

Passive Transfer of Anti-Laminin 5 Antibodies Induces Subepidermal Blisters in Neonatal Mice

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Abstract

Patients with a recently identified subepithelial blistering disease have IgG anti-laminin 5 autoantibodies. To determine if such antibodies can be pathogenic in vivo, we developed and characterized rabbit anti-laminin 5 IgG, and passively transferred these antibodies to neonatal mice. Immune rabbit IgG specifically bound human and murine epidermal basement membranes, immunoblotted and immunoprecipitated all laminin 5 subunits from extracts of human and murine keratinocytes, and showed no reactivity to other keratinocyte proteins or epithelial basement membranes that do not contain laminin 5. Mice ($n = 29$) receiving purified anti-laminin 5 IgG developed, in a dose-related fashion, circulating anti-laminin 5 antibodies, deposits of rabbit IgG and murine C3 in epidermal basement membranes, and subepidermal blisters of skin and mucous membranes. No alterations developed in controls ($n = 14$) receiving identical amounts of normal rabbit IgG. Passive transfer of anti-laminin 5 (but not control) IgG to neonatal C57BL/6 ($n = 3$) or mast cell-deficient ($n = 3$) mice produced subepidermal blisters with the same clinical, histologic, and immunopathologic features as those documented in BALB/c mice. These studies establish an animal model of a human blistering disease that can be used to define disease mechanisms and treatment modalities. (*J. Clin. Invest.* 1996. 98:1509–1518.) Key words: autoimmunity • laminin • animal models

Introduction

Cicatricial pemphigoid is an acquired, autoimmune, subepithelial blistering disease that predominates on mucous membranes. Recent studies have shown that ~5% of patients with well characterized cicatricial pemphigoid have IgG anti-basement membrane (BM)¹ autoantibodies directed against lami-

nin 5 (also called laminin isoform 5, nicein, epiligrin, and kalinin) (1–7). Laminin 5 ($\alpha 3\beta 3\gamma 2$) is a heterotrimeric adhesion molecule that is associated with anchoring filaments in the lamina lucida of human epidermal BM (8–10). These filaments comprise part of an adhesion complex that anchors basal keratinocytes to epidermal BM. Like many other constituents of epidermal BM, laminin 5 is produced by human keratinocytes (HK) (8–11). In vitro, HK deposit laminin 5 in their extracellular matrix (ECM) where this protein serves as the major integrin ligand for these cells (8, 9, 12). Interestingly, certain monoclonal anti-laminin 5 antibodies have been shown to impair HK adhesion to ECM in vitro (9). These experimental findings have raised the hypothesis that anti-laminin 5 autoantibodies in patients with cicatricial pemphigoid may impair the adhesion of basal keratinocytes to epidermal BM in a similar manner and hence are pathogenic in vivo. To address this hypothesis, we immunized rabbits with laminin 5 purified from the ECM of HK, purified and characterized the resulting rabbit anti-laminin 5 IgG, and then passively transferred the latter into neonatal mice. Studies using this animal model of disease demonstrate that anti-laminin 5 (but not normal, control) IgG induces subepithelial blisters in newborn mice at doses as low as 1 mg/g body wt. Moreover, these studies demonstrate that such lesions develop in newborn mice independent of the activation of terminal complement components or the degranulation of dermal mast cells. These studies establish an animal model of a human blistering disorder that can be used to investigate disease pathomechanisms and experimental therapies.

Methods

Reagents

FITC-conjugated goat anti-rabbit IgG (Tago, Inc., Burlingame, CA), FITC-conjugated goat anti-rabbit IgM (Cappel Laboratories, West Chester, PA), FITC-conjugated goat anti-mouse C3 (Cappel Laboratories), FITC-conjugated goat anti-mouse IgM (Cappel Laboratories), rabbit anti-laminin 5 antiserum (a control reagent generously provided by Dr. Peter Marinkovich, Stanford University, Stanford, CA, and Dr. Robert Burgeson, Harvard Medical School, Cambridge, MA), normal rabbit serum (GIBCO BRL, Grand Island, NY), serum from a reference patient with antiepiligrin cicatricial pemphigoid who has IgG anti-BM autoantibodies directed against laminin 5, and normal human serum were used as described below (1–3). Protein A-bearing formalin-fixed staphylococci (Pansorbin) were obtained from Calbiochem-Behring Corp. (San Diego, CA). Collagen types I, III, V, and VII (Calbiochem-Behring Corp.), human plasma fibronectin, laminins 1 ($\alpha 1\beta 1\gamma 1$) and 2 ($\alpha 2\beta 1\gamma 1$) (the latter formerly called merosin), and type IV collagen (GIBCO BRL), and human as well as bovine serum albumin (Sigma Chemical Co., St. Louis, MO) were used as described below.

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1. Abbreviations used in this paper: BM, basement membrane; ECM, extracellular matrix; HK, human keratinocyte; IF, immunofluorescence.

Cell cultures

HK were derived from newborn human foreskins and cultured in Keratinocyte SFM (GIBCO BRL) (13). The skin of neonatal BALB/c mice was excised and treated with 0.25% trypsin in Hank's balanced salt solution containing Ca^{++} and 1 mM Hepes at 4°C for 16 h. Sheets of murine epidermal cells were recovered, disaggregated, and cultured in Keratinocyte SFM.

Laminin 5

Laminin 5 was isolated from the ECM of cultured HK as described previously (3, 8). In brief, confluent cultures of HK were sequentially extracted with 1% Triton X-100 in PBS, 2 M urea in 1 M NaCl, and 8 M urea to yield culture dishes coated with laminin 5. Urea-insoluble proteins were detached from the surface of culture dishes with a disposable cell scraper (Costar Corp., Cambridge, MA) and suspended in 0.5% (wt/vol) SDS; all extraction buffers contained 1 mM phenylmethylsulfonyl fluoride and 2 mM *N*-ethylmaleimide. Laminin 5 derived from HK ECM was dialyzed against 0.1% SDS in Tris-buffered saline at 4°C, concentrated by solvent recovery (Sephadex G-75; Pharmacia Fine Chemicals, Uppsala, Sweden), and stored at -70°C.

Immunization of rabbits

Sera from 10 New Zealand White rabbits were tested for evidence of circulating IgG reactive with human epidermis or epidermal BM by indirect immunofluorescence (IF) microscopy (14). Preimmune sera were collected from four rabbits showing the lowest background reactivity to human skin, and these subjects were immunized subcutaneously with purified laminin 5 (100 µg/ml) suspended in Freund's complete adjuvant. 14 d later, each subject was boosted once with the same preparation of laminin 5 (100 µg/ml) suspended in incomplete Freund's adjuvant. Immune sera were obtained at regular intervals, characterized separately, and used as individual reagents in the passive transfer studies described below.

Characterization of rabbit anti-laminin 5 antibodies

Indirect IF microscopy. Serial dilutions of immune (and preimmune, control) rabbit sera were tested for IgG reactive with intact human and neonatal murine skin as well as 1 M NaCl split human skin by indirect IF microscopy (14). The same samples were also tested for IgG reactive with laminin 5 in the ECM of HK by indirect IF microscopy as described previously (1, 2). The tissue distribution of the reactivity of anti-laminin 5 antisera was determined in indirect IF microscopy studies of tissues from humans (skin, vagina, colon, kidney, gall bladder, and synovium), BALB/c mice (skin, oral mucosa, conjunctiva, cornea, tongue, and kidney), and New Zealand White rabbits (skin).

Complement fixation. Immune (and preimmune, control) rabbit sera were tested for their ability to elicit deposition of human C3 in normal human epidermal BM using a previously described indirect IF microscopy complement fixation technique (15, 16).

Immunoblot. Immune (and preimmune, control) rabbit sera were characterized for their pattern of IgG reactivity against all proteins extracted from HKs and HK ECM by immunoblotting. In these experiments, cultured HK were extracted with Tris-buffered saline containing 1% SDS, 5% β-mercaptoethanol, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 5 mM each of leupeptin, antipain, chymostatin, and pepstatin A; HK ECM was isolated as described above. Samples of HK extracts or ECM were reduced and applied to 6% SDS-polyacrylamide minigels (Novex, San Diego, CA), transferred to nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA) by electrophoresis, and immunoblotted with immune and preimmune rabbit sera (3, 17, 18). Alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000) was used as the second-step antibody in these studies; immunoblots were developed for 3 min (alkaline phosphatase-conjugate substrate kit; Bio-Rad Laboratories), and then washed extensively with Tris-buffered saline. Reference rabbit anti-laminin 5 antiserum and normal rabbit serum were used as additional controls to specifically identify all laminin 5 subunits (i.e., unproc-

essed and/or processed α, β, and γ subunits) in these samples. In related experiments, immune (and preimmune, control) rabbit sera were immunoblotted against all proteins extracted from subconfluent monolayer cultures of BALB/c epidermal cells. Murine epidermal cells were extracted and analyzed in the same manner as HK with the exception that chemiluminescence (ECL™; Amersham Corp., Life Sciences, Little Chalfont, UK) was used to develop the immunoblots; again, the reference anti-laminin 5 antiserum served as the positive control for these experiments.

To further characterize the reactivity of anti-laminin 5 IgG, immune (and preimmune, control) sera were tested against collagen types I, III, IV, V, and VII, laminins 1 and 2, human plasma fibronectin, and human as well as bovine serum albumin by dot immunoblot.

Immunoprecipitation. Subconfluent monolayers of HKs were metabolically radiolabeled with ³⁵S-methionine (50 µCi/ml; specific activity ~ 1,100 Ci/mmol; New England Nuclear, Boston, MA) in methionine-free media. Radiolabeled HK extracts and culture medium were processed as described previously and studied by immunoprecipitation using immune and preimmune rabbit sera, reference rabbit anti-laminin 5 antiserum, serum from a reference patient with anti-epiligrin cicatricial pemphigoid, and normal human serum (19, 20). Using the same techniques, monolayer cultures of BALB/c epidermal cells were metabolically radiolabeled, extracted, and studied in immunoprecipitation experiments using anti-laminin 5, preimmune, and reference positive control rabbit sera. Immunoprecipitation samples were studied by gradient SDS-PAGE and fluorography.

Purification of rabbit IgG

Immune (and normal, control) rabbit IgG was purified by chromatography on Affi-Gel Blue Gel (Bio-Rad Laboratories), concentrated by ultrafiltration (Centricon 30; Amicon Corp., Danvers, MA), and analyzed on Coomassie blue-stained SDS-polyacrylamide gels containing standards of known concentration. These studies demonstrated that the immune and normal rabbit IgG preparations used in these studies were purified to homogeneity. Final IgG concentrations were determined by OD₂₈₀ using an extinction coefficient (1%, 1 cm) of 13.6.

Neonatal mice

Neonatal BALB/c and DBA/2Ncr mice were obtained from the National Institutes of Health animal care facility (Frederick Cancer Research and Development Center, Frederick, MD). DBA/2Ncr mice are known to be C5-deficient on the basis of being homozygous for the He^c allele. Female C57Bl/6J (W^v/+) and male W^bReJ (W/+) mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and bred to produce W/W^v (mast cell-deficient) neonates; littermates served as controls for these subjects. In passive transfer studies, W/W^v neonates and their littermates were studied in a completely blinded manner since the distinguishing features of the former are first manifest clinically at 2–3 d of age. For confirmation of phenotype, the relative number of dermal mast cells in these mice was determined in light microscopy studies of skin sections stained with toluidine blue and Giemsa.

Passive transfer studies

Neonatal BALB/c, DBA/2Ncr, W/W^v, and littermate control mice, 24–36 h old with a body weight of 1.3–1.8 g were used in these studies. Purified anti-laminin 5 (or normal, control) rabbit IgG was administered subcutaneously or intraperitoneally to neonatal mice as previously described (21–23); the majority of these animals received IgG via subcutaneous injection. IgG doses ranged from 0.01 to 10 mg/g body wt/d for 2–4 d. To avoid administration of excessive fluid volumes, IgG concentrations as high as 100 mg/ml were used; the maximal injection volume for any subject was 100 µl.

Evaluation of neonatal mice

Mice were examined daily for determination of their general well being as well as evidence of cutaneous or mucosal alterations (specifi-

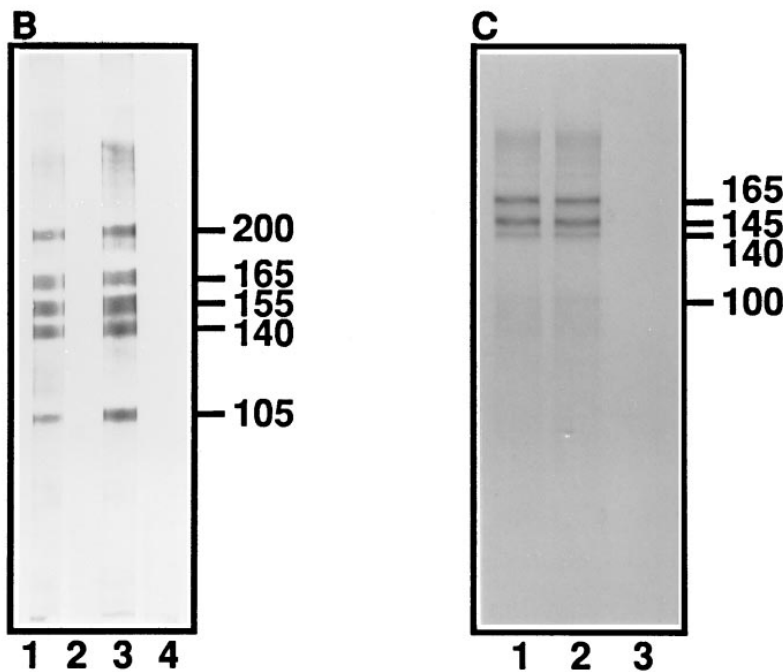
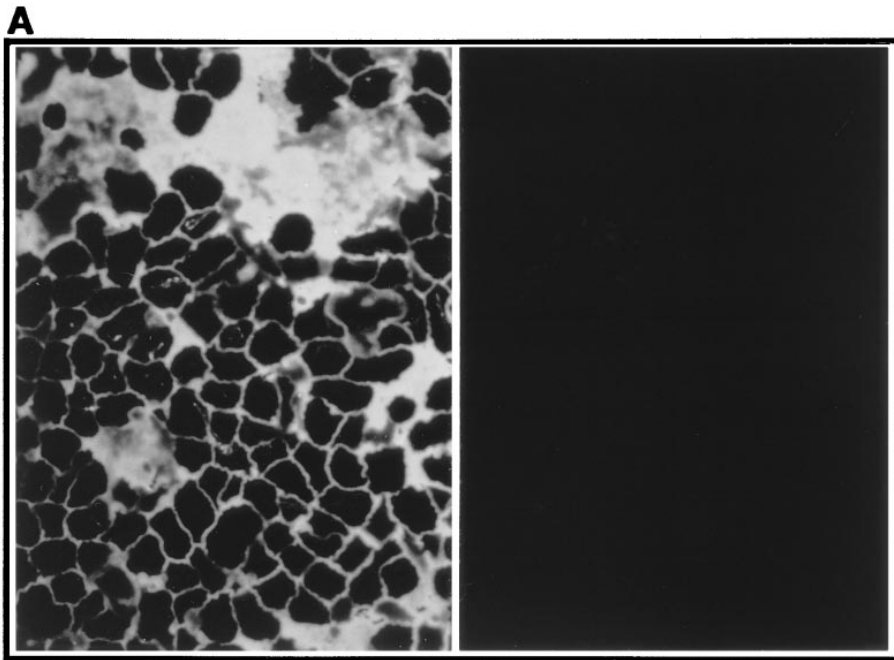


Figure 1. IgG in immune rabbit serum specifically binds laminin 5 in HK ECM and cell extracts. (A) By immunofluorescence microscopy, laminin 5 between and beside colonies of subconfluent and nonpermeabilized cultured HKs is recognized by IgG in immune (left) but not preimmune (right) rabbit sera. (B) IgG in immune (lane 3) and reference positive control (lane 1) rabbit sera specifically immunoblot the unprocessed and processed α subunits (200 and 165 kDs, respectively), the β subunit (140 kD), and the unprocessed and processed γ subunits (155 and 105 kDs, respectively) of laminin 5 in total protein extracts of cultured HK. IgG in normal (lane 2) and preimmune (lane 4) rabbit sera do not immunoblot any HK proteins on these reduced 6% SDS-polyacrylamide gels. The markers in the right margin indicate the $M_r \times 10^{-3}$. (C) In studies of metabolically radiolabeled human keratinocyte media, IgG in immune (lane 2) and reference positive control (lane 1) rabbit sera specifically immunoprecipitate a set of disulfide-linked polypeptides of 165, 145, 140, and 100 kDs that comigrate on a reduced 4–12% gradient SDS-polyacrylamide gel. Preimmune rabbit serum (lane 3, control) shows no reactivity to any HK proteins in these immunoprecipitation studies. The markers in the right margin indicate the $M_r \times 10^{-3}$. (Note: the minor variance in the calculated molecular weights of the unprocessed and processed γ subunits of laminin 5 in B and C is related to the differences in the acrylamide content of these respective gels.)

cally, erythema, edema, blisters, erosions, or crusts). At 3, 6, 12, 24, and 48 h, as well as other selected time points after the passive transfer of IgG, animals were killed and pieces of skin as well as oral and nasal mucosa were fixed in 10% buffered formalin, stained with hematoxylin and eosin, and studied by light microscopy. Samples of kidney and heart were also obtained from experimental and control mice and processed for light microscopy studies. Direct IF and immunogold electron microscopy of skin from experimental and control subjects was performed as described previously (14, 24). The former employed reagents directed against rabbit IgG, rabbit IgM (control), murine C3, and murine IgM (control), while the latter used 10-nm gold-labeled goat anti-rabbit conjugate (Janssen Pharmaceuticals, Piscataway, NJ). Sera were obtained from mice at the time of killing and studied by indirect IF microscopy to detect the presence (and ti-

ters) of circulating rabbit IgG directed against epidermal BM in 1 M NaCl split normal human skin. All passive transfer experiments included mice that received purified normal rabbit IgG (i.e., controls); these subjects were studied in the same manner as mice receiving anti-laminin 5 IgG.

Results

Laminin 5

SDS-polyacrylamide gel electrophoresis and immunoblot studies using the previously described reference anti-laminin 5 antiserum demonstrated that the laminin 5 purified from HK ECM was recovered as a complex of five disulfide-linked

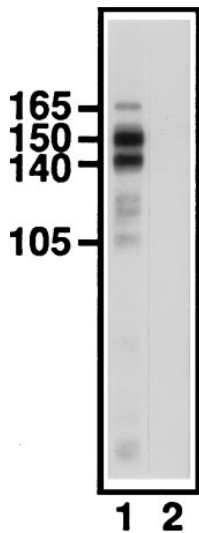


Figure 2. IgG in immune rabbit serum specifically binds laminin 5 in total protein extracts of cultured BALB/c epidermal cells. IgG in immune (lane 1) but not preimmune (lane 2) rabbit sera specifically immunoblots the processed α subunit (165 kD), the β subunit (140 kD), and the unprocessed and processed γ subunits (150 and 105 kDs, respectively) in total protein extracts of cultured BALB/c epidermal cells. The same proteins were identified by the reference positive control anti-laminin 5 antiserum, but required a longer exposure of the chemiluminescent substrate (data not shown). The markers in the left margin indicate the $M_r \times 10^{-3}$.

polypeptides corresponding to the unprocessed and processed α subunits (200 and 165 kDs, respectively), the 140 kD β subunit, and the unprocessed and processed γ subunits (155 and 105 kDs, respectively). Laminin 5 was free of contaminating proteins or degradation fragments.

Characterization of rabbit anti-laminin 5 IgG

Indirect IF microscopy. Circulating IgG from immune (but not preimmune or normal) rabbits bound epidermal BM in intact human and neonatal murine skin at a titer of 10,240 and the dermal side of 1 M NaCl split human skin at a titer of 20,480. After purification, IgG from immune rabbits bound the latter IF test substrate with a titer of 40,960, while purified normal rabbit IgG remained free of reactivity. IgG in immune (but not preimmune) sera also bound laminin 5 in HK ECM by indirect IF microscopy (Fig. 1 A). Anti-laminin 5 antisera bound epithelial BMs in human epidermis, vagina, and colon as well as murine epidermis, oral mucosa, conjunctiva, and cornea. Anti-laminin 5 antisera did not bind BMs in human or murine blood vessels (a tissue site of laminin 1 [$\alpha 1\beta 1\gamma 1$]), nerves (synapses containing laminin 3 [$\alpha 1\beta 2\gamma 1$]), or kidney (a site of laminins 1 and 3), BMs in human gall bladder or synovium, the basal lamina of striated muscle in murine tongue (a site of laminin 2 [$\alpha 2\beta 1\gamma 1$]), or any BMs in rabbit skin.

Complement fixation. Sera from immune (but not preimmune or normal) rabbits activated human complement in vitro and elicited deposition of C3 in human epidermal BM at the site corresponding to the localization of anti-laminin 5 IgG (data not shown).

Immunoblot. IgG in immune and positive control rabbit sera (but not preimmune or normal rabbit sera) specifically identified polypeptides that correspond to the previously described unprocessed and/or processed α , β , and γ subunits of laminin 5 in total protein extracts of cultured HKs and BALB/c epidermal cells (Figs. 1 B and 2, respectively) as well as HK ECM (data not shown). No other proteins in extracts of HKs, BALB/c epidermal cells, or HK ECM were recognized by IgG from rabbits immunized with laminin 5.

In direct comparative immunoblot studies, anti-laminin 5 (but not preimmune, control) sera specifically bound laminin 5 in extracts of HK ECM, but showed no reactivity to collagen

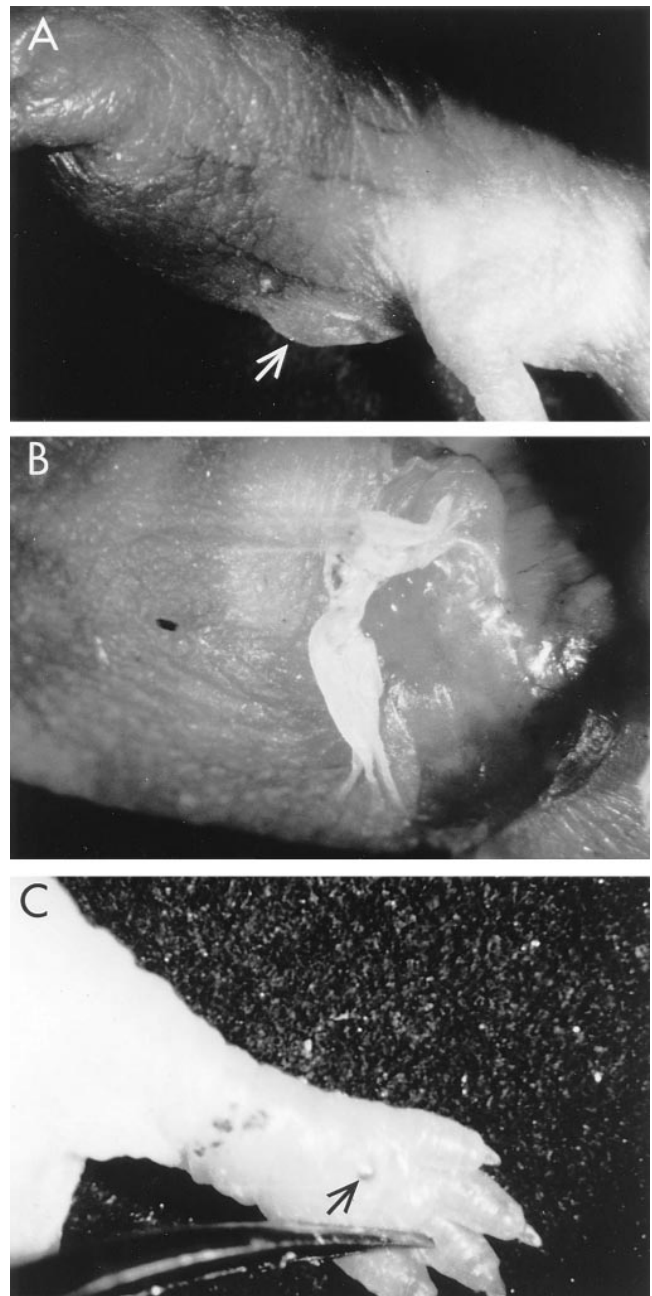


Figure 3. Anti-laminin 5 IgG specifically induces subepidermal blisters and peeling in vivo. (A) A large, tense blister is evident on the abdomen of a neonatal BALB/c mouse 24 h after passive transfer of 10 mg/g body wt of purified anti-laminin 5 IgG. A control subject that received an identical dose of normal rabbit IgG showed no clinical or microscopic evidence of epidermal separation. (B) Full-thickness peeling of epidermis away from the margin of incised skin is apparent in a BALB/c mouse killed 24 h after passive transfer of 10 mg/g body wt of anti-laminin 5 IgG. (C) A small blister is present on the plantar surface of an experimental subject 24 h after the passive transfer of 10 mg/g body wt of anti-laminin 5 IgG. Light microscopy studies confirmed that this lesion signified a subepidermal blister (see Fig. 3 C).

types I, III, IV, V, or VII, laminins 1 or 2, human plasma fibronectin, or human as well as bovine serum albumin.

Immunoprecipitation. Further evidence of the specificity of the anti-laminin 5 IgG in immune sera of these four rabbits

Table I. Passive Transfer Studies

IgG*	Dose [‡] mg/gm	Findings [§]		Direct IF [¶]	Indirect IF [¶]	Number
		Clinical	Microscopic			
BALB/c mice						
R/L5	20	Blisters, peeling	Subepidermal blister	IgG, C3	40,960	2
R/L5	5–10	Vesicles, peeling	Subepidermal blister	IgG, C3	20,480–40,960	15
R/L5	1–2.5	Peeling	Subepidermal blister	IgG, C3	5,120	4
R/L5	0.01–0.20	Normal	Normal	IgG, C3	20–320	8
R IgG	2–20	Normal	Normal	Negative	Negative	14
DBA/2NCR mice						
R/L5	10	Vesicles, peeling	Subepidermal blister	IgG, C3	40,960	3
R IgG	10	Normal	Normal	Negative	Negative	2
W/W ^v mice						
R/L5	5	Vesicles, peeling	Subepidermal blister	IgG, C3	20,480	3
R IgG	5	Normal	Normal	Negative	Negative	2
W/W ^v littermates						
R/L5	5	Vesicles, peeling	Subepidermal blister	IgG, C3	20,480	8
R IgG	5	Normal	Normal	Negative	Negative	5
						Total = 66

*Type of purified IgG passively transferred to neonatal mice; R/L5 signifies purified rabbit anti-laminin 5 IgG, R IgG signifies purified normal rabbit IgG. [‡]Total doses of purified IgG passively transferred to neonatal mice in divided doses over 2–4 d. [§]Summary of major clinical and light microscopy findings in experimental and control mice (see text for details). [¶]Direct IF microscopy results refer to the immunoreactants identified in the epidermal BMs of mice receiving IgG by passive transfer. [¶]Indirect IF microscopy results refer to the titer of circulating rabbit IgG directed against the dermal side of 1 M NaCl split normal human skin.

was demonstrated in immunoprecipitation studies of metabolically radiolabeled extracts of HK and murine epidermal cells as well as the conditioned media of cultured HKs. In these studies, immune (but not preimmune) rabbit sera identified disulfide-linked polypeptides that comigrated in SDS-polyacrylamide gels with the same polypeptides immunoprecipitated by reference laminin 5 antiserum (Fig. 1 C) as well as serum from a patient with anti-epiligrin cicatricial pemphigoid (data not shown).

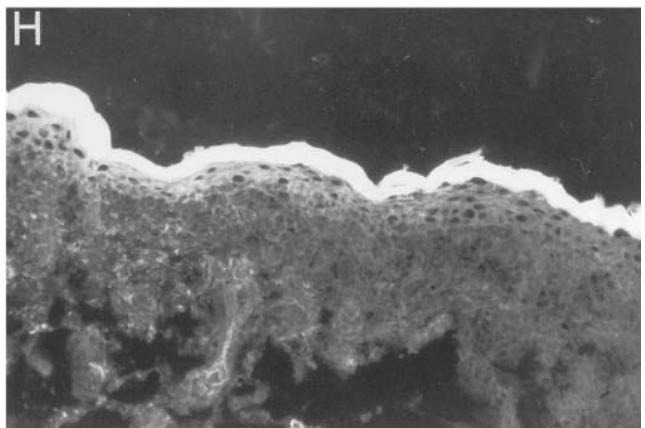
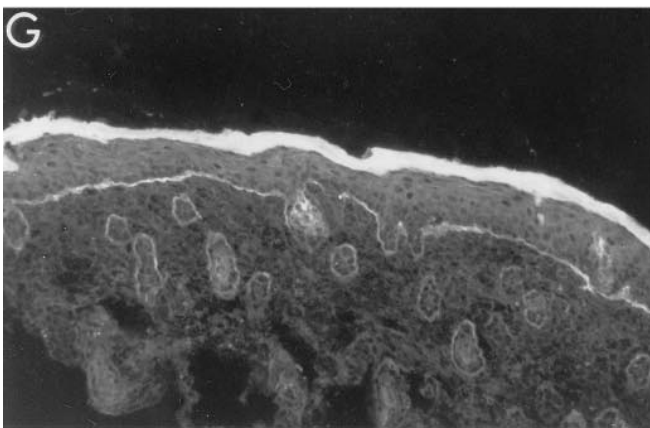
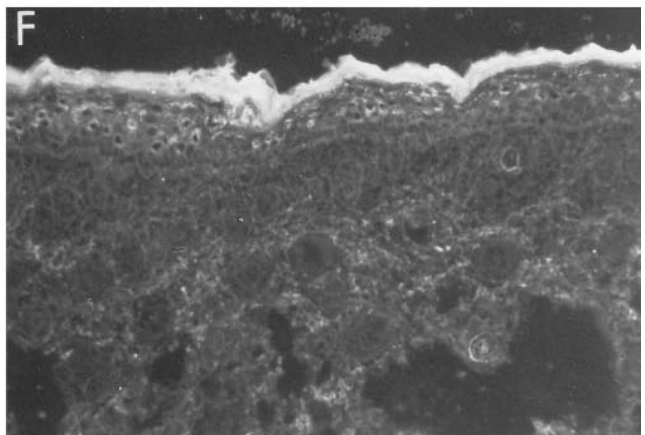
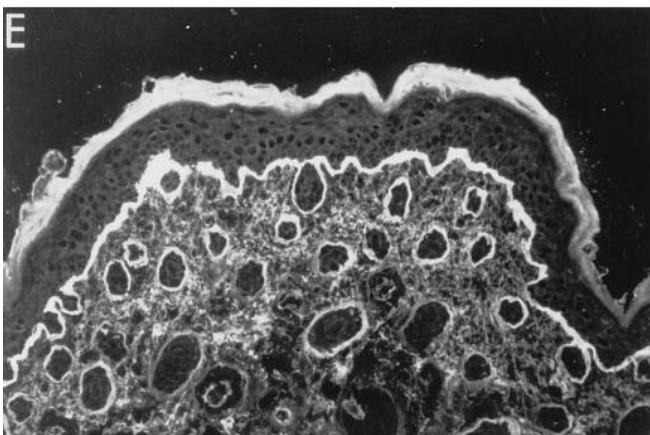
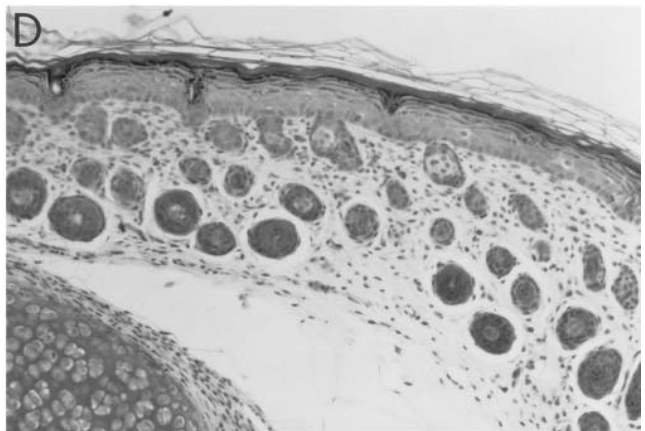
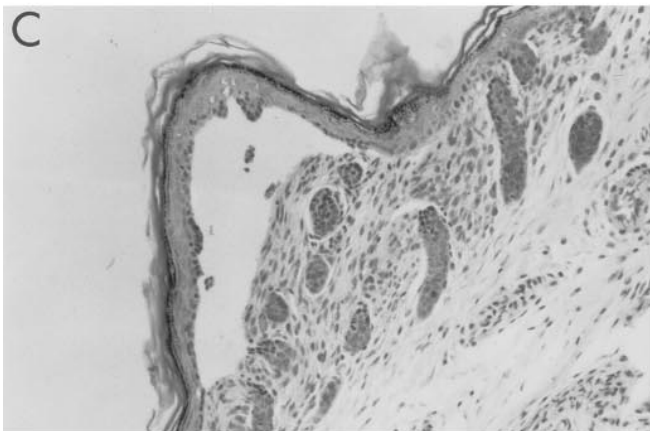
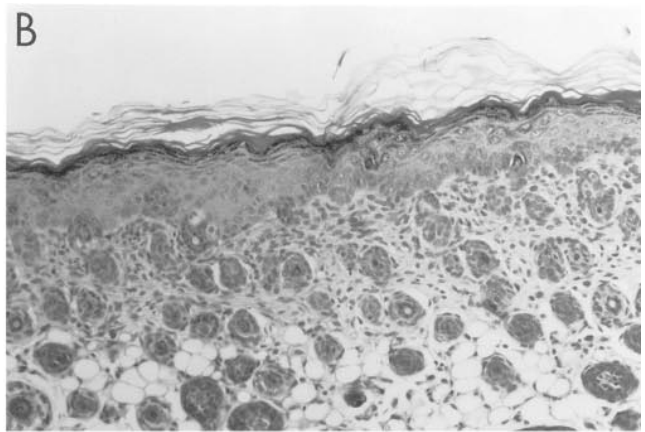
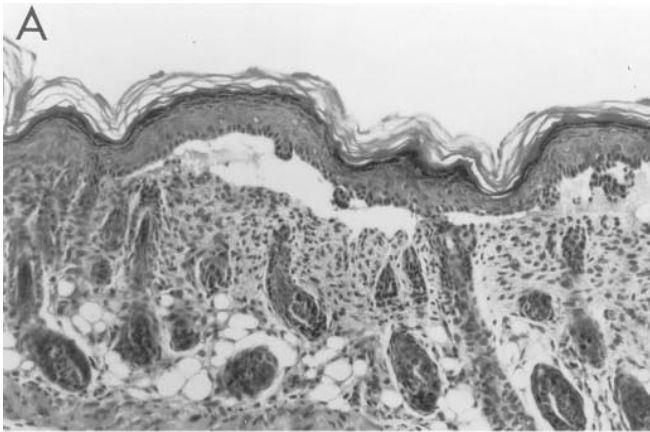
Passive transfer studies

Clinical findings. Neonatal BALB/c mice (24–36 h of age) were injected subcutaneously (along the back) or intraperitoneally with purified rabbit anti-laminin 5 (or normal rabbit) IgG daily for 2–4 d. Within 24–48 h, mice that received total doses of anti-laminin 5 IgG exceeding 5 mg/g body wt ($n = 17$) developed tense blisters on their abdomen, feet, and/or ears (Fig. 3, A and C). The number and size of blisters in these mice developed in a dose-related manner and were often accompanied by faint, localized erythema (Table I). Blisters were not localized to injection sites, but rather predominated in areas exposed to friction or trauma. The same pattern of lesions developed in mice that received anti-laminin 5 IgG via subcutaneous or intraperitoneal injections. While these mice were not frankly “Nikolsky’s positive” (i.e., subject to epidermal loss secondary to lateral mechanical pressure), incision of their skin within 1–4 d of anti-laminin 5 administration created epidermal detachment and peeling that allowed sheets of epidermis to be lifted away from the underlying nonadherent dermis (Fig. 3 B).

Time course studies after passive transfer of 5 mg/g body wt of anti-laminin 5 IgG to neonatal BALB/c mice indicated that while no clinical or histologic alterations were evident within 3 h microscopic subepidermal blisters were detectable at 6 h (see below), epidermal peeling developed within 12 h, and frank, tense blisters were present within 24–48 h after the administration of specific immunoglobulin. Interestingly, BALB/c mice that received > 5 mg/g body wt of anti-laminin 5 IgG continued to develop new blisters as late as 8 d after passive transfer of antibody. Blisters at these and earlier time points were replaced by erosions and/or crusts that were followed within a few days by reepithelization. Subsequent alterations documented in eight mice followed daily for as long as 12 wk after passive transfer of these doses of anti-laminin 5 IgG consisted only of coat abnormalities and diminished growth (early findings that proved to be relatively minor and transient).

In BALB/c mice that received total doses of 1–2.5 mg/g body wt of anti-laminin 5 IgG ($n = 4$), clinical findings were limited to peeling of epidermis after skin incision (Table I). There was no evidence of clinical lesions or peeling in mice that received < 1 mg/g body wt of anti-laminin 5 IgG ($n = 8$) or in any BALB/c mice that received 2–20 mg/g body wt of normal rabbit IgG (controls, $n = 14$).

Passive transfer of 5 or 10 mg/g body wt of anti-laminin 5 IgG also induced blisters, erosions, and peeling in newborn DBA/2NCR (C5-deficient) mice ($n = 3$) as well as W/W^v (mast cell-deficient) mice ($n = 3$) and W/W^v littermate controls ($n = 8$) (Table I). Again, no alterations were induced in neonatal DBA/2NCR ($n = 2$), W/W^v ($n = 2$), or W/W^v littermates ($n = 5$) that received equivalent doses of purified normal rabbit IgG.



Light microscopy. Light microscopy studies of flank and abdominal skin obtained from neonatal BALB/c mice 1–2 d after subcutaneous or intraperitoneal injection of anti-laminin 5 IgG (1–20 mg/g body wt; $n = 21$) revealed broad areas of subepidermal blister formation (Fig. 4 A). Such lesions were present even in what was clinically considered to be intact skin (epidermal separation and peeling being a common development during necropsy). Similar lesions were also evident in histologic studies of plantar skin (Fig. 4 C) as well as oral and nasal epithelia. As noted earlier, microscopic subepidermal blisters were present in BALB/c neonates as early as 6 h after passive transfer of 5 mg/g body wt of anti-laminin 5 IgG. Subepidermal blisters were free of leukocytic infiltrates, dermal edema, or necrotic keratinocytes (Figs. 4, A and C). Moreover, these studies found a correlation between the amount of passively transferred IgG and the extent of subepidermal blister formation in subjects receiving 1–20 mg/g body wt of anti-laminin 5 IgG. Light microscopy studies of skin from experimental mice that received < 1 mg/g body wt of anti-laminin 5 IgG ($n = 8$) or control subjects injected with 2–20 mg/g body wt of normal rabbit IgG ($n = 14$) showed normal intact murine skin devoid of any infiltrates or other histopathological alterations (Fig. 4, B and D). There was no evidence of vasculitis in skin biopsies from any experimental or control BALB/c mice. In addition, light microscopy studies of kidneys and hearts from representative experimental and control mice were identical and found no evidence of hemorrhage, vasculitis, or tissue alteration.

Light microscopy studies of skin from neonatal DBA/2NCR ($n = 3$), W/W^v ($n = 3$), and W/W^v littermate ($n = 8$) mice 1–2 d after passive transfer of 5–10 mg/g body wt of anti-laminin 5 IgG also revealed noninflammatory subepidermal blisters free of leukocytic infiltrates, dermal edema, necrotic keratinocytes, or vasculitis. Light microscopy studies of DBA/2NCR ($n = 2$), W/W^v ($n = 2$), and W/W^v littermates ($n = 5$) that received equivalent total doses of normal rabbit IgG (controls) revealed normal intact murine skin.

IF microscopy. Direct IF microscopy of skin from all neonatal BALB/c mice that received anti-laminin 5 IgG revealed continuous deposits of rabbit IgG in epidermal BMs (Fig. 4 E) (Table I). The relative intensity of these immunoreactants directly correlated with the dose of passively transferred anti-laminin 5 IgG. All mice that received total doses of anti-laminin

5 IgG exceeding 1 mg/g body wt had very bright in situ deposits of rabbit IgG in their epidermal BMs; in blistered skin, such deposits were almost exclusively localized to the dermal side of the sample. Sequential studies of BALB/c mice that received 5 mg/g body wt of anti-laminin 5 IgG ($n = 6$) found that in situ deposits of IgG in epidermal BM persisted for as long as 7 wk (the longest time point examined). In addition to deposits of rabbit IgG, continuous deposits of murine C3 were also found in the epidermal BMs of all neonatal BALB/c mice that received anti-laminin 5 IgG (Fig. 4 G). Direct IF microscopy of skin from neonatal DBA/2NCR, W/W^v, and W/W^v littermates that received anti-laminin 5 IgG also revealed continuous deposits of rabbit IgG and murine C3 in epidermal BMs. There were no deposits of rabbit IgG or murine C3 in the epidermal BMs of any mice that received normal rabbit IgG (Fig. 4, F and H).

Indirect IF microscopy studies of sera from neonatal BALB/c, DBA/2, W/W^v, and W/W^v littermates 1–2 d after passive transfer of anti-laminin 5 IgG identified circulating rabbit IgG that bound the dermal side 1 M NaCl split human skin. Titers of circulating rabbit anti-BM IgG in these mice correlated with the dose of passively transferred antibody and ranged from 5,120 to 40,960 in mice receiving total doses of 1–20 mg/g body wt of anti-laminin 5 IgG (Table I). Sequential studies of sera from BALB/c mice that received 5 mg/g body wt of anti-laminin 5 IgG found that circulating anti-BM antibodies were present in their circulation for as long as 4 wk (the longest time point examined). All indirect IF microscopy studies of sera from mice that received normal rabbit IgG were negative.

Direct immunogold electron microscopy. Direct immunogold electron microscopy studies of intact skin from a representative BALB/c mouse that received 5 mg/g body wt of anti-laminin 5 IgG localized deposits of rabbit IgG to the interface of the lamina lucida and the lamina densa (Fig. 5 A). Some deposits of IgG were also localized to the adjacent lamina densa itself. There were no deposits of gold beads in the intact skin of a representative BALB/c mouse that received equivalent total doses of normal rabbit IgG (Fig. 5 B).

Discussion

The stratified squamous epithelium of human epidermis is derived from proliferating basal keratinocytes that are attached to epidermal BM. Adhesion of basal keratinocytes to epidermal BM is critical for epithelial polarization, differentiation, morphogenesis, and wound healing. Laminin 5 is a recently identified laminin isoform that has been localized to the lamina lucida of epidermal BM where it is associated with anchoring filaments, ultrastructural elements that link basal keratinocytes to epidermal BM in vivo. In vitro, laminin 5 is one of the major components of HK-derived ECM (8, 9). Laminin 5 promotes the adhesion of HK to ECM by serving as the preferred ligand for integrin $\alpha_3\beta_1$ in plasma membranes and focal adhesions, and colocalizing with integrin $\alpha_6\beta_4$ in hemidesmosome-like stable anchoring complexes (8, 12). Interestingly, monoclonal antibodies directed against laminin 5 have been shown to impair the adhesion of HKs to their ECM (experimental findings that attest to the important role that this protein plays in HK adhesion in vitro) (9). Relatedly, recent studies have identified abnormalities of laminin 5 in vivo in patients with inherited and autoimmune subepidermal blistering diseases (10). For example, it has been shown that laminin

Figure 4. Anti-laminin 5 IgG passively transferred to neonatal BALB/c mice binds epidermal BMs, fixes complement, and induces noninflammatory subepidermal blisters in vivo. Light and immunofluorescence microscopy studies of skin from neonatal BALB/c mice 24 h after administration of 10 mg/g body wt of purified anti-laminin 5 IgG (A, C, E, and G) or normal, control IgG (B, D, F, and H). Hematoxylin- and eosin-stained sections of flank and plantar skin (A and C, respectively) from experimental subjects show separation of epidermis from epidermal BM and the resulting subepidermal blisters; lesions have developed in the absence of leukocytic infiltrates, dermal edema, or necrotic keratinocytes. Immunofluorescence microscopy studies of flank skin from an experimental subject show in situ deposits of rabbit IgG (E) and murine C3 (G) in BMs of the epidermis and hair follicles. In contrast, the flank (B) and plantar (D) skin of mice that received equivalent doses of purified normal rabbit IgG is intact and free of in situ deposits of rabbit IgG (F) and murine C3 (H) in epidermal BMs.

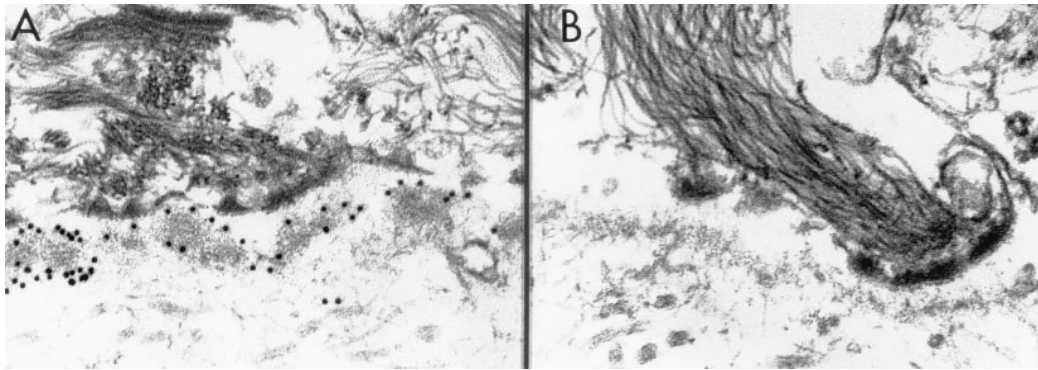


Figure 5. In situ deposits of rabbit anti-laminin 5 IgG in murine epidermal BM are specifically localized to the lamina lucida-lamina densa interface and the underlying lamina densa. (A) Direct immunogold electron microscopy of skin from a BALB/c mouse that received 5 mg/g body wt of anti-laminin 5 IgG shows specific binding of gold particles to the lamina lu-

cida-lamina densa interface and the underlying lamina densa. There is no specific binding of gold particles to the cytoplasm, hemidesmosomes, or plasma membranes of overlying basal keratinocytes or to sites in the sublamina densa region. (B) Direct immunogold electron microscopy of skin from a BALB/c mouse that received 5 mg/g body wt of normal rabbit IgG shows no specific binding of gold particles to any aspect of basal keratinocytes, epidermal BM, or the sublamina densa region.

5 is absent or diminished in the epidermal BM of patients with Herlitz's junctional epidermolysis bullosa, a potentially lethal inherited blistering disease (25–27). Moreover, mutations in genes encoding laminin 5 subunits have recently been identified in these patients (28–30). Another indication of the key role that this protein plays in epidermal adhesion *in vivo* was provided by studies showing that patients with one form of cicatricial pemphigoid, an acquired subepithelial blistering disease of mucous membranes and skin, have IgG anti-BM autoantibodies directed against laminin 5 (1–5). Thus, acquired and inherited abnormalities in laminin 5 have been directly linked to diseases characterized by separation of epidermis from epidermal BM in human skin.

The ability of monoclonal anti-laminin 5 antibodies to impair HK adhesion *in vitro* raised the possibility that anti-laminin 5 autoantibodies in patients with cicatricial pemphigoid may be pathogenic *in vivo*. To investigate this hypothesis, we immunized rabbits with laminin 5 purified from the ECM of HK, purified and characterized the resulting rabbit anti-laminin 5 IgG, and then passively transferred the latter into neonatal mice. Studies using this animal model of disease demonstrate that anti-laminin 5 (but not normal) IgG induces subepithelial blisters of skin and mucous membranes that are noninflammatory, form independent of the activation of terminal complement components or the degranulation of dermal mast cells, and continue to develop as long as 8 d after the administration of antibody. Both intraperitoneal and intradermal administration of anti-laminin 5 IgG produced subepidermal blisters in this model. Interestingly, intradermal injections of anti-laminin 5 IgG did not induce localized inflammatory reactions at injection sites, but rather produced noninflammatory blisters that predominated elsewhere (largely at sites of friction or trauma). The anti-laminin 5 IgG employed in these studies bound all subunits of human and murine laminin 5, showed a profile of tissue reactivity that is identical to the known distribution of laminin 5 *in vivo*, and did not react with other matrix proteins present in epidermal or other epithelial BMs. Correspondingly, the tissue specificity of the injury produced in this animal model (i.e., the induction of subepithelial blisters in epidermal, oral, and nasal epithelia as well as the absence of alterations at sites devoid of laminin 5) further attests to the specificity of the anti-laminin 5 IgG used in these passive transfer studies.

The experimental approach used in these studies is analogous to the recently developed passive transfer animal model of bullous pemphigoid (22, 31). Both of these models provide important insights into the pathophysiology of human blistering diseases despite the fact that passive transfer of human IgG autoantibodies directed against bullous pemphigoid antigens or laminin 5 do not cause blisters in mice. As previously reported, human anti-laminin 5 autoantibodies do not bind murine epidermal BM *in vitro* (32, 33) or after passive transfer to mice *in vivo* (Lazarova and Yancey, unpublished observations). Such findings are not unexpected since several murine monoclonal anti-human laminin 5 antibodies do not bind the epidermal BMs of nonprimates. Our passive transfer studies used extensively characterized polyclonal anti-laminin 5 IgG directed against epitopes present on all subunits of human and murine laminin 5 (including the α subunit of the former, which is targeted by IgG autoantibodies from patients with this form of cicatricial pemphigoid). In contrast, the recently described passive transfer model of bullous pemphigoid required the use of rabbit IgG specifically directed against the murine homologue of the immunodominant epitope of human bullous pemphigoid antigen 2 (22, 31).

Passive transfer of anti-laminin 5 IgG to neonatal mice induced microscopic subepidermal blisters within 6 h, peeling of epidermis at 12 h, and frank, tense blisters by 24 h. Though subepidermal blisters continued to develop in neonates for as long as 8 d after passive transfer of > 5 mg/g body wt of anti-laminin 5 IgG, none of these animals developed generalized disease, multiorgan failure, or vasculitis. Anti-laminin 5 IgG induced subepithelial blisters *in vivo* in a dose-dependent manner and as little as 1 mg/g body wt of anti-laminin 5 IgG caused separation of murine epidermis. While the doses of anti-laminin 5 IgG administered in these studies are comparable to those used in passive transfer models of other human blistering diseases, there are several notable differences between our model and other previously described experimental systems (21–23). For example, in the passive transfer model of pemphigus vulgaris, blisters develop in neonatal mice within 24 h; development of subsequent lesions requires another injection of pathogenic antibodies. In the recently established murine model of bullous pemphigoid (22, 31), specific IgG elicits subepidermal blisters in a complement-dependent manner; lesions fail to develop when pathogenic antibodies are

passively transferred to C5-deficient or complement-depleted mice. Finally, passive transfer of high doses of purified IgG from a patient with severe epidermolysis bullosa acquisita has been shown to produce alterations in neonatal BALB/c mice that mimic some aspects of this disease (i.e., deposits of immunoreactants in epidermal BM, granulocyte-rich infiltrates in dermal papillae, and pronounced dermal edema), yet failed to produce clinical or microscopic subepidermal blisters in vivo (23). All of these experimental findings differ significantly from those documented in our passive transfer studies and further suggest that anti-laminin 5 IgG produces separation of epidermis from epidermal BM in vivo by a specific rather than an inflammatory and potentially nonspecific reaction. Moreover, these experimental findings are in many ways comparable to clinical findings in patients with cicatricial pemphigoid (specifically, the minimal inflammation present in their mucosal lesions, the cell-poor infiltrate observed in light microscopy studies of their lesional tissue, and their occasional poor response to treatment with systemic glucocorticosteroids).

Induction of blisters in this animal model has been very reproducible and readily elicited by IgG isolated from four of four rabbits immunized with human keratinocyte-derived laminin 5. These results imply that the immunodominant portion of this protein plays a key role in maintaining adhesion of basal keratinocytes to epidermal BM in vivo. While rabbits immunized with laminin 5 developed high titers of IgG directed against human and murine epidermal BMs, these subjects had no in situ deposits of IgG in their epidermal BMs, and their sera showed no reactivity to epidermal BMs in multiple samples of normal rabbit skin. Moreover, upon breeding, two immune females produced litters of normal pups despite the sustained presence of high-titer anti-laminin 5 IgG that was passaged transplacentally to the circulation (but not to the intact epidermal BMs) of their newborns. While the exact explanation for this observation is unknown, it is suspected that the rabbits deleted their clones of autoreactive lymphocytes.

These studies establish the first animal model of the human blistering disease cicatricial pemphigoid and create an experimental system that can be used to investigate disease pathomechanisms and experimental therapies. This model can also be used to assess the pathogenic activity of antibodies raised against specific subunits of laminin 5, as well as portions of these subunits that are recognized to possess important biologic activities. Similarly, when mapping studies have defined the epitopes within laminin 5 that are targeted by circulating autoantibodies from patients with cicatricial pemphigoid, it should be possible to use this model to determine if such immunodominant sites harbor pathogenic epitopes as well. This approach should also further elucidate the mechanism whereby laminin 5 promotes the adhesion of epidermis to epidermal BM in vivo.

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