Epitope Mapping of the Laminin Molecule in Murine Skin Basement Membrane Zone: Demonstration of Spatial Differences in Ultrastructural Localization

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Results of studies performed to date with polyclonal anti-laminin antibodies have been conflicting as to the ultrastructural localization of this glycoprotein in skin basement membrane zone (BMZ). Whereas initial reports suggested its presence solely within the lamina lucida (LL), others have suggested that laminin is instead an exclusive component of the lamina densa (LD). In an attempt to more critically address this issue, we have examined both intact and partially separated (via 1 M NaCl) murine skin BMZ by indirect immunoelectron microscopy via a two-step immunoperoxidase technique on unfixed cryopreserved tissue, utilizing nine well-characterized monoclonal antibodies with binding specificity for laminin. Localization of the sites of the epitopes recognized by these antibodies on isolated laminin molecules was previously determined by rotary shadowing and by biochemical analyses on enzymatic fragments of laminin. Whereas at least faint immunoreactants were detected in both regions with eight of nine antibodies, predominant staining was noted within the LL with three of eight and within (and even sparsely below) the LD in three of eight. One antibody bound solely to the LL; another bound equally within both regions. Although some overlap was noted, it appears that the epitope on the distal portion of the long arm of the laminin molecule resides primarily within the skin LD, whereas epitopes on more central portions of the short arms are present within the LL or within both LL and LD. The findings of stratification of laminin epitopes within skin BMZ supports a similar recent observation in mouse kidney and suggests that portions of the laminin molecule span both LD and LL, and that there may be a non-random spatial orientation for the laminin molecule within murine skin BMZ. *J Invest Dermatol 96:309–313, 1991*

Laminin, a large (~900 kD) glycoprotein composed of three disulfide-linked subunits, is a ubiquitous component of all basement membranes, including skin [1]. By rotary shadowing electron microscopy, it has been shown that the laminin molecule is cross-shaped, and is composed of one long arm and three short arms, each having one or more globular domains [2]. A variety of different functional cell binding domains [3–7] have now been identified in separate fragments of the laminin molecule following partial proteolysis, and a series of monoclonal antibodies have been produced that recognize specific epitopes within such fragments. By immunohistochemical study of the binding sites of several intraveneously injected anti-laminin monoclonal antibodies, it has been shown recently that spatial differences exist in the ultrastructural localization of specific laminin epitopes in mouse kidney BMZ [8].

At the present time, there is conflicting data as to the precise localization of laminin in skin BMZ. Whereas Foidart et al [9] originally showed an intra-lamina lucida localization within human dermoepidermal junction, Laurie et al [10] and Fleischmajer et al [11] suggested that laminin was primarily or exclusively present within the lamina densa in this organ. In each of these studies, polyclonal antibodies of different sources were utilized. Given the availability of a series of well-characterized monoclonal antibodies to specific regions of the mouse laminin molecule, in the present study we have sought to re-address the issue of where, within at least mouse skin BMZ, portions of the laminin molecule ultrastructurally reside.

MATERIALS AND METHODS

Source of Antibodies Nine rat × mouse anti-laminin IgG class monoclonal antibodies were employed as tissue-culture supernatants in the present study. Each monoclonal antibody was produced by immunization of a Sprague-Dawley rat with laminin isolated and purified from Englebreth-Holm-Swarm tumor and was previously characterized as to binding site(s) by rotary shadowing and immunoblot analysis of pepsin and elastase digestions, as described in detail elsewhere [8]. Binding characteristics of each of these antibodies are summarized in Table I. Normal rat IgG (Cappel Laboratories, West Chester, PA) served as the matched control for each monoclonal

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Abbreviations:

BMZ: basement membrane zone
IgG: immunoglobulin G
kD: kilodaltons
LD: lamina densa
LL: lamina lucida
NaCl: sodium chloride
PBS: phosphate-buffered saline

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Table I. Binding Site(s) of Each Anti-Laminin Monoclonal Antibody Employed, as Previously Determined by Rotary Shadowing, Immunoblot, and Immunoprecipitation Analyses, and via Ultrastructural Localization Along the Dermoepidermal Junction of Intact and NaCl-Separated Mouse Skin

Laminin Epitope	Binding Sites (via Rotart Shadowing)	Binding Site(s) of Monoclonal Antibody to Specific Epitope of Murine Laminin Molecule (via Immunoelectron Microscopy and Immunoperoxidase Staining)			
		Intact Mouse Skin		NaCl-Split Mouse Skin	
		Lamina Lucida	Lamina Densa	Lamina Lucida	Lamina Densa
9B5	P1 fragment (unreduced)	1+	−	1+	−
3A4	P1 fragment (unreduced	1+	1+	2+	2+
8D3	P1 fragment (unreduced)	2+	1+	2+	+/−
8B3	P1 fragment (unreduced)	−	−	−	−
6D6	P1 fragment	1+	1+	2+	+/−
5C1	P1 fragment (unreduced); lateral arms near the center of the intact laminin cross	1+	1+	1+	2+
5A2	Inner globule on short arms of intact laminin molecule and elastase-treated fragments containing globular domains; immunoprecipitates only B chains	2+	1+	2+	+/−
9D2	B chains (specific site unknown)	1+	1+	+/−	1+
5D3	Near terminal domain of long arm of intact and E8 fragment (~140 kD) of molecule	2+	2+	1+	2+
Negative control		−	−	−	−

antibody. Fluorescein- and horseradish peroxidase-conjugated anti-rat IgG (Cappel) served as the secondary antibodies for all immunofluorescence and immunoperoxidase studies, respectively. Unless otherwise specified, all dilutions were performed in 0.0067 M phosphate-buffered saline (PBS), pH 7.4.

Source and Preparation of Tissues Manually epilated adult BALB/c mouse skin was surgically removed from the thorax of euthanized animals and all visible subcutaneous tissue removed. For intact skin preparations, such tissue was directly embedded in OCT medium (Lab-Tek Products, Napersville, IL), snap frozen in liquid nitrogen, and stored at −70°C until needed. Other skin specimens were instead first partially separated at the level of the lamina lucida by incubation on the surface of a 1 M NaCl solution (48–72 h, 4°C), as previously described elsewhere [12]. Uniform separation at the level of the lamina densa was confirmed by immunofluorescence localization of bullous pemphigoid antigen and type IV collagen to the roof and base, respectively, of separated skin, as previously described [13,14].

Indirect Immunofluorescence Studies (Intact and NaCl-Separated Skin) Indirect immunofluorescence was performed on cryopreserved intact and NaCl-separated BALB/c mouse skin. In brief, 6–8 μm-thick cryostat sections were prepared from each specimen and placed on albuminized glass slides. Each specimen was first exposed to one of the nine anti-laminin monoclonal antibodies (undiluted; 30 min; room temperature; humidified chamber), rinsed extensively with PBS, and then incubated in the presence of fluorescein-conjugated goat anti-rat IgG (1:20; 30 min; room temperature). Following further rinsing with PBS, 50% glycerol in PBS was placed over each tissue section, each slide overlaid with a coverslip, and the tissue sections examined with a Leitz Laborlux 12 immunofluorescence microscope by epi-illumination.

Indirect Immunoelectron Microscopy Indirect immunoelectron microscopy was performed with each anti-laminin monoclonal antibody, using 10 μm-thick cryosections of unfixed intact and NaCl-separated BALB/c skin as tissue substrates. Tissue sections were serially incubated with normal goat serum (1:10; 20 min; room temperature), anti-laminin monoclonal antibody (1:3–1:12; 18 h; 4°C), and horseradish peroxidase-conjugated goat anti-rat IgG (1:2–1:8; 2 h; room temperature). As a negative control, normal rat serum (1:2–1:8) was substituted for each monoclonal antibody. After brief fixation with 1% glutaraldehyde, tissue sections were then incubated with diaminobenzidine and hydrogen peroxide, post-fixed with 1% osmium tetroxide, dehydrated with a gradient alcohol series, and embedded in Spurr's resin. Ultrathin sections were counterstained with lead citrate and then examined under a Phillips 300 electron microscope.

RESULTS

At the light microscopic level, eight of nine anti-laminin monoclonal antibodies bound uniformly to all basement membranes in intact skin (Fig 1), and to the dermal side of the partially separated der-

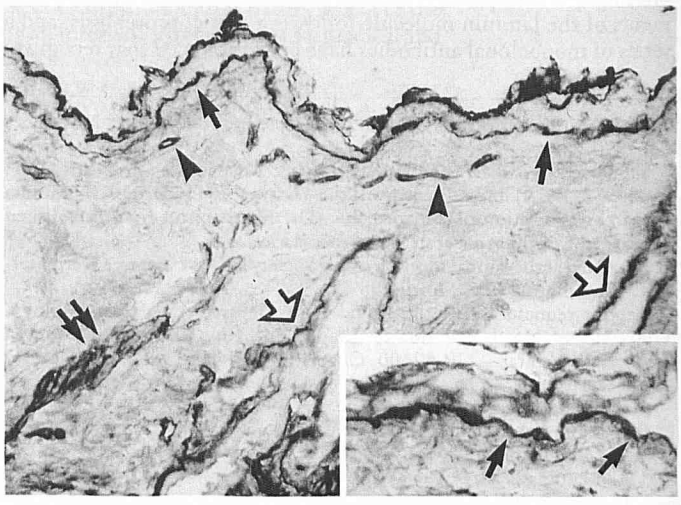

Figure 1. At the light microscopic level, immunoperoxidase staining of BALB/c mouse skin with each of eight anti-laminin monoclonal antibodies demonstrated linear deposition of immunoreactants along the dermoepidermal junction *(arrows)*, and around hair follicles *(empty arrows)*, arrector pili muscles *(double arrows)*, and dermal vasculature *(arrowheads)*. Magnification, ×250. *Inset,* following partial separation of the epidermis from the dermis with 1 M NaCl, laminin was seen exclusively on the dermal side *(arrows)* of the dermoepidermal junction. Magnification ×400.

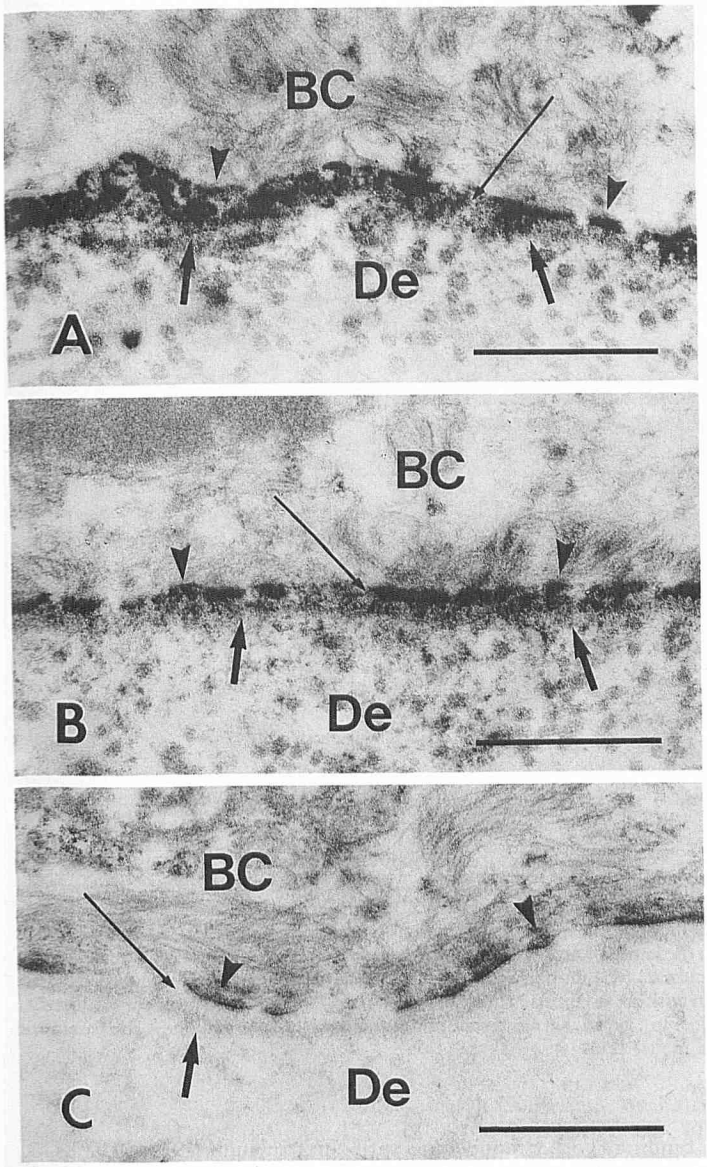

Figure 2. Indirect immunoelectron microscopy of intact BALB/c skin, following incubation with specific anti-laminin monoclonal antibodies and horseradish peroxidase-conjugated anti-rat IgG. BC, basal cell; De, dermis; *arrowheads,* hemidesmosomes; all magnification *bars,* 0.5 μm. *A,* 5D3 monoclonal antibody: immunoreaction products are distributed almost equally in both the lamina densa *(thick arrows)* and the lamina lucida *(long arrow),* with sparse amounts of reaction products also visible focally within the sublamina densa region. *B,* 5A2 monoclonal antibody: immunoreaction products are more strongly localized within the lamina lucida *(long arrow)* than along the lamina densa *(arrows),* especially in areas directly underneath hemidesmosomes *(arrowheads). C,* Normal rat serum (negative control): no immunoreactants are noted within the lamina lucida *(long arrow)* or along or beneath the lamina densa *(arrow).* Note, however, the intrinsic electron density of the hemidesmosomes, when visualized by this approach.

moepidermal junction in all NaCl-treated specimens (insert, Fig 1). One monoclonal antibody (8B3) did not bind to any of the skin specimens, although the latter antibody has been previously shown to still readily bind to some mouse kidney tubular BMZ and mesangial matrices [8].

At the immunoelectron microscopic level in intact mouse skin (Fig 2), five of the eight anti-laminin monoclonal antibodies that bound to skin basement membrane revealed apparent equal amounts of immunoreactants within both lamina lucida and lamina densa. In contrast, two of the monoclonal antibodies (5A2; 8D3) appeared to preferentially bind to the lamina lucida, and one monoclonal antibody (9B5) appeared to bind exclusively to the lamina lucida.

In contrast, greater differences in spatial staining by several of the antibodies were uncovered in those skin specimens partially separated at the level of the lamina lucida by pre-exposure to 1 M NaCl (Fig 3). Specifically, predominantly lamina lucida staining (Fig 3*A*) was noted with three of eight antibodies (5A2; 6D6; 8D3), exclusive intra-lamina lucida staining with one antibody (9B5), primarily lamina densa and to a much lesser extent also sub-lamina densa staining (Fig 3*B, C*) in three of eight (5C1; 5D3; 9D2), and essentially equal distribution of staining with one antibody (3A4). These results are detailed in Table I.

DISCUSSION

Considerable controversy still exists in the literature as to the actual localization of the laminin molecule in skin basement membranes [9–11]. To add to this confusion, little, if any, published information exists regarding the spatial binding sites for each of the anti-laminin polyclonal antibodies that have been previously utilized in skin-related ultrastructural studies. Based upon the results of our studies with a panel of well-characterized anti-laminin monoclonal antibodies, however, it is now quite evident that the laminin molecule does not reside within a single portion of the basement membrane that comprises the dermoepidermal junction. Rather, different epitopes on the laminin molecule appear to have preferential spatial orientation, although some can be found in both the lamina lucida and lamina densa in varying amounts. Taken together, however, it is clear that the entire laminin molecule must span at least a portion of both the lamina lucida and the lamina densa. As such, these findings may explain the apparent discordance among previous observations in human skin, if in fact marked differences in representation of specific epitopes existed in those polyclonal antibody preparations previously employed.

When all of the data in the present study are collectively considered, it appears that although some overlap in epitope distribution was observed, in at least mouse skin basement membrane, some epitopes on the distal portion of the long arm of laminin (as exemplified by 5D3) reside primarily within the lamina densa; similarly, at least some epitopes on the short arms of the molecule (such as 5C1 and 9D2) also insert into the lamina densa, although different conformations of the molecule would also allow for the detection of the latter epitopes to a lesser extent within the lamina lucida. In contrast, other epitopes on the laminin molecule appear to reside primarily (i.e., 5A2; 6D6; 8D3) or exclusively (i.e., 9B5) within the lamina lucida, although lesser amounts of immunoreactants detectable within the lamina densa with several of the corresponding monoclonal antibodies suggest that some laminin molecules may be so oriented within the dermoepidermal junction as to embed even those more central portions of the molecule within the lamina densa as well. This suggests the likelihood that the orientation of the laminin molecule within the dermoepidermal junction is non-random, and presumably reflects the optimal orientation for interactions with other matrix components (e.g., type IV collagen and heparan sulfate proteoglycan) that are present within skin basement membrane.

Recently, Schittny et al performed a similarly designed study in mouse cornea, utilizing immunogold technique with antibodies having binding specificity for three distinct portions of the murine laminin molecule (central segments; end of the long arm; end of one or more of the short arms) [15]. Analogous to our findings in skin dermoepidermal junction, definite differences in epitope localization were noted in corneal epithelial basement membrane. Whereas epitopes of central segments of the molecule were most often noted within the very uppermost portion of the lamina densa, epitopes residing on the ends of the short arms were more heavily detectable within the lamina lucida than the adjacent lamina densa. In contrast, epitopes on the end of the long arm of the laminin molecule could

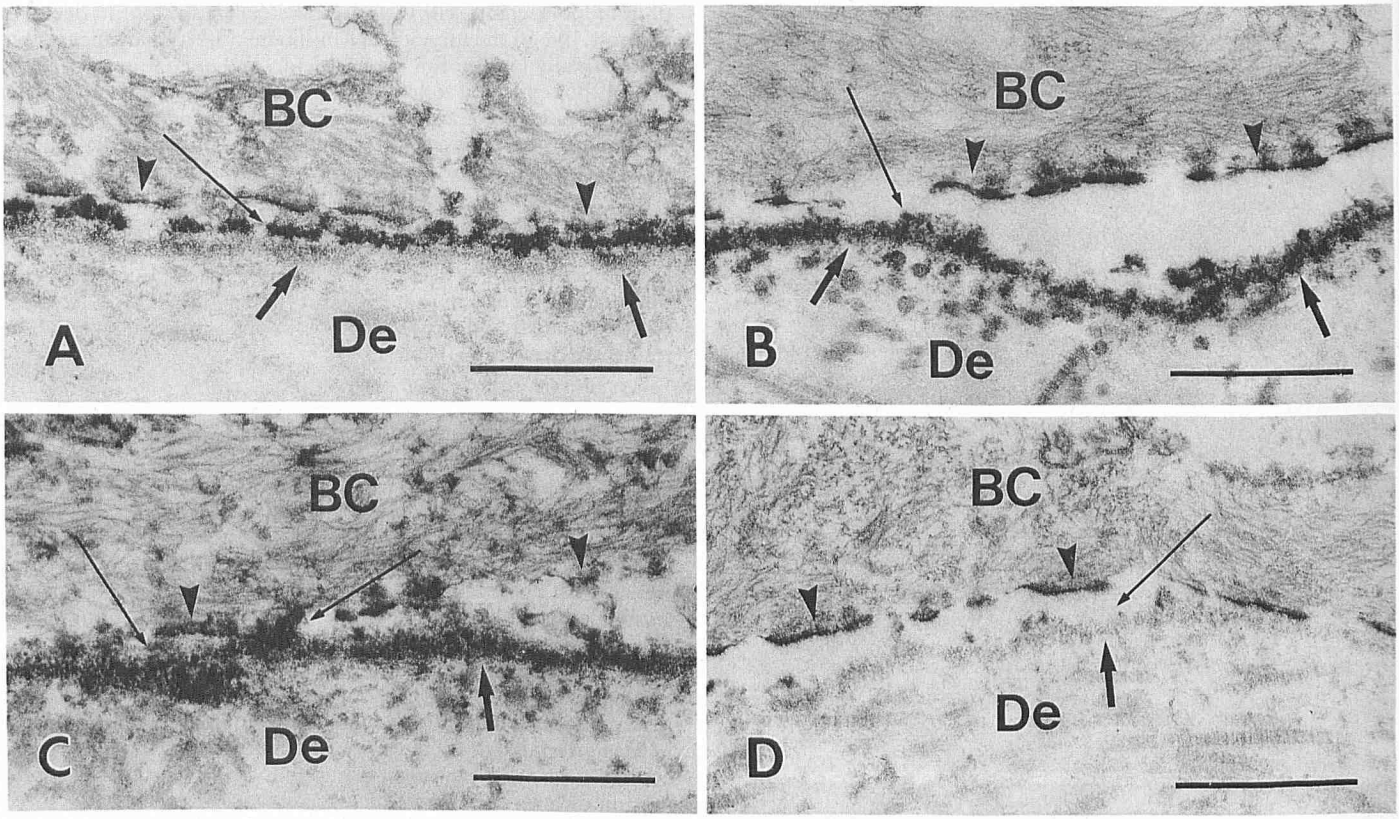

Figure 3. More distinctive differences were noted in the spatial localization of specific laminin epitopes when indirect immunoelectron microscopy was performed on BALB/c skin partially separated through the lamina lucida via prior exposure to 1 M NaCl. BC, basal cell; De, dermis; *arrowheads,* hemidesmosomes; all magnification *bars,* 0.5 μm. *A,* 5A2 monoclonal antibody: immunoreaction products are essentially exclusively confined to the mid-lamina lucida *(long arrow).* The lamina densa *(thick arrows)* is virtually devoid of staining. *B,* 9D2 monoclonal antibody: in marked contrast to that seen with 5A2 monoclonal antibody, 9D2 immunoreaction products are almost exclusively localized within the lamina densa *(arrows),* with only sparse, focal remnants of reactants *(long arrow)* visible within the lowermost portion of the NaCl-exaggerated lamina lucida. *C,* 5C1 monoclonal antibody: primary localization of immunoreactants remains within the lamina densa *(arrow)* although sparser deposits can also be seen within the lamina lucida *(long arrows)* and even within the upper sub-lamina densa region. *D,* Normal rat IgG (negative control): identical to Fig 2*C* (intact skin), no background staining is noted within the region of the basement membrane (lamina densa, *arrow;* lamina lucida, *long arrow*) of partially separated mouse skin.

be detected in three different locations: within the center of the basement membrane, at the boundary of the overlying cells and the lamina lucida, or at the boundary of the connective tissue stroma and the lamina densa. Of particular interest, these findings were contrasted with diffuse distribution of each portion of the molecule within Descemet's membrane. It was concluded in this latter study that the laminin molecule did indeed have a rather limited repertoire of orientations within the corneal epithelial basement membrane, with its center portion most often located near the junction of the lamina lucida and lamina densa, and with the end of its long arm either close to the overlying cells (presumably attaching via specific cell membrane receptor molecules) or in apposition with the underlying stroma, thereby facilitating interaction with matrix components.

It is also of considerable interest to contrast the findings in the present study with those observed with the same monoclonal antibodies in mouse kidney [8]. In the latter study, horseradish peroxidase-conjugated anti-laminin monoclonal antibodies were injected intravenously, and then kidneys humanely harvested 1–3 h later. In the mouse kidney, the monoclonal antibody (5D3) that localized to the terminal end of the long arm was variably present within peripheral loop glomeruli and exclusively present within the lamina rara of the thick BMZ of Bowman's capsule and proximal tubules, rather than primarily within the lamina densa, its site of residence within the dermoepidermal junction. On the other hand, 5D3 densely labels the lamina densa of distal tubular basement membranes in the kidney, which, like those of skin BMZ, are generally quite thin. Binding of other monoclonal antibodies, including 9B5, 9D2, 8D3, 6D6, 5A2, and 3A4, was completely absent within peripheral loop glomerular BMZ, but was focally detectable within both lamina rara and lamina densa of the BMZ of the proximal tubule. In contrast, all of these monoclonal antibodies readily labeled skin BMZ. Taken together, these latter findings would suggest almost completely different orientations for the laminin molecule in some kidney and skin BMZ, possibly reflecting organ-specific differences in ultrastructure (e.g., two lamina lucida regions in kidney glomeruli as opposed to one in skin; absence of hemidesmosomes, anchoring filaments, and anchoring fibrils in kidney), in overall antigenic composition of BMZ, and in function of resident BMZ.

It is also of interest that one anti-laminin monoclonal antibody (8B3) lacked any binding to skin BMZ. Whereas this antibody bound to some kidney BMZ, previous studies have demonstrated that this antibody does not recognize BMZ surrounding peripheral nerves, muscles (skeletal, cardiac, smooth), and structures within intestinal tissue, despite attempts at chemical "unmasking" of the epitope. Analogous restrictions in the expression of selected epitopes of human laminin have been noted by others using monoclonal antibodies on selected genitourinary tract tissues [16]. This and our findings already discussed in mouse skin provides additional support to the accumulating body of evidence (reviewed in [7]) that different laminin isotypes may exist and differ in their tissue distribution. Alternatively, it is possible that the particular ultrastructural and biochemical features present within the dermoepidermal junction may so alter the spatial orientation of the laminin molecule as to

prevent recognition of a specific conformational site, rather than a primary sequence, by the 8B3 monoclonal antibody.

Unfortunately, none of the monoclonal antibodies employed in this study bound sufficiently strongly to human skin basement membrane zone to permit direct examination of the latter tissue. As such, it is still possible that a distinctly different distribution of the laminin molecule might occur in human skin, although we believe that the latter is unlikely. Rather, based upon the rather striking differences in staining seen in murine skin, we would anticipate at least the same degree of compartmentalization in higher vertebrates. However, confirmation of the latter hypothesis must await the performance of identical studies on human skin when a panel of anti-human laminin monoclonal antibodies representing a similarly diverse spectrum of epitopes can be produced.

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ANNOUNCEMENT

The Gordon Research Conference on Barrier Function of Mammalian Skin will be held on August 12–16, 1991 at Plymouth State College in Plymouth, New Hampshire, USA. Topics to be discussed include epidermal biophysics, percutaneous absorption, and epidermal cell culture. All interested researchers are encouraged to apply. Applications are available from the Gordon Conference office or from the Chairman, Dr. Russell O. Potts, Cygnus Therapeutic Systems, 400 Penobscot Drive, Redwood City, CA 94063.