The Alloantigenic Sites of α3α4α5(IV) Collagen

PATHOGENIC X-LINKED ALPORT ALLOANTIBODIES TARGET TWO ACCESSIBLE CONFORMATIONAL EPITOPES IN THE α5NC1 DOMAIN*†‡

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Anti-glomerular basement membrane (GBM) antibody nephritis is caused by an autoimmune or alloimmune reaction to the NC1 domain of α5(IV) collagen. Some patients with X-linked Alport syndrome (XLAS) develop post-transplant nephritis mediated by pathogenic anti-GBM alloantibodies to collagen IV chains present in the renal allograft but absent from the tissues of the patient. In this work, the epitopes targeted by alloantibodies from these patients were identified and characterized. All XLS alloantibodies recognized conformational epitopes in the NC1 domain of α5(IV) collagen, which were mapped using chimeric α1/α5 NC1 domains expressed in mammalian cells. Allograft-eluted alloantibodies mainly targeted two conformational alloepitopes mapping to α5NC1 residues 1–45 and 114–168. These regions also encompassed the major epitopes of circulating XLS alloantibodies, which in some patients additionally targeted α5NC1 residues 169–229. Both kidney-eluted and circulating alloantibodies to α5NC1 structurally sequestered epitopes accessible in the α3α4α5NC1 hexamers of human GBM, unlike anti-GBM autoantibodies, which targeted sequestered α3NC1 epitopes. The results identify two immunodominant α5NC1 epitopes as major alloantigenic sites of α3α4α5(IV) collagen specifically implicated in the pathogenesis of post-transplant nephritis in XLS patients. The contrast between the accessibility of these alloepitopes and the crypticity of autoepitopes indicates that distinct molecular forms of antigen may initiate the immunopathogenic processes in the two forms of anti-GBM disease.

Glomerulonephritis is an important cause of renal injury leading to end-stage kidney failure. Some of the most aggressive forms of rapid progressive glomerulonephritis are mediated by IgG anti-glomerular basement membrane (anti-GBM) antibodies, which circulate in patient plasma and bind along the glomerular capillary loops in a linear pattern. Although anti-GBM antibody nephritis can be triggered by either autoimmune or alloimmune mechanisms, both autoantibodies and alloantibodies target the α3α4α5(IV) collagen network of the GBM, producing similar pathology findings and clinical presentations (1).

Studies of the epitopes targeted by anti-GBM antibodies and their relationship to the tertiary and quaternary structures of the antigen can provide insights into the pathogenic mechanisms at the molecular level. For instance, most patients with autoimmune anti-GBM nephritis and its variant, Goodpasture disease, have circulating autoantibodies targeting two conformational epitopes, E\textsubscript{A} and E\textsubscript{P}, within the non-collagenous (NC1) domain of α3(IV) collagen (2). These epitopes are structurally sequestered (cryptic) in the GBM by quaternary interactions among NC1 domains (3, 4), which form hexamer complexes in basement membranes. In the α3α4α5 NC1 hexamer found in the GBM, the accessibility of the α3NC1 autoepitope is limited by its proximity to the α4NC1 and α5NC1 subunits (5). Abnormal exposure of these cryptic epitopes by putative pathogenic factors has been postulated to circumvent immune self-tolerance to α3(IV) collagen, inducing autoantibodies and autoimmune glomerulonephritis.

Anti-GBM alloantibodies also target the NC1 domains of α3α4α5(IV) collagen but are elicited by a distinct mechanism, an alloimmune reaction to “foreign” collagen IV chains in the renal allograft of transplanted Alport patients. In these patients, mutations in the COL4A3 or COL4A4 genes (in autosomal recessive Alport syndrome) or in the COL4A5 gene (in X-linked Alport syndrome, XLAS) prevent the normal assembly of α3α4α5(IV) collagen in the GBM (6), causing progressive glomerulonephritis and eventually end-stage renal failure. Among patients receiving a kidney transplant, about 3–5% develop Alport post-transplant nephritis mediated by anti-GBM alloantibodies, a serious complication resulting in allograft loss in the majority (~88%) of cases (7). The alloantibodies target various

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The abbreviations used are: GBM, glomerular basement membrane; APTN, Alport post-transplant nephritis; NC1, the non-collagenous domain of type IV collagen; XLAS, X-linked Alport syndrome; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney.
subunits of the α3α4α5 NC1 hexamer, depending on the underlying genetic defect. Autosomal recessive Alport syndrome patients produce alloantibodies to α3NC1 and α4NC1 domains (8–11). XLAS patients tend to elicit alloantibodies to α5NC1, although other NC1 subunits may also be targeted (8, 9, 12). Thus far, the alloantigenic sites recognized by X-linked alloantibodies have not been identified, although the majority of cases of post-transplant nephritis occur in patients with XLAS, the most common form of Alport syndrome (~85% cases).

The aim of this study was to identify and characterize the major alloantigenic sites of α3α4α5(IV) collagen in XLAS patients with post-transplant anti-GBM nephritis. Using newly engineered chimeric α1/α5 NC1 domains, two immunodominant conformational alloepitopes within the NC1 domain of α5(IV) collagen were identified as major targets of kidney-bound and circulating X-linked alloantibodies. The accessibility of the XLAS alloepitopes within the α3α4α5 NC1 hexamers of human GBM, contrasting with the crypticity of autoepitopes, implicates distinct molecular forms of the antigen as initiators of the pathogenic process in alloimmune versus autoimmune anti-GBM disease.

**EXPERIMENTAL PROCEDURES**

**Patients**—All alloantibodies characterized in this work were from transplanted male XLAS patients with clinically proven Alport post-transplant nephritis (APTN). Patient APTN-1 had hearing loss and developed end-stage kidney disease at age 20; acid-eluted alloantibodies were available from the fourth allograft. From patient APTN-2 (same as “AS-I” in Ref. 13), the second allograft was stored frozen after nephrectomy. Pieces of allograft were thawed, homogenized, and washed with cold phosphate-buffered saline, pH 7.2, and GBM-bound alloantibodies were eluted with 0.1 M glycine at pH 2.8 and 2.2. The acid eluates were pooled and neutralized with Tris buffer, pH 8.0. By SDS-PAGE analysis, the wash fraction contained plasma proteins (albumin, IgG) trapped in the blood vessels of the renal allograft, including alloantibodies not bound to the allograft GBM. Alloantibodies in the wash fraction had distinct proper-ties from those eluted at low pH, likely representative of the circulating alloantibodies of this patient. From patient APTN-3 (same as “patient c” in Ref. 9), protein A-bound antibodies from therapeutic immunoabsorption were used. Serum positive for anti-GBM alloantibodies by indirect immunofluorescence was available from patient APTN-4, who developed proteinuria and hematuria at 35 days after transplantation; the initial biopsy showed crescentic glomerulonephritis, and linear IgG along the allograft GBM was found at 4 and 7 months post-transplant (14). Another serum was also available from patient APTN-5, described in Ref. 15, and “patient h,” described in Ref. 9. Sera or plasma exchange fluid from patients with autoimmune anti-GBM (Goodpasture) disease were previously described (14) or purchased from Wieslab AB (Lund, Sweden).

**Materials**—NC1 hexamers of collagen IV were isolated from human kidney cortex basement membranes (4); subsets of α1α2, α3α4α5, and α1α2/α5α6 NC1 hexamers were affinity-purified using immobilized mAbs (5). Recombinant human NC1 monomers were expressed in human 293 cells (16). Monoclonal antibody Mab5 to α5NC1 (clone A7) was from Wieslab AB.

**DNA Constructs**—The cDNAs for human α1NC1 and α5NC1 were amplified by PCR using Pfu polymerase (Stratagene) for its low error rate. The α1NC1 cDNA was subcloned with the 5’ end into the Nhel and the 3’ end into the SacII site of a pRC-X expression vector containing the BM40 signal peptide and FLAG peptide (2). The α5NC1 cDNA was ligated into the Nhel and Apal sites of the same vector. Because α1NC1 cDNA contained unique restriction sites for ApaI and NarI and α5NC1 cDNA was from Wieslab AB.

**Cell Transfection and Expression of Recombinant Proteins**—Human embryonic kidney (HEK)-293 cells were cultivated in 90-mm cell culture plates in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 5% fetal bovine serum. For each construct, 4 μg of plasmid DNA was transfected by calcium phosphate co-precipitation into 70% confluent HEK 293 cells. After 2 days, transfected cells were seeded onto a new plate and selected with 250 μg/ml G418. Resistant cells were screened for expression of recombinant proteins by Western

**TABLE 1**

| Chimera | Primer | Sequence 5’ to 3’ direction |
|---------|--------|----------------------------|
| 5111    | A1 F   | aga ccc aag ctt ctc gct gcc t |
| 1511    | A3 F   | ata aaa ggg ccc acg tga act a |
| 1151    | A4 F   | atg tgg cgg ctc aca tcc tgg gcc t |
| 1115    | A6 F   | taa tac gac tca tta tag gg |
|         | B6 R   | aca ggt acc acg gcc gtt aca tcc |

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blot using anti-FLAG mAb M2. After G418-resistant cells reached confluence, culture supernatants were collected every 2 days. Recombinant proteins were affinity-purified on immobilized anti-FLAG M2 mAb columns, concentrated by ultrafiltration, and stored frozen in aliquots.

Electrophoresis and Immunoblotting—Proteins were separated by SDS-polyacrylamide gel electrophoresis in 4–20% gradient gels under non-reducing conditions followed by staining with Coomassie Brilliant Blue or transfer to Immobilon P membranes for Western blotting with antibodies to FLAG or α1–α6 NC1 domains.

Enzyme-linked Immunosorbent Assays (ELISA)—Indirect ELISA was performed in 96-well plastic plates (Nunc-Immuno) coated overnight with purified antigens (100 ng/well) in 50 mM sodium carbonate buffer, pH 9.5, and blocked with 1% bovine serum albumin. In some experiments, the antigen was reduced prior to coating by treatment with 50 mM Tris(2-carboxyethyl)phosphine hydrochloride for 10 min at 60 °C. Binding of human IgG or mouse mAbs to the immobilized antigens was detected with alkaline phosphatase-conjugated antibodies to human or mouse IgG. Hydrolysis of p-nitrophenyl phosphate was monitored at 405 nm using a SpectraMax 190 plate reader (Molecular Devices). For inhibition ELISA, alloantibodies were preincubated overnight at room temperature with various concentrations of NC1 antigens before measuring binding to immobilized α5NC1 or chimeras. Capture ELISA was adapted from Ref. 4, using different pairs of capture and detecting antibodies. NC1 hexamers from human kidney cortex (1 μg), native or dissociated by brief exposure to low pH, were incubated in wells precoated with 300 ng of mAb 8D1 (recognizing α3NC1 epitopes accessible in NC1 hexamers). The binding of human anti-GBM allo- or autoantibodies to captured antigen was detected with alkaline phosphatase-conjugated anti-human IgG.

ELISA measurements were performed in duplicate determinations and repeated three times. The results are shown as means and standard deviations. For each alloantibody, the significance of differences in binding to various antigens was analyzed by one-way analysis of variance followed by Dunnett’s test for multiple comparisons (versus control α1NC1). For overall comparisons among all alloantibodies, the binding data were analyzed by repeated measures analysis of variance. Significance was inferred when p was < 0.05. GraphPad Prism version 4.03 was used for all statistical analyses.

RESULTS

Anti-GBM Alloantibodies from XLAS Patients with Post-transplant Nephritis Primarily Target Conformational Epitopes within the NC1 Domain of α5(IV) Collagen—Before selecting a strategy for mapping the XLAS alloepitopes, the specificity of alloantibodies for recombinant human NC1 monomers expressed in HEK-293 cells was determined by indirect ELISA (Fig. 1). Allograft-eluted XLAS alloantibodies from patient APTN1 (Fig. 1a) reacted strongly and specifically with α5NC1, whereas those from APTN2 (Fig. 1b) also exhibited weak binding to α3NC1 and α4NC1 (~6% relative to α5NC1). Circulating XLAS alloantibodies from all four patients (Fig. 1c–f) bound to α5NC1, and two patients (APTN-4 and -5) also had alloantibodies to α3NC1. Reduction of disulfide bonds in the reactive NC1 monomers strongly decreased the binding of XLAS alloantibodies from all patients by 50–90% (Fig. 1, solid bars), verifying the conformational requirements of the alloepitopes. For comparison, the binding of Mab5 to α5NC1 was not affected by reduction (less than 3% decrease in reactivity; data not shown), indicating that Mab5 recognizes linear epitopes. An overall comparison among all patients revealed that the binding of XLAS alloantibodies to α5NC1 was significantly higher than that to any other NC1 domain; other differences were not statistically significant. To map the conformational α5NC1 alloepitope(s), a strategy based on chimeric α1/α5 NC1 domains was chosen, analogous to those used for mapping conformational α3NC1 epitopes (2, 17). The α1NC1 was selected as inert scaffolding because it is highly homologous to α5NC1 (~83% sequence identity) but did not bind XLAS alloantibodies.

Expression of Chimeric NC1 Constructs for Mapping Conformational α5NC1 Epitopes—The alignment of α1NC1 and α5NC1 sequences identified 38 different residues (17%) clustered in four regions (Fig. 2A). To identify which of these regions encompass the alloepitopes of XLAS alloantibodies, the α5NC1 domain was divided into four quarters, which were substituted into the α1NC1 scaffold, yielding four chimeras designated “5111,” “1511,” “1151,” and “1115” (Fig. 2B). Recombinant α1/α5 NC1 chimeras containing an amino-terminal FLAG-tag were expressed in human 293 cells for correct folding and purified by affinity chromatography (Fig. 3).

Identification of α5NC1 Regions Containing XLAS Alloepitopes and the Conformational Dependence of Alloantibody Binding—The relative binding of XLAS alloantibodies to α1/α5 NC1 chimeras was determined by indirect ELISA, assigning
100% reactivity to α5NC1 (Fig. 4). Three chimeras exhibited significant reactivity for alloantibodies, but the patterns of specificity varied among patients. Alloantibodies from all but one patient bound to chimera 1151 (α5NC1 residues 114–168), which was the sole target of eluted APTN-1 alloantibodies (Fig. 4a) and a major target of circulating alloantibodies APTN-2, -3, and -4 (Fig. 4, c–e). Chimera 5111 (α5NC1 residues 1–45) reacted most strongly with kidney-eluted APTN-2 (Fig. 4b) and circulating APTN-5 (Fig. 4f) alloantibodies. Circulating alloantibodies from patients APTN-3 and APTN-5 also bound to chimera 1115. The alloantibody binding to any of the three chimeras was abolished after the reduction of disulfide bonds, whereas Mab5 binding to chimera 5111 was not affected (see supplemental Fig. 1), indicating that all immunoreactive chimeras harbor conformational XLS alloepitopes.

The inhibition of alloantibody binding to immobilized α5NC1 was fully inhibited only by the same chimera in soluble form, which was as effective as a mixture of all chimeras or the whole α5NC1 (Fig. 5, e–g). The binding of APTN-3 to immobilized α5NC1 (Fig. 5h) was more strongly inhibited by chimera 1151 (46%) than by 5111 (22%) or 1115 (21%). The combined inhibition toward all chimeras was 65%, as compared with 90% by α5NC1, indicating that ~27% of APTN-2 alloantibodies target epitopes spanning two or more α5NC1 regions.

Because serum IgG from patient APTN-3 reacted with three α1/α5 chimeras, the existence of alloantibodies targeting three distinct epitopes was verified by reciprocal inhibition. The binding of APTN-3 serum IgG to each of the immobilized chimeras 5111, 1151, and 1115 was determined after incubation with soluble immunoreactive chimeras (5111 and 1151 in most patients), relative to soluble α5NC1. For kidney-eluted APTN-1 alloantibodies (Fig. 5a), inhibition by chimera 1151 was similar to that produced by α5NC1 (93% versus 97% inhibition at 10 μg/ml antigen), indicating that α5NC1 residues 114–168 fully encompass the alloepitope(s). This epitope region was also immunodominant in the serum APTN-4 (Fig. 5d), the binding of which was inhibited 70% by 1151 but only 7% by 5111 (as compared with 84% by α5NC1). For patient APTN-2, whereas circulating alloantibodies (Fig. 5c) were inhibited 30% by 5111 and 39% by 1151, allograft-bound alloantibodies (Fig. 5b) were significantly more reactive toward 5111 (57%) than 1151 (12%). The inhibition by the combined chimeras was less than that by α5NC1 (60% versus 79% for circulating and 63% versus 89% for kidney eluted alloantibodies), indicating that ~27% of APTN-2 alloantibodies target epitopes spanning two or more α5NC1 regions.

Binding of XLAS Alloantibodies to Human NC1 Hexamers and the Accessibility of Alloepitopes in α3α4α5NC1 Hexamers—Differences in epitope specificity between kidney-eluted and circulating alloantibodies, as in patient APTN-2, suggest that subsets of alloantibodies with higher affinity for the native form of the alloantigen are enriched in the allograft GBM. Consistent with this hypothesis, human kidney NC1 hexamers strongly inhibited the binding to immobilized α5NC1 of kidney-eluted alloantibodies (Fig. 6). The binding to α5NC1 of circulating alloantibodies was also inhibited but at higher concentrations of hexamer, indicat-
ing lower affinity of interaction. Because NC1 hexamers could inhibit the binding to α5NC1 of kidney-bound and circulating XLAS alloantibodies, the α5NC1 alloepitopes must be accessible in the quaternary structure of the NC1 hexamers.

The subsets of NC1 hexamers from human kidney cortex targeted by XLAS alloantibodies were identified by analyzing the composition of NC1 fraction bound or not bound to

**FIGURE 3.** SDS-PAGE and Western blot analysis of α1NC/α5 chimeras. Recombinant α1/α5 chimeras isolated from the culture medium were separated by SDS-PAGE in 4–20% gradient gels under non-reducing conditions and then stained with Coomassie Brilliant Blue (A) or transferred to Immobilon-P membranes for immunoblotting with anti-FLAG mAb (B).

**FIGURE 4.** Epitope specificity of anti-α5NC1 XLAS alloantibodies. Alloantibody binding to immobilized α1/α5NC1 chimeras was measured by indirect ELISA. Binding to α5NC1 was assigned 100%. Chimeras significantly more reactive (p < 0.05) than the control α1NC1 domain (1111, dotted reference line) are indicated by an asterisk. a and b, kidney-eluted APTN-1 and -2 alloantibodies; c–f, circulating APTN-2, -3, -4, and -5 alloantibodies.

**FIGURE 5.** Inhibition of alloantibody binding to α5NC1 by α1/α5 chimeras. XLAS alloantibodies eluted from the kidney allografts of patients APTN-1 (a) and -2 (b) or circulating from patients APTN-2 (c), -3 (e–h), and -4 (d) were incubated overnight with α1/α5 NC1 chimeras or NC1 monomers at the concentration indicated (10 μg/ml for e–g). Alloantibody binding to immobilized α5NC1 (a–d and h), 5111 (e), 1151 (f), and 1115 (g) in the presence of inhibitor was then assayed by indirect ELISA and compared with the binding in the absence of inhibitor, assigned 100%.

**FIGURE 6.** Inhibition of alloantibody binding to α5NC1 by human NC1 hexamers. The binding of kidney-eluted alloantibodies APTN-1 and -2 (filled symbols) and circulating alloantibodies APTN-2 and -3 (open symbols) to immobilized α5NC1 was assayed by indirect ELISA after incubation with various concentrations of human NC1 hexamers.
APTN-1 alloantibody (Fig. 7A). All α3, α4, α5, and α6 NC1 monomers and dimers, along with small amounts of α1NC1 and α2NC1, were found in the alloantibody-bound fraction. The unbound fraction contained only α1NC1 and α2NC1 domains. This result indicates that the alloantibodies bind both α3α4α5 and α1α2α5α6 NC1 hexamers but not α1α2 NC1 hexamers. The reactivity of XLAS alloantibodies with both sets of α5NC1-containing hexamers was also verified by indirect ELISA using affinity-purified human NC1 hexamers (Fig. 7B), for comparison, anti-GBM (Goodpasture) autoantibodies selectively targeted α3α4α5NC1 hexamers. Based on the location of collagen IV networks in normal human kidneys, XLAS alloantibodies bind to α1α2/α5α6(IV) collagen in the Bowman’s capsule basement membrane and to α3α4α5(IV) collagen in the GBM of the renal allograft.

The accessibility of XLAS alloepitopes in α3α4α5 NC1 hexamers was verified by capture ELISA. To avoid alterations of the quaternary structure by harsh elution conditions, native α3α4α5 NC1 hexamers were purified in situ by using immobilized mAb 8D1, which recognizes accessible α3NC1 epitopes. The relative binding of human anti-GBM allo- and autoantibodies to native α3α4α5 NC1 hexamers or their isolated subunits (generated by acid dissociation) was then compared (Fig. 8). For all patients, XLAS alloantibodies reacted strongly with native α3α4α5NC1 hexamers, demonstrating that the alloepitopes are fully accessible in the hexamer structure. In contrast, anti-GBM autoantibodies bound significantly less to native α3α4α5 NC1 hexamers than to dissociated subunits captured by mAb 8D1 (α3NC1 monomers and α3/α5 NC1 dimers), confirming that the autoepitopes are cryptic in the NC1 hexamer. Similar results were obtained by using for capture other mAbs that bind native α3α4α5 NC1 hexamers (not shown).

FIGURE 8. Comparison of the accessibility of anti-GBM alloepitopes and autoepitopes within the α3α4α5 NC1 hexamers. Human α3α4α5NC1 hexamers (N) or their NC1 subunits generated by acid dissociation (D) were captured to immobilized mAb 8D1 (300 ng/well). Binding of XLAS alloantibodies (alloAb), anti-GBM autoantibodies (autoAb), and control sera to captured NC1 domains was then detected with specific secondary antibodies to human IgG.

DISCUSSION

This study identifies for the first time the major alloantigenic sites of α5(IV) collagen, targeted by both kidney-bound and circulating anti-GBM alloantibodies from XLAS patients with post-transplant nephritis. All XLAS alloantibodies characterized in this work reacted predominantly (and some exclusively) with conformational epitopes within the α5NC1 domain. Within the α5NC1 domain, three alloreactive regions were identified based on the binding of XLAS alloantibodies to α1/α5 NC1 chimeras. All three XLAS alloepitopes were conformation-dependent, as shown by the loss of alloantibody binding to α1/α5 chimeras after the reduction of disulfide bonds. Furthermore, the XLAS alloepitopes were found to be accessible in the native α3α4α5NC1 hexamers, and thus, in the α3α4α5(IV) collagen network occurring in the normal human GBM.

In all patients, the immunodominant XLAS alloepitopes were encompassed by chimeras 1151 or 5111 (α5NC1 residues 114–168 and 1–45, respectively). Together, these regions accounted for between 60 and 97% of the α5NC1 immunoreactivity present in kidney-bound and circulating alloantibodies. The alloepitope present in chimera 1151 was exclusively targeted by kidney-bound APTN-1 alloantibodies and a major target of circulating APTN-2, -3, and -4 alloantibodies. The allo-
residues clustered in two narrow regions of the NC1 hexamer (18). The candidate alloepitope residues are: Asp-3, Thr-15, Ala-17, Gln-22, Leu-25, Gln-26, Glu-20, Val-116, His-129, Gln-132, and Asp-135. Notably, most of these residues are accessible in the NC1 hexamer by residues 15–29 and 125–139, designated E_A and E_B (Fig. 9), based on their homology to the respective epitope regions of α3NC1 (2, 3).

A distinctive feature of the XLAS alloepitopes is their accessibility in the α3α4α5 NC1 hexamer. This was demonstrated by three lines of evidence: (a) the ability of human kidney NC1 hexamers to inhibit alloantibody binding to immobilized α5NC1; (b) the complete binding of α3-α6NC1 monomers and dimers to XLAS alloantibodies; and (c) strong reactivity of XLAS alloantibodies with native rather than dissociated α3α4α5NC1 hexamers in capture ELISA. Except for kidney-eluted alloantibodies from one patient, APTN-2 in this study, the accessibility of XLAS alloepitopes in NC1 hexamers has not previously been investigated.

A comparison of the properties of the α5NC1 alloepitopes with those of α3NC1 autoepitopes reveals commonalities and distinctions. The immunodominant alloepitopes and autoepitopes both reside at homologous E_A and E_B regions. However, the alloepitopes are accessible on the surface of the two known subsets of α3α4α5 hexamers, non-cross-linked and cross-linked, whereas the autoepitopes are sequestered within the cross-linked hexamers (4). The contrast between the accessibility of alloepitopes and the crypticity of autoepitopes implicates distinct molecular forms of antigen as initiators of the pathogenic process in anti-GBM disease.

In XLAS patients who develop post-transplant nephritis, the likely antigen is the native form of α3α4α5(IV) collagen present in the renal allograft. Because this network is not assembled in the basement membranes of the affected XLAS patients, B cells specific for epitopes within the α3α4α5NC1 hexamers (whether accessible or sequestered in the quaternary structure) cannot undergo deletion, anergy, or receptor editing. In contrast, anti-GBM autoantibodies do not target exposed epitopes in the α3α4α5NC1 hexamer, suggesting that central or peripheral tolerance to this epitope is normally established in the B cell compartment. Autoimmune anti-GBM (or Goodpasture) disease may be triggered if immune self-tolerance to α3(IV) collagen is circumvented by putative pathogenic modifications that expose cryptic autoepitopes in the NC1 hexamers, for instance by reactive oxygen species (19).

The repertoire of alloreactive T helper cells in XLAS patients may also shape the specificity of the alloantibody response. Because the defect in the COL4A5 gene that causes the loss of α5(IV) chains also prevents the stable incorporation of α3, α4, and α6(IV) chains in the basement membranes of XLAS patients, it is unlikely that B cells reactive with any of these chains undergo negative selection. However, XLAS alloantibodies consistently target α5NC1 epitopes and only occasionally react with α3NC1, whereas α4NC1 or α6NC1 do not appear to be significant targets. The overall pattern of alloantibody specificity is consistent with the primary defect in the COL4A5 gene, leading to a failure to establish T cell tolerance to the missing α5(IV) collagen chain. However, operational tolerance to α3, α4, and α6(IV) chains may still be established in the T cell compartment because at least some, if not all, of these chains continue to be produced intracellularly (20). Alloantibodies to the α3NC1 domain found in some XLAS patients may...
be elicited by epitope spreading; for instance, if B cells recognizing α3NC1 epitopes process α3-α5NC1 dimers and present α5NC1 peptides to activated Th cells specific for α5NC1 epitopes. This mechanism is consistent with the observation that XLAS alloantibodies are initially specific for α5NC1 in the early stages of post-transplant nephritis, but then reactivity to α3NC1 arises as the disease progresses (21).

Detailed knowledge of the B cell and T cell alloepitopes implicated in the pathogenesis of post-transplant nephritis may facilitate the development of preemptive therapeutic strategies aiming to restore immune tolerance to normal α3α4α5(IV) collagen in X-linked Alport patients prior to a renal transplant. Protocols for inducing immune tolerance are widely used to prevent the production of inhibitory alloantibodies against transfused factor VIII in patients with hemophilia A (22). In principle, similar strategies may be developed to induce tolerance in Alport patients before a kidney transplant. Such therapeutic approaches would be particularly beneficial for Alport patients with a history of post-transplant anti-GBM nephritis, who have a very high risk of disease recurrence in subsequent allografts. The availability of dog and mouse models of Alport syndrome provides the necessary platform for experimental studies seeking to understand how immune tolerance to α3α4α5(IV) collagen can be modulated for therapeutic purposes.

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The Alloantigenic Sites of α3α4α5(IV) Collagen: PATHOGENIC X-LINKED ALPORT ALLOANTIBODIES TARGET TWO ACCESSIBLE CONFORMATIONAL EPITOPES IN THE α5NC1 DOMAIN

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