Laminin-6 and Laminin-5 Are Recognized by Autoantibodies in a Subset of Cicatricial Pemphigoid

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We characterized basement membrane zone (BMZ) autoantigens targeted by autoantibodies (AAb) from patients with cicatricial pemphigoid. Serum from a patient with severe oral cicatricial pemphigoid contained IgG anti-BMZ AAb. The AAb labeled a lower BMZ component on salt-split skin and localized to the lower lamina lucida/lamina densa by direct and indirect immunoelectron microscopy (IEM) but did not label blood vessels. The AAb did not react with EHS laminin-1 and type IV collagen, pepsinized human type IV collagen, recombinant entactin, or NC1 domain of type VII collagen by dot blotting and western blotting. We focused our studies on the laminin family, as laminin-5 was identified as an autoantigen in cicatricial pemphigoid. Culture-conditioned media from normal keratinocytes (containing laminin-6 and laminin-5) and JEB keratinocytes (containing laminin-6 but not laminin-5) were studied by western blotting. Under nonreducing conditions, the patient's AAb recognized a 600-kDa protein (laminin-6) intensely and a 400-kDa protein (laminin-5) weakly in normal keratinocyte medium even though abundant laminin-5 was present. In JEB keratinocyte medium, however, the 600-kDa protein (laminin-6) alone was recognized by the patient's AAb. The AAb also immunolabeled BMZ of JEB skin that lacked laminin-5. The AAb from this patient and two other patients with anti–laminin-5 cicatricial pemphigoid immunoprecipitated both laminin-6 and laminin-5. Taken together, the results of IEM, nonreducing western blotting, immunoprecipitation, and JEB skin BMZ immunolabeling indicate that laminin-6, as well as laminin-5, is identified by the AAb from a subset of cicatricial pemphigoid patients. We propose the name "*anti-laminin cicatricial pemphigoid*" for this subset. ***Key words: blister/epidermolysis bullosa. J Invest Dermatol 108:848–853, 1997***

Skin basement membrane zone (BMZ) autoantigens characterized include the hemidesmosome/upper lamina lucida–located bullous pemphigoid antigens (BP230 and BP180) (Stanley *et al*, 1981; Labib *et al*, 1986) and LAD-1, the linear IgA bullous dermatosis antigen (Zone *et al*, 1990; Marinkovich *et al*, 1996), the lower lamina lucida–located laminin-5 (previously named kalinin, epiligrin, nicein, BM600) (Domloge-Hultsch *et al*, 1992; Kirtschig *et al*, 1995), and p105 (Chan *et al*, 1993; 1995a; Chan and Woodley, 1996), and the sub-lamina densa–located type VII collagen (epidermolysis bullosa acquisita antigen) (Woodley *et al*, 1984). Other well-defined BMZ components, including laminin-1, $\alpha 6\beta 4$ integrin, type IV collagen, perlecan, entactin/nidogen, plectin, and laminin-6 (previously named k-laminin), have not been reported as being targeted by autoantibodies (AAb) in blistering skin diseases (Stepp *et al*, 1990; Tsao *et al*, 1990; Marinkovich *et al*, 1992a; Iozzo, 1994; Kuhn, 1994; Timpl and Brown, 1994; Liu *et al*, 1996).

Manuscript received May 14, 1996; revised February 24, 1997; accepted for publication March 1, 1997.

This paper was presented in part at the Annual Meeting of the Society for Investigative Dermatology, Washington, DC, May 1–5, 1996.

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The laminin family consists of a group of heterotrimers of various combinations of three chains, α, β, and γ (Burgeson *et al*, 1994). Laminin-6 is a laminin variant with an expression restricted to anchoring filament–containing basement membranes (Marinkovich *et al*, 1992a). Laminin-6, a heterotrimer of $\alpha 3\beta 1\gamma 1$-chains secreted by epidermal cells, shares structural and immunologic similarities with laminin-1 (heterotrimer of $\alpha 1\beta 1\gamma 1$) and laminin-5 (heterotrimer of $\alpha 3\beta 3\gamma 2$) (Marinkovich *et al*, 1992a). Laminin-6 is recognized by anti–laminin-1 antibody due to its reactivity with $\beta 1$- and $\gamma 1$-chains and is recognized by monoclonal antibody (MoAb) BM165 due to its reactivity with $\alpha 3$-chain (Marinkovich *et al*, 1992a). Polyclonal anti–laminin-5 antibody recognizes both laminin-5 and laminin-6 under nonreducing conditions, but recognizes only laminin-5 under reducing conditions (Marinkovich *et al*, 1992a). In this paper, we describe BMZ components identified by AAb from a patient with oral cicatricial pemphigoid, an immune-mediated subepithelial blistering disease. Because laminin-5 has been identified as a target antigen in cicatricial pemphigoid (Domloge-Hultsch *et al*, 1992), we focused our investigations on the laminin family and demonstrate that laminin-6 and laminin-5 are the co-target antigens for the AAb. We further confirm these co-target antigens with AAb from two additional patients who had been diagnosed to have "anti–laminin-5 cicatricial pemphigoid." Because a pure, non–cross-reacting, anti–laminin-6 antibody is not

available, the identification of laminin-6 is based on comparative studies using antibodies that cross-react with laminin-6 and laminin-5 as probes.

In junctional epidermolysis bullosa (JEB), an inherited blistering disease characterized by hemidesmosomal anchoring filament abnormalities (Marinkovich, 1993), many patients show absence of laminin-5 expression in skin BMZ (Meneguzzi *et al*, 1992). The genetic defects of Herlitz type JEB have been documented at the molecular level (Aberdam *et al*, 1994; Pulkkinen *et al*, 1994). MoAb BM165, which cross-reacts with both laminin-5 and laminin-6 (Marinkovich *et al*, 1992a), shows reduced but positive labeling of the BMZ in JEB skin that shows absence of laminin-5 staining by non–cross-reacting laminin-5 antibody (Meneguzzi *et al*, 1992). It has been shown that the labeling of MoAb BM165 in the JEB skin BMZ is due to the presence of laminin-6 in these patients as demonstrated by radioimmunoprecipitation of keratinocyte-conditioned medium derived from these patients (Marinkovich *et al*, 1993). For this reason, we also included JEB skin sections in our study.

CASE REPORT

A 43-y-old woman had painful blisters and erosions in her oral cavity for 19 y. Her past medical history included allergic rhinitis and asthma. Physical examination revealed a few blisters and extensive erosions of her pharynx, dorsal tongue, and some erythema on her buccal mucosae. Her ocular mucosae and skin were free of disease. Histopathologic examination of an oral mucosal lesion demonstrated a subepithelial blister and a prominent mononuclear inflammatory cell infiltrate within the lamina propria. Routine direct immunofluorescence microscopy perfomed on the patient's lesional and perilesional biopsy specimens detected a linear pattern of IgG deposits at the epithelial BMZ. Direct immunofluorescence microscopy performed on 1 M sodium chloride-split patient's epithelial tissue localized the IgG deposits exclusively to the dermal side of the BMZ (Gammon *et al*, 1984). The patient's disease did not respond to treatments of topical or systemic corticosteroids, topical or systemic retinoids, dapsone, colchicine, or topical cyclosporin A. The patient's epithelial tissue and serum were obtained for further studies as outlined below.

MATERIALS AND METHODS

BMZ Proteins and Anti-BMZ Antibodies The following purified or recombinant BMZ proteins were either purchased from commercial sources or obtained as gifts from other investigators: EHS type IV collagen and pepsinized human type IV collagen (Sigma Chemical Co., St. Louis, MO), EHS laminin-1 (Rao Chilikuri, Northwestern University, Chicago, IL), an eukaryotic recombinant human type VII collagen (full length NC1, Chen *et al*, 1997a), recombinant entactin (Tsao *et al*, 1990). Primary antibodies against basement membrane components were either purchased commercially or obtained as gifts: polyclonal anti–laminin-1 (GIBCO-BRL, Grand Island, NY), MoAb anti–laminin-1, anti-type IV and VII collagens (Sigma), MoAb K140 and BM165 (specific for laminin β3-chain and α3-chain, respectively, Rouselle *et al*, 1991; Marinkovich *et al*, 1992b), polyclonal anti-type IV collagen and monoclonal anti-perlecan (John Couchman, University of Alabama), polyclonal anti-entactin (Tsao *et al*, 1990), and polyclonal anti–laminin-5 (Marinkovich *et al*, 1992b). In addition, goat anti-human factor VIII–related antigen was purchased from Atlantic antibodies (Stillwater, MN).

Immunofluorescence Microscopy To determine the immunoreactivity of the patient's circulating AAb to BMZ, indirect immunofluorescence microscopy was performed on various tissue substrates including normal neonatal foreskin, monkey esophagus, and mouse skin. In addition, we also included skin substrates from a patient with Herlitz junctional epidermolysis bullosa (JEB) totally lacking BMZ expression of laminin-5 (β3-chain mutation[1]), and skin substrates from patients with dystrophic epidermolysis bullosa (n = 3) and epidermolysis bullosa simplex (n = 1). The patient's serum (at 1:10 dilution) was used as the first step of incubation, followed by incubation with fluorescein-conjugated goat anti-human IgG (Cappel, Durham, NC) (Chan *et al*, 1993). To determine the IgG subclasses of the patient's anti-BMZ AAb, the incubation of the patient's serum was followed by incubation with fluorescein-conjugated MoAb to human IgG1, IgG2, IgG3, and IgG4 (Sigma). To determine the relative location of the autoantigen targeted by the patient's IgG AAb, 1 M sodium chloride-split normal human skin was used as substrate (Gammon *et al*, 1984). Normal human serum diluted at 1:10 was used as negative control throughout the experiment. Sera from patients with typical epidermolysis bullosa acquisita and bullous pemphigoid were used as positive controls to ensure the proper split of the substrate and to ensure the preservation of the BMZ antigens. Fluorescein-conjugated goat antibodies to rabbit IgG (Cappel) and mouse IgG (γ-chain–specific, Kirkegaard and Perry, Gaithersburg, MD), and fluorescein-conjugated rabbit anti-goat IgG (Cappel) were used as secondary antibodies.

Keratinocyte Cultures Primary keratinocytes were cultured from normal foreskin and immortalized keratinocytes were established from skin from a patient with JEB as previously described (γ2-chain mutation, Miquel *et al*, 1996; Marinkovich *et al* 1992a). Conditioned medium from confluent culture was collected, concentrated by serial centrifugation with Centricon concentrator (W.R. Grace and Co., Beverly, MA), and stored at −80°C until immunoblotting was performed.

Western Blotting and Dot Blotting Analyses Western blotting experiments were carried out by 5 or 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) followed by electrophoretic transfer to nitrocellulose membranes (Towbin *et al*, 1979). The electrophoresis was carried out with a mini gel apparatus (Novex, San Diego, CA), and the proteins were vertically separated with a constant 125 voltage mode. After the completion of electrophoresis, the separated proteins were horizontally transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) with a constant 33 voltage mode. After the transfer efficiency was assessed by a reversible Ponceau S stain (Sigma), and the nonspecific binding was blocked by 5% powdered milk, the membranes then were reacted with primary antibodies (diluted in Tris-buffered saline plus 1% bovine serum albumin) at 4°C overnight. The membranes were subsequently reacted at room temperature for 1 h, with peroxidase-conjugated antibodies: goat anti-human IgG (γ-chain–specific, Kirkegaard and Perry), goat anti-rabbit IgG (Cappel), or goat anti-mouse IgG (γ-chain–specific, Kirkegaard and Perry). The immunoreactions were visualized with a peroxidase substrate 4-chloro-1-naphthol (Bio-Rad) (Chan *et al*, 1995a). When nonreducing conditions were desired, β-mercaptoethanol was omitted from the sample buffer. For dot blotting experiments, 3 to 7 μg of purified or recombinant proteins were vacuum-blotted onto nitrocellulose membranes with a dot-blot apparatus (Bio-Rad). After the blotting efficiency was checked by Ponceau S stain, the nitrocellulose membranes were blocked with powdered milk, incubated with primary and secondary antibodies, and reacted with enzyme substrate as described above.

Immunoprecipitation Immunoprecipitation experiments were carried out with [^{35}S]methionine/cysteine–metabolically labeled keratinocyte-conditioned media as previously described (Marinkovich *et al*, 1992a, 1992b). Briefly, keratinocytes derived from normal donor and a patient with JEB lacking laminin-5 were grown and subpassaged in keratinocyte growth medium. Cells were subpassaged, allowed to attach for 12 h, and then incubated in methionine/cysteine–free keratinocyte growth medium, plus ^{35}S-labeled methionine/cysteine mixture (50 μCi per ml, Amersham Corp. Danvers, MA) for an additional 24 h. Immunoprecipitation experiments were carried out as described previously (Marinkovich *et al*, 1992a) except that the irreversible serine protease inhibitor AEBSF (Calbiochem, La Jolla, CA) was added to a final concentration of 1 mM to the radiolabeled conditioned medium samples after collection and prior to immunoprecipitation. Fibronectin was removed by pre-cleansing with gelatin-Sepharose. Sera from two patients with "anti–laminin-5 cicatricial pemphigoid" (Domloge-Hultsch *et al*, 1992) and MoAb 165 were also included in the studies.

Immunoelectron Microscopy Direct IEM was performed with a post-embedding immunogold method on epithelial tissue obtained from the patient, as described previously (Schaumburg-Lever *et al*, 1994). Indirect IEM was performed with the patient's serum on neonatal foreskin substrate using an established method with some modifications (Holubar *et al*, 1975; Chan *et al*, 1995b). Briefly, 14 μm-thick cryosections of skin placed on gelatin-coated glass slides were incubated with the patient's serum at 37°C for 45 min, followed by incubation of fluorescein-labeled goat anti-human IgG (γ-chain–specific, Kirkegaard and Perry) under the same conditions. The slides were then subjected to serial reactions at room temperature for 30 min: rabbit anti-goat IgG (Cappel), goat anti-horseradish peroxidase (Cappel), then type IV horseradish peroxidase (Sigma). After the slides were

[1] Lanigan C, Hoeffler WK, Cantrell C, Coberly S, Tzou C, Snively G, Brimhall E, Woodley DT, Bauer EA: Mutations in the β3 (B1) chain of laminin 5 (nicein/kalinin) in Herlitz's junctional epidermolysis bullosa. *J Invest Dermatol* 104:603A, 1995 (abstr).

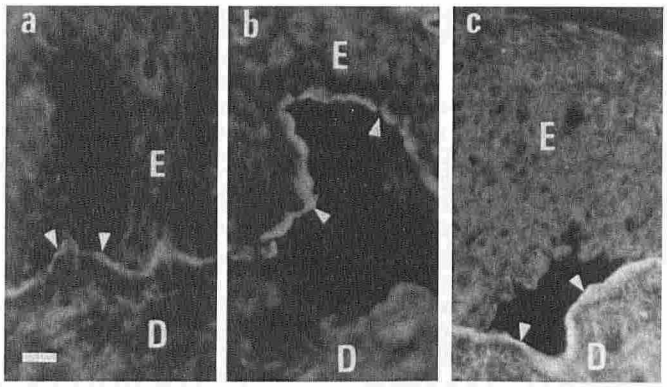

Figure 1. The patient's IgG AAb labeled the dermal side of BMZ in 1.0 M sodium chloride-split normal human skin substrate. Salt-split skin substrates were incubated with serum from a patient with oral cicatricial pemphigoid *(a)*, a patient with typical bullous pemphigoid *(b)*, and a patient with typical epidermolysis bullosa acquisita *(c)*, followed by incubation with fluorescein-conjugated goat anti-human IgG. E, epidermis; D, dermis; arrowheads indicate IgG-binding sites. *Scale bar*, 9 μm.

fixed in 2.5% glutaradelhyde, they were reacted with 0.15% Hanker-Yates solutions (Polysciences, Warrington, PA) in the presence of 0.01% hydrogen peroxide. The slides were then post-fixed with 1% osmium tetroxide in cacodylate buffer, dehydrated through graded ethanol, and embedded in a Spurr/Araldite mixture (EM Sciences, Warrington, PA). Ultrathin sections were post-stained with 0.3% uranyl acetate–lead citrate and examined and photographed under a transmission electron microscope (model HU-12A, Hitachi Ltd. Tokyo, Japan).

RESULTS

The Patient's IgG Autoantibodies Labeled Normal Skin BMZ and Laminin-5–Deficient Skin BMZ On salt-split normal adult human skin substrates, the patient's IgG AAb labeled a BMZ component at the dermal side of the split, similar to that of epidermolysis bullosa acquisita, but distinct from that of bullous pemphigoid **(Fig 1)**. The patient's IgG anti-BMZ AAb were identified to be IgG2, IgG3, and IgG4 subclasses (data not shown). The patient's IgG AAb labeled, in addition to adult skin, the BMZ of neonatal human foreskin and monkey esophagus, but not of mouse skin. In nonlesional skin obtained from a Herlitz JEB patient with total absence of BMZ laminin-5 expression, this patient's IgG AAb labeled the BMZ with reduced intensity compared to normal human BMZ **(Fig 2)**. This pattern of immunostaining is similar to the pattern with MoAb BM165, which recognizes both laminin-5 and laminin-6 (Marinkovich *et al*, 1993). In contrast, laminin-5 as detected by MoAb K140, which recognizes laminin-5 but not laminin-6, was completely absent in the skin BMZ of this JEB patient **(Fig 2)**. In addition, the patient's AAb labeled BMZ of skin sections from patients with dystrophic epidermolysis bullosa (n = 3) and epidermolysis bullosa simplex (n = 1) with the same intensity as that of normal neonatal foreskin sections (data not shown). Unlike antibodies to BMZ components laminin-1, type IV collagen, entactin, and perlecan, the patient's AAb did not label dermal blood vessels. This lack of immunolabeling of blood vessel components was verified by direct comparison with antibodies to factor VIII-related antigen, a prominent blood vessel component (data not shown).

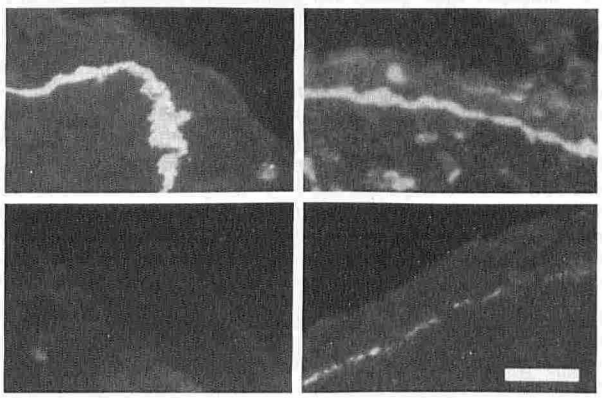

Figure 2. The patient's IgG AAb labeled a BMZ component in a Herlitz JEB patient skin that lacks laminin-5 expression (due to β3-chain mutation) with reduced intensity. The MoAb specific for laminin-5 β3-chain (K140) *(left column)* and the patient's serum *(right column)* were reacted with a normal human skin *(top row)* and the JEB patient skin *(bottom row)*, followed by reaction with fluorescein-conjugated antibodies to mouse IgG *(left column)* and human IgG *(right column)*. *Scale bar*, 50 μm.

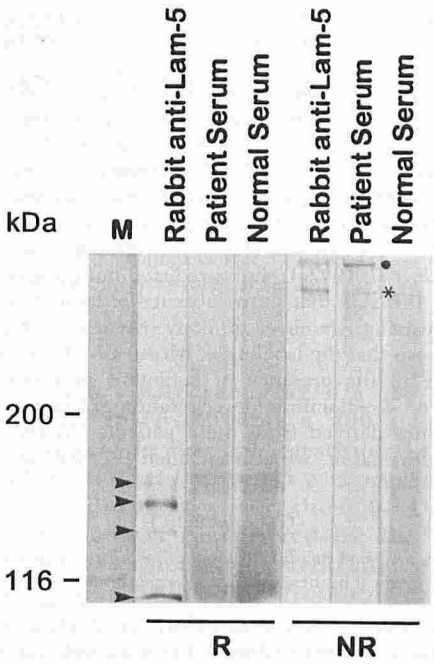

Figure 3. The patient's IgG AAb recognized laminin-6 and laminin-5 in normal keratinocyte-conditioned medium. Normal keratinocyte-conditioned medium proteins electrophoretically separated by a 5% sodium dodecyl sulfate-PAGE under reducing (R) or nonreducing (NR) conditions were transferred to nitrocellulose membranes, reacted with polyclonal anti–laminin-5, the oral cicatricial pemphigoid patient serum, or normal human serum, then visualized with peroxidase-conjugated goat antibodies to rabbit IgG or human IgG. The patient's IgG AAb did not react under reducing conditions, but under nonreducing conditions, the patient's IgG AAb reacted strongly with a 600-kDa laminin-6 protein (●) that was weakly reacted by anti–laminin-5 and reacted weakly with a 400-kDa laminin-5 protein (*) that was strongly identified by anti–laminin-5. M, molecular weight standards.

The Patient's IgG Autoantibodies Recognized both Laminin-6 and Laminin-5 Proteins on Nonreducing Western Blots On dot blotting and western blotting experiments, the patient's AAb failed to recognize the following purified or recombinant BMZ proteins under native or denatured conditions: EHS laminin-1 and type IV collagen, pepsinized human type IV collagen, recombinant entactin, and NC1 domain of type VII collagen, when compared to positive control antibodies (data not shown). Under reducing conditions, the patient's IgG AAb was nonreactive with laminin-5 in normal keratinocyte-conditioned medium, even though laminin-5 was clearly present in the sample as detected by polyclonal anti–laminin-5 **(Fig 3)**. Nonreduced western blots of normal keratinocyte medium demonstrated 600-kDa and 400-kDa protein bands **(Fig 3)**. The patient's AAb reacted intensely with the

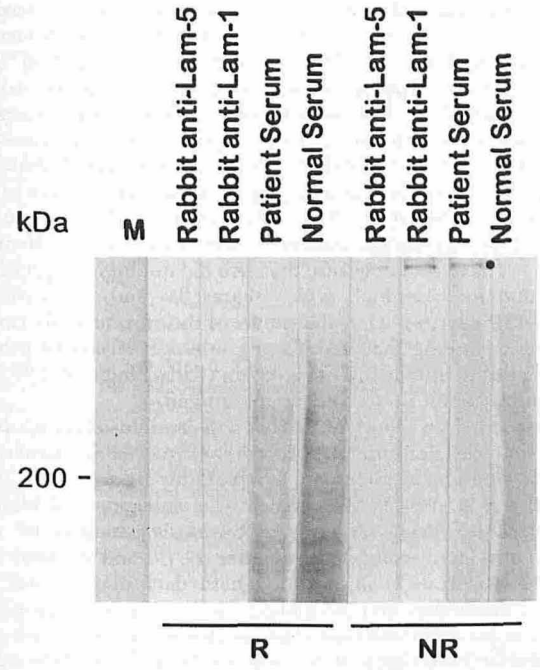

Figure 4. The patient's IgG AAb recognized laminin-6 in keratinocyte-conditioned medium from a Herlitz JEB patient lacking laminin-5 (due to γ2-chain mutation). The JEB keratinocyte-conditioned medium proteins electrophoretically separated by sodium dodecyl sulfate–PAGE under reduced (R) or nonreducing conditions (NR) were transferred to nitrocellulose membranes, reacted with polyclonal anti–laminin-5, anti–laminin-1, the oral cicatricial pemphigoid patient serum, or normal human serum, then visualized with peroxidase-conjugated goat antibodies to rabbit IgG or human IgG. The patient's IgG AAb reacted with a 600-kDa laminin-6 protein (●) that co-migrated with a protein band weakly recognized by anti–laminin-5 and with the lower of the doublet bands recognized by anti–laminin-1 only under nonreducing conditions. M, molecular weight standards.

upper band and weakly with the lower band, whereas polyclonal anti–laminin-5 reacted intensely with the lower band and weakly with the upper band. Similarly, using a keratinocyte-conditioned medium derived from a patient with Herlitz JEB in whom absence of BMZ laminin-5 secretion was demonstrated, the patient's AAb recognized a 600-kDa protein band co-migrating with a band weakly recognized by polyclonal anti–laminin-5 and the lower of the doublet protein bands recognized by polyclonal anti–laminin-1, under nonreducing conditions **(Fig 4)**. The reactivity of the 600-kDa protein band clearly identifies it as laminin-6 (Marinkovich *et al*, 1992a).

The Patient's IgG Autoantibodies' Binding Sites Were Localized to the Lower Lamina Lucida/Lamina Densa Direct IEM localized the gold particles to the lower lamina lucida/lamina densa area. No gold particles were observed within the upper lamina lucida or the sub-lamina densa area **(Fig 5)**. Similarly, indirect IEM using a peroxidase technique revealed that the patient's autoantibodies bound to an antigenic site at the lower lamina lucida/lamina densa area. No immune deposits were observed in the upper lamina lucida or sub-lamina densa zone (data not shown). The localization of the antigenic site of this patient's AAb is somewhat similar to the antigenic site in the lower lamina lucida for AAb to laminin-5 in patients with a variant of cicatricial pemphigoid (Domloge-Hultsch *et al*, 1992) and to that for AAb against a 105-kDa lower lamina lucida protein p105 (Chan *et al*, 1993). This lamina densa AAb-binding site is distinct from that of the AAb for bullous pemphigoid (Holubar *et al*, 1975), linear IgA bullous dermatosis (Chan *et al*, 1995b), and epidermolysis bullosa acquisita (Woodley *et al*, 1984).

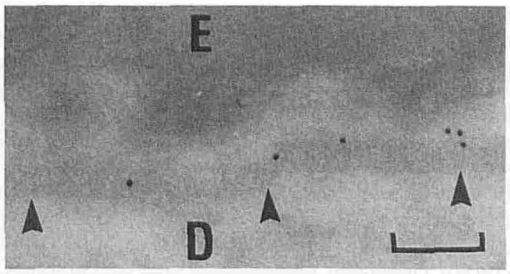

Figure 5. The antigenic site of the patient's IgG AAb is at the lower lamina lucida/lamina densa of BMZ. Direct IEM using a post-embedding immunogold method localized the *in vivo*-bound IgG deposits (as indicated by the gold particles) to the lower lamina lucida and lamina densa (indicated by ▲) of the BMZ. E, epidermis; D, dermis. *Scale bar*, 0.46 μm.

IgG Autoantibodies from the Index Patient and Two Other Patients with Anti–Laminin-5 Cicatricial Pemphigoid Recognized Both Laminin-6 and Laminin-5 Native Proteins Using normal human and JEB keratinocyte-conditioned media as substrates, the patient's AAb immunoprecipitated a 600–700 kDa band that was also immunoprecipitated by polyclonal anti–laminin-1 and MoAb BM165 when the precipitants were analyzed in nonreducing gels. This 600–700 kDa disulfide-bonded complex was reduced to triple protein bands of a doublet of 220 and 210 kDa and a 190-kDa band when these precipitates were analyzed on reducing gels **(Fig 6)**. These immunoprecipitation patterns further

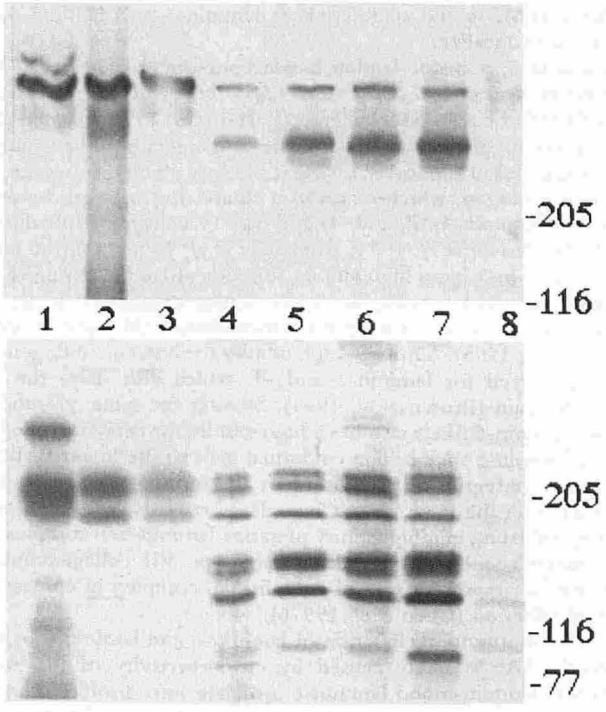

Figure 6. AAb from multiple cicatricial pemphigoid patients recognize laminin-6. Cultured Herlitz JEB keratinocytes (γ2-chain mutation; *lanes 1,2,3*) and normal neonatal foreskin keratinocytes *(lanes 4,5,6,7,8)* were radiolabeled, and the conditioned medium was immunoprecipitated, separated on a 3 to 5% gradient sodium dodecyl sulfate-polyacrylamide gel under nonreducing conditions *(upper panel)* or a 6% gel under reducing conditions *(lower panel)*, using the following antibodies: polyclonal anti–laminin-1 *(lane 1)*; index patient's serum *(lanes 2,4)*; MoAb BM165 (anti-α3; *lanes 3,7*), sera from two additional patients with "anti–laminin-5 cicatricial pemphigoid" *(lanes 5,6)*, and no primary antibody *(lane 8)*. Molecular weight marker positions are shown on the *right*.

confirmed that the patient's AAb recognized laminin-6, as described previously (Marinkovich *et al*, 1992a). The possibility that these bands were laminin-5 is precluded by their presence in conditioned medium derived from a JEB patient keratinocyte, which totally lacks laminin-5 secretion. With normal human keratinocyte-conditioned medium as substrate, the patient's AAb immunoprecipitated, in addition to laminin-6, the laminin-5 bands (400–450 kDa on nonreducing gels; 165, 140, and 105 kDa on reducing gels) **(Fig 6)** (Rousselle *et al*, 1991; Domloge-Hultsch *et al*, 1992; Kirtschig *et al*, 1995). Sera from two other patients with anti–laminin-5 cicatricial pemphigoid also immunoprecipitated both laminin-6 and laminin-5, as did MoAb BM165 **(Fig 6)**.

DISCUSSION

We have demonstrated that the IgG AAb from three patients with cicatricial pemphigoid bind a laminin variant, laminin-6, as well as laminin-5. One patient, whom we studied in detail, produced AAb that did not recognize laminin-1, type IV or VII collagens, perlecan, or entactin, but did recognize laminin-6 on nonreducing western blots; immunoprecipitated laminin-6 in normal keratinocyte-conditioned medium as well as in JEB keratinocyte-conditioned medium, which lacks laminin-5 secretion; and localized to the lower lamina lucida/lamina densa area of BMZ by direct and indirect IEM. The reduced but positive labeling of the Herlitz JEB patient skin BMZ by the AAb of this patient indicates that the autoantigen is present in a BMZ that lacks laminin-5, thus further supporting the notion that laminin-6 is one of the target antigens.

MoAb BM165, which recognizes both laminin-5 and laminin-6, ultrastructurally localized to anchoring filaments in the mid to lower lamina lucida area of BMZ. The AAb of this patient, which recognized both laminin-6 and laminin-5, localized to an antigenic site at the lower lamina lucida/lamina densa by both direct and indirect IEM, in the region where laminin-5 and laminin-6 are believed to localize.

Laminin-5, a major lamina lucida component that serves as a preferred ligand for $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins in the plasma membrane of basal epithelial cells (Carter *et al*, 1991; Niessen *et al*, 1994), seems to be a good candidate in connecting other components situated in the lower BMZ. It has low affinity, however, for entactin/nidogen, which serves as a connecting network between laminins (laminin-1, -2, and -4) and type IV collagen (Aumailley *et al*, 1989; Brown *et al*, 1994; Aumailley *et al*, 1996). On the other hand, laminin-1 has a high affinity for entactin/nidogen due to the fourth epidermal growth factor-like repeat domain III in its $\gamma 1$-subclass chain, which is not present in laminin-5 (Mayer *et al*, 1993; Mayer *et al*, 1995). Similarly high affinity for entactin/nidogen has been observed for laminin-2 and -4, which also share the $\gamma 1$-subclass chain (Brown *et al*, 1994). Sharing the same $\gamma 1$-subclass chain, laminin-6 likely also has a high affinity for entactin/nidogen. Thus, laminin-5 may bridge epidermal cells to the lower BMZ by linking the integrins on the outer part and forming a complex with laminin-6 on the inner part (Champliaud *et al*, 1996). Our recent finding of strong binding affinity of native laminin-5/6 complex for the noncollagenous (NC1) domain of type VII collagen further indicates an important role of laminin-5/6 complex in epidermal-dermal adhesion (Chen *et al*, 1997b).

The co-immunoprecipitation of laminin-6 and laminin-5 by this patient's AAb is likely caused by cross-reactivity of the AAb. Whereas laminin-5 and laminin-6 associate into disulfide-bonded complex in tissue (Champliaud *et al*, 1996), this complex does not occur in cell culture, as is evidenced by the fact that multiple anti–laminin-5 MoAbs including MoAbs K140 and GB3 (anti-$\beta 3$ and anti-$\gamma 2$, respectively; Marinkovich *et al*, 1993; Matsui *et al*, 1995) clearly do not co-precipitate laminin-6. Also, polyclonal anti–laminin-1 antibodies and MoAbs against laminin-1 $\beta 1$- and $\gamma 1$-chains immunoprecipitate laminin-1 and laminin-6 from radiolabeled keratinocyte-conditioned medium but clearly show no evidence of co-precipitating laminin-5 (Marinkovich *et al*, 1992a). If laminin-5 and laminin-6 were present as a disulfide-bonded complex in culture medium, co-precipitation of laminin-6 would occur with all antibodies against any of the laminin-5 or laminin-6 chains. This is clearly not the case. Only in the instance of anti–$\alpha 3$-chain antibody such as BM165 is co-precipitation noted. Therefore, this co-precipitation can be attributed to antibody cross-reactivity alone. In light of this observation, it is not surprising that cicatricial pemphigoid AAb precipitated both laminin-6 and laminin-5, as previous studies already demonstrated that anti–laminin-5 AAb in cicatricial pemphigoid patients recognize laminin $\alpha 3$-chain (Kirtschig *et al*, 1995). The data from the current study indicate that the index patient's AAb bind to the laminin-6 molecule at its α-chain because the AAb did not immunoprecipitate or immunoblot laminin-1, which shares $\beta 1$- and $\gamma 1$-chains with laminin-6 **(Figs 4, 6)**. Thus the results of the current study confirm the previous finding that AAb from a subset of cicatricial pemphigoid patients identify laminin-5 α-chain (Kirtsching *et al*, 1995) and also identify laminin-6 as a co-target antigen.

It has been shown previously that anti-laminin $\alpha 3$-chain MoAb BM165 induces detachment of keratinocytes from culture substrates *in vitro* and detachment of epidermis from dermis *in vivo* (Rousselle *et al*, 1991). More recently, a subepidermal blister in neonatal mouse skin was induced by passive transfer of rabbit anti–laminin-5 antibodies that recognize α-, β-, and γ-chains of the molecule (Lazarova *et al*, 1996). These data suggest that anti–laminin-5 antibodies may be pathogenic in cicatricial pemphigoid patients. It has been assumed that inactivation of laminin-5 is the mechanism of blistering in these patients, but in light of the current observation, it is quite possible that inactivation of laminin-6 (by anti–$\alpha 3$-chain antibody) may contribute equally significantly to the blistering process in these patients as the inactivation of laminin-5.

The clinical implication of the current findings is that more BMZ components than we previously were aware of can be targeted by autoimmune reactions. Ultrastructural localization of antigenic sites by IEM and identification of autoantigens by immunoblotting and immunoprecipitation are essential for the accurate determination of autoimmune targets in this group of immune-mediated subepithelial blistering diseases. We propose the name "anti-laminin cicatricial pemphigoid" for this subset of patients to account for the observation that both laminin-5 (previously named epiligrin) and laminin-6 are identified by these patients' AAb.

The authors would like to thank Thomas Traczyk for his technical assistance. This work is supported, in part, by an Annenberg Foundation Career Development Award (L.S.C., Dermatology Foundation, Evanston, IL), a Dermatology Foundation Career Development Award (M.P.M., Dermatology Foundation, Evanston, IL), a Merit Review Research Grant (L.S.C., VA Research Committee, Long Beach, CA), a Clinical Investigator Award (L.S.C., K08 AR01961–01A1, National Institutes of Health, Bethesda, MD), and research support from Palo Alto VA Medical Center (M.P.M., Palo Alto, CA).

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