Immunogold Localization of the 97-kD Antigen of Linear IgA Bullous Dermatosis (LABD) Detected with Patients' Sera

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The classification of linear IgA bullous dermatosis in the group of subepidermal blistering diseases is still a matter of controversy. This situation is due partly to the considerable clinical heterogeneity of the disease but also results from the difficulties in characterization and localization of the specific basement membrane zone antigen(s) recognized by immunoglobulin (Ig)A antibodies. In the present study, we have combined the Western blot detection of circulating autoantibodies with an ultrastructural immunogold labeling of human skin antigens using the same patients' sera. Our results, obtained with a short series of sera showing exclusive IgA class reactivity with the epidermal portion of salt-split skin, indicate that the antibodies recognizing the 97-kD antigen on immunoblot bind to the hemidesmosomal plaques of basal keratinocytes and the adjacent lamina lucida. These homogeneous laboratory results remain in striking contrast to the heterogeneity of clinical pictures in the patients studied, suggesting a participation of complementary, possibly not humoral, phenomena in the pathogenesis of linear IgA bullous dermatosis. ***Key words: immunogold labeling/basement membrane zone/hemidesmosome. J Invest Dermatol 103:656–659, 1994***

Linear IgA bullous dermatosis (LABD) is an acquired subepidermal blistering disease characterized by the presence of linear IgA at the dermo-epidermal junction (DEJ) of perilesional skin [1,2]. Bullae and the underlying dermis usually contain infiltrate cells, predominantly neutrophils. Clinically, the patients demonstrate a vesicobullous eruption that may resemble skin lesions of bullous pemphigoid or dermatitis herpetiformis. Involvement of mucous membranes is less frequent. The lesions usually heal with low-dose corticosteroid and sulfone treatment. However, scarring lesions, particularly ocular, have been observed in some cases, leading to the diagnosis of immunoglobulin (Ig)A cicatricial pemphigoid [3–5]. Another clinical subtype of LABD is chronic bullous disease of childhood (CBDC), occurring predominantly in preschool children. In CBDC there is frequently an annular distribution of grouped blisters observed in the groin and inner thighs and spontaneous remission usually occurs within 2 to 3 years.

Biochemical studies of cutaneous antigens involved in the pathogenesis of LABD have to date given divergent results, which are likely related to patient selection criteria and the methodology employed. The most reproducible and reliable data concern the detection by LABD sera of an IgA-specific 97-kD protein in epidermal preparations from ethylenediaminetetraacetic acid (EDTA)-separated normal human skin [6]. Immunoglobulins reactive with the 97-kD band can be eluted from immunoblots and their binding to the basement membrane zone of normal epidermis confirms the specificity of the detected autoantibody. Another putative LABD antigen of 285 kD has been described in a dermal skin extract (after 1 M NaCl split) [7]. However, its detection with sera reactive on indirect immunofluorescence with the epidermal portion of salt-split skin raises questions about its potential significance. Immunoblot reactivity with a dermal protein of 255 kD was obtained with two of four LABD sera binding to the dermal portion of salt-split skin [8], once again indicating the heterogeneity of the cases. Selected sera from patients with "linear IgA bullous dermatoses" reacting with the dermal side of NaCl-separated normal human skin have been observed to recognize a 290-kD dermal protein [9] or a dermal antigen co-migrating with an enriched pro-collagen VII band.‡ These findings suggest the existence of an IgA form of epidermolysis bullosa acquisita, previously not distinguished from LABD.

Publications describing the ultrastructural localization of antigens recognized by LABD sera are infrequent and, again, show divergent results. Using the indirect immunoperoxidase method, we have recently reported three patterns of reactivity of LABD sera with normal human skin.§ Most of the studied sera reacted with the lamina lucida (LL) and often displayed a hemidesmosome-related distribution. We could detect a sub-lamina densa (LD) antigen recognition only in isolated cases. These results confirmed previously published case reports on the immunoreactivity of LABD sera with

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Abbreviations: LABD, linear IgA bullous dermatosis; CBDC, chronic bullous disease of childhood; LL, lamina lucida; LD, lamina densa.

‡Rusenko KW, Gammon WR, Briggaman RA: Type VII collagen is the antigen recognized by IgA anti-sub lamina densa autoantibodies (abstr). *J Invest Dermatol* 92:510, 1989.

§Kowalewski C, Haftek M, Schmitt D: Ultrastructural localization of binding sites for linear IgA bullous dermatosis (LABD) antibodies (abstr). *J Invest Dermatol* 101:404, 1993.

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an antigen localized in the LL [6,10], but remain in opposition with another immunoelectron microscopy (IEM) immunoperoxidase study performed on a large group of patients in which circulating IgA autoantibodies consistently recognized the sub-LD zone of normal human skin [11].

In the present report on LABD antigen(s) studied with selected patients' sera, we describe correlations between the results obtained using indirect immunogold labeling IEM and immunoblotting with proteins extracted from EDTA-separated human epidermis.

MATERIALS AND METHODS

Patient Sera Patients with a subepidermal blistering disease diagnosed as LABD according to the current clinical or immunohistochemical criteria [1,2,12] were included in this study. We have selected six sera showing high titers of circulating autoantibodies with strong, linear, exclusive IgA class reactivity with the basement membrane zone on indirect immunofluorescence. Two patients showed classical adult form of the disease, one presented with cicatricial pemphigoid-like scarring lesions including ocular involvement, and the remaining three were CBDC patients. Serum samples were obtained during active phases of the disease. Controls included a bullous pemphigoid serum with IgG anti-180-kD antigen immunoblot reactivity, a human monoclonal IgG antibody against the 230-kD bullous pemphigoid antigen [13], two epidermolysis bullosa acquisita sera (one of IgG, another of IgA class [9], exclusively) reactive with the 290-kD dermal antigen, and two normal sera from healthy blood donors.

Indirect Immunofluorescence on NaCl- and EDTA-Split Skin Normal human skin fragments (mammoplasty) were incubated either in 1 M NaCl or in 20 mM EDTA for 18 h at 4°C, snap-frozen, and cut into 5-μm sections on a cryomicrotome, according to the methods previously described [6,14]. These chemically separated skin samples, showing a split along the lower LL, were used as substrates for evaluation of immunoreactivity of the sera. Briefly, frozen tissue sections collected on glass slides were first incubated with progressive dilutions of the tested sera (up to 1:320 in phosphate-buffered saline [PBS], pH 7.2), washed in PBS, stained with fluorescein-conjugated anti-human IgA or anti-human IgG rabbit antibodies diluted 1:20 (Zymed Labs, San Francisco, CA), washed again with PBS, and finally observed with an epifluorescent microscope.

Indirect Immunogold Post-Embedding Labeling Using Autoimmune Human Sera Small pieces of normal human skin (flexor surface of a male forearm) were cryoprotected with 20% glycerol/PBS (pH 7.4; 30 min at 4°C) and plunge-frozen in liquid propane using an HM-80 cryofixation device (Reichert-Jung-Leica, Vienna, Austria). The tissue was subsequently dehydrated with methanol (−80°C to −60°C), cryosubstituted, and embedded at −60°C in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg, Germany) using CS-Auto (Reichert-Jung-Leica) and ultraviolet light polymerization. Ultra-fine sections collected on formvar-coated nickel grids were used for immunolabeling, basically following the method of Shimizu *et al* [15]. The sections were first incubated for 15 min on drops of blocking buffer (5% normal goat serum, 0.1% gelatin, 0.8% bovine serum albumin [BSA] in PBS, pH 7.4), then 1 h with a patient's serum (diluted 1:20 in incubation buffer: 1.0% normal goat serum, 0.1% gelatin, 0.8% BSA in PBS, pH 7.4), washed (6 × 2 min in washing buffer: PBS with 0.1% gelatin, 0.8% BSA), reacted with rabbit anti-human IgA or IgG (Fab')2 (Dako SA, Trappes, France; diluted 1:500 in incubation buffer; 1 h), and washed again (6 × 2 min). Localization of the autoantibody binding sites was revealed with goat anti-rabbit IgG immunogold conjugate (GAR IgG, G-5; Amersham, Amersham, UK) applied at 1:20 dilution in incubation buffer, for 1 h. Following final washes in washing buffer (2 × 4 min) in PBS (3 × 4 min), and in H_2O (2 × 4 min), the sections were counterstained with uranyl acetate in methanol for 5 min.

Immunoblot of IgA Class Autoantibodies Reactive with Epidermal Proteins Reactivity of IgA class antibodies present in sera was evaluated by Western immunoblot of sera against epidermal extract containing the 230- and 180-kD bullous pemphigoid antigens as well as an epidermal wash solution enriched for the 97-kD LABD antigen, as described previously [6,16,17]. Briefly, freshly obtained abdominoplasty skin was immediately frozen at −70°C stored up to 1 month and thawed immediately prior to use. The epidermis was easily separated from dermis after soaking for 18 h in PBS containing 20 mM EDTA at 4°C. Epidermis was washed 3 times for 15 min in PBS and the wash solution pooled. The wash solution was dialyzed against water at 4°C, lyophilized, and resuspended in sample buffer. Preparation of epidermal extract containing the 230- and 180-kD antigens was performed by the extraction of sheets of epidermis in sodium dodecyl sulfate (SDS) and β-mercaptoethanol, as previously reported [16]. Eighty micrograms of epidermal extract or epidermal wash was applied to each lane of separate 6.5% SDS polyacrylamide gel electrophoresis gels [16]. The separated proteins from both gels were electrophoretically transferred onto nitrocellulose sheets and their reactivity with test sera at 1:10 dilution was established using biotin-labeled anti-alpha chain and anti-gamma chain antibody and avidin-biotin-peroxidase complex [18].

Table I. Results of Indirect Immunolabeling Studies Indicating Localization of the 97-kD Antigen in Hemidesmosome-Associated Cell Membrane

Clinical Type of LABD[a]	IIF on EDTA Split Skin (Titer)[b]	Indirect Immunogold IEM[c]	Western Blot (Epidermal Extract)[d]
CBDC[e]	Roof (1:80)	HD/underlying LL[e]	97-kD
CBDC	Roof (1:160)	HD/underlying LL	97-kD
CBDC	Roof (1:320)	HD/underlying LL	Negative
Classic adult	Roof (>1:320)	HD/underlying LL	97-kD
Classic adult	Roof (1:20)	HD/underlying LL	97-kD
CP-like adult	Roof (1:80)	HD/underlying LL	97-kD

[a] Patients were selected according to the following criteria: subepidermal blistering disease, linear deposits on DEJ exclusively of the IgA class, and high titer of circulating IgA autoantibodies.
[b] These sera were also reactive on 1 M NaCl-split skin.
[c] Normal human skin cryofixed and low-temperature embedded in Lowicryl K11 M was used as substrate. Mixed pattern of immunogold labeling was obtained in all cases. Observation of several sections revealed that the predominant localization was the hemidesmosome-associated portion of the plasma membrane. Appropriate positive controls were included and confirmed the reliability of our labelings.
[d] None of the LABD sera reacted with the 180- or 230-kD bullous pemphigoid antigens present in epidermal extract.
[e] CBDC, chronic bullous disease of childhood; CP, cicatricial pemphigoid; LL, lamina lucida; HD, hemidesmosome.

RESULTS

Indirect immunolabeling studies with the selected LABD sera gave the results summarized in **Table I**. Irrespective of the clinical subtype, sera from patients with LABD demonstrated exclusively IgA class immunoreactivity with the epidermal portion of NaCl- and EDTA-split skin on indirect immunofluorescence in the titers noted. All sera recognized antigen(s) localized ultrastructurally to the hemidesmosomal plaques and the adjacent LL **(Fig 1)**. Both structures were labeled by the same serum on the same specimen but with different labeling intensity, most probably related to the DEJ cutting angle. Most of the immunogold particles were found in a linear disposition within the basal cells, directly over the hemidesmosomal portion of the plasma membrane. Some periodic extracellular labeling was also noted. These findings suggest predominant antigen distribution along the cell membrane within the hemisdesmosomes. There were no major differences among the six sera concerning their ultrastructural pattern of reactivity, even though the intensity of the labeling varied. All but one sera were reactive with the 97-kD protein present in epidermal wash **(Fig 2)**. None of the LABD sera reacted with the 180- or 230-kD bullous pemphigoid antigens present in epidermal extract. None of the control sera showed reactivity with the 97-, 180-, or 230-kD antigens when reacted with an anti-IgA secondary antibody (not shown). When studied with IEM, the control sera gave immunoglobulin-class-specific labeling of predictable structures, typical for the antigens against which they were directed. Hemidesmosomal plaques or adjacent portions of the LL were recognized by antibodies against the 230- and 180-kD bullous pemphigoid antigens, respectively. The sera of epidermolysis bullosa acquisita (IgG or IgA type, reactive with a 290-kD dermal antigen [9]) labeled preferentially the LD and sub-LD zone in a continuous band-like pattern **(Fig 3)**.

DISCUSSION

All the selected LABD sera with anti-DEJ reactivity of the IgA class exclusively gave a positive immunogold labeling on normal human skin. LABD antibodies directed to the epidermal portion of split skin were demonstrated to bind to an antigen localized to the hemidesmosomal plaques and the LL. Preferential expression of this antigen in the hemidesmosomes and the underlying LL suggests its

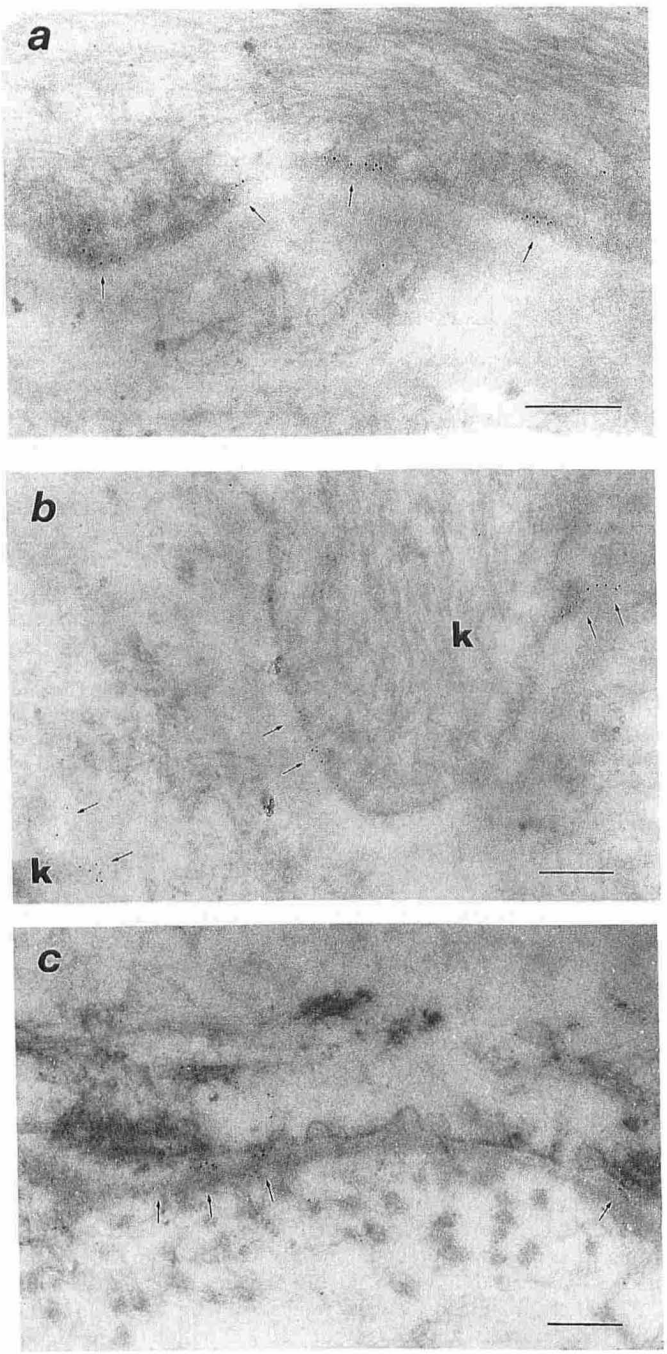

Figure 1. Basement membrane zone antigen(s) recognized by the LABD sera used in this study are localized to hemidesmosome plaques *(a)* or the underlying lamina lucida of normal human epidermis *(b,c)*. *Arrows,* structures specifically labeled with 5 nm gold granules on ultrathin sections of Lowicryl K11M-embedded normal human skin. k, basal keratinocyte. *Bars,* 200 nm.

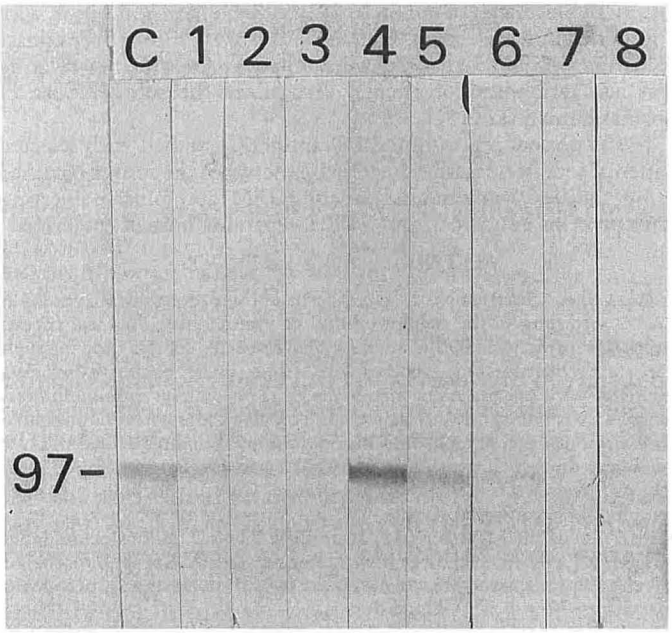

Figure 2. Western blot reactivity of LABD sera with the 97 kD antigen *(lanes 1, 2, 4, 5, and 6)*. Epidermal wash preparation from EDTA-separated skin was incubated with patient sera and developed with anti-alpha-chain-specific antibody. The positive control from an LABD patient is labeled *C*. *Lanes 1–6* correspond in order with the patient results listed in **Table I**, and a negative control serum is present in *lane 8*. Patient 3 failed to react with the 97-kD antigen, and also failed to react with the 180- and 230-kD antigens in epidermal extract (not shown). *Lane 7* is from the patient with sub-lamina densa LABD whose serum reacts with the 290-kD dermal epidermolysis bullosa acquisita antigen but is negative on this immunoblot against epidermal wash.

possible implication in the cohesive function of the basement membrane zone. Alternatively, its co-localization with other structural proteins playing a role in the epidermal-dermal adhesion may be of possible pathogenic importance in initiating the autoimmune reaction, which is then followed by a cellular inflammatory response. The nature of the 97-kD protein remains unknown; however, its extractibility during the PBS wash procedure suggests that the antigen is not strongly anchored in the plasma membrane and the hemidesmosome attachment plaque. Our findings remain in opposition to some previously published results where indirect immunoperoxidase reactivity obtained with 17 sera from patients with various clinical forms of LABD was always localized to the sub-LD region of normal human skin [11]. Because basement membrane zone split skin was not used as a substrate, it was impossible to directly compare the results of that study with our own results.

The serum containing IgA autoantibodies reactive with the floor of an artificial bullae and decorating the sub-LD/LD region in IEM was negative on blot with our epidermal extract and is known to recognize a 290-kD dermal antigen localized to anchoring fibrils [9,19,20]. Such cases could be regarded as sub-LD LABD or, better, referred to as IgA-epidermolysis bullosa acquisita. A typical IgG-epidermolysis bullosa acquisita serum used in this study confirmed an identical localization of the target antigen, as observed with anti-subLD autoantibodies of the IgA class.

The majority of the studied sera recognizing epidermal antigen(s) in indirect immunohistochemical methods (indirect immunofluorescence on split skin, IEM) were also positive on Western blot for the 97-kD protein, considered as an epidermal antigen characteristic for LABD [6]. Furthermore, besides the 97-kD protein, no other epidermal antigen could be detected with the LABD patient sera. Specifically, there was no reactivity with either the 180- or 230-kD bullous pemphigoid antigens. One CBDC serum was unreactive on blot but gave a similar pattern of indirect immunogold labeling as the rest of the 97-kD antigen-positive autoantibodies. This suggests a considerable homogeneity of the group, from the antigenic point of view, and remains in striking contrast to its clinical diversity. Indeed, no particular relationship between the clinical type of LABD (adult, cicatricial, infantile) and the indirect immunogold labeling pattern could be detected. The patient presenting with cicatricial pemphigoid-like ocular lesions was clinically very similar to the IgA-epidermolysis bullosa acquisita case but responded well to sulfones [9,21]. However, he could not be distinguished from the other LABD cases with indirect immunogold IEM. These findings confirm the clinical heterogeneity of LABD but indicate that the

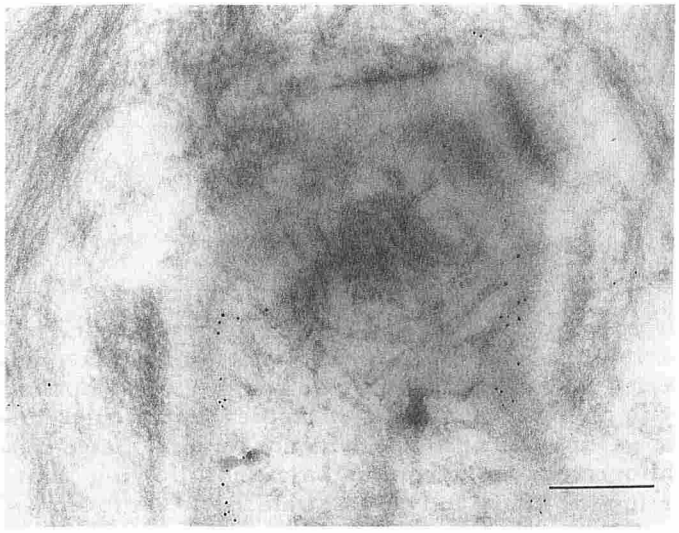

Figure 3. Immunogold labeling of the LD/sub-LD zone. Reactivity of the epidermolysis bullosa acquisita serum recognizing a 290-kD dermal antigen exclusively in IgA antibody class [9]. *Bar,* 200 nm.

antigen recognized by circulating IgA autoantibodies may be the same, irrespective of the clinical picture. A similar conclusion has been proposed on the basis of tissue- and phylogenetic-distribution studies of the adult LABD and CBDC antigens [22]. Furthermore, results of *in vitro* studies on normal human skin explants suggest that IgA antibody deposition at the DEJ and proteolytic activity are directly involved in blister formation in LABD [23]. It may be suggested that accompanying cell-mediated phenomena of variable intensity may influence the disease course and be essential for expression of a particular clinical subtype of LABD. This hypothesis is further supported by the fact that the *in vivo* IgA deposits display a considerable case-to-case variability and by the observations of changing immunodeposit sites in the same patient studied at various time intervals. In fact, different authors have reported various direct IEM patterns of localization of *in situ* immunodeposits in LABD: in the LL, under the LD, or a combined "mirror image" pattern on both sides of the LD [11,12,24–27].

Further comparative studies of clinically well-documented cases using IEM and immunoblotting simultaneously on various kinds of protein extracts are necessary for better comprehension of LABD.

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