



Keratinocyte footprint assay discriminates antilaminin-332 pemphigoid from all other forms of pemphigoid diseases*

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Linked Comment: Bilgic and Murrell. *Br J Dermatol* 2020; **182**:270–271.

Summary

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Accepted for publication

10 May 2019

Funding sources

None.

Conflicts of interest

None to declare.

*Plain language summary available online

DOI 10.1111/bjd.18129

Background Antilaminin-332 mucous membrane pemphigoid is a chronic severe pemphigoid disease characterized by autoantibodies to laminin-332. At present no commercial assay is available to demonstrate antilaminin-332 antibodies, and diagnosis relies on in-house techniques with limited sensitivities.

Objectives In order to move, keratinocytes cultured *in vitro* secrete laminin-332 to attach to the culture dish. In that way, they leave behind a unique footprint trail of laminin-332. We aimed to develop a sensitive and specific laboratory assay to determine antilaminin-332 autoantibodies in patient serum based on binding of patient IgG to these unique footprints.

Methods Normal human keratinocytes were grown on glass coverslips and incubated with patient or control serum for 1 h. The binding of IgG was then investigated by immunofluorescence. After validating the test for its ability to identify antilaminin-332 autoantibodies it was converted into a daily available test based on binding of IgG to dried coverslips that can be stored frozen. The staining patterns of sera from patients with antilaminin-332 pemphigoid were then compared with those of sera from patients with other autoimmune bullous diseases and normal human sera.

Results IgG of all antilaminin-332 pemphigoid sera ($n = 16$) bound to laminin-332 footprints, while all normal human controls ($n = 55$) were negative. From the sera of patients with other diseases ($n = 72$) four sera tested positive. The footprint assay was also positive for sera that were negative by salt-split skin analysis, demonstrating that it is a very sensitive technique.

Conclusions The keratinocyte footprint assay is a fast and specific assay to confirm or rule out the presence of antilaminin-332 autoantibodies.

What's already known about this topic?

- Antilaminin-332 mucous membrane pemphigoid is a severe form of pemphigoid, and patients may have an increased risk of malignancies.
- The diagnosis of antilaminin-332 mucous membrane pemphigoid is complicated by the lack of specific commercial tests for antilaminin-332 antibodies and can be confirmed only in specialized laboratories.
- Keratinocytes in culture need laminin-332 for adhesion and migration and therefore deposit it on the bottom of the culture dish.

What does this study add?

- The keratinocyte footprint assay detects antilaminin-332 autoantibodies in patient serum using the native laminin-332 produced by cultured keratinocytes.

What is the translational message?

- The keratinocyte footprint assay is a fast and specific assay to confirm or rule out the presence of antilaminin-332 autoantibodies.

Mucous membrane pemphigoid (MMP) is a subepidermal autoimmune blistering disease affecting single or multiple mucous membranes of the mouth, eyes, nasopharynx, oropharynx, larynx and anogenital region, with associated skin involvement in about 30% of cases.¹ Formerly termed cicatricial pemphigoid because of the often observed scarring evolution of the lesions, it is associated with autoantibodies directed against different antigens located at the basement membrane zone (BMZ) level.² These are the intracellular hemidesmosomal protein BP230, the transmembrane hemidesmosomal proteins BP180 and integrin $\alpha 6\beta 4$, and laminin-332 (Ln-332). Recent reviews found that 6.4–23.3% of patients with MMP have autoantibodies against Ln-332, the pathogenicity of which has been proved by passive transfer in mice and human skin graft models.^{1,3–8}

Ln-332, formerly known as kalinin, epiligrin, nicein and laminin-5, is a heterotrimer that consists of the laminin chains $\alpha 3$, $\beta 3$ and $\gamma 2$. In a systematic review of 57 studies, Amber *et al.* found that for 59 patients the IgG was mostly directed to the $\alpha 3$ subunit (86%), followed by the $\gamma 2$ subunit (46%) and the $\beta 3$ subunit (29%).⁹ Most Ln-332 is found in the lamina densa and lower lamina lucida underneath hemidesmosomes, and is an essential component of the anchoring filaments that connect the hemidesmosomes via the adhesion receptors integrin $\alpha 6\beta 4$ and BP180 to the underlying anchoring fibrils. Similarly, it binds to integrin $\alpha 3\beta 1$ to bridge focal adhesions and anchoring fibrils.^{10,11} Genetic absence of the protein leads to severe generalized junctional epidermolysis bullosa, formerly named type Herlitz, a devastating blistering disease fatal in early childhood.¹²

Apart from its adhesion function Ln-332 also has roles in cell migration, tissue maturation and wound repair.^{13,14} Since 1997, 36 anti-Ln-332 cases have been reported in association with an underlying malignancy.^{15–31} Early studies reported that 20–30% of patients had a solid cancer.^{30,32–34} However, later studies found no statistically significant differences for the occurrence of cancer between Ln-332 and non-Ln-332 MMP, and according to Bernard *et al.* the prevalence rate of cancer in patients with MMP did not differ from that of the general population in the same age range in France.^{3–5}

Laboratory diagnosis of anti-Ln-332 is complicated. Immunofluorescence analysis of a skin biopsy and serum can give clues but not a definitive diagnosis. The IgG deposition at the BMZ of a skin biopsy is n-serrated as in pemphigoid diseases, with antibodies to BP180 or p200. Indirect immunofluorescence (IIF) on salt-split skin (SSS) substrate serum shows binding of IgG to the floor of the blister, as do antibodies to type VII collagen and p200. The combination of n-serrated deposition

and floor binding thus leaves a diagnosis of either anti-p200 or anti-Ln-332 pemphigoid. The final diagnosis therefore relies on the identification of anti-Ln-332 antibodies, and as commercial tests for this are not available, this is mainly achieved in specialized laboratories that have in-house techniques such as immunoprecipitation, immunoblot, enzyme-linked immunosorbent assay (ELISA) or knockout analysis.^{3,35,36}

As elegantly shown by Frank and Carter, keratinocytes in culture need Ln-332 to attach to the surface of the culture dish.¹⁴ When migrating, the cells polarize and deposit precursor Ln-332 at the rear of the moving cell. This Ln-332 is left behind and forms a unique footprint trail. We made use of this phenomenon to develop a fast and simple serum assay that discriminates anti-Ln-332 MMP from all other forms of pemphigoid diseases.

Materials and methods

Human sera

Sixteen sera from patients with anti-Ln-332 MMP were included in the study. One (patient 16) had IgG to both Ln-332 and type VII collagen, and one (patient 12) had Orf-induced anti-Ln-332 pemphigoid.^{37,38} IIF on SSS was positive for dermal IgG binding in 15 cases, and one was judged as dubious. The final diagnosis was achieved by either immunoblot analysis, immunoprecipitation, ELISA or IIF knockout analysis on type VII collagen-deficient skin and Ln-332-deficient skin (Table 1). We further included 25 sera that also stained the floor of SSS, 12 sera from patients with epidermolysis bullosa acquisita (EBA) and 13 sera from patients with anti-p200 pemphigoid. All patients with EBA had u-serrated IgG deposition on the BMZ on direct immunofluorescence and dermal staining on SSS IIF. The anti-p200 pemphigoid had been characterized by immunoblot and IIF on type VII collagen and Ln-332-deficient skin.³⁹

We also included 55 sera from patients who also had mucosal lesions but who had not been diagnosed with anti-Ln-332 MMP. These were 30 MMP sera from which 15 labelled the roof of SSS and 15 were negative on SSS, 10 sera from patients with erosive lichen planes, 11 sera from patients with paraneoplastic pemphigus, two sera from patients with erythema exudativum multiforme, and two sera that stained both the roof and floor of SSS. Also included were seven sera from patients with bullous pemphigoid (BP) that stained the epidermal side of SSS and recognized BP180 and/or BP230 by immunoblot and/or ELISA. As controls we used 55 normal human sera.

Table 1 Diagnostic findings in 16 patients with antilaminin-332 mucous membrane pemphigoid

Sample	Sex	Age (years)	DIF (BMZ)	SSS dermal binding	IB Ln-332	IP Ln-332	ELISA		IIF (KO skin)		Cancer
							Ln-332	Col7	Col7	Ln-332	
1	M	54	IgG 4+, C3c 3+, IgA 2+, IgM 2+	IgG 3+, IgA+	Neg	np	np	–	+	–	Yes
2	F	59	Neg	IgG 2+	$\alpha 3, \beta 3$	np	np	–	+	–	No
3	M	65	IgG 3+, C3c 2+, IgA 1+	IgG 1+	$\alpha 3$	np	np	np	np	np	No
4	F	86	IgG 3+, C3c 3+, IgA 2+, IgM \pm	IgG 1+	$\gamma 2$	np	np	–	+	–	No
5 ^a	M	27	IgG 2+, C3c 3+	IgG 2+	$\alpha 3$	np	+	–	np	np	No
6 ^a	M	25	IgG 3+, C3c 3+	IgG 2+	$\alpha 3$	np	+	–	np	np	No
7 ^a	F	65	IgG 3+, C3c +/2+	IgG 2+	$\alpha 3$	np	+	–	np	np	No
8 ^a	F	42	IgG 3+, C3c 2+	IgG 2+	$\alpha 3$	np	+	–	np	np	No
9 ^a	M	70	IgG 2+	IgG 1+	$\alpha 3$	+	np	np	np	np	Yes
10	M	82	IgG 3+, C3c 3+	IgG 3+	$\alpha 3, \beta 3, \gamma 2$	np	np	–	np	np	No
11	M	77	np	IgG 2+	$\alpha 3, \beta 3$	np	np	–	+	–	na
12 ^b	F	52	IgG 3+, C3c 3+, IgA 1+	IgG 2+	Neg	np	np	–	+	–	No
13	M	81	np	IgG 3+	$\alpha 3$	np	np	np	np	np	na
14 ^a	M	56	IgG 2+, IgA 2+	IgG dubious	$\alpha 3$	np	+	np	np	np	Yes
15	M	63	IgG 3+, C3c 2+	IgG 1+	$\alpha 3$	np	np	np	np	np	na
16 ^{a, c}	F	64	IgG 3+/4+, C3c +/2+	IgG 3+	$\alpha 3$	–	+	np	np	np	No

BMZ, basement membrane zone; Col7, type VII collagen; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; F, female; IB, immunoblot; IIF, indirect immunofluorescence; IP, immunoprecipitation; KFA, keratinocyte footprint assay; Ln-332, laminin-332; M, male; na, not available; Neg, negative; np, not performed; SSS, salt-split skin. IP was performed with radiolabelled human keratinocyte extract.³⁴ IB was performed for all sera on extract of cultured keratinocytes, and for sera of patients 7, 9 and 16 also on human keratinocyte matrix proteins. ELISA for anti-Col7 was performed with a kit from MBL (Nagoya, Japan) and ELISA for anti-Ln-332 was performed on affinity-purified Ln-332 as described previously.^{34 a-c} Described previously by ^aTerra *et al.*,^{34 b} van den Bos *et al.*³⁸ and ^cJonkman *et al.*³⁷

Keratinocyte footprint assay on living keratinocyte substrate

Primary normal human keratinocytes (NHKs) were isolated from redundant healthy skin obtained from breast reduction surgery upon written informed consent. NHKs were grown on 12-mm-diameter glass coverslips (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) in 24-well plates using CnT-Prime medium (CELLnTEC, Bern, Switzerland) at 37 °C and 5% CO₂. Keratinocytes were seeded at a density of 1 × 10⁴ cells in 500 µL medium in a 1.9-cm² well, and after 3 days of culture the coverslips were incubated with 200 µL 2.5% serum in medium for 1 h at 37 °C. The cells were then rinsed three times with phosphate-buffered saline (PBS), fixed in 2% formaldehyde for 10 min and stored frozen at –80 °C until staining. Fixed keratinocytes were stained using 1 : 400 DyLight488-labelled goat antihuman IgG (Thermo Fisher Scientific Inc.) in PBS containing 1% ovalbumin (PBS/OVA).

Keratinocyte footprint assay on air-dried frozen keratinocyte substrate

NHKs were cultured as described above and after 3 days they were rinsed three times with PBS and air dried. Cells were stored frozen at –80 °C until further use. After thawing, the coverslips were incubated with 1 : 10 serum in PBS/OVA for 1 h at 37 °C. After three washes in PBS, they were stained with 1 : 400 DyLight488-labelled goat antihuman IgG in

PBS/OVA. Of five sera where the corresponding biopsy had additional linear IgA BMZ deposition, incubated coverslips were also stained with 1 : 400 DyLight488-labelled goat anti-human IgA (Bethyl Laboratories, Montgomery, TX, U.S.A.) in PBS/OVA.

Immunofluorescence microscopy

To visualize Ln-332 we used monoclonal antibodies for all of the separate chains: clone 546215 for the $\alpha 3$ subunit, K140 for the $\beta 3$ subunit and D4B5 for the $\gamma 2$ subunit. Type VII collagen was stained with monoclonal LH7.2 and BP180/LAD-1 with monoclonal 233. As a secondary step we used Alexa 488-conjugated goat antimouse IgG, and in case of double staining Alexa 568-conjugated goat antimouse IgG (Thermo Fisher Scientific Inc.). All coverslips were examined with a Leica DMRA fluorescence microscope and images were acquired with a Leica DFC350 FX digital camera (Leica, Wetzlar, Germany).

Results

Keratinocytes in culture deposit laminin-332 but not type VII collagen or BP180

When we stained coverslips with monoclonal antibodies to Ln-332, binding was observed outside cells in a pattern described before as migration trails. The same pattern was

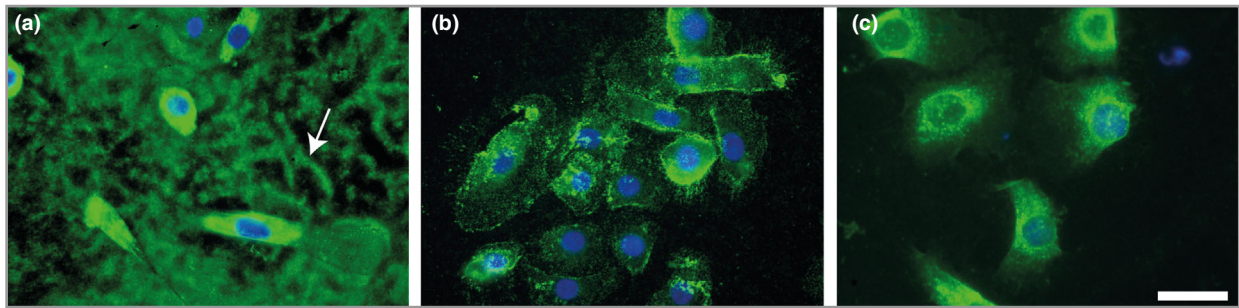


Fig 1. Keratinocytes deposit laminin-332 but not type VII collagen or the ectodomain of BP180 on the bottom of the culture dish. Air-dried coverslips were stained for (a) the laminin-332 β 3 subunit, (b) the BP180 ectodomain and (c) type VII collagen. Only laminin-332 is present as footprints. The white bar represents 20 μ m.

obtained for the α 3, β 3 and γ 2 chains, showing that each subunit is available on the coverslip. In contrast, monoclonal antibodies to the ectodomain of BP180, which is shed in culture, and type VII collagen did not bind outside cells (Fig. 1). Therefore, cultured keratinocytes could potentially be used in a diagnostic assay for anti-Ln-332 MMP.

Keratinocyte footprint assay on a living keratinocyte substrate

The anti-Ln-332 patient IgG that bound to the coverslips in the footprint pattern colocalized with the binding of monoclonal antibodies to all three laminin chains (Fig. 2 shows overlap with the γ 2 subunit). Such footprints were observed for all anti-Ln-332 pemphigoid sera, including those that were negative in SSS IIF (Fig. 3a–c). All of the EBA, anti-p200, bullous pemphigoid and normal control group sera did not show any binding to the Ln-332 footprints, except one (Fig. 3d–l). The exception was a patient with anti-p200 pemphigoid who was positive for anti-p200 IgG by immunoblot and whose serum also bound to Ln-332-deficient skin (not shown). Sera from six of the seven patients with bullous pemphigoid bound to the membrane of the cells, in line with the accessibility of the BP180 ectodomain.

Keratinocyte footprint assay on air-dried frozen keratinocyte substrate

As living cell incubation is labour intensive we tested whether patient IgG would also bind to footprints on coverslips that after 3 days had been air dried and frozen. The results were identical to the living cell incubation in terms of binding to laminin (Fig. 4). Tests performed on coverslips stored for 18 months at -30°C gave the same results as freshly frozen coverslips (not shown). The only difference from living cell assays was that all sera also bound to the cells, as the cytoplasm of dried cells is accessible to IgG and this contains newly synthesized but not yet secreted antigens. We also tested the sera of five patients who had, besides IgG, deposition of IgA along the BMZ, although only one serum sample bound with IgA to the floor of SSS. Of these five sera the IgA

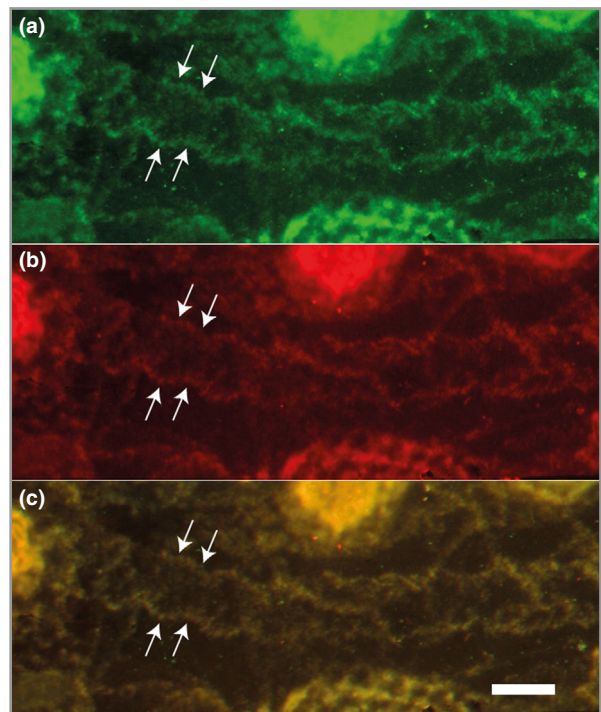


Fig 2. Patient IgG binds to deposited laminin-332. Double staining of living cells incubated with antilaminin-332 patient IgG for (a) human IgG and (b) the laminin-332 γ 2 subunit shows complete colocalization in the merged panel (c). The white bar represents 50 μ m.

of two bound to the laminin footprints. Furthermore, we screened the 55 sera of patients with mucosal lesions that had not been diagnosed as anti-Ln-332 MMP. We found that in four cases IgG was present that bound to the Ln-332 on the coverslip.

Discussion

Specific commercial tests to detect anti-Ln-332 autoantibodies are lacking, which results in missed or delayed diagnoses of anti-Ln-332 MMP. Of tests that have been developed in house, immunoprecipitation using radiolabelled keratinocyte extracts

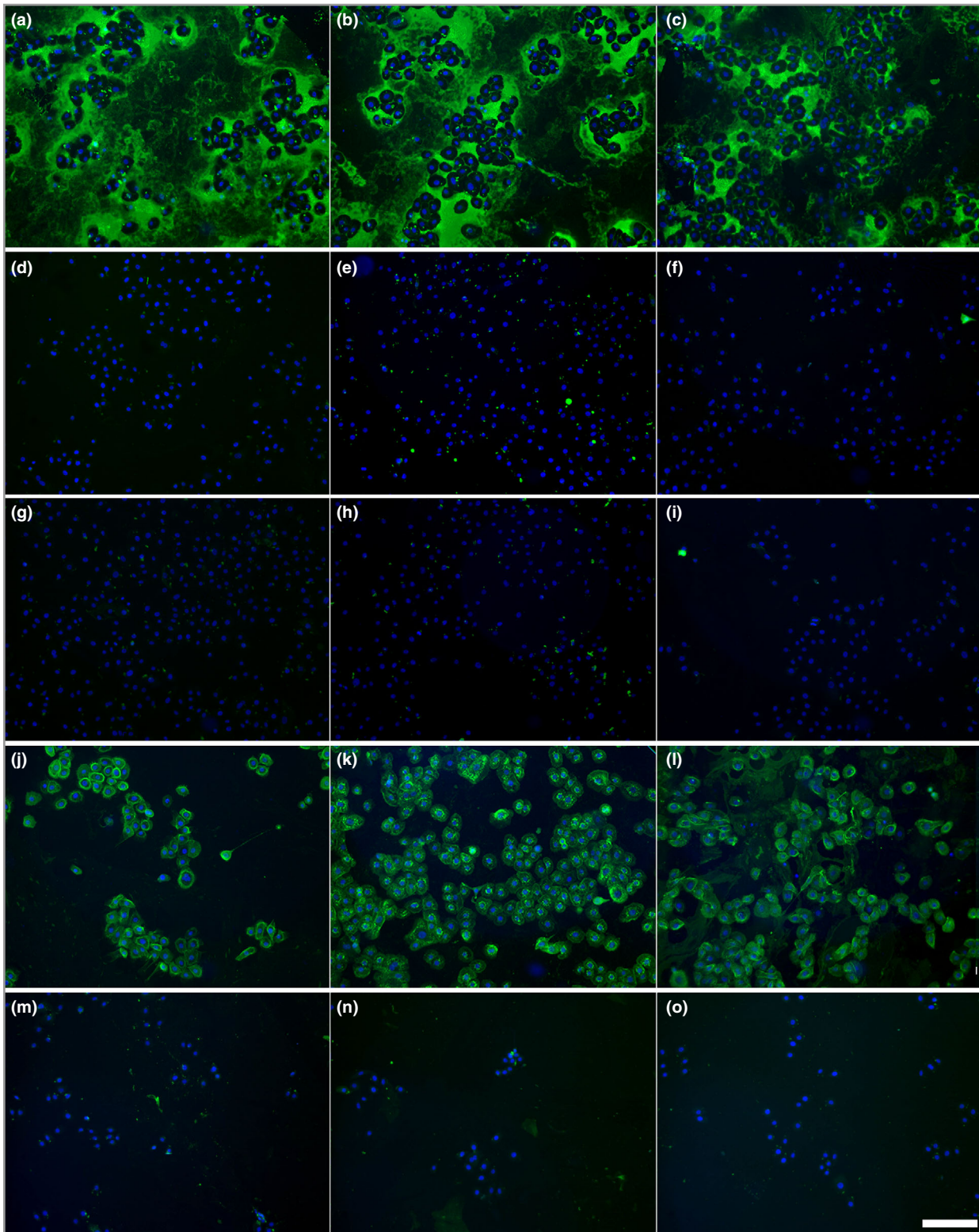


Fig 3. IgG binding patterns after incubation of different types of pemphigoid sera with living keratinocytes. Binding patterns after 1-h incubation with sera of patients with (a–c) antilaminin-332 mucous membrane pemphigoid, (d–f) epidermolysis bullosa acquisita, (g–i) anti-p200 pemphigoid and (j–l) bullous pemphigoid and (m–o) normal human control sera. The white bar represents 150 μ m.

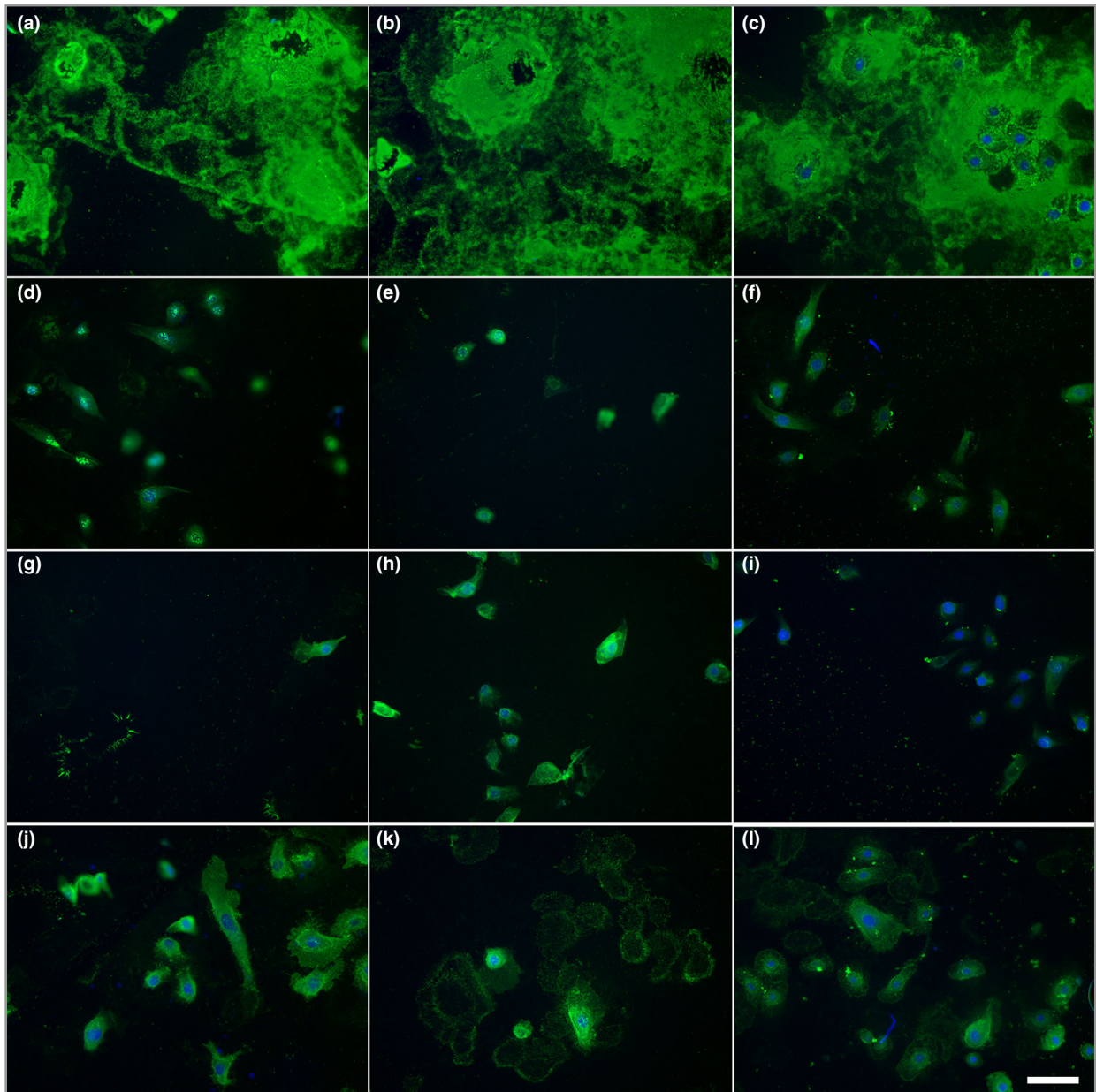


Fig 4. IgG binding patterns after incubation of different types of pemphigoid sera with air-dried coverslips. Binding patterns of sera of patients with (a–c) antilaminin-332 mucous membrane pemphigoid, (d–f) epidermolysis bullosa acquisita, (g–i) anti-p200 pemphigoid and (j–l) bullous pemphigoid. The white bar represents 60 μm .

is the most sensitive and specific, but it is restricted to specialized laboratories. It is labour intensive and its high costs and risks do not make it a practical test. Immunoblot analysis has a lower and also variable sensitivity due to the amount of Ln-332 in the substrate and the absence of conformational epitopes.^{35,40,41} A commercial ELISA for anti-Ln-332 IgG is not available, but a few in-house ELISAs have been developed by independent laboratories with varying success. The first reported ELISA for anti-Ln-332 used a recombinant form of Ln-332. Its sensitivity was 91% but specificity was low as it was also positive for 40% of tested bullous pemphigoid sera.⁴²

Extraction of the extracellular matrix that keratinocytes deposit on the bottom of the culture dish results in a substrate that is extremely rich in Ln-332.⁴³ When this substrate was used in ELISA a high number of false-positives were again found in the bullous pemphigoid group when polyclonal secondary antibodies were used, but when a monoclonal antibody to IgG4 was used as a secondary antibody the sensitivity became 91% with a specificity of 98%, although here also 13% of the bullous pemphigoid patient sera tested positive. More recently an ELISA based on purified Ln-332 from SCC25 cells found only 2% positives in BP and 9% in hospitalized patients without autoimmune

bullous disease; however, the sensitivity and specificity were not provided.⁵

The keratinocyte footprint assay we developed is based on the exclusive binding of anti-Ln-332 IgG to the deposited extracellular matrix Ln-332. No deposition of type VII collagen or the shed ectodomain of BP180 was found on the plate, although it is known that both proteins are secreted in the medium.^{44,45} Also, the laminin $\gamma 1$ chain is not deposited on the culture dish, as reported before.⁴⁶ In our series, all sera from patients diagnosed with anti-Ln-332 MMP tested positive for footprint binding, while all 55 normal human control sera tested negative. IgG of 24 EBA and anti-p200 pemphigoid sera, which, as with anti-Ln-332, also bind the floor of SSS, did not bind to the footprints. For one case that had proven anti-p200 IgG antibodies we found that the serum also contained antibodies to Ln-332 as it bound to the footprints, making this case mixed anti-p200 and anti-Ln-332 pemphigoid. IgG of six bullous pemphigoid sera bound to living cells in line with the accessibility of the BP180 ectodomain. One case that by immunoblot and ELISA only demonstrated IgG to BP230 did not bind to the cells in accordance with the cytoplasmic location of BP230 (not shown).

Together these results demonstrate that the specificity of the assay is probably close to 100%. When we analysed patients with non-Ln-332 MMP we found no binding for sera from patients with oral erosive lichen planus or erythema exudativum multiforme. One of the 11 cases of paraneoplastic pemphigus also demonstrated anti-Ln-332 IgG. A similar case of paraneoplastic pemphigus with additional anti-Ln-332 IgG has been described before.⁴⁷

One of the two sera that stained both the roof and floor of SSS was also positive for anti-Ln-332. Such cases have been described before by Hayakawa *et al.*³ Two of the other 30 MMP sera also contained anti-Ln-332 IgG antibodies. One serum sample was from a patient who had oral, nasal, pharyngeal and laryngeal mucous membranes affected without skin involvement. A skin biopsy demonstrated BMZ deposition of IgG and IgA; however, all serology was negative. Based on the positive keratinocyte footprint assay a final diagnosis of anti-Ln-332 MMP was made. The other serum sample bound the roof of SSS and was anti-NC16A ELISA positive; immunoblotting demonstrated both anti-BP180 IgG and IgA antibodies.

The concomitant presence of antibodies to Ln-332 and BP180 has been described already several times.^{2,4,48,49} These last two patients show that a small percentage of patients with MMP who are not initially diagnosed with anti-Ln-332 MMP may have antibodies to Ln-332. Although the titres are below the detection level of SSS IIF the antibodies might still contribute to the clinical phenotype. However, this has to await a larger series of patients to compare the clinical details of those with and without additional anti-Ln-332 antibodies.

The possibility to perform the keratinocyte footprint assay on freezer-stored coverslips makes it a practical assay that within a few hours will confirm or rule out anti-Ln-332 MMP. If a biopsy is available, it is possible to discriminate most cases with IIF SSS floor-binding sera between EBA, anti-

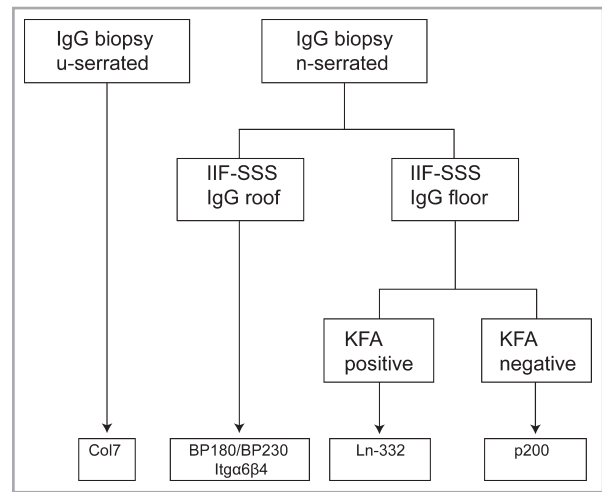


Fig 5. Flowchart for the diagnosis of different subtypes of pemphigoid diseases. By combining (i) the serration pattern of basement membrane zone deposition in the skin, (ii) indirect immunofluorescence (IIF) of serum on salt-split skin (SSS) and (iii) the keratinocyte footprint assay (KFA), rapid identification of the antigens recognized can be achieved. Col7, type VII collagen; Itg $\alpha 6\beta 4$, integrin $\alpha 6\beta 4$; Ln-332, laminin-332.

Ln-332 pemphigoid and anti-p200 pemphigoid. In EBA the IgG BMZ deposition in the biopsy will be u-serrated; if the deposition in the biopsy is n-serrated then sera with a positive keratinocyte footprint assay are from patients with anti-Ln-332 pemphigoid and sera that are negative are from patients with anti-p200 (Fig. 5).

The keratinocyte footprint assay that we report here represents a simple and reliable assay for routine diagnostics that rapidly identifies the presence of anti-Ln-332 antibodies. The keratinocyte footprint assay could be of help for prospective case-control studies to demonstrate or not the reality of the association of Ln-332 MMP with cancer.

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