Quaternary Epitopes of \( \alpha345(IV) \) Collagen Initiate Alport Post-Transplant Anti-GBM Nephritis

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ABSTRACT
Alport post-transplant nephritis (APTN) is an aggressive form of anti-glomerular basement membrane disease that targets the allograft in transplanted patients with X-linked Alport syndrome. Alloantibodies develop against the NC1 domain of \( \alpha5(IV) \) collagen, which occurs in normal kidneys, including renal allografts, forming distinct \( \alpha345(IV) \) and \( \alpha1256(IV) \) networks. Here, we studied the roles of these networks as antigens inciting alloimmunity and as targets of nephritogenic alloantibodies in APTN. We found that patients with APTN, but not those without nephritis, produce two kinds of alloantibodies against allogeneic collagen IV. Some alloantibodies targeted alloepitopes within \( \alpha5NC1 \) monomers, shared by \( \alpha345NC1 \) and \( \alpha1256NC1 \) hexamers. Other alloantibodies specifically targeted alloepitopes that depended on the quaternary structure of \( \alpha345NC1 \) hexamers. In \( Col4a5 \)-null mice, immunization with native forms of allogeneic collagen IV exclusively elicited antibodies to quaternary \( \alpha345NC1 \) alloepitopes, whereas alloimmunogens lacking native quaternary structure elicited antibodies to shared \( \alpha5NC1 \) alloepitopes. These results imply that quaternary epitopes within \( \alpha345NC1 \) hexamers may initiate alloimmune responses after transplant in X-linked Alport patients. Thus, \( \alpha345NC1 \) hexamers are the culprit alloantigen and primary target of all alloantibodies mediating APTN, whereas \( \alpha1256NC1 \) hexamers become secondary targets of anti-\( \alpha5NC1 \) alloantibodies. Reliable detection of alloantibodies by immunoassays using \( \alpha345NC1 \) hexamers may improve outcomes by facilitating early, accurate diagnosis.


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We addressed the roles of these networks as antigens inciting alloimmunity and targets of nephritogenic alloantibodies in APTN.

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Sera and kidney eluates from APTN patients, but not post-transplant sera from X-linked Alport syndrome patients without nephritis, contained IgG alloantibodies staining the GBM and Bowman’s capsule basement membranes by
indirect immunofluorescence (Figure 1A). By indirect ELISA and Western blot, IgG alloantibodies from APTN sera or allograft eluates bound to recombinant (r-) α5NC1 monomers, α345NC1 hexamers from the GBM, and α1256NC1 hexamers from bladder basement membranes, but not to purified α12NC1 hexamers (Figure 1, B and C). Post-transplant sera from Alport patients without nephritis did not contain significant amounts of IgG binding to α5NC1 monomers or GBM hexamers (Figure 1D). In vivo targets of APTN alloantibodies were identified by analyzing immune complexes solubilized from a nephrectomized allograft. Most α3-α6 NC1 monomers and dimers were found in the protein G-bound fraction comprising IgG-bound NC1 hexamers, whereas the unbound free hexamers contained α1-α2NC1 domains (Figure 1E). This implies that most α345NC1 and α1256NC1 hexamers, but not α12NC1 hexamers, in APTN allografts are complexed to IgG alloantibodies.

In mice, low doses of anti-α5NC1 antibodies bind in vivo exclusively to the GBM, whereas higher doses additionally bind to Bowman’s capsule and tubular basement membranes (Supplemental Figure 1). That anti-α5NC1 alloantibodies initially target the GBM likely reflects the greater accessibility of this site to circulating IgG antibodies.11 Larger amounts of alloantibodies saturate available GBM binding sites, affording binding to other basement membranes containing α5 (IV) collagen. Therefore, IgG alloantibodies bound to Bowman capsule basement membranes are pathogenically significant not only as mediators of inflammation at this site, but also by implying excessive production of pathogenic anti-GBM alloantibodies. Notably, about 30% of transplanted Alport patients have linear fixation of IgG in the allograft GBM but no GN, nor circulating anti-GBM alloantibodies detectable by indirect immunofluorescence staining.12,13 These patients probably produce smaller amounts of alloantibodies, insufficient to cause antibody-mediated nephritis.

We next addressed whether APTN alloantibodies target identical epitopes within α5NC1 monomers and hexamers. In cross-inhibition experiments, GBM hexamers essentially abolished the binding of alloantibodies to α5NC1 monomers, whereas α5NC1 monomers inhibited alloantibodies binding to GBM hexamers by only 30%–60% (Figure 2A). This implies that GBM α345NC1 hexamers contain all alloepitopes present in α5NC1 monomers and also additional distinct alloepitopes. In contrast, α1256NC1 hexamers and α5NC1 monomers were antigenically similar in cross-inhibition studies. Both were as effective as native GBM (α345NC1) hexamers at inhibiting the binding of alloantibodies to immobilized r-α5NC1, bladder basement membrane (α1256NC1) hexamers, and dissociated GBM hexamers, but significantly less effective at inhibiting alloantibodies binding to native GBM hexamers. (Figure 2B). Thus, r-α5NC1 monomers and α1256NC1 hexamers share common alloepitopes also present within α345NC1 hexamers, whereas native α345NC1 hexamers harbor additional alloepitopes encoded by the quaternary structure, targeted by distinct alloantibodies.

Two subsets of APTN alloantibodies equally reactive toward α345NC1...
Quaternary epitopes are created by protein-protein interactions that occur during multimerization; antibodies recognizing true quaternary epitopes do not bind to individual monomeric subunits. This explains why APTN alloantibodies, unlike Goodpasture autoantibodies, exhibit reduced binding to dissociated GBM NC1 hexamers. Upon hexamer dissociation, NC1 monomer subunits cross-linked by sulfilmine bonds yield NC1 dimers that preserve part of the original trimer-trimer interface. We asked whether NC1 dimers contain any dissociation-resistant quaternary epitopes. In immunoblotting experiments, preincubation of APTN alloantibodies with α5NC1 monomers abolished binding to α5NC1 monomers but not to α5NC1-containing dimers from human GBM, implying that some quaternary epitopes are preserved within α3-α5NC1 dimers.

Quaternary alloepitopes were reconstituted by in vitro assembly of recombinant NC1 monomers. The α5NC1-unbound alloantibodies, reactive toward NC1 monomers, bound to complexes of α3NC1-α4NC1 or α3NC1-α5NC1 monomers and to r-α345NC1 hexamers assembled from NC1 monomers. Reconstituted r-α345NC1 hexamers were antigenically identical to those isolated from tissues, because they completely inhibited the binding of APTN alloantibodies to native NC1 hexamers from human GBM. These results suggest that α345NC1 hexamers harbor several quaternary epitopes, some residing at the inter-molecular α3-α5NC1 interface, others at intra-molecular α3NC1-α4NC1 and α3NC1-α5NC1 interfaces. Anti-α5NC1 alloantibodies likewise target several epitopes within α5NC1 monomers. Polyclonal alloantibodies targeting multiple tertiary α5NC1 and quaternary α345NC1 alloepitopes maximize the density of GBM-bound IgG, exacerbating GN by promoting complement activation and ligation of low-affinity activating Fcy receptors.

A practical application of these findings could be improved immunosassays...
Figure 3. Analysis of quaternary α345NC1 alloepitopes and immunoassays for optimal detection of APTN alloantibodies. (A) NC1 hexamers harbor three types of epitopes differentiated by their susceptibility to dissociation into subunits. Dissociation of M-hexamers lacking sulfonimine crosslinks into NC1 monomer subunits (top) reversibly destroys all quaternary epitopes, but does not affect epitopes encoded by the tertiary structure of NC1 monomers (solid ovals). Dissociation of cross-linked D-hexamers (bottom) selectively destroys quaternary epitopes located at the NC1 interfaces within trimers (checkered ovals), but not those at the trimer-trimer interface within cross-linked NC1 dimers (striped ovals). NC1 cross-links are represented by horizontal lines. (B) APTN alloantibodies bind to dissociation-resistant quaternary epitopes in α3-α5NC1 dimers. Western blot is performed using r-α5NC1 monomers (lane a) and human GBM α345NC1 hexamers (lane b). Staining with Mab5 reveals the position of α5NC1 monomers (M) and α3-α5NC1 dimers (D) subunits. Preincubation of APTN alloantibodies with soluble α5NC1 monomers (5 μg/ml) inhibits binding of APTN alloantibodies to α5NC1 monomers but not to α3-α5NC1 dimers. Preincubation of APTN alloantibodies with α345NC1 hexamers from human GBM (10 μg/ml) inhibits binding to both monomeric and dimeric NC1 subunits. Normal human serum (NHS) was used as negative control. (C) Reconstitution of quaternary APTN epitopes by in vitro association of NC1 monomers. Indirect ELISA showing the binding of α5NC1-unbound alloantibodies (solid bars) to complexes formed by α3NC1 and α4NC1 monomers, by α3NC1 and α5NC1 monomers, and to r-α345NC1 hexamers assembled in vitro. The binding of a α5NC1-bound APTN alloantibodies (open bars) is shown for comparison. (D) Inhibition ELISA showing the binding of APTN alloantibodies to human GBM α345NC1 hexamers in the presence of various concentrations of soluble α3NC1 (●), α4NC1 (▲), and α5NC1 (▲) monomers, as well as in vitro assembled α345NC1 hexamers (▼) and human GBM NC1 hexamers (○). Binding of APTN alloantibodies is completely inhibited by r-α345NC1 hexamers, but not by their component NC1 monomer subunits, with half-maximal inhibition at about 12 ng/ml (apparent kD=0.08 nM). (E–G) Comparison of three immunoassays for APTN alloantibodies. The binding of human IgG alloautoantibodies from Goodpasture patient sera (n=5), sera or allograft eluted alloantibodies from X-linked Alport syndrome patients with APTN (n=5), sera from transplanted X-linked Alport syndrome patients without nephritis (n=12), and NHS (n=6) is assessed by indirect ELISA using immobilized α3NC1 monomers (E), indirect ELISA using immobilized r-α345NC1 hexamers (F), and by sandwich ELISA using α345NC1 hexamers from human GBM captured onto immobilized mAb 26–20 (G). The significance of differences among groups is assessed by one-way ANOVA followed by Bonferroni post tests for pairwise comparisons (ns, not significant; ***P<0.001).
BRIEF COMMUNICATION

Labeled EA and EB) yields cross-reactive alloantibodies that initially bind to PEC, parietal epithelial cells. Parietal epithelial cells may provide another route for alloantibodies. Podo, podocytes; producing anti-GBM alloantibodies. Epitope spreading to tertiary damage to the glomerular reach the interstitium from peritubular capillaries and then bind to hexamers in GBM. When produced in sufficient amounts, anti-α5NC1 alloantibodies may reach the interstitium from peritubular capillaries and then bind to α1256NC1 hexamers in GBM. When produced in sufficient amounts, anti-α5NC1 alloantibodies may reach the interstitium from peritubular capillaries and then bind to α1256NC1 hexamers in the Bowman’s capsule basement membranes (BCBM) of the allograft. During disease, damage to the glomerular filtration barrier and to the “second filtration barrier” formed by parietal epithelial cells may provide another route for alloantibodies. Podo, podocytes; PEC, parietal epithelial cells.

Figure 4. Culprit and victim: A model depicting the pathogenesis of APTN. When a normal kidney is transplanted into X-linked Alport syndrome patients, allogenic collagen IV in the allograft GBM activates alloreactive B cells recognizing quaternary epitopes (striped and checkered ovals) within α345NC1 hexamers, which differentiate into plasma cells producing anti-GBM alloantibodies. Epitope spreading to tertiary α5NC1 epitopes (ovals labeled Eα and Eβ) yields cross-reactive alloantibodies that initially bind to α345NC1 hexamers in GBM. When produced in sufficient amounts, anti-α5NC1 alloantibodies may reach the interstitium from peritubular capillaries and then bind to α1256NC1 hexamers in the Bowman’s capsule basement membranes (BCBM) of the allograft. During disease, damage to the glomerular filtration barrier and to the “second filtration barrier” formed by parietal epithelial cells may provide another route for alloantibodies. Podo, podocytes; PEC, parietal epithelial cells.

Figure 2). Intact mouse GBM collagen IV was not immunogenic in wild type mice expressing α345(IV) collagen, likely reflecting immune self-tolerance toward autologous antigens. Thus, the absence of immune tolerance toward α345(IV) collagen in X-linked Alport syndrome fosters production of anti-GBM alloantibodies. Colda5–null mice also produced anti-α5NC1 antibodies, but only when challenged with antigens lacking native quaternary structure—α5NC1 monomers or dissociated GBM hexamers. These results suggest that quaternary epitopes of α345NC1 hexamers play two key roles in APTN: inciting alloimmunity and providing a target for pathogenic alloantibodies.

We propose the following model for APTN pathogenesis (Figure 4). In transplanted X-linked Alport syndrome patients, native α345(IV) collagen in the allograft activates alloreactive B cells. These, with cognate T help, differentiate into plasma cells producing alloantibodies targeting quaternary epitopes of α345NC1 hexamers in the GBM. Consistent with quaternary epitopes being the initial target of APTN alloantibodies, an analysis of serial serum samples from one APTN patient showed that early in the disease, alloantibodies bound strongly to human GBM hexamers, but poorly to “sheep α3NC1” (an antigen preparation likely containing α5NC1 monomers/dimers), yet the difference lessened as the disease progressed.5 This broadening of the immune response suggests that alloantibodies to tertiary α5NC1 epitopes arise secondarily, possibly by intramolecular epitope spreading, which may be correlated with disease severity. Thus, α345NC1 hexamers are the culprit alloantigen and primary target of all APTN alloantibodies, whereas α1256NC1 hexamers become secondary targets of alloantibodies binding to shared α5NC1 epitopes.

CONCISE METHODS

Proteins
Human GBM NC1 hexamers were purified as described.19 Due to the limited availability of human tissues, we also used homologous bovine hexamers, which have similar subunit organization.23,24 NC1 hexamers from bovine GBM and bovine bladder basement membranes were solubilized by digestion with bacterial collagenase (Worthington, Lakewood, NJ) and purified by passage through a DE-52 ion-exchange column and gel-filtration chromatography.25 Total NC1 hexamers from GBM comprised a mixture of α12NC1 and α345NC1 hexamers, and those from bladder basement membranes comprised a mixture of α12NC1 and α1256NC1 hexamers. For some experiments, α12NC1, α345NC1, and α1256NC1 hexamers were purified by affinity chromatography using rat IgG mAbs b14 (anti-α5NC1) and B66 (anti-α6NC1).23 Recombinant human NC1 monomers were expressed in 293 cells and purified by affinity chromatography on immobilized anti-FLAG mAb.26 In vitro assembly of NC1 monomers into dimers and r-α345NC1 hexamers was performed as previously described.27

Antibodies
Anti-GBM alloantibodies were analyzed in sera (n=3) and allograft eluates (n=2) from five X-linked Alport syndrome patients with APTN, described in previous publications.8 For comparison, we used previously described Goodpasture sera.28 Normal human sera (Innovative Research, Novi, MI) were used as negative controls. We also evaluated the presence of alloantibodies in archived serum samples from 12 transplanted X-linked Alport syndrome patients who did not develop APTN.

ELISA Immunoassays
For indirect ELISA, Maxisorp microtiter plastic plates were coated overnight with r-NC1 monomers (100 ng/well) or NC1 hexamers (300 ng/well) in carbonate buffer, pH 9.6, or NC1 hexamers (300 ng/well) in PBS, pH 7.4, and then blocked with 1% BSA. For capture ELISA, wells precoated with mAb 26–20 (300 ng/well), which specifically binds α345NC1 hexamers,29 were incubated with NC1 hexamers from human GBM (1 μg/well). For inhibition ELISA, alloantibodies were preincubated overnight at room temperature with various concentrations of NC1 antigens before measuring binding to immobilized NC1 monomers and hexamers.
IgG binding was detected with alkaline phosphatase-conjugated goat anti-human or antimouse IgG (Rockland Immunochemical, Gilbertsville, PA) followed by chromogenic substrate. The absorbance values were corrected for background by subtracting the nonspecific binding of human IgG to wells coated with BSA alone (in indirect ELISA) or mAb 26-20 alone (in capture ELISA). The statistical significance was analyzed using GraphPad Prism (GraphPad Software, San Diego, CA), by one-way ANOVA followed by Bonferroni post tests for pairwise comparisons.

**Western Blot Analyses**
NC1 hexamers (500 ng/lane) and r-NC1 monomers (300 ng/lane) were separated by SDS-PAGE in 6%–20% gradient gels under nonreducing conditions and transferred to Immobilon P. Membranes were blocked with 5% blotting grade nonfat dry milk and sequentially incubated with diluted sera, alkaline phosphatase-conjugated secondary antibodies, and chromogenic substrate.

To determine the composition of NC1 hexamers targeted by APTN alloantibodies in the allograft, renal cortex basement membranes isolated from a nephrectomy specimen were digested with collagenase to solubilize NC1 hexamers. Immune complexes consisting of IgG bound to NC1 hexamers were separated from free NC1 hexamers by absorption to protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Science, Piscataway, NJ). After solubilization in sample buffer, separation by SDS-PAGE, and transfer onto Immobilon P, the NC1 domains were analyzed by immunoblotting with mAbs specific for α1-α6NC1 domains, as previously described.10

**Indirect Immunofluorescence**
Cryostat sections (5 μm) of snap-frozen mouse, human, or monkey kidneys embedded in OCT were fixed in acetone at -20°C for 10 minutes. Frozen kidney sections from Col4a3−/− mice transgenically expressing human COL4A3 were used to analyze the specificity of IgG antibodies from mouse sera, as previously described.30 After blocking with 3% normal goat serum and 3% bovine albumin, appropriately diluted primary antibodies were added for 1 hour, and then the sections were stained with AlexaFluor488 goat anti-human or anti-mouse IgG (H+L) (Invitrogen Molecular Probes, Eugene, OR). Before staining, human kidney sections were treated with 0.5 U/ml Ig degrading enzyme IdeS (FabRICATION; Genovis AB, Lund, Sweden) to reduce endogenous IgG background. For inhibition assays, human alloantibodies were preincubated with r-α5NC1 monomers (50 μg/ml). Stained sections were observed with an Axioplan 2 fluorescence microscope (Carl Zeiss MicroImage, Thornwood, NY) and images were captured with AxioVision 4.8 software.

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**DISCLOSURES**
None.

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