

and $\alpha3$NC1 monomers, with little binding to $\alpha1/\alpha2$NC1 dimers (D1 dimers) and monomers or to $\alpha4$NC1 monomers, as determined by ELISA (Fig. 4*B*). Among the individual dimers comprising D2, the tissue-bound antibodies bound to $\alpha3$NC1 dimers but not to $\alpha4$NC1 or $\alpha5$NC1 dimers, as determined by two-dimensional gel electrophoresis and immunoblotting (data not shown). These results establish the $\alpha3$NC1 domain as the target for kidney- and lung-bound autoantibodies, indicating that $\alpha3$NC1 antibodies are pathogenic and mediate the glomerulonephritis and pulmonary hemorrhage. The organ-specific form of the experimental GP syndrome is consistent with the restricted tissue distribution of the $\alpha3$(IV) chain, occurring mainly in glomerulus of the kidney (20) and alveolus of the lung (10).

In contrast, renal and lung function was unaffected in rabbits immunized with D1 dimers (Fig. 2), despite the production of antibodies. Antibodies were detected in blood but not in kidney or lung (Fig. 3). Circulating antibodies were targeted to D1 dimer, and to $\alpha1/\alpha2$NC1 monomers, as well as to D2 dimers (Fig. 4*A*), again indicating crossreactivity among the various NC1 dimers. These results, however, directly establish that antibodies elicited against $\alpha1/\alpha2$NC1 dimers (D1 dimers) are nonpathogenic.

Overall, the results show that the $\alpha3$NC1 dimer contains a pathogenic epitope which is not shared by the NC1 domains of the other four chains of type IV collagen. This pathogenic epitope, putatively assigned to 36 aa at the carboxyl terminus (17), also appears to be serologically similar to rabbit, on the basis that kidney-bound rabbit antibodies compete (>90%) against human GP antibodies for binding to $\alpha3$NC1 domain (Fig. 4*C*). Hence, this GP epitope is most likely the structural feature that lacks tolerance and confers pathogenicity on the $\alpha3$NC1 domain, distinguishing it from other NC1 domains.

However, the $\alpha3$NC1 dimer arranged in the native hexamer configuration is nonpathogenic despite the production of antibodies. The renal and pulmonary functions of rabbits immunized with NC1 hexamers were unaffected (Fig. 2), but the rabbits produced antibodies that bound to all five kinds of NC1 domains ($\alpha1$–$\alpha5$) (Fig. 4*A*). The lack of pathogenicity of $\alpha3$NC1 hexamer—in contrast to the pathogenicity of $\alpha3$NC1 dimer, which is derived by hexamer dissociation (Fig. 1)—implies sequestration of a pathogenic epitope in hexamer which must be exposed to elicit the production of pathogenic antibodies. The pathogenic epitope is likely to be identical to the GP epitope, which is also sequestered and becomes accessible for binding to GP antibodies when exposed by hexamer dissociation (11) (Fig. 1). The exposure of this epitope could be a crucial factor in the etiology of GP syndrome and could be the result of precipitating environmental factors, such as exposure to respiratory viruses (21) or to hydrocarbons and smoke (22). Since most humans do not get GP syndrome, however, those that do also most likely express a genotypically appropriate profile of immune-susceptibility genes.

Here we report an experimental autoimmune model of GP syndrome that mimics the triad of clinical features of human autoimmune GP syndrome and in which both autoantigen and autoantibodies have been characterized. The model further establishes a causal relationship between anti-$\alpha3$(IV) collagen autoantibodies and the pulmonary and renal lesions. The findings advance a molecular explanation for several previous landmark studies, including the demonstration that the passive transfer of GP antibodies into monkeys will produce nephritis (7) and that sheep immunized with large amounts of human GBM will develop anti-GBM nephritis (23, 24). These findings also provide an explanation for the anti-GBM ne-

phritis in sheep and rats induced by human GBM (25) and soluble bovine GBM (26), respectively.

Our experimental autoimmune model of GP syndrome has pathogenic features similar to experimental autoimmune myasthenia gravis (27, 28) and anti-tubular basement membrane disease (12). These autoimmune models can be induced by a heterologous autoantigen, mimic the organ/site specific nature of the human disease, and present single pathogenic autoantibody. The model described in the present study, however, provides a prototype for delineating autoimmune mechanisms involving the type IV collagen in extracellular matrix. The model should offer an approach to understanding the role of T cells mediating the production of pathogenic anti-α3(IV) collagen autoantibodies, the mechanisms by which these pathogenic antibodies gain access to the sequestered epitope and trigger pathological changes involving immune cells, and the efficacy of new forms of therapy.

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