Anti-Basement Membrane Autoantibodies in Patients with Anti-Epiligrin Cicatricial Pemphigoid Bind the α Subunit of Laminin 5

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Recent studies have identified a group of cicatricial pemphigoid patients who have IgG anti–basement membrane autoantibodies that recognize epiligrin, a set of disulfide-linked polypeptides closely related if not identical to laminin 5 (formerly called kalinin, nicein, or BM600). To further understand the pathophysiology of blister formation in these patients, we have sought to identify the specific polypeptide(s) targeted by their autoantibodies. Comparative studies show that sera from these patients (nine of nine), P1E1 monoclonal anti-epiligrin antibody, and polyclonal as well as monoclonal anti–laminin 5 antibodies immunoprecipitate the same set of disulfide-linked polypeptides from media of biosynthetically radiolabeled human keratinocytes. Moreover, sera from eight of nine patients with anti-epiligrin cicatricial pemphigoid immunoblot the α subunit of laminin 5 but show no reactivity to its β or γ subunits. In addition, circulating IgG from a representative patient was affinity-purified against the α subunit of laminin 5 and shown to bind the dermal side of 1 M NaCl split skin in the same manner as autoantibodies from all patients with anti-epiligrin cicatricial pemphigoid. Sera from patients with bullous pemphigoid (n = 5), other forms of cicatricial pemphigoid (n = 5), epidermolysis bullosa acquisita (n = 4), or bullous systemic lupus erythematosus (n = 1) show no reactivity against any subunit of this laminin isoform in immunoprecipitation or immunoblot experiments. These findings correlate with prior reports showing that a monoclonal antibody directed against the α subunit of laminin 5 (i.e., laminin subunit α3) induces detachment of human keratinocytes from extracellular matrix *in vitro* as well as epidermis from human skin *in situ*. Together, these studies suggest that laminin subunit α3 mediates attachment of basal keratinocytes to epidermal basement membrane and that autoantibodies directed against it may be pathogenic. *J Invest Dermatol 105:543–548, 1995*

Epiligrin is a set of disulfide-linked polypeptides in the extracellular matrix (ECM) of cultured human keratinocytes (HKs) that was initially recognized for its role in promoting cell adhesion [1,2]. *In vitro*, epiligrin binds integrin $\alpha_3\beta_1$ in plasma membranes and focal adhesions and co-localizes with integrin $\alpha_6\beta_4$ in hemidesmosome-like stable anchoring contacts. *In vivo*, epiligrin is found in the lamina lucida of human epidermal basement membrane (BM). Recent studies have shown that this integrin ligand is a laminin isoform that is closely related (if not identical) to laminin 5 [3]. Laminin 5 (formerly called kalinin, nicein, or BM600) is a laminin isoform that has been localized to anchoring filaments in human epidermal BM [4–6]. Like "classical" laminin 1, laminin 5 consists of disulfide-linked α, β, and γ subunits (specifically, $\alpha_3\beta_3\gamma_2$) [7]. The α and γ subunits exist as cell-associated precursors of 200 and 155 kD, respectively; these precursors are rapidly processed following secretion to 165 and 105 kD, respectively [8]. The β subunit exists as a 140-kD polypeptide that does not undergo processing [8]. In human skin, laminin 5 is thought to most closely resemble the fully processed form of this heterotrimer. Although the subunits of laminin 5 show substantial deduced amino acid sequence homology to the corresponding polypeptides in laminin 1, the α, β, and γ subunits of laminin 5 have significant truncations that are thought to determine how this molecule uniquely binds HKs to epidermal BM [9]. The recent identification of mutations in genes encoding laminin 5 subunits in patients with the potentially lethal, Herlitz's junctional epidermolysis bullosa attests to the importance of this complex in maintaining adhesion of human epidermis to epidermal BM [10–12].

Recent studies have identified a group of patients with a form of cicatricial pemphigoid (CP) who have IgG autoantibodies that bind the lower lamina lucida of epidermal BM and immunoprecipitate a distinct set of disulfide-linked polypeptides from HK extracts and culture medium [13–15]. This set of polypeptides co-migrates in sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with the same complex immunoprecipitated by monoclonal antibodies directed against epiligrin or laminin 5. To further

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Abbreviations: AP, alkaline phosphatase; BSLE, bullous systemic lupus erythematosus; CP, cicatricial pemphigoid; HK, human keratinocyte; WBS, 0.5% Triton X-100 in TBS.

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understand the pathophysiology of blister formation in these patients, we have sought to determine the precise identity of the polypeptide(s) targeted by their autoantibodies. These studies show that autoantibodies from eight of nine patients with anti-epiligrin CP specifically target the α subunit of laminin 5 (i.e., laminin subunit $\alpha 3$). Moreover, circulating IgG from a representative patient was affinity-purified against laminin subunit $\alpha 3$ (but not control polypeptides) and shown to bind the dermal side of 1 M NaCl separated human skin in the same manner as autoantibodies from all patients with anti-epiligrin CP. These findings suggest that laminin subunit $\alpha 3$ plays a key role in HK adhesion to epidermal BM and that autoantibodies directed against it are involved in the pathogenesis of subepithelial blister formation in patients with this form of CP.

MATERIALS AND METHODS

Patients Serum samples were obtained from nine patients with anti-epiligrin CP. The clinical, histologic, and immunopathologic features of five of these patients have been described in detail elsewhere [14]; the salient features of the other four patients are the same. In brief, all of these patients have a mucosal-predominant subepithelial bullous disease and IgG anti-basement membrane autoantibodies that bind the dermal side of 1 M NaCl split skin and immunoprecipitate a distinct set of disulfide-linked polypeptides from the extracts or media of biosynthetically radiolabeled human keratinocytes. Control serum samples were obtained from well-characterized patients with bullous pemphigoid (BP, n = 5), epidermolysis bullosa acquisita (EBA, n = 4), bullous systemic lupus erythematosus (n = 1), other forms of CP (n = 5), and normal volunteers (n = 12). Criteria used to classify bullous disease control subjects were as follows: serum samples from patients with BP immunoprecipitated BPAG1 (n = 5) and BPAG2 (n = 3) from radiolabeled HK extracts; serum samples from all patients with EBA immunoblotted type VII collagen in extracts of lamina densa/dermis; the patient with bullous systemic lupus erythematosus satisfied clinical, histologic, and immunopathologic criteria for this diagnosis and had circulating IgG autoantibodies against type VII collagen; all patients with other forms of CP demonstrated subepithelial blisters of their oral, nasal, laryngeal, conjunctival, and/or esophageal mucosa, *in situ* deposits of immunoreactants in epithelial BMs, and circulating IgG anti-BM autoantibodies directed against the epidermal side of 1 M NaCl split skin that did not immunoprecipitate BPAG1 or BPAG2; clinically, the latter patients are indistinguishable from those with anti-epiligrin CP.

Reagents Fluorescein isothiocyanate–conjugated goat anti-human IgG (Tago, Inc., Burlingame, CA), alkaline phosphatase (AP)–conjugated goat anti-human IgG (Tago, Inc.), AP-conjugated goat anti-rabbit IgG (Tago, Inc.), rabbit anti–laminin 5 antiserum [8], normal rabbit serum (GibcoBRL, Grand Isle, NY), and murine monoclonal antibodies directed against epiligrin (P1E1 [1]), laminin 5 (BM165 [4]), and human IgG_1 (Southern Biotechnology Associates, Birmingham, AL) were used as described below. Protein A–bearing formalin-fixed staphylococci (Pansorbin) were obtained from Calbiochem Behring (San Diego, CA).

Cell Cultures HK were derived from newborn human foreskins and cultured in keratinocyte serum-free medium (GibcoBRL) as described previously [16]. SCC-25 cells (American Type Culture Collection, Rockville, MD, CRL 1628) were cultured in 50% Ham's F-12 medium, 50% Dulbecco's minimal essential medium, hydrocortisone 0.5 mg/ml, 10% heat-inactivated fetal bovine serum, and antibiotics (all from GibcoBRL).

Biosynthetic Radiolabeling of HK Culture Medium Subconfluent monolayers of HKs were metabolically radiolabeled with ^{35}S-methionine (50 μCi/ml; specific activity ~1100 Ci/mmol, New England Nuclear, Boston, MA) in methionine-free medium as described previously [17]. Radiolabeled HK culture medium was made 1 mM in ethylene diaminetetraacetic acid and phenylmethylsulfonyl fluoride, centrifuged at 750 × *g* for 10 min, recovered, dialyzed against 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 (Tris-buffered saline [TBS]) at 4°C, and then stored at −70°C.

Immunoprecipitation Immunoprecipitation studies of radiolabeled HK culture medium were performed as described previously [17,18]. Preclearance studies were performed by preabsorption of radiolabeled culture medium with either 50 μl of rabbit anti–laminin 5 antiserum or normal rabbit serum (control). Immunoprecipitation samples were studied by gradient SDS-PAGE and fluorography as described previously [17].

Laminin 5 Laminin 5 was isolated from the ECM of cultured HK as well as the culture media of SCC-25 cells and used in immunoblot studies as described below. In brief, confluent cultures of HK were sequentially extracted with 1% Triton X-100 in phosphate-buffered saline, 2 M urea in 1 M NaCl, and 8 M urea to yield culture dishes coated with ECM rich in laminin 5. Urea-insoluble proteins were detached from the surface of culture dishes with a disposable cell scrapper (Costar Corporation, Cambridge, MA) and suspended in 0.5% w/v SDS as described previously [1]. All extraction buffers contained 1 mM phenylmethylsulfonyl fluoride and 2 mM N-ethylmaleimide. The HK ECM extract was dialyzed against 0.1% SDS in TBS at 4°C, concentrated by exposure of dialysis units to Sephadex G-75 (Pharmacia, Uppsala, Sweden), and stored at −70°C. The conditioned medium of SCC-25 cells was used as another source of laminin 5. In brief, over a period of 2 to 3 d, SCC-25 cells (80% density) were adapted to their basal medium free of fetal bovine serum. Following 24 to 48 h of additional culture, the conditioned medium of these cells was recovered, centrifuged at low speed to remove cell debris, and then incubated with ammonium sulfate (300 mg/ml) overnight on ice. After centrifugation (~10,000 × *g* for 60 min), the pellet was resuspended in 65 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 25 mM Tris, pH 7.5, made 4 μg/ml with diisopropyl fluorophosphate, dialyzed against the same Tris buffer, microfuged at 14,000 rpm for 30 min at 4°C, and stored at −70°C.

Immunoblotting Protein electrophoresis was performed according to the method established by Laemmli using 6% SDS-PAGE gels [19]. Resolved proteins were transferred to nitrocellulose paper as described by Towbin using the NOVEX system (San Diego, CA) [20]. Nitrocellulose sheets were blocked overnight at 4°C in TBS containing 3% bovine serum albumin and then cut into strips. Experimental and control sera were diluted 1:200 in 0.5% Triton X-100 in TBS (WBS) and applied to nitrocellulose strips containing laminin 5 for 2 h at room temperature. Rabbit anti–laminin 5 antiserum and normal rabbit serum (both at 1:1000) were used as positive and negative controls, respectively, to specifically identify all laminin 5 subunits (i.e., processed and unprocessed α, β, and γ subunits). Following their incubation with first-step antibodies, nitrocellulose strips were washed five times with an excess volume of WBS (5 min per wash). Second-step antibodies (AP-conjugated goat anti-human IgG or AP-conjugated goat anti-rabbit IgG in the case of controls) were diluted 1:1000 in WBS and incubated with strips for 2 h at room temperature and then washed in the same manner described above. Immunoblots were developed for 3 min (AP-conjugate substrate kit, Bio-Rad Laboratories, Hercules, CA) then washed extensively with TBS.

Affinity-Purification Studies To show that anti–laminin 5 antibodies in the sera of patients with anti-epiligrin CP are specifically reactive with human epidermal BM, IgG from a representative patient was affinity purified against laminin subunit $\alpha 3$ (or control polypeptides) immobilized on nitrocellulose paper and tested against 1 M NaCl split skin by indirect immunofluorescence microscopy as described previously [21,22]. In brief, HK ECM was resolved by electrophoresis and transferred to nitrocellulose paper as described above. Horizontal strips of nitrocellulose paper containing laminin subunit $\alpha 3$ (or 140-kD ECM polypeptides, control) were incubated with patient serum (1:5 in TBS) overnight at 4°C and then washed extensively with 0.5% polysorbate 20 in TBS. Bound antibody was eluted from experimental and control nitrocellulose strips with 50 mM glycine in 500 mM NaCl, pH 2.3, neutralized immediately with 500 mM Na_2HPO_4, pH 6.5, dialyzed against TBS for 16 h at 4°C, and concentrated by ultrafiltration (Centricon 30, Amicon Corporation, Danvers, MA). Affinity-purified antibodies were tested against 1 M NaCl split skin by indirect immunofluorescence microscopy using fluorescein isothiocyanate–conjugated goat anti-human IgG as a second-step antibody [22].

RESULTS

Circulating IgG from Patients with Anti-Epiligrin CP Immunoprecipitate Laminin 5 Comparative studies demonstrate that serum from a representative patient with anti-epiligrin CP, P1E1 monoclonal anti-epiligrin antibody, and polyclonal as well as monoclonal anti–laminin 5 antibodies all immunoprecipitate the same set of disulfide-linked polypeptides from the media of biosynthetically radiolabeled HKs **(Figs 1 and 2)**. The predominant polypeptides (and corresponding laminin 5 subunits) in this complex consist of the following: a doublet of ~200 and ~190 kD (the unprocessed α subunit and a similar polypeptide [see below], respectively); a doublet of ~165 and ~155 kD (the processed α and the unprocessed γ subunits, respectively); a doublet of ~145 and ~140 kD (a minor fragment of the α subunit [variably present in such immunoprecipitation studies [8]] and the β subunit, respectively); and a polypeptide of ~105 kD (the processed γ subunit). This complex is not recognized by a control monoclonal antibody

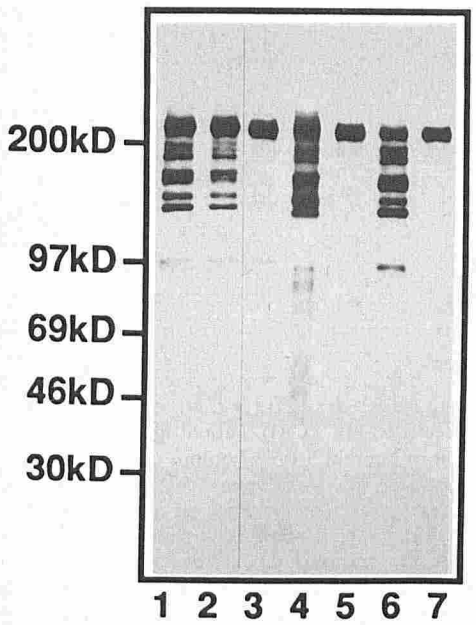

Figure 1. Circulating IgG from patients with anti-epiligrin CP immunoprecipitates laminin 5. Medium from ^{35}S-methionine biosynthetically radiolabeled human keratinocytes was preabsorbed with normal human, mouse, and rabbit serum, twice mixed with aliquots of freshly washed Pansorbin, and then studied in immunoprecipitation experiments as previously described [17,18]. Immunoprecipitation samples were applied to SDS-polyacrylamide gels and studied by fluorography. The comparative immunoprecipitation study shown here utilized the following: BM165 monoclonal anti–laminin 5 antibody (*lane 1*); P1E1 monoclonal anti-epiligrin antibody (*lane 2*); monoclonal anti-human IgG_1 antibody (negative control, *lane 3*); rabbit anti–laminin 5 antiserum (*lane 4*); normal rabbit serum (negative control, *lane 5*); serum from a representative patient with anti-epiligrin CP (*lane 6*); and normal human serum (negative control, *lane 7*). BM165 (*lane 1*), P1E1 (*lane 2*), rabbit anti–laminin 5 antiserum (*lane 4*), and serum from our representative patient with anti-epiligrin CP (*lane 6*) all immunoprecipitate the same set of disulfide-linked polypeptides of 200, 165, 155, 140, and 105 kD. This complex is not immunoprecipitated by monoclonal anti-human IgG_1 antibody, normal rabbit serum, or normal human serum (negative controls, *lanes 3, 5, and 7*, respectively). Preclearance of media with rabbit anti-fibronectin antibodies removed the intense band of 240 kD seen in all of these immunoprecipitation samples.

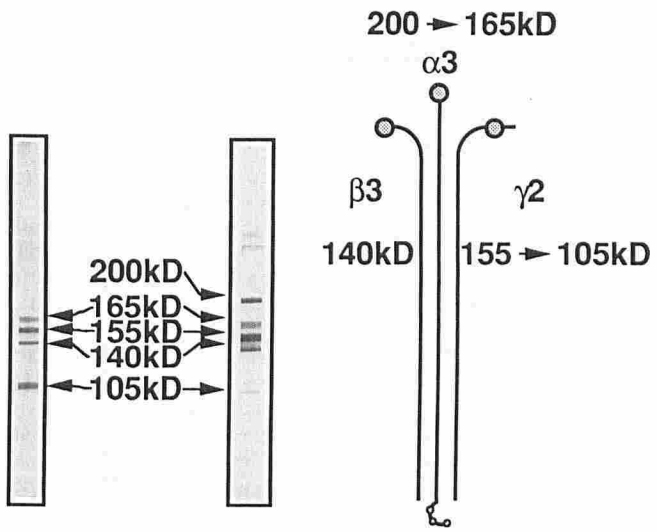

Figure 2. Laminin 5 is a heterotrimeric protein composed of disulfide-linked α, β, and γ subunits. The α subunit exists as unprocessed and processed polypeptides of 200 and 165 kD, respectively. The β subunit is a 140-kD polypeptide that does not undergo processing following synthesis or secretion. The γ subunit of laminin 5 exists as unprocessed and processed polypeptides of 155 and 105 kDs, respectively. Serum-free conditioned media of SCC-25 cells was treated with ammonium sulfate (300 mg/ml) to yield a precipitate that was resuspended in Tris buffer, reduced, applied to SDS-polyacrylamide gels, transferred to nitrocellulose paper, and then immunoblotted with rabbit anti–laminin 5 antiserum (*left*). Similarly, confluent cultures of human keratinocytes were sequentially extracted with 1% Triton X-100 in phosphate-buffered saline, 2 M urea in 1 M NaCl, and 8 M urea to yield ECM proteins that were scrapped from the surface of culture dishes, concentrated, reduced, applied to SDS-polyacrylamide gels, and then studied by immunoblot with rabbit anti–laminin 5 antiserum (*right*). The *insets* show the various laminin 5 subunits in the media of SCC-25 cells (*left*) and the ECM of human keratinocytes (*right*). The same rabbit anti–laminin 5 antiserum was used as a positive control in the experiments shown in **Figs 3 and 4**.

or sera from a normal rabbit or normal human volunteer. Moreover, preclearance of radiolabeled HK media with rabbit anti–laminin 5 antiserum (but not control normal rabbit serum) removed all polypeptides reactive with serum from a representative patient with anti-epiligrin CP (data not shown). Sera from all patients with anti-epiligrin CP immunoprecipitate this same set of polypeptides. In contrast, serum samples from control patients with BP (n = 5), CP (n = 5), EBA (n = 4), or bullous systemic lupus erythematosus (n = 1) as well as normal volunteers (n = 12) do not.

All comparative immunoprecipitation studies utilized biosynthetically radiolabeled media from subconfluent monolayers of HKs to minimize the presence of other laminin isoforms (e.g., laminin 6, $\alpha 3\beta 1\gamma 1$) that tend to accumulate in postconfluent cultures. Nonetheless, the ~190-kD polypeptide immunoprecipitated by all experimental (but not control) antibodies is thought to represent a crossreactive or co-precipitating laminin subunit. The intense band of ~240 kD seen in all immunoprecipitation samples **(Fig 1)** was confirmed in preclearance studies to be fibronectin.

Circulating IgG from Patients with Anti-Epiligrin CP Immunoblot the α Subunit of Laminin 5 In immunoblot studies of HK ECM, rabbit anti–laminin 5 antiserum identifies five polypeptides corresponding to the unprocessed and processed α subunits (~200 and 165 kD, respectively), the β subunit (~140 kD), and the unprocessed and processed γ subunits (~155 and 105 kD, respectively) of this laminin isoform **(Fig 2)**. None of these polypeptides are bound by normal rabbit serum (negative control, data not shown). Circulating IgG from eight of nine patients with anti-epiligrin CP bind polypeptides of 200 and 165 kD that comigrate in SDS-PAGE with unprocessed and processed α subunits of laminin 5, respectively **(Fig 3)**. None of these sera bound polypeptides corresponding to the β or γ subunits; circulating IgG autoantibodies from the patient that did not immunoblot any laminin 5 subunit do immunoprecipitate this protein, presumably via recognition of a conformational determinant. Serum samples from patients with other forms of CP (n = 5), BP (n = 5), EBA (n = 4), or bullous systemic lupus erythematosus (n = 1) as well as 12 normal volunteers show no reactivity to polypeptides corresponding to any laminin 5 subunit in these immunoblot studies.

Because the conditioned medium of SCC-25 cells represents a well-characterized source of laminin 5, additional immunoblot studies were conducted on this material. In these experiments, rabbit anti–laminin 5 antiserum identifies four polypeptides of ~165, 155, 140, and 105 kD that correspond to the laminin 5 subunits described above **(Fig 2)**. Circulating IgG from five of five patients with anti-epiligrin CP bind a single 165-kD polypeptide in SCC-25 medium that corresponds to the processed α subunit of laminin 5 **(Fig 4)**; sera from the four other patients with anti-epiligrin CP were not tested against this antigen source. Serum samples from patients with other subepidermal bullous diseases (n = 15) or 11 normal volunteers do not bind any subunit of laminin 5 in these immunoblot studies. Interestingly, circulating IgG from one normal volunteer binds a polypeptide in the media of

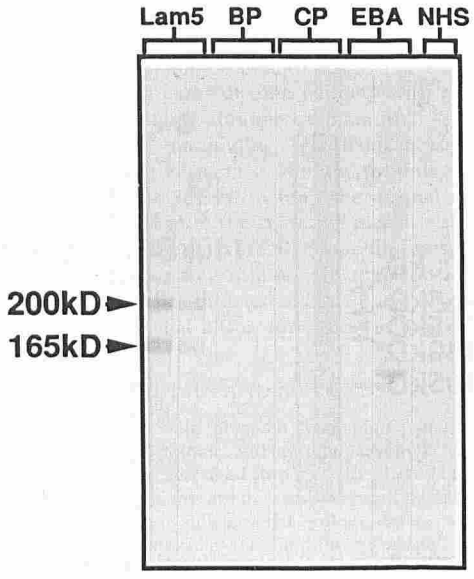

Figure 3. Circulating IgG from patients with anti-epiligrin CP identifies the α subunit of laminin 5 isolated from the ECM of human keratinocytes on immunoblots. The ECM of human keratinocytes was isolated, reduced, applied to SDS-polyacrylamide gels, and studied by immunoblot as described in the legend of **Fig 2**. Sera from representative patients with anti-epiligrin CP (*lanes 1 and 2*) specifically immunoblot the 200-kD unprocessed and 165-kD processed α subunits of laminin 5 in HK ECM. Sera from patients with BP (*lanes 3 and 4*), other forms of CP (*lanes 5 and 6*), EBA (*lanes 7 and 8*), or a normal volunteer (*lane 9*) do not bind any laminin 5 subunits.

SCC-25 cells that appears to co-migrate in SDS-PAGE with the processed α3 chain of laminin 5. However, when this serum sample was tested by indirect immunofluorescence microscopy using 1 M NaCl separated human skin, no IgG anti-BM autoantibodies were detected. Moreover, this volunteer's serum did not immunoprecipitate any polypeptides from radiolabeled HK extracts and media or immunoblot laminin 5 in HK ECM (see above). Solitary polypeptides in this antigen source that are bound by circulating IgG from certain subjects signify patient-specific but not disease-specific reactivity.

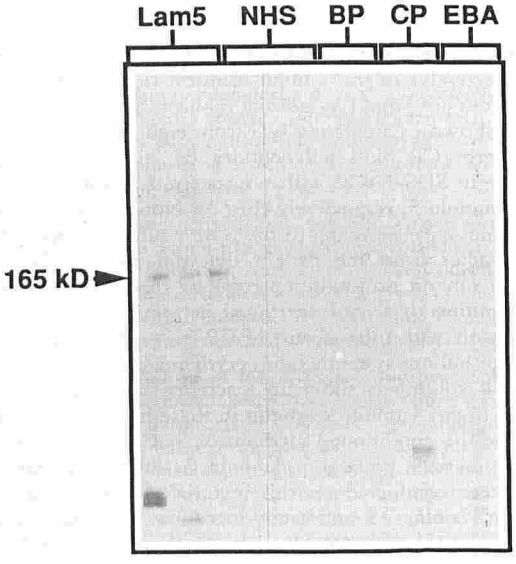

Figure 4. Circulating IgG from patients with anti-epiligrin CP identifies the α subunit of laminin 5 isolated from the conditioned media of SCC-25 cells on immunoblots. The serum-free conditioned media of SCC-25 cells was isolated, reduced, applied to SDS-polyacrylamide gels, and studied by immunoblot as described in the legend of **Fig 2**. Sera from representative patients with anti-epiligrin CP (*lanes 1, 2, and 3*) bind the 165-kD processed α subunit of laminin 5. Sera from normal volunteers (*lanes 4, 5, and 6*) as well as patients with BP (*lanes 7 and 8*), other forms of CP (*lanes 9 and 10*), and EBA (*lanes 11 and 12*) do not immunoblot any laminin 5 subunits.

Affinity-Purified Anti–Laminin 5 IgG Autoantibodies (α Subunit Specific) Bind the Dermal Side of 1 M NaCl Split Skin To determine if circulating anti–laminin 5 antibodies in our patients are reactive with human epidermal BM, IgG from a patient with higher titers of these autoantibodies was affinity purified against the α subunit of laminin 5 immobilized on nitrocellulose paper and tested against 1 M NaCl split skin by indirect immunofluorescence microscopy. As a control, serum from the same patient was affinity-purified against 140-kD polypeptides in the same antigen source (i.e., HK ECM). Patient IgG affinity purified against the α subunit of laminin 5 (i.e., laminin α3) specifically bound the dermal side of 1 M NaCl split skin in the same manner as autoantibodies in the sera of this and other patients with anti-epiligrin CP **(Fig 5*A*)**. In contrast, eluates from control polypeptides showed no staining of 1 M NaCl split skin **(Fig 5*B*)**. Moreover, because the molecular weight of these control polypeptides corresponds to the β subunit of laminin 5, negative findings in these indirect immunofluorescence microscopy experiments confers even greater specificity about the reactivity of these patients' circulating autoantibodies against the α subunit of this heterotrimer.

DISCUSSION

Patients with anti-epiligrin CP have IgG autoantibodies that bind the extracellular matrix of cultured HK as well as the lower lamina lucida of human epidermal BM [13–15]. In addition, these patients' anti-BM autoantibodies have been shown to immunoprecipitate a distinct set of disulfide-linked polypeptides from extracts and media of HK [13,14]. Comparative immunoprecipitation and preclearance studies have shown that this complex is the same as that identified by monoclonal antibodies directed against epiligrin or laminin 5. These autoantibodies have been used as a disease-specific marker for patients with this form of CP, because sera from patients with other forms of CP, other subepidermal bullous diseases, and normal volunteers do not immunoprecipitate this complex [13,14]. Immunoprecipitation studies such as those described above assess the binding of antibodies to antigens under nonreduced and nondenaturing conditions (i.e., conditions in which disulfide-bonded polypeptides remain associated and retain their tertiary structure). In such studies, it is not possible to discern which polypeptide (or polypeptides) within a complex are specifically targeted by a patient's autoantibodies (or similarly, if all patients are targeting the same polypeptide within a complex). The objective of this study was to determine which polypeptide(s) within this complex is (are) bound by autoantibodies from patients with anti-epiligrin CP. The rationale is that this information would further our understanding of the pathophysiology of this disease and elucidate the function of the adhesion molecule targeted by these patients' autoantibodies. To address these questions, we performed additional comparative immunoprecipitation and preclearance studies to confirm that these patients' autoantibodies and well-characterized polyclonal and monoclonal anti–laminin 5 antibodies identify the same complex of disulfide-linked HK polypeptides. These studies confirm that multiple antibodies directed against laminin 5 or epiligrin immunoprecipitate the same set of polypeptides from biosynthetically radiolabeled HK culture media. We next tested these patients' sera (and that of controls) against reduced laminin 5 by immunoblotting—an experimental system in which disulfide-bonded polypeptides are separated and denatured. These studies were practical because the synthesis, processing, and isolation of laminin 5 has been recently detailed and because a polyclonal anti–laminin 5 antiserum reactive

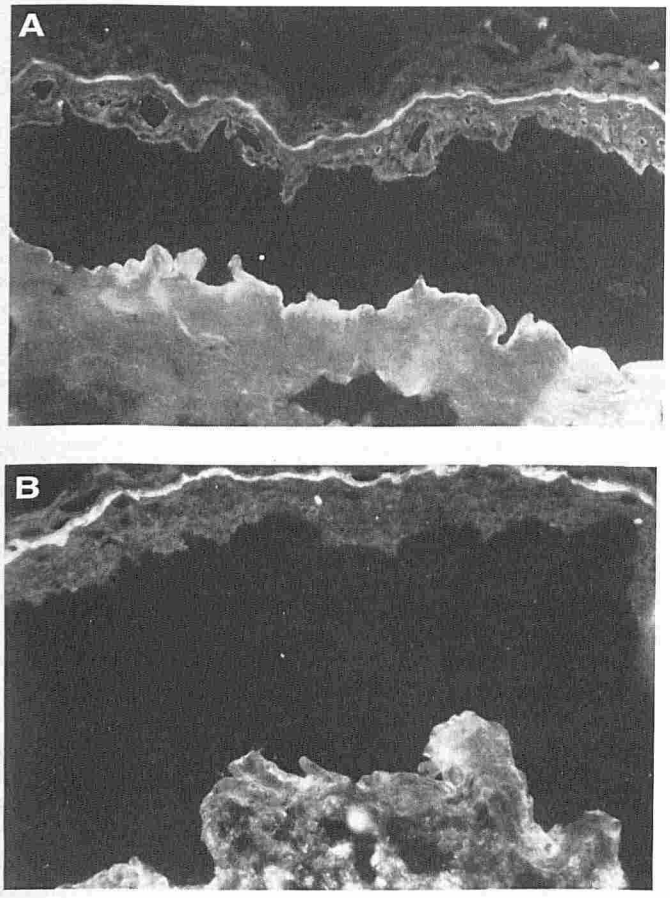

Figure 5. Affinity-purified anti-laminin 5 IgG autoantibodies (α subunit specific) bind the dermal side of 1 M NaCl split skin. Monolayers of cultured human keratinocytes were sequentially extracted to yield ECM proteins that were solubilized, concentrated, separated on SDS-polyacrylamide gels, and then transferred to nitrocellulose paper. Horizontal strips of nitrocellulose paper containing the α subunit of laminin 5 (or identical amounts of nitrocellulose paper containing 140-kD polypeptides, negative control) were incubated with serum from a representative patient with anti-epiligrin CP (diluted 1:5 in Tris-buffered saline) overnight at 4°C. The strips were washed extensively with 0.5% polysorbate 20 in Tris-buffered saline and the bound antibody was eluted from experimental and control nitrocellulose strips with 50 mM glycine in 500 mM NaCl, pH 2.3. Eluates were neutralized immediately with 500 mM Na_2HPO_4, pH 6.5, dialyzed against Tris-buffered saline for 16 h at 4°C, and concentrated by ultrafiltration. Affinity-purified autoantibodies were tested against 1 M NaCl split skin by indirect immunofluorescence microscopy using fluorescein isothiocyanate–conjugated goat anti-human IgG as a second-step antibody. IgG from a representative patient with anti-epiligrin CP affinity purified against the α subunit of laminin 5 binds the dermal side of 1 M NaCl split skin in the same manner as circulating autoantibodies in the sera of this and other patients with this disorder (*A*). IgG from the same patient that is affinity purified against 140-kD polypeptides in the same antigen source does not bind epidermal BM (*B*).

with all subunits of this laminin isoform was available for use as a positive control [4,8]. These studies found that autoantibodies from eight of nine patients with anti-epiligrin CP bind the α subunit of laminin 5, and that none of these patients' sera show reactivity to the β or γ subunits of laminin 5. The one patient that did not immunoblot any laminin 5 subunit presumably has circulating autoantibodies directed against a conformational determinant on this protein (i.e., a determinant detectable by immunoprecipitation but not by immunoblot). The reactivity of these patients' autoantibodies against the α subunit of laminin 5 is further specific in that no laminin 5 subunits were immunoblotted by autoantibodies from patients with other forms of CP or other subepidermal bullous diseases. Moreover, it was possible to affinity purify the circulating autoantibodies of a representative patient against the α subunit of laminin 5 immobilized on nitrocellulose paper and show that this specific IgG binds the dermal side of 1 M NaCl split skin in the same manner as autoantibodies in the sera of all patients with anti-epiligrin CP. These findings all indicate that the α subunit of this laminin isoform (i.e., laminin α3) is specifically targeted by autoantibodies in patients with this form of CP. On a practical level, these studies also demonstrate that autoantibodies in patients with this form of CP can be identified by immunoblotting—a laboratory technique that is more widely available than immunoprecipitation and that hopefully can be used to identify additional patients with this disorder.

Findings in this study correlate with several prior observations. First, it has been previously shown that the stoichiometric ratio of the polypeptides immunoprecipitated by autoantibodies from patients with this form of CP is always the same [13,14]. This consistent finding has suggested that these patients' autoantibodies are targeting the same polypeptide within this disulfide-linked complex, a suggestion confirmed by the immunoblot findings presented here. Second, our finding that patients with this form of CP target the α subunit of laminin 5 correlates with prior studies showing that this particular subunit plays an important role in promoting HK adhesion to extracellular matrix *in vitro* and epidermal BM *in vivo*. Specifically, Rousselle *et al* have shown that a monoclonal antibody (i.e., monoclonal antibody BM165) directed against this laminin subunit causes HKs to round and detach from culture dishes *in vitro* and skin fragments to split within the lamina lucida *in situ* [4]. Findings in our current study support the suggestion that this subunit of laminin 5 mediates attachment of HKs to epidermal BM and further suggest that autoantibodies in patients with this form of CP may be pathogenic. Third, prior studies have identified mutations in laminin 5 subunits in patients with Herlitz's junctional epidermolysis bullosa [10–12], an inherited and potentially lethal subepidermal bullous disease. Interestingly, patients with anti-epiligrin CP share some clinical features seen in patients with this form of junctional epidermolysis bullosa. For example, both groups of patients have noninflammatory, subepithelial bullous lesions that frequently affect the mouth, nasopharynx, larynx, and esophagus. In contrast, patients with anti-epiligrin CP have lacked extensive cutaneous involvement or abnormalities in their nails and teeth that are characteristically seen in patients with Herlitz's disease. These unexplained phenotypic differences may relate to tissue-specific or developmental variances in the relative amounts or biologic activities of laminin 5 in mucosal versus cutaneous or neonatal versus adult BMs. In any event, acquired or inherited abnormalities in this anchoring filament associated protein result in separation of human epidermis from epidermal BM.

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ANNOUNCEMENT

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With the support of this grant, a testing kit, including appropriate oligomer primers, PCR amplification conditions, and other technical information, is now available. The Department of Dermatology at Jefferson will provide, free of charge, eight of these kits to investigators for the diagnostic purpose, allowing a universally accessible strategy for accurate diagnosis and first-trimester DNA-based prenatal diagnosis to families at risk for recurrence of the dystrophic forms of EB.

Investigators interested in this offer should contact Jefferson Dermatology at the address below, with information indicating the potential usefulness of this kit for their research and/or clinical service.

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