Anti-plectin autoantibodies in subepidermal blistering diseases

Jacqueline JA Buijsrogge, Marcelus CJM de Jong, Guus J Kloosterhuis, Maarten H Vermeer,* Jan Koster,† Arnoud Sonnenberg,† Marcel F Jonkman and Hendri H Pas

Center for Blistering Diseases, Department of Dermatology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
*Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands
†Netherlands Cancer Institute, Amsterdam, the Netherlands

Published in Br J Dermatol 2009; 161(4):762-71
SUMMARY

Background: Hemidesmosomal proteins may become targets of autoimmunity in subepidermal blistering diseases. Well-known recognized auto-antigens are the intracellular plaque protein BP230, the transmembrane BP180 and its shed ectodomain LAD-1.

Objectives: The aim of the study was to establish the prevalence of autoimmunity against plectin, another intracellular plaque protein, and to investigate its antigenic sites.

Patients/methods: Two hundred and eighty two patients with subepidermal blistering diseases, investigated by routine immunoblot analysis for possible anti-plectin antibodies, were included in the study. Epitope mapping was performed using recombinantly produced overlapping plectin domains from the actin binding domain to the rod domain. The COOH-terminal region of plectin was not included in the study.

Results: In 11 out of 282 (3.9 %) patients an immunoblot staining pattern identical to that of anti-plectin monoclonal antibody HD121 was found. Affinity-purified antibodies bound back to normal human skin in a pattern typical for plectin, i.e. to the epidermal basement membrane zone as well as to keratinocytes in the epidermis, and to myocytes. No binding was seen to plectin-deficient skin of a patient with epidermolysis bullosa simplex with muscular dystrophy. Epitope mapping of the plectin molecule showed that the central coiled-coil rod domain is an immunodominant hotspot as 92% of the sera with anti-plectin antibodies reacted with it. Most patients with anti-plectin antibodies also had antibodies to other pemphigoid antigens.

Conclusions: Plectin is a minor pemphigoid antigen with an immunodominant epitope located on the central rod domain.
### INTRODUCTION

Plectin is a widely expressed high molecular weight protein found in different cell types, including muscle and epithelial cells. In keratinocytes the plectin 1a isoform, a full length plectin variant (> 500 kDa) containing the sequence encoded by the alternative first exon 1a, is localized in hemidesmosomes and desmosomes, and in muscle cells it is associated with the sarcolemma.\(^1\)\(^-\)\(^3\) Plectin is involved in anchoring of intermediate filaments to the plasma-, and nuclear membrane, as well as the cross-linking of intermediate filaments, and interlinking intermediate filaments with microtubules and microfilaments.\(^4\)\(^,\)\(^5\) Plectin is part of the plakin protein family together with desmoplakins I and II, envoplakin, BP230, periplakin, epiplakin and MACF,\(^4\)\(^,\)\(^6\) that all probably arose from a single precursor gene.\(^7\) The protein contains a long central rod domain with an alpha-helical coiled coil conformation that is flanked by globular NH2- and COOH-terminal domains.\(^5\) The central rod domain is also found in four other family-members: desmoplakin, envoplakin, periplakin, and BP230. The N-terminal part harbours an actin-binding domain and a plakin domain which both are also involved in binding to the integrin β4 subunit.\(^8\)\(^-\)\(^10\) The COOH-terminal is made up of six plakin repeat domains, a linker subdomain located between the 5\(^{th}\) and 6\(^{th}\) repeat and a glycine-serine-arginine domain. Intermediate filament proteins vimentin and cytokeratins (K8/18) bind to the COOH-terminal plectin domain at the linker region between the 5\(^{th}\) and 6\(^{th}\) repeat domain and the 5\(^{th}\) repeat contributes to binding.\(^8\)\(^,\)\(^9\)\(^,\)\(^11\)\(^,\)\(^12\)

Pemphigoid is a group of subepidermal bullous autoimmune diseases of the skin caused by autoantibodies against the epidermal basement membrane zone (BMZ).\(^13\) Autoantibodies against BP230 and BP180 are associated with bullous pemphigoid (BP), the most common variant of pemphigoid, as well as with pemphigoid gestationis (PG), lichen planus pemphigoides (LPP) and mucous membrane pemphigoid (MMP), whereas autoantibodies against the linear IgA disease antigen (LAD-1), which is the soluble shed ectodomain of BP180, are found in linear IgA disease (LAD).\(^14\)\(^-\)\(^16\)

Plectin also appeared to be recognized by autoantibodies in immunobullous diseases. Paraneoplastic pemphigus (PNP), a rare form of pemphigus with an underlying neoplasm, is characterized by an unique immune response against plakin family proteins, and plectin was found among these.\(^17\)\(^,\)\(^18\) In 2000 Ohnishi et al. had described a patient with a vesicular form of pemphigoid and IgG antibodies against a 450 kDa protein.\(^19\) Originally thought to be plectin this protein was later identified as epiplakin instead.\(^20\) Although epiplakin belongs to the plakin family it is structurally different from plectin and encoded by a different gene. At the same time using a combination of immunoprecipitation and immunoblotting Laffitte et al.\(^21\) were able to show IgG anti-plectin antibodies in a five-month-old child who clinically suffered from bullous pemphigoid. We recently described a case of epidermolysis bullosa aquisita (EBA) with concomitant IgA antibodies to plectin.\(^22\) To date, no other reports exist of immunobullous diseases with plectin autoantibodies.
Chapter 3

In our laboratory sera from patients suspected of autoimmune bullous diseases are routinely screened by immunoblotting for presence of pemphigoid autoantibodies. We then noticed that some patients bound to the blot in high molecular weight patterns similar to the pattern obtained with plectin monoclonal antibodies. Here we therefore set to demonstrate that plectin indeed is an autoantigen in bullous pemphigoid, to identify its incidence in immunobullous diseases, and to identify the site of binding on the plectin molecule.

MATERIALS AND METHODS

Patient sera
As part of the Center for Blistering Diseases at the University Medical Center Groningen our laboratory performs diagnostic assays on biopsies and sera of patients suspected of an autoimmune bullous disease. In this study we used sera that we received over a time span of seven years (1997 to 2003). All sera used were initial sera of our own clinic or earliest diagnostic samples sent to our referral centre. We retrospectively analyzed the immunoblot patterns of these sera and selected those sera that displayed reactivity with one or more of the hemidesmosomal antigens BP230, BP180, LAD-1 or the suspected plectin. To ascertain that we only included sera from patients with proven subepidermal autoimmune diseases, we further selected only those samples for which immunofluorescence analysis confirmed such diagnosis. Therefore the included sera had to meet to the following criteria: if both serum and biopsy were available then the serum was included if the biopsy demonstrated a linear deposition of IgG, IgA or both along the epidermal BMZ, if only serum was available then the serum had to display binding along the BMZ on monkey oesophagus and/or along the roof of salt-split human skin. Two hundred eighty two sera met the inclusion criteria; of these 226 had only IgG antibodies, 11 had only IgA antibodies and 45 had both IgG and IgA antibodies. The case files of the patients seen at our own clinic showed that majority of the patients had BP while the others had PG, LPP, MMP or LAD. Eleven sera, that displayed a possible plectin pattern on immunoblot, were selected for further characterization. In these additional studies we also included, as a positive control, the first patient that we discovered with possible plectin antibodies, and who was a special case of EBA with concomitant IgA to plectin.22 We used two sera from this patient (#7 in this study); a serum from 1979 and a serum from 1985. Both sera displayed the same immunoblot plectin binding characteristics.

Recombinant plectin constructs
The generation of the recombinant, hemagglutinin (HA) epitope tagged, plectin proteins used in this study has been described before.10 In brief, cDNAs encoding polypeptide sequences of the plectin 1a variant were subcloned into the multicloning sites of the eukaryotic expression vector pcDNA-HANII (Invitrogen), which allows expression of a recombinant protein that
is tagged with HA at the N terminus. All nucleotide and amino acid positions are numbered with the ATG initiation codon at position one (GenBank accession no. U53204). Plasmid inserts were generated by restriction enzyme digestion or polymerase chain reaction (PCR) by using the proofreading Pwo DNA polymerase (Roche Diagnostics, Indianapolis, IN) and gene-specific sense and antisense primers containing restriction site tags. Seven recombinant plectin-constructs were generated: (a) plec\textsuperscript{1–339} containing the actin-binding domain (ABD), (b) plec\textsuperscript{1–606}, (c) plec\textsuperscript{1–1154} containing both the ABD and the plakin domain, (d) plec\textsuperscript{1–2532} containing the ABD, the plakin domain and the coiled-coil central rod domain, (i) plec\textsuperscript{284–1154} containing the plakin domain, (e) plec\textsuperscript{284–2532} containing the plakin domain and the rod domain, and (h) plec\textsuperscript{1390–2532} containing solely the rod domain (figure 1). Numbers in superscript correspond to the amino acid residues of subclones. The African monkey kidney cell line COS-7 was grown in Dulbecco’s modified essential medium (DMEM) containing 10% (v/v) fetal calf serum. Cells were grown to 70% confluence in 10-cm culture dishes and transiently transfected with 7.5 µg of cDNA by using Diethylaminoethyl-dextran.\textsuperscript{23} Cells were incubated with transfection medium for 3 hours, which was then replaced by DMEM medium. After 24 hours, this medium was replaced by DMEM containing 5 mM sodium butyrate. After another 24 hours incubation, the cells were washed twice with phosphate-buffered saline and directly lysed in Laemmli sample buffer. After heating at 95°C for 5 min the extracts were stored at –20°C until used.

**Figure 1.** Schematic diagram of plectin 1a and the recombinant constructs used in the study. Underneath the full length plectin 1a the recombinant constructs are depicted, preceded by their corresponding character. C = COOH-terminal/intermediate filament binding domain (IFBD), N = N-terminal part of plectin 1a. On the right side of each construct the number of sera positive to each construct are shown. The cartoonized domains of plectin 1a as used in this figure are shown at the bottom. * Construct C was tested for 11 of the 12 sera.

**Immunoblot analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and routine immunoblot analysis of patient sera on keratinocyte extracts was performed as described previously.\textsuperscript{16} Immunoblot analysis with recombinant plectin constructs was done for all sera suspected of containing plectin autoantibodies and was performed as follows. After SDS-PAGE and transfer of the proteins onto nitrocellulose, the correct position of all constructs on the blots was identified with monoclonal 12CA5 that is directed against the HA-tag. For analysis of patient sera the blots were blocked in 1% milk in TBS (50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-
HCl), pH 7.5 containing 500 mM NaCl) for 1 hour at room temperature. Incubation with patient serum was done overnight at 37 °C with the serum diluted 1:300 in 1% milk in TBS in case IgG was detected and in 0.66% milk in TBS when IgA was present. The blots were washed three times for 5 minutes with TBS containing 0.05% Tween-20 (TTBS). Next, the blots were incubated for one hour at room temperature with AffiniPure mouse anti-human IgG, Fcγ-fragment-specific (Jackson ImmunoResearch, West Grove, PA, USA) 1:500 in TTBS or AffiniPure goat anti-human serum IgA, α-chain-specific (Jackson ImmunoResearch) 1:600 in TTBS. After another three 5-minute washes with TTBS the blots were incubated for another hour with blotting grade alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (BioRad, Hercules, CA, USA) 1:250 in TTBS for detection of IgG, and with alkaline phosphatase-conjugated rabbit anti-goat IgA, Fc-fragment-specific (Jackson ImmunoResearch) 1:250 in TTBS for detecting IgA. Finally, the blot was developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 1 M Tris-HCl buffer, pH 9.5 containing 100 mM NaCl and 10 mM MgCl₂.

Affinity purification of serum antibodies
From two sera we affinity-purified the antibodies that bound to the blot in a series of bands resembling a ladder and matching the binding pattern of the anti-plectin monoclonal antibody.22,24 As these purified antibodies were destined to prove by immunofluorescence that they were capable of binding back to human skin substrate, we specifically chose the IgA containing sera as background in immunofluorescence is generally lower for IgA than for IgG. Specific areas of the immunoblot were eluted using glycine-HCl, as described before.16 In brief, keratinocyte cell extract was separated by SDS-PAGE and electroblotted to 0.2 μm pore size nitrocellulose (Bio-Rad Laboratories, CA). Small vertical strips were excised and these were stained to identify the position where the patient antibodies bound. The corresponding area was then excised from the unstained blot. Similar-sized areas were cut from three other regions where no binding was observed, and served as controls for the elution process. The cut areas were incubated overnight in 1 ml of 1:20 diluted patient serum in TTBS at room temperature followed by two extensive washes with TTBS. Bound immunoglobulin was then eluted with 750 μl of 20 mM sodium citrate, pH 3.2, and 0.01% ovalbumin during a four-minute incubation at room temperature, and then immediately neutralized by mixing in 150 μl of 2 M Tris-HCl, pH 7.5. After overnight dialysis at 4°C against phosphate buffered saline containing 0.01% Tween 20, the antibodies were concentrated by ultrafiltration to 100 ul (Vivaspin concentrator 30,000 MWCO PES, Vivascience, UK) and stored at –80°C until used.

Immunofluorescence microscopy
IIF microscopy was performed as previously described, using the following substrates: normal human skin, salt-split normal human skin, and plectin-deficient skin from a patient with epidermolysis bullosa simplex with muscular dystrophy (EBS-MD) homozygous for a null mutation in the plectin gene (c.G13187T, p.E1914X).25 Analysis of serum on a substrate that
misses the antigen of interest is also known as knock-out IF-analysis. In brief, 4 μm thick cryostat sections were placed on microscope slides and air-dried before a fan for 15 minutes. Sections were washed in phosphate-buffered saline (PBS, 10 mM pH 7.3) and then incubated with patient serum for 30 minutes at room temperature. For salt-split skin analysis we used the serum at a 1:8 dilution, for knock-out analysis at a 1:4 dilution, and the affinity-purified antibodies were used undiluted. After thorough washing in PBS for 15 minutes, the sections were incubated for a further 30 min in fluorescein-conjugated Fcγ-specific goat F(ab’)2 anti-human IgA (Protos Immunoresearch, Burlingame, CA, USA) or fluorescein-conjugated Fcγ-specific goat F(ab’)2 anti-human IgG (Protos Immunoresearch, Burlingame, CA, USA) in PBS supplemented with 1% bovine serum albumin. After another 15 minutes of washing in PBS the sections were coverslipped under PBS-glycerol (1:1 v/v) and examined at 40 times magnification with a Leica DMRA fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany). On each occasion, positive and negative serum controls were included.

DIF microscopy was performed as described before.

RESULTS

Prevalence of plectin binding patient sera
Routine immunoblot analysis of the 282 patient sera revealed that 11 (3.9 %) displayed the characteristic high molecular weight “ladder” binding pattern (figure 2). The binding immunoglobulins were of the IgG isotype in 10 patients and of both the IgA and IgG isotype in one (patient #8). Nine of the eleven sera bound also to other bullous pemphigoid autoantigens, i.e. BP230, BP180 and LAD-1 by immunoblot (table I). In the table we included our control patient (#7) that has been described by us before as having both anti-plectin IgA and anti-type VII collagen IgA and IgG antibodies.

![Figure 2. Immunoblot analysis on keratinocyte extract shows binding of patient sera in a typical high molecular weight ladder pattern. Patient sera: lane 2, IgA patient #7 (1979 sample); lane 3, IgG patient #4; lane 4, IgG patient #5; lane 5, IgG patient #12; lane 6, IgG patient #11. The same pattern was obtained with anti-plectin monoclonal HD121 (lane 1). Additional binding to bullous pemphigoid antigens BP230 and/or BP180 is seen in lanes 3, 4 and 6. As substrate we used keratinocytes directly extracted with Laemmli sample buffer. The observed pattern depends on the detergent used for extraction: lane 7, Laemmli sample buffer extract; lane 8, Triton X-100 extract. Both lanes were stained with monoclonal HD121.](image-url)
The plectin binding sera show a typical “ladder”-pattern

The immunoblot binding pattern consists of at least ten different bands and is identical to the pattern seen with mouse monoclonal antibody HD121 that is specific to HD1/plectin (figure 2, lane 1). The ladder pattern was observed when cells were extracted with Laemlli sample buffer, which contains 2% SDS, while extraction with the mild detergent Triton-X-100 gave only one prominent higher molecular weight band (figure 2, lane 7-8).

The blot-binding antibodies bind to human skin in a plectin distribution pattern

To investigate if the “ladder”-pattern IgA antibodies were indeed capable of binding to skin antigens, we eluted the bound antibodies of patients #7 and #8. Both bound to the roof of salt-split skin (not shown). When incubated on a section of normal human skin IIF showed that the eluted immunoglobulin bound around the keratinocytes and along the epidermal BMZ (Fig. 3a and c), in line with the distribution of plectin in epidermis in both hemidesmosomes and desmosomes. The staining is most prominent along the epidermal BMZ, and in IIF microscopy plectin staining along the epidermal BMZ may stand out whereas the intraepidermal staining may become weak on lower antibody concentrations (personal observation). In addition, binding was also observed around myocytes of the arrector pili muscles (figure 3e) in a pattern of dots and short lines at the cell periphery resembling the distribution pattern of plectin in smooth muscle tissue observed by Wiche et al. who stained urinary bladder smooth muscle cells with rabbit anti-plectin sera. No binding was seen with the control eluates (figure 3b, d and f).

Plectin-deficient skin demonstrates absent or diminished binding of anti-plectin containing patient sera

If the circulating antibodies were directed to plectin only, they should not bind to skin or muscle of a patient with plectin-deficient EBS-MD. Indeed the whole serum IgA of patient #7 did not bind to the BMZ of plectin-deficient skin nor around myocytes of the arrector pili myocytes of plectin-deficient skin (figure 4a-d). When we incubated EBS-skin with whole serum of patient #8 we observed severely decreased binding (figure 4e-j). The remaining weak signal therefore must be due to other antigen-specificity IgA serum antibodies being present, that were not detected by immunoblotting or IgA NC16A-ELISA as both these assays were negative.
Table I. Immunoblot and salt-split skin analysis of the anti-plectin sera.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)/sex</th>
<th>Immunoblot</th>
<th>Salt-Split skin</th>
<th>Working diagnosis</th>
<th>Final diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plectin</td>
<td>BP180</td>
<td>BP230</td>
<td>LAD-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30/F</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP</td>
</tr>
<tr>
<td>2</td>
<td>78/F</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP/DH</td>
</tr>
<tr>
<td>3</td>
<td>82/M</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>PV/BP</td>
</tr>
<tr>
<td>4</td>
<td>88/M</td>
<td>IgG</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
</tr>
<tr>
<td>5</td>
<td>72/F</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP</td>
</tr>
<tr>
<td>6</td>
<td>81/F</td>
<td>IgG</td>
<td>IgG, IgA</td>
<td>Roof, IgG</td>
<td>BP</td>
</tr>
<tr>
<td>7</td>
<td>81/F</td>
<td>IgA</td>
<td>IgG</td>
<td>LAD</td>
<td>EBA</td>
</tr>
<tr>
<td>8</td>
<td>83/F</td>
<td>IgG, IgA</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>LAD</td>
</tr>
<tr>
<td>9</td>
<td>81/M</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP/PV</td>
</tr>
<tr>
<td>10</td>
<td>64/M</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP</td>
</tr>
<tr>
<td>11</td>
<td>57/F</td>
<td>IgG</td>
<td>IgG</td>
<td>Roof, IgG</td>
<td>BP</td>
</tr>
<tr>
<td>12</td>
<td>1/F</td>
<td>IgG</td>
<td>IgA</td>
<td>Roof, IgG</td>
<td>LAD</td>
</tr>
</tbody>
</table>

Patient #7 has been described by us before as having additional antibodies to type VII collagen. BP = bullous pemphigoid, DH = dermatitis herpetiformis, PV = pemphigus vulgaris, EBA = epidermolysis bullosa acquisita, LAD = linear IgA dermatosis.

In vivo depositions in hair muscle tissue and staining along the BMZ of muscle cells

Muscle cells contain plectin but not BP230, BP180 or LAD-1. We therefore looked for evidence of IgG or IgA deposition in hair muscle. Only one of our patient biopsies of perilesional skin (patient #7) contained a hair muscle. DIF of a cross-section of this biopsy showed a fine dotted granular pattern (figure 3g). We do not have any evidence that this deposition is due to anti-plectin antibodies, but we never observed any such deposition before in hair muscle.

Binding of anti-plectin antibodies to recombinant plectin domains

All suspected anti-plectin sera were tested for binding of antibodies to recombinant fragments of plectin (figure 1). The antibodies from eleven patients reacted with the central coiled-coil rod domain (fragment Plec1390-2532). Two of the sera also contained antibodies that bound to the actin-binding domain (fragment Plec1-339; table II). None of the sera bound the plakin domain (fragment Plec284-1154). The serum of one patient was negative for all constructs used and may have specificity to epitopes not present in the used recombinant fragments (patient #5). Control sera from BP230 or BP180 positive pemphigoid patients did not react with the constructs (not shown). We also tested sera from 4 PNP patients, as plectin is reported in the literature as a PNP autoantigen, but none of our sera reacted with any of the recombinant plectin domains nor with native plectin from keratinocyte cell extracts (not shown).
### Table II. Immunoblot reactivity of anti-plectin sera with recombinant plectin domains

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plec&lt;sup&gt;1-339&lt;/sup&gt; (a)</th>
<th>Plec&lt;sup&gt;1-606&lt;/sup&gt; (b)</th>
<th>Plec&lt;sup&gt;1-1154&lt;/sup&gt; (c)</th>
<th>Plec&lt;sup&gt;1-2532&lt;/sup&gt; (d)</th>
<th>Plec&lt;sup&gt;284-2532&lt;/sup&gt; (e)</th>
<th>Plec&lt;sup&gt;1390-2532&lt;/sup&gt; (h)</th>
<th>Plec&lt;sup&gt;284-1154&lt;/sup&gt; (l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
</tr>
</tbody>
</table>

Patients #1-6 and #9-12 IgG, patients #7 and #8 IgA.
+ strong positive, +/- weak positive, - negative, n.d. not done
Anti-plectin autoantibodies in subepidermal blistering diseases

Figure 3. IgA affinity-purified antibodies bind back to human skin in a plectin-like pattern.

IIF of affinity-purified IgA (patient #7, 1985 sample) on normal skin substrate shows linear BMZ and intercellular staining of epidermal (a) and hair follicle keratinocytes (c), and also around the arrector pili myocytes (e). No binding was seen with the control eluate; epidermal (b) and hair follicle keratinocytes (d), and arrector pili myocytes (f).

Scale bar is 10 mm. DIF of oblique cross-section of smooth muscle arrector pili tissue (patient #7) shows in vivo deposition of IgA in a fine dotted granular pattern (g). Scale bar is 10 mm.
Figure 4. Absent or diminished binding of anti-plectin serum IgA to plectin-deficient EBS-MD skin. IIF of whole serum of patient #7 (a and c with 1985 sample, b and d with 1979 sample) on EBS-MD skin (a,b) and normal skin (c,d). Note that perinuclear binding is not lost on EBS-MD skin (a). The myocytes of the arrector pili muscle of plectin-deficient skin do not bind IgA (b) in contrast to those of control skin (d). Scale bar is 10 mm. IIF of normal human control serum (e,h), whole serum of patient #8 (f,i) and serum from a juvenile LAD patient (g,j) on EBS-MD skin (e,f,g) and normal skin (h,i,j). The IgA staining along the BMZ is severely diminished on EBS-MD substrate for the anti-plectin serum (compare f and i) while the serum of the juvenile LAD patient, that had IgA antibodies to the LAD-1 antigen, does bind to the EBS-MD substrate with similar intensity as to normal skin (compare g and j). Scale bar is 25 mm.
DISCUSSION

The experiments described here demonstrated undoubtedly the existence of anti-plectin autoantibodies in patients with subepidermal blistering diseases. Serum immunoblot staining patterns were identical to that of the monoclonal anti-plectin; affinity-purified antibodies stained skin - and muscle - in a plectin distribution pattern; binding of whole sera to plectin-deficient skin was absent or diminished; and sera specifically bound recombinant plectin constructs. The finding of immunodepositions on arrector pili muscle by DIF is also a sign for the existence of anti-plectin antibodies.

In a series of 282 consecutive patients with subepidermal blistering diseases we found a prevalence of 3.9 % (11/282) which is similar to the one out of sixteen reported before by Laffitte et al. The patient with EBA, used as a positive control, was not included in the calculation of the prevalence as the sera of this patient dated back to before the inclusion period. We had four sera of her available that were collected over a period of nine years and all these contained anti-plectin IgA antibodies. This one particular patient proved the diagnostic potential of new laboratory assays as immunoblot and served as a trigger for the current study. In PNP the reported prevalence seems much higher with reported incidence figures of 81% (13/16) by immunoprecipitation and 82% (23/28) by immunoblot, however we could not confirm these figures as our four PNP patients reacted negative by immunoblot.

In all but one case we also demonstrated that concomitant antibodies to other antigens were present, in nine cases these were the classical BP antigens, in one case the EBA antigen (#7) and in one case an unidentifiable antigen (#8). The one patient (#3) that seemed to have only anti-plectin antibodies appeared to have anti-BP230 antibodies two years later (not shown). Due to this multi-antigen response the direct pathogenicity and the clinical manifestation of the anti-plectin antibodies cannot be determined. This ‘more than one antigen’ response is common to BP where IgG reactions to both BP230 and BP180 and/or LAD-1 are commonly seen and is explained by ‘epitope spreading’, which is a secondary autoimmune response to other hemidesmosomal antigens during a chronic autoimmune or inflammatory response. In this regard, plectin, being part of the hemidesmosome, could be exposed to the immune system during a primary inflammatory response to BP230 or BP180. In line with such vision is that all but one of our patients had antibodies to other antigens.

The reactivity of the patient sera with the recombinant plectin constructs not only confirmed their plectin-specificity but also allowed partial mapping of the recognized epitopes. Eleven out of twelve sera (92%) bound to the coiled-coil rod domain, implying that this rod domain harbours one or more major epitopes. Two sera (17%) also bound to the actin-binding domain, implying that this is another but minor antigenic site. The plakin domain did not contain epitopes. One serum reacted strongly with the keratinocyte extract but did not react with any of the recombinant fragments. This may be due to post-translational modifications not present on the recombinant constructs, but it is also conceivable that the epitope here is present on
the COOH-terminal intermediate filament binding domain (IFBD). As the IFBD was not present in our constructs we cannot exclude that the other sera also might recognize epitopes on this domain. BP230, a major bullous pemphigoid antigen and also a plakin family member, has a similar structure containing both a rod domain and an IFBD domain. Skaria et al., using COS7 cells produced BP230 fragments, found multiple antigenic reactive sites on BP230 mostly situated (84% of the sera) on the IFBD, but not more than 36% of the sera bound the rod domain. Therefore the plectin rod domain may be more immunoreactive than the BP230 rod domain, although the immunoreactivity of the BP230 rod domain may be underestimated as Skaria et al. missed a mid-rod 300 amino acid sequence (11.3 %) in their constructs. Other plakin family members desmoplakin, envoplakin and periplakin have also been recognized as autoantigens. Desmoplakin, as BP230, was demonstrated to harbour major epitopes on the COOH-terminal part. Periplakin and envoplakin are antigens in PNP, and sera of these patients were demonstrated to bind to multiple epitopes, not only COOH-terminal but distributed over the whole of both proteins including the plakin domain.35-39

Earlier studies had demonstrated either a ladder pattern or one band for plectin.18,21,40 The explanation for this inconsistency is most likely the nature of the detergent used: the heavily denaturing SDS will dissolve entire protein complexes where the non-ionic Triton-X-100 will only extract loosely bound protein. We agree with McLean et al. that is unlikely that the lower molecular weight bands arise from the higher molecular weight bands trough proteolytic degradation.40 We observed the exact same pattern irrespective of whether we extracted the cells immediately in Laemlli sample buffer containing an additional mix of proteolytic inhibitors, or left them first for 15 minutes lysed in water without proteolytic inhibitors (not shown). Also extracts prepared from another source -human epidermis- gave a similar pattern (not shown). The two most plausible explanations for the multiple bands are either a high cellular turnover of plectin, visible as a series of degraded fragments, or the presence of different isoforms encoded for by alternative spliced transcripts. The latter idea is supported by molecular analyses of human, mouse and rat cDNA that have already demonstrated up to 16 different transcripts.3,41,42 Also rodless isoforms have been identified in rat and man of which the human form was visible as a 360 kDa minor protein band next to the 520 kDa full-length protein.43,44

Our clinical data are limited to a retrospective review of case notes. Reviewing the patient files did not reveal other obvious clinical manifestations than blisters. We also contacted the physicians who treated the patients at the time of serum collection but none of them remembered any specific clinical aberrations unusual for bullous disease. Close examination of new patients that present with anti-plectin antibodies might eventually reveal unexpected or unusual features. The following final conclusions can be drawn from the experiments described in this study. First, plectin indeed has to be grouped with the hemidesmosomal proteins that may become autoantigenic targets in immunobullous diseases. Second, its rod domain harbours one or more immunodominant epitopes. Third, based on its prevalence it is a relatively minor antigen compared to BP230 and BP180.
ACKNOWLEDGMENTS

We thank Dr. K. Owaribe (Nagoya, Japan) for supplying monoclonal antibody HD-121.
REFERENCES


12. Spurny R, Abdoulrahman K, Janda L et al. Oxidation and nitrosylation of cysteines proximal to the intermediate filament (IF)-binding site of plectin: effects on structure


25. Koss-Harnes D, Hoyheim B, Jonkman MF, et al. Life-long course and molecular characterization of the original Dutch family with epidermolysis bullosa simplex with
Chapter 3


