The α5 Chain of Type IV Collagen Is the Target of IgG Autoantibodies in a Novel Autoimmune Disease with Subepidermal Blisters and Renal Insufficiency*

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We describe a novel autoimmune disease characterized by severe subepidermal bullous eruptions and renal insufficiency with IgG autoantibodies directed against the NC1 domain of the α5(IV) collagen chain. In vivo deposits of IgG and C3 were found along the dermal-epidermal junction of skin lesions. The identity of the target antigen was determined by immunohistochemical analyses of candidate antigens using the patients’ autoantibodies. The patients’ IgG autoantibodies reacted with a 185-kDa polypeptide that was distinguished from the known autoantigens of the extracellular matrix including type XVII collagen, type VII collagen, or the α3, β3, and γ2 chains of laminin 5. Preincubation of the serum with recombinant α5(IV)NC1 domain of type IV collagen abolished immunoreactivity with the 185-kDa antigen. The serum reacted specifically with the α5(IV)NC1, among the six NC1 domains of type IV collagen, by Western blot and enzyme-linked immunosorbent assay analyses. The patients’ autoantibodies reacted with normal skin and renal glomerulus but not with skin and glomerulus of a patient with Alport syndrome in which the basement membranes are devoid of the α5(IV) collagen chain. This study provided for the first time unambiguous evidence for the α5(IV) collagen chain as the target antigen in a novel autoimmune disease characterized by skin and renal involvement.

Collagens comprise a family of proteins that constitute the major structural components of the extracellular matrix in a variety of tissues. To date, at least 19 different collagen types have been characterized in vertebrates, with different subunits representing as many as 30 distinct genes (1). The collagens usually comprise a central triple helical collagenous domain flanked by noncollagenous amino- and carboxyl-terminal domains (NC1 and NC2).

Three collagen types have been shown thus far to be the target of autoimmune response in human pathologies: (i) the NC1 domain of α5 chain of type IV collagen in Goodpasture syndrome (2), (ii) the NC1 domain of type VII collagen in epidermolysis bullosa acquisita (EBA) (3), and (iii) a noncollagenous segment of type XVII collagen in bullous pemphigoid (BP) (4). The α3(IV) collagen chain, present in glomerular and alveolar basement membranes, is the target antigen in Goodpasture syndrome, a lethal form of autoimmune disease characterized by glomerulonephritis and pulmonary hemorrhage. Type VII collagen, which forms the anchoring fibrils at the cutaneous basement membrane zone (BMZ), is the target antigen in EBA, a subepidermal blistering skin disease. Type XVII collagen, also known as the 180-kDa bullous pemphigoid antigen and BPAG2, a constituent of anchoring filaments at the cutaneous BMZ, is recognized by IgG autoantibodies from patients with BP, a subepidermal blistering skin disease.

Type IV collagen molecules form a network structure primarily in the basement membranes of various tissues. To date, six genetically distinct type IV collagen polypeptide chains, α1(IV)–α6(IV), have been described (Fig. 1) (5). The α1(IV) and α2(IV) chains, encoded by COL4A1 and COL4A2, respectively, are ubiquitous, whereas α3(IV), α4(IV), α5(IV), and α6(IV) chains, encoded by COL4A3, COL4A4, COL4A5, and COL4A6, respectively, are present in a restricted tissue distribution. The α5(IV) and α6(IV) chains are both present at the dermal-epidermal junction (6), whereas α3(IV), α4(IV), and α5(IV) make up the renal glomerular basement membrane (5, 6). We here show that the α5 chain of type IV collagen is the target antigen in an autoimmune disease distinguishable from other autoimmune diseases described thus far on the basis of its clinical presentation and the antigenic specificity of the autoantibodies.

EXPERIMENTAL PROCEDURES

Patients and Sera—Serum and lesional skin from two patients (patients 1 and 2) with subepidermal blisters and renal insufficiency were used in this study. The patients’ clinical features are discussed elsewhere. The patients had a progressive bullous eruption, covering over 70% of the skin surface. The patients developed uremia during the course of disease, and urinalysis revealed 3+ protein and 1+ red blood; the urine sediment for both patients contained dysmorphic red cells and granular casts. Skin biopsy revealed a subepidermal blister with marked eosinophilic infiltrates. In addition to the patients’ serum samples, sera from the following patients were used in the present study: (i) patients with Goodpasture syndrome with IgG autoantibodies directed against the α5 chain of type IV collagen; (ii) two patients with X-linked Alport syndrome transplanted with normal kidney with circulating antibodies to the α5(IV); (iii) two cases of EBA with circulating antibodies directed against the α1(IV) chain of type IV collagen.

The abbreviations used are: EBA, epidermolysis bullosa acquisita; BMZ, basement membrane zone; BP, bullous pemphigoid; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

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antibodies to the 290-kDa antigen; (iv) two pemphigoid gestationis patients with circulating antibodies to the 180-kDa BPAG2; (v) two cases of linear IgA bullous dermatosis (LABD); (vi) four pemphigus vulgaris patients with circulating antibodies to the 130-kDa desmoglein-3; (vii) a patient with IgG antibodies to the 168-kDa epithelial antigen and (viii) eight unrelated healthy individuals. The monoclonal and polyclonal antibodies used in this study were as follows: (i) a rabbit polyclonal antibody to type IV collagen purified from human placenta (Institut Pasteur de Lyon, Lyon, France); (ii) monoclonal antibodies (mAb) to the α1, α5, and α6 chain of type IV collagen (Wiselab, Lund, Sweden); (iii) a mouse mAb to type IV collagen (Dako SA, Trappes, France); (iv) a mouse mAb to type VII collagen (Serotec, Oxford, United Kingdom); (v) a human mAb to the 230-kDa BP antigen (BPAG1) (7); (vi) a guinea pig polyclonal antibody to rBP55, a BPAG1 recombinant protein (8); and (vii) a rabbit polyclonal antibody to the immunodominant epitopes of the BPAG2 ectodomain (rET7) (9) (the last three items were produced in our laboratory). Secondary antibodies included fluorescein isothiocyanate or rhodamine isothiocyanate-conjugated rabbit antibodies to either human IgG, human IgA, or human IgM (Dako, Glostrup, Denmark); a monoclonal mouse anti-human IgE (Advanced Immuno Chemical, Long Beach, CA); a biotinylated goat anti-human and anti-mouse IgG (Amersham Pharmacia Biotech); alkaline phosphatase and peroxidase-conjugated anti-human and anti-rabbit IgG (Amersham Pharmacia Biotech); a biotinylated anti-rabbit/guinea pig IgG (Dako, Carpinteria, CA).

Immunofluorescence Microscopy—Circulating anti-BMZ antibodies were detected by IF performed on 4-μm cryostat sections of the following tissue substrates: normal human skin, normal human salt-split skin (see below), normal human kidney, and skin and kidney from a patient with X-linked Alport syndrome characterized by the absence of the α5(IV) chain. The dermal-epidermal junction was separated by 1 mM NaCl containing 50 μM phenylmethylsulfonyl fluoride and 0.1 mM EDTA (10).

Skin Antigens—To define further the antigens targeted by the patients’ autoantibodies, the sera were tested against several different BMZ protein extracts or purified proteins. Two different protocols were used for extraction of skin antigens, as described previously (11). Epidermal proteins were obtained from normal human keratinocytes in culture, and dermal proteins were extracted from human dermis after removing the epidermis.

In order to evaluate the immunoreactivity of the patients’ sera against the tissue-specific chains of type IV collagen, we produced fusion proteins corresponding to the NC1 domains of the α1(IV)–α6(IV) chains. These fusion proteins are a Flag fusion containing a Flag epitope (Research Genetics, Huntsville, ALBI Systems, New Haven, CT). The expression vectors for the α3(IV)NC1, α4(IV)NC1, and α5(IV)NC1 were constructed by polymerase chain reaction amplification of the genes from plasmids pD5a3, MM19-14, and MD-6 (12). The construction of the expression vectors for α1(IV)NC1, α2(IV)NC1, and α6(IV)NC1 was accomplished by polymerase chain reaction rearrangement amplification of the genes from a commercially available cDNA library (CLONTECH, Palo Alto, CA), respectively. The following primers were used: 5′-GCT AGC ATC TGT TGA TCA CGG CTT CC-3′ and 5′-CCG CAG TAG CTT AGT GAG CAG GCC CAT CTT CCA CCT-3′ for α1(IV)NC1; 5′-GCT AGC CGT CAG CTT CTA CTT CCT CCA CCT GCC CAG CCG CCG CTT AGC AGG-3′ for α2(IV)NC1; 5′-TAT ATG CTA GCC CAC TAC AGG GAC GAC GAT CAA AAA CCT GGA GAC CAC-3′ and 5′-GCC GAG GCC CCC TAG CTA TAC TTT CCA ACC CAG CAG-3′ for α3(IV)NC1; 5′-ATA ATG CTA GCC GCC TAC AAC AGG GAC GAT GAC GAC CCT GGA GAC CC-3′ and 5′-GCC GAG GCC CCC TAG CTA TAC TTT CCA ACC CAG CAG-3′ for α4(IV)NC1; 5′-TGT GCG CTA GCT GAC GAC GAT GAC GAC GAC GAC CCC CCT GGT GCC GGT CCC CTT TG-3′ and 5′-TAG AAT AGG GCC CTC TAG ATG CTC CGT GGA GCC CAT GGT GGC ACC CCA CAG CTC GCA-3′ for α5(IV)NC1; and 5′-GCT AGC CGT CAG CTT CCA CCT GCC CAG CCG CCG CTT AGC AGG-3′ for α6(IV)NC1.

The secreted proteins carried the Flag sequence fused to the amino terminus of the respective full-length NC1 domain. The proteins were purified by a single affinity chromatographic step using anti-Flag agarose columns.

To evaluate whether the patients’ sera contain antibodies reactive with the other previously identified skin antigens, we used a fusion protein that harbors the carboxyl-terminal part of the BPAG1 (rBP55), a peptide (RG14) that corresponds to the NC16a domain of BPAG2 (type XVII collagen) (RSILPYGDSMDRIE), and a purified form of laminin-5. rBP55 and RG14 contain the major antigenic epitopes for antibodies to BPAG1 and BPAG2, respectively (9). Laminin-5 was purified by affinity chromatography from culture media of squamous carcinoma cells (SCC25 cells) (13). Briefly, culture medium (500 ml) was passed sequentially over 25 ml of gelatin-Sepharose (Amersham Pharmacia Biotech) and 10 ml of BM165-Sepharose (G1 fraction of monoclonal Ab to BM165) equilibrated in PBS. Material bound to BM165-Sepharose was eluted using 1 mM acetic acid.

immunodominant antigen—Gas Chromatography and Electrophoresis and Immunoblot Assay—Proteins were separated by SDS-polyacrylamide gel electrophoresis (4–22% acrylamide gradient gel) under reducing conditions, transferred onto nitrocellulose membranes, and incubated with the sera, as described elsewhere (13).

Immunofluorescence of Nitrocellulose Immunoaffinity-purified Antibodies—The protein bands transferred onto nitrocellulose membranes were cut and immunochemically stained with the serum to identify the band of interest (185 kDa). Horizontal strips of the membrane were cut and immunochemically stained with the patient’s sera to identify the band of interest (185 kDa) and different dilutions (1:20, 1:100). The serum was dialyzed against the buffer, concentrated, and used for immunofluorescence studies.

Enzyme-linked Immunosorbent Assay (ELISA)—To evaluate possible reactivity of the patients’ sera to α1(IV)–α6(IV) chains, we performed an ELISA. We used recombinant proteins corresponding to α1(IV)–α6(IV) NC1, rBP55, and RG14. Microtiter plates (96-well) were coated with the proteins at a concentration of 1.5 μg/ml in PBS buffer, overnight at 4 °C. The plates were washed with PBS-Tween 20% and then incubated with 3% bovine serum albumin at room temperature for 1 h. After incubation, the plates were incubated at 4 °C, with Flag peptide (final concentration, 5 μg/ml) in 1% bovine serum albumin. After washing, the plates were blocked with 1% bovine serum albumin. Coated wells were then incubated with antibodies (dilution 1:100) and patients’ sera (dilution 1:20) for 1 h, washed, and incubated for 45 min with peroxidase-conjugated anti-human or anti-rabbit IgG diluted 1:1000 in PBS. The plates were then washed, and phenylenediamine dihydrochloride was added and allowed to react for 30 min. The absorbance was measured at 405 nm.
Antigen Recognized by Patients' IgG Autoantibodies Is a Normal Component of the Epithelial Basement Membrane Zone—IF microscopy of the patients' perilesional skin revealed linear IgG and C3 deposits along the dermal-epidermal junction in a pattern similar to that obtained from the patients' circulating antibodies with the BMZ of normal human skin (data not shown). Autoantibodies were mainly directed against epitopes residing on the dermal side as revealed by examination of lesional and normal skin treated by 1 M NaCl (Fig. 2). Immunoglobulin class analysis showed that the anti-BMZ antibodies from the patients' sera contained IgG but lacked IgA, IgM, or IgE.

Glomerular Basement Membrane IF Staining of the Patients' Sera Differentiates It from Other Blistering Skin Diseases—Because the patients developed nephropathy during the course of their disease, we sought to determine whether the patients' autoantibodies recognize any component of renal glomerular basement membrane. Interestingly, the patients' sera reacted with normal kidney sections and stained the basement membranes of glomeruli (Fig. 3). This is in contrast to autoantibodies from other blistering diseases including EBA and BP that fail to react with glomerular basement membrane.

Patients' Autoantibodies Label a Unique 185-kDa Polypeptide Corresponding to the α5(IV) Chain of Type IV Collagen—Immunoblots of the patients' sera on dermal extracts revealed a band of 185 kDa (Fig. 4B). This reactivity could be abolished by preincubation of the serum with recombinant α5(IV)NC1 domain of type IV collagen. The specific immunoreactivity of the patients' sera against the α5(IV) NC1 domain was further confirmed by immunoblot assay (Fig. 4C). The anti-α5(IV) alloantibodies clearly reacted with the recombinant α5(IV) NC1 recombinant protein. IgG antibodies eluted from the 185-kDa band further bound the skin and renal BMZ by IF microscopy. These findings confirmed that the IgG autoantibodies that recognize the 185-kDa antigen are indeed antibodies directed against a BMZ component. The patients' sera did not show any reactivity with either the 230- or the 180-kDa BP antigens (Fig. 4A) by immunoblot assay or rBP55 and RG14 by ELISA. There was no IgG reactivity against the type VII collagen (EBA antigen), the 185-kDa cicatricial pemphigoid antigen, or the laminin-5 subunits using the patients' sera by immunoblot assay.

By ELISA, autoantibodies from the patient 1 reacted specifically with α5(IV) NC1 domain with an absorbance value of 1.2 (Fig. 5). The absorbance value was down to 0.2 using a serum sample that was obtained after therapeutic control of blister formation. No specific reactivity was observed with the α1(IV)–α4(IV)NC1 or α6(IV) NC1 domains by either ELISA (values less than 0.3) or immunoblot assay. Control sera from normal individuals or patients with BP or EBA did not react with the α1–α6(IV) NC1 domains by ELISA (values less than 0.3) or by immunoblot assay.

Patients' Autoantibodies Do Not React with the Skin and Glomerular Basement Membranes Deficient in the α5(IV) Collagen Chain—X-linked Alport syndrome is a hereditary renal disease with mutations in genes encoding the α5(IV) collagen chain (5). The skin and glomerulus of the Alport patient are deficient in the α5(IV) collagen chain. We examined by IF the skin (Fig. 6, A–C) and glomerulus (Fig. 6, D–F) using the circulating antibodies from the patients in the present study. The patients' sera did not stain the skin (Fig. 6B) or the glomerular basement membrane (Fig. 6E) from the Alport patient. The absence of the α5(IV) chain in the Alport skin (Fig. 6C) and glomerulus (Fig. 6F) was verified by the absence of reactivity with the α5(IV)NC1 monoclonal antibody. Alport tissues strongly reacted with anti α1(IV) collagen antibody (Fig. 6, A and D).

Loss of Continuous Linear Expression of α5(IV) along the Dermal-Epidermal Junction of Lesional Skin—We stained the patients' lesional skin with mAb to α5(IV) in order to evaluate the expression pattern of α5(IV) as compared with normal human skin (Fig. 7). Anti-α5(IV) revealed a continuous linear staining along the dermal-epidermal junction of a normal skin section (Fig. 7B). This pattern was quite different from that observed in the patients' lesional skin, in which a discontinuous expression of α5(IV) was detected along the dermal-epidermal junction using an anti-α5(IV) mAb (Fig. 7A). This finding
confirmed that the α5(IV) collagen chain is the target antigen within the lesional tissue.

DISCUSSION

In this study, we report, for the first time, the identification of the α5(IV) chain of type IV collagen as the target antigen in a novel autoimmune disease, characterized by sudden onset of extensive bullous eruptions of skin accompanied by mucosal lesions and nephropathy.

The identity of the target antigen was established by immunochemical analyses of several candidate components. The patients’ antibodies reacted with a 185-kDa protein that was distinguished from the 230-kDa or the 180-kDa BP antigens, the type VII collagen EBA antigen (3), the 168-kDa cicatricial pemphigoid antigen (13) or the α3, β3, and γ2 chains of laminin 5 (15, 16) or the β1 integrin (17). Antibodies eluted from the 185-kDa protein, fractionated by electrophoresis, and reacted with the epithelial and renal basement membrane in a fashion analogous to that of whole serum. Preincubation of the patients’ sera with the α5(IV)NC1 domain of type IV collagen abolished the reactivity of the 185-kDa antigen. Furthermore, the sera specifically reacted with the α5(IV)NC1 domain, among the six NC1 domains, by Western blot and ELISA analysis. Targeting the α5(IV) chain was also confirmed at the tissue level, at which an altered expression of α5(IV) had been demonstrated at the lesional skin. These data clearly established the α5(IV) collagen chain as the target autoantigen in our cases.

This unique autoimmune disease represents the second primary human disease in which one of the type IV collagen α chains serves as the target antigen (Fig. 1). In Goodpasture syndrome, in which α3(IV) chain is the target of IgG autoantibodies, no skin lesions develop because the α3(IV) chain is not
present in skin BMZ (18). Furthermore, as was clearly shown by ELISA, Goodpasture’s sera are specific for the α3(IV) NC1, whereas our patients’ sera do not react with this molecule.

Antibodies to α5(IV) chain have been recently identified in few transplanted Alport patients receiving a kidney that normally expresses α5(IV) chain (19). Their skin BMZ does not, however, express α5(IV), and consequently, no blistering can be seen. However, presence of autoantibodies in these cases attests to immunogenicity of α5(IV) chain and provides evidence for the presence of autoreactive T cells against this molecule.

Erythematosus base of the cutaneous lesions as well as marked eosinophil cell infiltrates observed in the lesional biopsy from our cases led us to postulate that these cells play an important role in skin BMZ disruption, as has been already suggested for BP. Eosinophils could play a crucial role in the dermal-epidermal junction of normal human skin (B). Because these patients had circulating antibodies reactive with both the skin and the kidney BMZ, their specific anti-α5(IV) reactivity merits testing.

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FIG. 7. Immunostaining of lesional skin with anti-α5(IV) antibody. A. A discontinuous expression of α5(IV) was observed along the dermal-epidermal junction of lesional skin (patient 1) using an anti-α5(IV) antibody (A). α5(IV) is normally expressed along the dermal-epidermal junction of normal human skin (B).