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Autoantibodies in a Subgroup of Patients with Linear IgA Disease React with the NC16A Domain of BP180[1]

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Linear IgA disease is an autoimmune subepidermal blistering disease characterized by IgA deposits at the cutaneous basement membrane zone. IgA antibodies from linear IgA disease sera react with antigens of 97 kDa (LABD97) and 120 kDa (LAD-1), both of which appear to be fragments of the extracellular domain of bullous pemphigoid 180 (type XVII collagen). The aim of this study was to determine whether linear IgA disease sera react with the immunodominant region of BP180 (NC16A domain), which is a major target of IgG autoantibodies produced by patients with bullous pemphigoid. Indeed, 11 of 50 linear IgA disease sera were found to contain IgA autoantibodies that recognized a recombinant form of NC16A by immunoblotting. The same sera also reacted with NC16A by enzyme-linked immunosorbent assay. An epitope mapping analysis uncovered four linear IgA disease-associated epitopes located within the 45 amino acid N-terminal stretch of NC16A, all of which were previously identified as antigenic sites targeted by bullous pemphigoid autoantibodies. Eight of the linear IgA disease sera that were reactive with NC16A also recognized LAD-1 secreted by the SCC-25 cell line, and five sera recognized BP180 extracted from keratinocytes. Linear IgA disease sera depleted of reactivity to NC16A by immunoadsorption continued to react with both the LAD-1 antigen and BP180 by immunoblotting and with the basement membrane zone by indirect immunofluorescence microscopy. Our results demonstrate that IgA autoantibodies from a subset of linear IgA disease patients react with the same sites on BP180 that are targeted by IgG autoantibodies in bullous pemphigoid. ***Key words: autoantigen/collagen/epitope/hemidesmosome. J Invest Dermatol 113:947–953, 1999***

Linear IgA disease (LAD) is a bullous autoimmune disorder characterized by subepidermal blisters and linear deposits of IgA at the cutaneous basement membrane zone (BMZ) (Chorzelski and Jablonska, 1979). Clinically, LAD of childhood, also referred to as chronic bullous disease of childhood, is differentiated from LAD of adulthood. Immunoelectron microscopic studies have uncovered what appears to be a heterogeneity in the autoimmune response among LAD patients. Some LAD sera show IgA reactivity to sites within the lamina lucida, others label the sublamina densa region, and still others react with both locations (Yaoita and Katz, 1977; Bhogal *et al*, 1987; Prost *et al*, 1989).

Multiple target antigens associated with LAD have been described. LAD sera with dermal binding on salt-split skin have been reported to react with a 255 kDa polypeptide present in dermal extracts (Dmochowski *et al*, 1993) and with type VII collagen, the autoantigen in epidermolysis bullosa acquisita (Zambruno *et al*, 1994). Wojnarowska *et al* (1991) reported reactivity of LAD sera with a 285 kDa protein, although this antigen has not been further characterized. Zone and coworkers were the first to report that IgA autoantibodies associated with the lamina lucida type of both the adult and childhood forms of LAD react with a 97 kDa protein (LABD97) extracted from epidermis and dermis (Zone *et al*, 1990, 1996; Dmochowski *et al*, 1993). Based on biochemical studies and peptide sequence analyses, it appears that LABD97 and its 120 kDa precursor form (LAD-1) may be generated as proteolytic cleavage products of the extracellular domain of bullous pemphigoid (BP) 180, a transmembrane hemidesmosomal glycoprotein also known as type XVII collagen (Marinkovich *et al*, 1996; Pas *et al*, 1997; Hirako *et al*, 1998; Schäcke *et al*, 1998; Zone *et al*, 1998). BP180 has been identified as the target antigen of autoantibodies in LAD by other investigators as well (Ghohestani *et al*, 1997).

BP180 was first identified as a major antigenic target of IgG autoantibodies produced by patients with BP, another subepidermal bullous disease characterized by linear deposits of IgG at the BMZ (Labib *et al*, 1986; Diaz *et al*, 1990; Giudice *et al*, 1992). BP180 is a transmembrane glycoprotein with a type II orientation, i.e., its amino-terminal domain localizes to the cytoplasm whereas the carboxy-terminus projects into the extracellular space and spans the lamina lucida of the BMZ (reviewed in Zillikens and Giudice, 1999). The pathogenic relevance of antibodies to murine BP180 has been demonstrated using a passive transfer mouse model (Liu

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Abbreviations: BMZ, basement membrane zone; BP, bullous pemphigoid; GST, glutathione S-transferase; IB, immunoblotting; NC, non-collagenous; TBST, Tris-buffered saline plus Tween-20.
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et al, 1993). The ectodomain of human BP180 consists of 15 interrupted collagen domains. Epitope mapping studies have shown that the NC16A domain of human BP180 harbors major extracellular antigenic sites recognized by BP sera (Giudice *et al*, 1993; Zillikens *et al*, 1997a). Four distinct BP-associated epitopes were identified (MCW-0, MCW-1, MCW-2, MCW-3) that were clustered within the N-terminal 45 amino acid stretch of NC16A (Zillikens *et al*, 1997a). Utilizing recombinant forms of NC16A as the antigen source, sensitive and specific assay systems for the detection of antibodies to BP180 have been developed (Matsumura *et al*, 1996; Zillikens *et al*, 1997b). In addition, reactivity of BP sera with other intracellular and extracellular sites on BP180 has been reported (Murakami *et al*, 1998; Egon *et al*, 1999; Nie and Hashimoto, 1999; Pirriard *et al*, 1999).

The aim of this study was to determine whether autoantibodies in LAD react with the immunodominant NC16A stretch on the BP180 ectodomain. Our findings indicate that, indeed, antibodies from a subgroup of LAD patients target this region of BP180.

MATERIALS AND METHODS

Human and rabbit sera This study included the sera of 50 LAD patients. Forty sera were from patients with LAD of adulthood and 10 were from patients with LAD of childhood. The mean age of adult patients was 65 y and the mean age of the children was 5 y. By direct immunofluorescence (IF) of perilesional skin, all patients showed a linear staining of IgA at the BMZ. Indirect IF analysis on 1 M NaCl-separated skin was performed as described (Zillikens *et al*, 1996). In sera of 35 patients, circulating IgA antibodies were detected that bound to the epidermal side of the split. Three sera showed dermal binding in addition. Rabbit serum R594 was raised against a glutathione S-transferase (GST) fusion protein containing 42 amino acids of the NC16A domain (GST-NC16A2–4) and rabbit serum R136 against a GST fusion protein encompassing a 49 amino acid stretch of the C-terminal portion of BP180 (**Fig 1**). Rabbit serum R58 was generated by immunization with recombinant GST (Balding *et al*, 1997). Generation of monoclonal antibody (MoAb) HD18 to the NC16A domain of BP180 and MoAb 123 directed to the 120 kDa keratinocyte-derived LAD-1 were described previously (Pohla-Gubo *et al*, 1995; Marinkovich *et al*, 1996). As controls, we used preimmune rabbit serum, MoAb CD45 (directed to the CD45RO protein on human T cells) (Dako, Glostrup, Denmark), and sera from 16 patients with dermatitis herpetiformis and 50 healthy controls. All sera from patients with dermatitis herpetiformis contained IgA antibodies that bound to endomysium of monkey esophagus.

BP180 recombinant proteins GST fusion proteins containing the full-length BP180 NC16A domain or various fragments of this domain were generated as reported previously (Zillikens *et al*, 1997a). In this study, we used GST fusion proteins containing the following fragments of the human BP180 ectodomain: NC16A1, NC16A2, NC16A2.5, NC16A3, NC16A1–3, NC16A2–5, and NC16A1–5. The designation of these fragments of NC16A followed a previous report (Zillikens *et al*, 1997a). GST fusion proteins were purified by glutathione agarose affinity chromatography (Giudice *et al*, 1993).

Production of the soluble ectodomain of BP180 and extraction of BP180 from cultured keratinocytes The 120 kDa soluble ectodomain of BP180 was isolated from culture medium of the SCC-25 line (ATCC CRL 1628). Immediately after collecting the medium, it was placed on ice, and phenylmethylsulfonyl fluoride (Sigma, Deisenhofen Germany) and ethylenediamine tetraacetic acid were added to final concentrations of 1 mM and 5 mM, respectively. Cellular debris was removed by centrifugation at $1000 \times g$ for 10 min. Subsequently, proteins from 10 ml medium were precipitated with 30% ammonium sulfate for 2 h at 4°C. After centrifugation at $15\,000 \times g$ for 90 min at 4°C, the pellet was resuspended in 100 μl of a buffer containing 65 mM NaCl, 25 mM Tris–HCl, pH 7.8, 1 mM aminoethylbenzene-sulfonylfluoride (Sigma), and 5 mM ethylenediamine tetraacetic acid and left on ice for 2 h (Marinkovich *et al*, 1992). After dialysis against resuspension buffer overnight, samples were centrifuged at $13\,000 \times g$ for 20 min at 4°C, a mix of protease inhibitors including leupeptin, pepstatin, chymostatin, and antipain (Sigma) was added to the supernatant, and samples were stored in aliquots at –80°C. Subconfluent cultured keratinocytes were extracted with a buffer containing 62.5 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and the mix of protease inhibitors. The extract was centrifuged $15\,000 \times g$ at 4°C and the supernatant stored at –80°C.

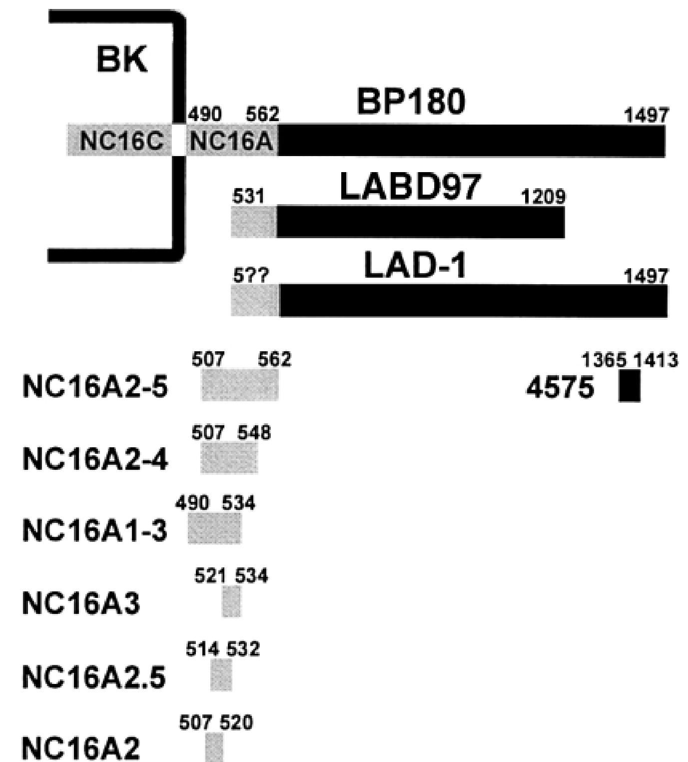

Figure 1. Schematic diagram of BP180, the epidermal 97 kDa linear IgA bullous disease antigen (LABD97), the keratinocyte-derived 120 kDa protein, and the recombinant fragments of BP180 used in this study. BP180 is a transmembrane protein of basal keratinocytes (BK) with a large ectodomain containing 15 interrupted collagen domains (black box). The major noncollagenous extracellular domain (NC16A) is located downstream from the transmembrane domain (NC16B). The amino-terminal NC16C domain localizes to the cytoplasm. Amino acid residue numbers are shown above the boxes. The amino-terminus of LABD97, as sequenced by Zone *et al* (1998), is positioned 42 amino acids downstream from the transmembrane domain [within region 3 of NC16A (Zillikens *et al*, 1997a)] and appears to be missing approximately 288 amino acids at the C-terminus of BP180. Neither terminus has been precisely defined for the 120 kDa LAD-1 antigen; however, it is thought that the N-terminus is located at or near the N-terminus defined for LABD97 and the LAD-1 C-terminus may be the same as that for the full-length BP180 protein. Recombinant fragments of NC16A are shown below. Rabbit 594 was immunized with a GST fusion protein encompassing 42 amino acids of the NC16A domain (NC16A2–4) (R594). Rabbit 136 was immunized with a GST fusion protein containing a 49 amino acid stretch of the C-terminus of BP180 (4575).

Immunoblotting Recombinant proteins, concentrated keratinocyte culture medium, and keratinocyte extracts were fractionated by 15%, 8%, and 6% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), respectively, and electrophoretically transferred to nitrocellulose (Giudice *et al*, 1993; Zillikens *et al*, 1997a). Blots were blocked for 45 min in 3% solution of skimmed milk powder in Tris-buffered saline plus Tween-20 (TBST) buffer [0.02 M Tris-(hydromethyl)-aminomethane, 0.14 M NaCl, Tween-20 0.01% (Sigma), pH 7.5]. Human sera were diluted 20-fold and rabbit sera were diluted 1000-fold in 1% bovine serum albumin (BSA) TBST. After a 12 h room temperature incubation with the diluted sera, the blots were washed and incubated with peroxidase-conjugated rabbit anti-human IgA or IgG (Jackson Immunoresearch Laboratories, West Grove, PA) diluted in 1% BSA TBST for 1 h. When diaminodiphenylbenzol was used as the chromogenic substrate, the secondary antibodies were diluted 1000-fold. In experiments where the Enhanced Chemiluminescence (Amersham Life Science, Little Chalfont, U.K.) protocol was used as the detection system, however, the secondary antibodies were used at 1:20 000.

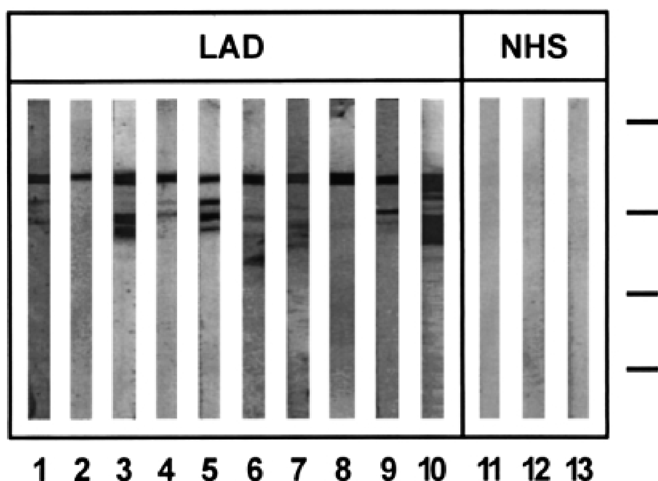

Figure 2. A subset of LAD sera reacts with recombinant BP180 NC16A. Affinity purified BP180 NC16A was fractionated by 15% SDS–PAGE and transferred to nitrocellulose. Strips were incubated with LAD (*lanes 1–10*) and healthy control sera (NHS) (*lanes 11–13*). Sera were used at a dilution of 1 : 20 and preadsorbed against recombinant GST to abolish nonspecific reactivity with the GST moiety of the fusion protein. Full-length GST-NC16A has a molecular weight of 35.5 kDa. Lower bands represent degradation products of the intact protein. The migration positions of the molecular weight markers of 45, 31, 21, and 14 kDa are indicated to the right.

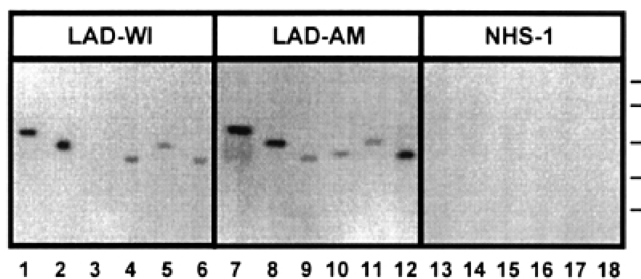

Figure 3. A subset of LAD sera reacts with epitopes within the N-terminal 45 amino acids of the BP180 NC16A domain that are also targeted by IgG antibodies in BP. Recombinant proteins were fractionated by 15% SDS–PAGE, transferred to nitrocellulose and labeled with sera LAD-WI, LAD-AM, and a normal control serum (NHS-1). The three immunoblots shown in this figure contained equal amounts of recombinant, affinity purified proteins NC16A1–5 (*lanes 1*, *7*, and *13*), NC16A1–3 (*lanes 2*, *8*, and *14*), NC16A3 (*lanes 3*, *9*, and *15*), NC16A2.5 (*lanes 4*, *10*, and *16*), NC16A2 (*lanes 5*, *11*, and *17*), and NC16A1 (*lanes 6*, *12*, and *18*). Sera were diluted 1 : 20 and completely preadsorbed against recombinant GST. The migration positions of the molecular weight markers of 66, 45, 31, 21, and 14 kDa are indicated to the right. The patterns of immunoreactivity are representative of patterns revealed by other LAD sera as shown in **Table I**.

Immunoadsorption procedures Immunoadsorptions were performed using a liquid phase protocol as described (Zillikens *et al*, 1997a). Human sera were diluted in 1% BSA in TBST buffer and incubated overnight with cell lysate containing recombinant GST or a cocktail of cell lysates containing GST-NC16A1, GST-NC16A1–3, and GST-NC16A2–5, respectively. The mixtures were centrifuged at 10 000 × *g* for 15 min at 4°C.

Enzyme-linked immunosorbent assay (ELISA) The procedure for development of the optimal working conditions of the ELISA to detect IgA autoantibodies to NC16A followed a previous protocol (Zillikens *et al*, 1997b). Briefly, analysis of the different plots generated by chessboard titrations of serial dilutions of antigen (10 μg to 10 ng), primary antibody (25–500-fold dilutions of a NC16A-reative LAD serum), and secondary antibody (500–5000-fold dilutions) demonstrated that coating of each well with 400 ng of GST-NC16A1–5, a 50-fold dilution of the primary antibody, and a 1000-fold dilution of the secondary antibody yielded the highest level of discrimination in this assay. As demonstrated previously (Zillikens *et al*, 1997b), maximal levels of protein were adsorbed when wells were coated with 400 ng of the target antigen. The optimized ELISA was run under the following conditions: Wells were coated for 2 h with 400 ng of purified recombinant GST-NC16A1–5 in 50 μl of 0.1 M carbonate/bicarbonate buffer, pH 9.6. After washing with phosphate-buffered saline, pH 7.2, and blocking with 100 μl phosphate-buffered saline containing 1% BSA, wells were incubated for 1 h with 50 μl of primary serum (LAD or control) diluted 50 fold in phosphate-buffered saline containing 1% BSA and 0.05% Tween-20, washed, and incubated for 1 h with 50 μl horseradish peroxidase-labeled goat anti-human IgA (Jackson) diluted 1000-fold in phosphate-buffered saline with 1% BSA and 0.05% Tween-20. After another washing, o-phenylenediamine in 0.1% H_2O_2 and, 10 min later, 50 μl 2 M H_2SO_4 were added. Each serum was assayed in triplicate for reactivity with both GST-NC16A1–5 and recombinant GST. For each serum tested, the mean OD_{492} reading obtained with GST was subtracted from the mean reading obtained with GST-NC16A1–5.

RESULTS

LAD sera react with recombinant BP180 NC16A LAD sera were assayed by immunoblotting for reactivity with the BP180 fusion protein, GST-NC16A1–5, which contains the entire NC16A domain of BP180. To eliminate any possible immunoreactivity with the GST moiety of this fusion protein, the patients' and control sera were preadsorbed with bacterial lysates containing recombinant GST. Of 50 LAD sera tested, 11 showed specific immunoreactivity with NC16A1–5 using anti-human IgA secondary antibodies (**Fig 2**). No IgA reactivity with BP180 NC16A1–5 was detected in sera of 16 patients with dermatitis herpetiformis or in 50 sera from healthy controls.

Subsequently, an ELISA system for the detection of IgA autoantibodies to NC16A was developed. Under the optimized assay conditions outlined in *Materials and Methods*, 25 LAD sera were tested. This group of sera included seven sera that demonstrated immunoblot reactivity with NC16A. The same seven sera also reacted with NC16A by ELISA (mean OD_{492} of 0.61) whereas the remaining 18 LAD sera and serum samples from 50 healthy controls had readings below the cut-off of the assay of OD 0.38 (mean OD of controls + 2SD).

IgA antibodies from LAD sera react with multiple NC16A epitopes To characterize the antigenic sites within the NC16A domain that are recognized by autoantibodies in LAD sera, immunoblotting was performed using the following six fusion proteins containing various fragments of the human BP180 NC16A domain (**Fig 3**): GST-NC16A1–5 (*lane 1*); GST-NC16A1–3 (*lane 2*); GST-NC16A3 (*lane 3*); GST-NC16A2.5 (*lane 4*); GST-NC16A2 (*lane 5*); and GST-NC16A1 (*lane 6*). Equal amounts of the different recombinant proteins were used for this analysis, as confirmed by immunoblotting with the anti-GST rabbit anti-serum, R58. This analysis was performed with seven LAD sera that showed reactivity with NC16A1–5. Results are summarized in **Table I** and representative patterns are shown in **Fig 3**. Of the seven LAD sera tested, all reacted with NC16A1–3 and NC16A1, two different subsets of six sera reacted with NC16A2 and NC15A2.5, and three sera reacted with NC16A3.

LAD sera, like MoAb 123, MoAb HD18, and rabbit anti-BP180 antibodies, react with a 120 kDa keratinocyte-derived protein (LAD-1) The NC16A-reactive LAD sera were analyzed by immunoblotting for reactivity with the LAD-1 antigen. Results of this analysis are summarized in **Table II** and representative findings are shown in **Fig 4**. As one set of controls, the 120 kDa LAD-1 antigen produced by cultured human keratinocytes was shown to react with MoAb 123 (*lane 1*). In addition, LAD-1 reacted with MoAb HD18 (directed against the NC16A domain) (*lane 2*), rabbit serum R594 (also directed against NC16A) (*lane 3*), and R136 (which recognizes the C-terminal portion of the BP180 ectodomain) (*lane 4*). No reactivity was seen with a normal rabbit serum (*lane 5*) and with a MoAb directed to the CD45RO protein. Of the 11 NC16A-reactive LAD sera, eight showed reactivity with LAD-1 (*lanes 6* and *7*), and five sera demonstrated IgA antibodies to

Table I. A subset of LAD sera reacts with the same antigenic sites within BP180 NC16A that are targeted by IgG antibodies in BP[a]

LAD sera	NC16A1-5	NC16A1-3	NC16A3	NC16A2.5	NC16A2	NC16A1
LAD-AM	++	++	+	+	+	++
LAD-BE	++	++	+	+	+	+
LAD-KU	+	+	–	+	–	+
LAD-LA	+	+	–	–	+	+
LAD-SC	+	+	+	+	+	+
LAD-WI	++	++	–	+	+	+
LAD-ZB	+	+	–	+	+	+
Positive sera	7/7	7/7	3/7	6/7	6/7	7/7

[a]LAD sera (n = 7) were depleted of anti-GST immunoreactivity and assayed for immunoblot reactivity with GST fusion proteins containing different portions of the BP180 NC16A domain.

BP180 extracted from keratinocytes (not shown). In contrast, IgA reactivity to LAD-1 or keratinocyte-derived full-length BP180 was not observed with any of the normal control human sera.

Binding of antibody R594 to LAD-1 is mediated by an epitope in region 4 of NC16A In a further set of experiments, we characterized the fine specificity of antibodies to LAD-1 in serum R594. This serum was generated by immunizing rabbits with a recombinant form of GST-NC16A2–4. By immunoblot analysis, after preadsorption against recombinant GST, R594 reacted with NC16A2, NC16A2.5, and NC16A3. After depletion of reactivity with NC16A2–3, R594 retained its reactivity with NC16A2–4 indicating that it also recognized an epitope in region 4 of NC16A. Subsequently, to identify the site on LAD-1 mediating the binding to R594, we assayed the immunoblot reactivity of R594 against LAD-1 after depleting the serum of reactivity with certain subfragments of the NC16A domain. Representative results of this analysis are shown in **Fig 5**. After preadsorption with GST-NC16A 2, NC16A2.5, NC16A 3, and NC16A 2–3 (*lane 7*), reactivity of R594 with LAD-1 did not change compared with the remaining reactivity after depletion with recombinant GST (*lane 5*). After preadsorption with NC16A2–4, however, R594 completely lost its ability to bind to LAD-1 (*lane 6*). These results indicate that binding of R594 to LAD-1 is mediated by an epitope in region 4 (amino acids 45–58) of NC16A.

Preadsorption of LAD sera against BP180 NC16A does not significantly alter their immunoblot reactivity with the LAD-1 antigen and BP180, or their indirect IF reactivity with the BMZ To investigate whether LAD sera that contain antibodies to NC16A also react with other epitopes on the BP180 ectodomain, we preadsorbed five NC16A-reactive sera (LAD-LI, LAD-LN, LAD-VO, LAD-WI, and LAD-YE) with a cell lysate mix containing GST-NC16A1, GST-NC16A1–3, and GST-NC16A2–5. A representative example of this analysis is shown in **Fig 6**. Preadsorption with a mix of these recombinant forms of NC16A completely abolished reactivity of the LAD sera with NC16A1–5 (**Fig 6*A***, *lane 2*). The five LAD sera reacted with LAD-1 protein from SCC-25 culture medium (**Fig 6*B***, *lane 4*; **Table II**), whereas normal control sera were unreactive (*lane 6*). Importantly, preadsorption of the LAD sera against the NC16A domain did not alter their reactivity with LAD-1 (**Fig 6*B***, *lane 5*). Subsequently, this strategy was used in three LAD sera that demonstrated IgA autoantibodies reactive with both recombinant NC16A and BP180 extracted from keratinocytes (LAD-LI, LAD-SC, and LAD-WI). Preadsorption of the three sera with the cell lysate mix of NC16A recombinants again did not alter their reactivity with BP180 extracted from keratinocytes (data not shown). These observations were confirmed by indirect IF analysis on 1 M NaCl-split skin. Five LAD sera, preadsorbed against recombinant GST, bound to the epidermal side of the split with titers of 1:160 (LAD-YE, LAD-VO), 1:80 (LAD-LN, LAD-WI), and 1:40 (LAD-LI), respectively. Preadsorption of the sera with the cell lysate mix of recombinant forms of NC16A did not alter their titer of indirect IF reactivity with the BMZ.

Table II. Some NC16A-reactive LAD sera also react with the keratinocyte-derived LAD-1 antigen and full-length BP180 extracted from keratinocytes[a]

LAD sera	NC16A	LAD-1	BP180	Indirect IF
LAD-AM	+	–	–	–
LAD-BE	+	–	–	–
LAD-KU	+	+	+	1 : 80
LAD-LA	+	+	+	1 : 160
LAD-LI	+	+	+	1 : 40
LAD-LN	+	+	–	1 : 80
LAD-SC	+	+	+	1 : 20
LAD-VO	+	+	–	1 : 160
LAD-WI	+	+	+	1 : 80
LAD-YE	+	+	–	1 : 160
LAD-ZB	+	–	–	1 : 10
Positive sera	11/11	8/11	5/11	9/11

[a]LAD sera were depleted of anti-GST reactivity and assayed for immunoblot reactivity with GST-NC16A, LAD-1 from concentrated conditioned SCC-25 culture medium, and BP180 extracted from keratinocytes. Indirect IF reactivity was determined on 1 M NaCl-split skin.

DISCUSSION

Over the past several years, our research groups and others have demonstrated that the basal keratinocyte surface protein, BP180, is a major antigenic target of autoantibodies produced by patients with several clinical variants of blistering diseases. Clinical entities that fall into this category include BP, pemphigoid/herpes gestationis, cicatricial pemphigoid, lichen planus pemphigoides, and, the focus of this study, LAD. Recent studies using a passive transfer mouse model system suggest that antibodies to BP180 might well be the causative factors in these diseases. An hypothesis that is just beginning to be explored is that the heterogeneity observed among this group of disorders might be accounted for by differences in the fine specificities and/or the effector functions of the anti-BP180 antibodies.

The biosynthetic relationship between BP180 and the major LAD autoantigens has long been under debate. Previous biochemical and immunologic findings documented significant differences between these proteins. For example, it is well established that certain LAD sera and MoAb react with LABD97 and LAD-1 (epidermal or keratinocyte-derived antigens of 97 and 120 kDa, respectively), but do not react with BP180 isolated from epidermis (Marinkovich *et al*, 1996; Zone *et al*, 1998). There is now compelling evidence, however, that both LABD97 and LAD-1 actually correspond to segments of the extracellular domain of BP180 (Pas *et al*, 1997, 1999; Hirako *et al*, 1998; Schäcke *et al*,

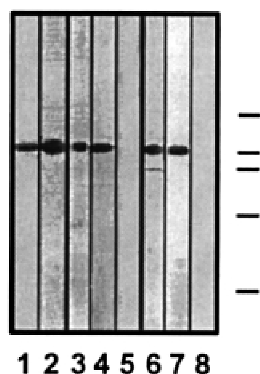

Figure 4. LAD sera, like MoAb 123, MoAb HD18, and rabbit anti-BP180 antibodies, react with LAD-1. Proteins of concentrated conditioned SCC-25 culture medium were fractionated by 8% SDS–PAGE, transferred to nitrocellulose, and incubated with MoAb 123 (*lane 1*), MoAb HD18 (*lane 2*), R594 (*lane 3*), R136 (*lane 4*), normal rabbit serum (*lane 5*), LAD sera LAD-VO (*lane 6*) LAD-LA (*lane 7*), and normal control serum (*lane 8*). The migration positions of molecular weight markers of 200, 116, 97, 66, and 45 kDa are indicated to the right.

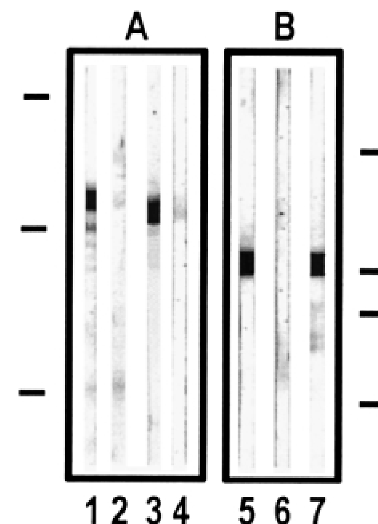

Figure 5. Rabbit serum 594 reacts with NC16A region 4 of LAD-1. In (*A*), recombinant GST-NC16A2–4 (*lanes 1* and *2*) and GST-NC16A1–3 (*lanes 3* and *4*) were fractionated by 15% SDS–PAGE. In (*B*), concentrated conditioned medium from SCC-25 cells was fractionated by 8% SDS–PAGE. The blots were labeled with serum R594 that had been preadsorbed with the following recombinant proteins: GST (*A*, *lanes 1* and *3*; *B*, *lane 1*), GST-NC16A2–4 (*A*, *lane 2*; *B*, *lane 2*), and GST-NC16A2–3 (*A*, *lane 4*; *B*, *lane 3*). After preadsorption of R594 against GST, the serum retained reactivity with GST-NC16A2–4, GST-NC16A1–3 (*A*, *lanes 1* and *3*), and LAD-1 (*B*, *lane 1*). Complete preadsorption of R594 against GST-NC16A2–4 (*A*, *lane 2*) abolished reactivity of this serum with LAD-1 (*B*, *lane 2*). In contrast, after complete preadsorption against GST-NC16A2–3 (*A*, *lane 4*), the serum retained its reactivity with LAD-1 (*B*, *lane 3*). The lines to the left of (*A*) indicate the positions of molecular weight markers of 45, 31, and 21 kDa, and those to the right of (*B*) represent the positions of molecular weight markers of 200, 116, 97, and 66 kDa.

1998; Zone *et al*, 1998). Based on these biochemical and molecular biologic studies, it appears that the 120 kDa LAD-1 antigen is shed from the basal keratinocyte surface by a proteolytic cleavage of BP180 near its membrane-spanning domain, and that the LABD97 is generated by further proteolysis which results in a loss of a segment at the BP180 C-terminus (**Fig 1**).

The NC16A domain has recently been shown to be a major target of autoantibodies from several distinct groups of patients. IgG autoantibodies from the sera of patients with BP target four tightly clustered epitopes within the N-terminal 45 amino acids of the BP180 NC16A domain (Zillikens *et al*, 1997a). No major reactivity was found with the remaining 31 amino acids on the C-terminal portion of NC16A (Zillikens *et al*, 1997a). A high percentage of pemphigoid gestationis sera also react with the same BP-related antigenic sites within the NC16A domain. A minor subset of pemphigoid gestationis sera recognized, in addition, an epitope on the very C-terminal portion of NC16A (Chimanovitch *et al*, 1999). In contrast, sera from patients with lichen planus pemphigoides were found to react with an unique epitope located on the C-terminal portion of NC16A neighboring BP-associated epitopes (Zillikens *et al*, 1999). Interestingly, the amino acid sequencing data provided by Zone *et al* (1998) indicates that LABD97 lacks the N-terminal 41 amino acids of NC16A. Thus, this protein is missing the major antigenic sites targeted by IgG antibodies in BP and pemphigoid gestationis. In this study, we demonstrate that a subset of LAD patients (11 of 50 patients) also reacts with a recombinant protein that corresponds to the NC16A domain.

Based on the data described above, we hypothesized that IgA antibodies in LAD sera bind to epitopes located within the C-terminal 36 amino acids of this domain. To address this question we performed an epitope mapping of IgA antibodies in LAD sera using various recombinant forms of NC16A. Somewhat unexpectedly, however, seven of seven LAD sera were found to react with an epitope located within the N-terminal 14 amino acid stretch of NC16A, and six of seven LAD sera reacted with an epitope within amino acids 15–28 of NC16A. Neither of these antigenic sites are located within the 97 kDa LAD protein as described by Zone *et al* (1998).

In contrast to our previous findings in BP, however, the NC16A domain does not appear to be the immunodominant region targeted by IgA antibodies in LAD. When LAD sera, reactive with both the BP180 NC16A domain and the 120 kDa soluble fragment of the BP180 ectodomain, were preadsorbed against the NC16A domain, all showed no diminution in their reactivity with the 120 kDa peptide. Likewise, when LAD sera, reactive with both NC16A and full-length BP180 were preadsorbed against NC16A, they showed no reduction in their reactivity with BP180. These results indicate that, whereas certain LAD sera react with the NC16A domain, they also exhibit major reactivity with additional epitopes on the remaining portions of the LAD-1 antigen and full-length BP180.

After analyzing the clinical data from our patients, we were unable to find any correlation between the presence of IgA antibodies to the NC16A domain and any major clinical feature. The group of LAD patients with NC16A reactivity includes both LAD of adulthood (nine of 40 patients) and LAD of childhood (two of 10 patients). In addition, there is no sex or age predisposition for this subset of LAD patients, and mucosal involvement was seen with the same frequency as in LAD patients that revealed no immune response to NC16A.

In another aspect of this study, we documented immunologic cross-reactivity between BP180 and the LAD-1 antigen using two rabbit anti-sera—one (R594) directed against the N-terminal region of the BP180 ectodomain and the other (R136) directed against the C-terminal portion of this molecule. Both of these well-characterized domain-specific anti-BP180 antibodies as well as MoAb HD18, directed to the NC16A domain of BP180, reacted with the same 120 kDa protein (LAD-1) that was also recognized by MoAb 123 and sera from LAD patients. LAD-1 was originally described as the target antigen of MoAb 123 and sera from LAD patients (Marinkovich *et al*, 1996). Our results are consistent with previous observations suggesting that the 120 kDa protein secreted from keratinocytes is a portion of the BP180 ectodomain (Hirako *et al*, 1998; Schäcke *et al*, 1998). These studies did not include antibodies from LAD patients or MoAb 123 and left the relationship between the 120 kDa BP180 fragment and the LAD-1 antigen unresolved. Data from other investigations (Pas *et al*, 1997, 1999; Zone *et al*, 1998) and the findings of this study strongly suggest, however, that this 120 kDa BP180 fragment and LAD-1

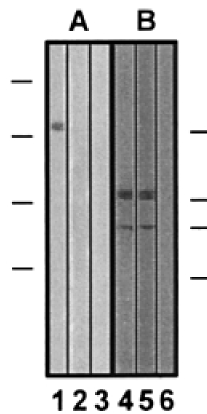

Figure 6. Preadsorption of LAD sera with the NC16A domain of BP180 does not alter their reactivity with LAD-1. In (*A*), *lanes 1–3*, affinity purified GST-NC16A was fractionated by 15% SDS–PAGE. In (*B*), *lanes 4–6*, concentrated conditioned medium from SCC-25 cells was fractionated by 8% SDS–PAGE. The blots were labeled with LAD-WI preadsorbed with either GST (*lanes 1* and *4*) or with a mix of bacterial cell lysates containing equal amounts of GST-NC16A1, GST-NC16A1–3, and GST-NC16A2–5 (*lanes 2* and *5*). The blots in *lanes 3* and *6* were incubated with NHS. The lines to the left of (*A*) indicate the positions of molecular weight markers of 45, 31, 21, and 14 kDa, and those to the right of (*B*) represent the positions of molecular weight markers of 200, 116, 97, and 66 kDa. The pattern of reactivity is representative of the pattern revealed by all five LAD sera analyzed by this procedure.

are identical. Amino acid sequencing data on LAD-1 are not available and the N-terminus of LAD-1 within the NC16A domain has not been precisely defined yet. In this study, we show epitope mapping data of serum from rabbit 594 that was immunized with a recombinant form of NC16A2–4. Although R594 contained antibodies to different antigenic sites on NC16A, including NC16A regions 2, 2.5, 3, and 4, preadsorption analyses demonstrated that only antibodies to NC16A region 4 appear to be relevant for the binding of R594 to LAD-1. Our finding is consistent with the assumption that the N-terminus of LAD-1 is located near or at the N-terminus defined for LABD97 (Zone *et al*, 1998). This circumstantial evidence, however, needs confirmation by amino acid sequencing of the LAD-1 protein. In addition, our results do not clarify the mechanism by which LAD-1 is generated.

Although most previous reports did not find LAD sera to react with BP180, some recent studies demonstrated that sera from patients with the clinical and immunofluorescence findings of LAD may recognize a 180 kDa protein extracted from skin or keratinocytes that comigrates with BP180 (Berard *et al*, 1996; Ghohestani *et al*, 1997; Arechalde *et al*, 1999; Schmidt *et al*, 1999). In addition, recently, a case with IgA and, to a lesser extent, IgG antibodies to BP180 NC16A was reported (Schmidt *et al*, 1999). We found that five of the 11 LAD sera that were reactive with BP180 NC16A recognized BP180 extracted from keratinocytes. The discrepancy between reactivity with NC16A and reactivity with full-length BP180 is likely to be due to the fact that the immunoblot assay is more sensitive in detecting anti-BP180 antibodies when recombinant BP180, rather than BP180 isolated from epidermis or keratinocytes, is used, as was previously shown (Zillikens *et al*, 1997b).

In summary, our results confirm that LAD is a heterogeneous disease. We demonstrate that a subset of patients with LAD produce autoantibodies to the BP180 NC16A domain. IgA autoantibodies in this subset of patients react with the same antigenic sites that are targeted by IgG autoantibodies in BP and pemphigoid gestationis. Further studies will address the pathogenic relevance of IgA autoantibodies to the BP180 NC16A domain.

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