

Human Amnion Membrane as a Substrate for the Detection of Autoantibodies in Pemphigus Vulgaris and Bullous Pemphigoid

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ABSTRACT: **Background:** Human amnion membrane (HAM) was suggested to be a superior antigenic substrate for immunoblotting in detecting autoantibodies of autoimmune bullous skin diseases. **Objectives:** To determine the properties of HAM as an antigenic substrate for the detection of autoantibodies in pemphigus vulgaris and bullous pemphigoid. **Methods:** Immunomapping and tandem liquid chromatography mass spectrometry were used to delineate the antigenic structure of HAM. Immunoblotting and indirect immunofluorescence were used to study the diagnostic utility of HAM in 25 pemphigus patients, 41 pemphigoid patients, and 36 controls, and the results were compared to those of indirect immunofluorescence on monkey esophagus, immunoblotting using normal human skin, and enzyme-linked immunosorbent assay. **Results:** Immunomapping demonstrated the presence of all the antigens known to be targeted in autoimmune bullous skin diseases, in both normal human skin and HAM, except for the absence of BP230, and low threshold levels of Dsg1, Dsg3 and Dsc3 in HAM. HAM indirect immunofluorescence demonstrated anti-basement membrane zone antibodies in 48.7% of the pemphigoid patients, and anti-intercellular space antibodies in 72.0% of the pemphigus patients. HAM immunoblotting did not demonstrate anti-BP230 antibodies, but detected anti-BP180 antibodies in 53.7% of the pemphigoid patients. It did not demonstrate anti-Dsg1 and/or anti-Dsg3 antibodies in any of the pemphigus patients. These results were inferior to those of ELISA and monkey esophagus indirect immunofluorescence. **Conclusions:** Compared to other studied methods, HAM does not offer advantages in detecting autoantibodies in bullous pemphigoid and pemphigus vulgaris.

IMAJ 2014; 16: 217–223

KEY WORDS: amnion, membrane, pemphigus, pemphigoid, antibodies

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Several techniques have been used for the detection of autoantibodies in autoimmune bullous skin diseases, including non-specific target antigen detection such as direct immunofluorescence and indirect immunofluorescence, and specific target antigen detection such as immunoblotting and enzyme-linked immunosorbent assay. Despite their lack of antigen specificity, direct immunofluorescence and indirect immunofluorescence are still widely used in the clinical diagnosis of autoimmune bullous skin diseases. ELISA allows the detection of autoantibodies against specific antigens, but it is presently limited to only a few commercial kits of known antigens. Immunoblotting using normal human skin as an antigenic source allows the detection of autoantibodies against a larger range of antigens, including novel targets. However, it requires human donors and mechanical, chemical or thermal separation methods which may distort the structure and distribution of some of the antigens [1,2]. Human amniotic membrane was suggested to be a more readily available and more useful substrate for immunoblotting analysis [3,4]. The results of previous studies on HAM immunoblotting demonstrated 100% sensitivity for the detection of the desmoglein 1 and Dsg3 antibodies in pemphigus vulgaris patients in one study [3] and only 64% sensitivity in another [4].

In the present study, we sought to further characterize the properties of HAM as a substrate for the diagnosis of pemphigus vulgaris and bullous pemphigoid. We used immunophenotyping and tandem liquid chromatography mass spectrometry to delineate the protein structure of HAM, and we studied HAM as a substrate by employing indirect immunofluorescence and immunoblotting on sera from 66 patients and 36 healthy controls. Ultimately, the accuracy of HAM immunoblotting and indirect immunofluorescence were compared to that of immunoblotting using normal human skin, indirect immunofluorescence using monkey esophagus, and ELISA.

ELISA = enzyme-linked immunosorbent assay
HAM = human amniotic membrane
Dsg = desmoglein

PATIENTS AND METHODS

We studied 41 consecutive patients with pemphigoid and 25 consecutive patients with pemphigus; 40 of the patients were females and 26 were males and their age ranged from 21 to 98 years (mean 70.4 years). The diagnosis in all these patients was based on the clinical, histological and direct immunofluorescence findings. Thirty-six healthy individuals, 19 females and 17 males aged 20–87 (mean 57.4 years), served as controls. All of the participants provided written and informed consent according to a protocol previously approved by the local Helsinki Committee of the Israel Ministry of Health.

TISSUE SAMPLE PREPARATION AND PROTEIN EXTRACTION

Fresh whole normal human placentas were obtained from the obstetrics department with the mothers' written and informed consent, according to a protocol approved by the local Helsinki Committee of the Israel Ministry of Health. Human amniotic membrane was separated from the placenta and washed extensively in 0.9% sodium chloride. It was then cut into small pieces of approximately 4 cm² and used for either the preparation of the amnion protein extract or snapped frozen for the immunofluorescence analysis. Adult normal human skin was obtained from patients undergoing cosmetic surgery, washed extensively in 0.9% sodium chloride, cut in pieces of approximately 15 cm², and exposed in 0.01 M phosphate-buffered saline (pH 7.2) solution at 56°C for 1 minute. Subsequently, the normal human skin fragments were stretched with the epidermal side facing upward, while gently scrubbing the epidermis with keratome. The obtained epidermis and the remaining dermal fragments were further used for preparation of epidermal and dermal protein extracts for immunoblotting, respectively. The proteins of HAM, human epidermis and human dermis were extracted based on a previously described protocol [4]. We obtained punch skin biopsies of normal human skin, 4 mm, to be used as frozen section substrates for the indirect immunofluorescence analysis.

ANTIBODIES

Mouse monoclonal and rabbit polyclonal antibodies to the following desmosomal and basement membrane zone antigens were used: rabbit anti-human immunoglobulin G antibodies to Dsg1 and 3, desmoplakin, laminin β 3, laminin γ 2, integrin β 4, envoplakin, periplakin, plakoglobin, laminin γ 1 and bullous pemphigoid antigen 1 (BP230), all purchased from Santa Cruz Biotechnology (CA, USA). Rabbit anti-human IgG desmocollin and collagen 7 antibodies and mouse anti-human IgG laminin γ 2 (specifically for indirect immunofluorescence) and BP230 antibodies were purchased from Life Span Biosciences (WA, USA), Abcam (Cambridge, UK), AbD Serotec (Oxford, UK) and Cosmo-

Bio Co., Ltd. (Tokyo, Japan), respectively. Rabbit anti-human BP180 antibody (clone 493) was kindly provided by Prof. M. Hertl (Marburg, Germany).

INDIRECT IMMUNOFLOURESCENCE AND IMMUNOPHENOTYPING

Circulating anti-skin antibodies were sought by using HAM as the antigenic substrate. The slides were incubated at room temperature for 1 hour with patient and control sera (diluted 1:10 in 0.01 M PBS). Following two washes with 0.01 M PBS, the slides were incubated at room temperature for 30 minutes with fluorescence isothiocyanate goat anti-human IgG Fc γ -specific antibody (Dakocytomation, Denmark). After extensive washing with tap water the slides were dried at room temperature, mounted and observed with an Olympus BX51 fluorescence microscope. Indirect immunofluorescence assay using monkey esophagus as the substrate was performed according to a standard technique. Immunophenotyping of tissue desmosomal and BMZ antigens in HAM and normal human skin was performed in a similar way using a panel of desmosomal and hemidesmosomal antibodies delineated above, as the primary antibodies. FITC swine anti-rabbit IgG F(ab')₂ and FITC goat anti-mouse IgG F(ab')₂ antibodies (Dakocytomation) were used as the secondary antibodies.

PROTEOMIC ANALYSIS

For identification of the extracted proteins from HAM, HEP and HDR, three bands corresponding to the molecular weights of 75–100 kDa, 100–150 kDa and 150–300 kDa were dissected from a 8% polyacrylamide gel and trypsinized. The tryptic peptides were analyzed by tandem liquid chromatography mass spectrometry analysis on the OrbitrapXL mass spectrometer (Thermo Scientific, USA). The data were analyzed with sequest 3.31 software versus the human section of the uniprot database using the Sequest search engine.

IMMUNOBLOTTING

Venous whole blood was collected onto a vacuette serum clot activator tube. Sera were obtained after centrifugation (1500 g x10 min at room temperature) and immediately frozen at -80°C until use. Circulating autoantibodies were detected by immunoblotting using HAM, HEP and HDR as the antigenic substrates, and rabbit anti-human IgG as the secondary antibody (Dakocytomation), according to a previously described protocol [5]. Subsequently, alkaline phosphatase conjugate substrate kit (Bio-Rad, USA) was employed for calorimetric detection of the bands.

PBS = phosphate-buffered saline
BMZ = basement membrane zone
FITC = fluorescence isothiocyanate
HEP = human epidermis
HDR = human dermis

Table 1. Distribution of the relevant antigens targeted in autoimmune bullous skin diseases in different substrates using LC-MS/MS

Protein substrate	Dsg1	Dsg3	Dsc3	Per	Env	Dp	Pkg	p170	BP180	BP230	Lam5 α3	Lam5 β3	Lam5 γ2	Int β4	Col7	Lam6 γ1
HAM	-/+	-/+	-/+	+	+	+	+	+	+	-	+	+	+	+	+	+
HEP	+	+	+	+	+	+	+	+	-/+	-/+	-	-	-	+	-	-
HDR	+	+	+	+	+	+	+	+	-/+	+	+	+	+	+	+	+

+ = significant identification, +/- = threshold detection level (sequest score ≤ 30, peak area ≤ 0.01), - = not identified

Dsg = desmoglein, Dsc = desmocollin, Per = periplakin, Env = envoplakin, Dp = desmoplakin, Pkg = plakoglobin, p170 = protease inhibitor alpha-2-macroglobuline-like-1, BP = bullous pemphigoid antigen, Lam = laminin, Int = integrin, Col = collagen, HAM = human amnion membrane, HEP = human epidermis, HDR = human dermis

ELISA

EUROIMMUN Medizinische Labordiagnostika AG (Lübeck, Germany) anti-BP180-NC16A-4X ELISA (IgG), anti-BP230-CF ELISA (IgG), anti-Dsg1 ELISA (IgG), and anti-Dsg3 ELISA (IgG) kits were used to determine the serological presence of autoantibodies to NC16A domain of BP180, C-terminal domain of BP230, Dsg1 and Dsg3, respectively. The ELISA assay was performed according to a protocol supplemented with the kit. The photometric measurements of the color intensity were performed at 450 nm on the Infinite F50 TECAN device (Neotec, Canada). The cutoff value for positive results was 20 RU/ml.

STATISTICAL ANALYSIS

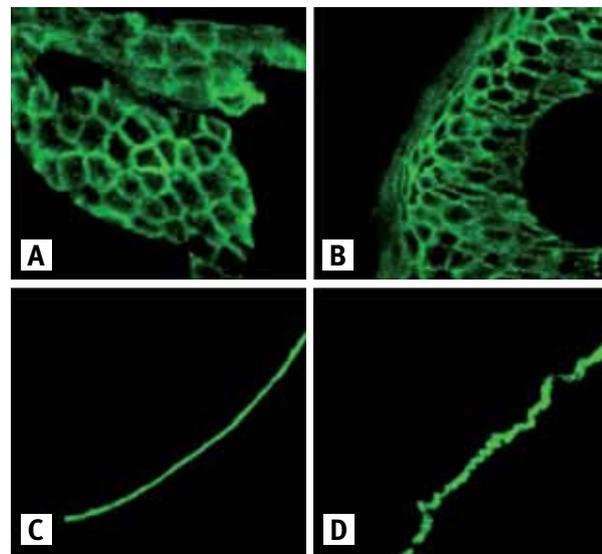
The diagnostic performance of immunoblotting, indirect immunofluorescence and ELISA methods for detection of pemphigus and pemphigoid major targeted antigens (e.g., Dsg1, Dsg3, BP180 and BP230) were evaluated based on sensitivity, specificity, positive and negative predictive values, and accuracy. These parameters and their 95% confidence intervals were evaluated using XLSTAT 2012 (Addinsoft, USA). Two-tailed *P* values for every diagnostic method were obtained by applying the Fisher exact test on a 2 x 2 contingency table. To test whether the differences between the sensitivities and specificities of a new proposed test and a non-reference test were statistically significant, the McNemar test for correlated proportions was applied. The *P* values are presented. All analyses were performed using the SPSS software package, version 20.0 (IBM, Armonk, NY, USA). *P* < 0.05 was considered statistically significant.

RESULTS

IMMUNOPHENOTYPING AND TANDEM LC-MS/MS ANALYSIS

Immunophenotyping of desmosomal and BMZ antigens in HAM and normal human skin demonstrated the presence of all the tested relevant antigens, except for BP230 which was absent altogether in HAM. An intercellular space pattern was obtained when applying IgG antibodies to the desmosomal components Dsg1, Dsg3, Dsc, Dp, Env and Pkg in HAM and in

Figure 1. Confocal images of immunomapping with anti-human plakoglobin IgGs localized to the ICS of HAM [A] and normal human skin [B]. Immunomapping with anti-human collagen 7 IgGs localized to the BMZ of HAM [C] and normal human skin [D]



normal human skin [Figure 1A and B]. A linear BMZ staining pattern was obtained in both HAM and normal human skin when IgG antibodies to BP180, Col7, Lam β3, Lam γ2, Lam γ1 and Int β4 were applied [Figure 1 C and D].

The identification of skin antigens in HAM and normal human skin protein extracts was performed by LC-MS/MS. All the relevant desmosomal and BMZ antigens, except for BP230, were present in HAM [Table 1], and Dsg1, Dsg3 and Dsc3 antigens were identified at threshold levels, as indicated by low peak area (≤ 0.01) and a low number of the identified peptides (n ≤ 3), showing an overall Sequest score of ≤ 30 [Table 1]. The HEP extract was rich mainly in antigens present in the epidermis, including Dsg1, Dsg3, Dsc3, Per, Env, Dp, Pkg and Int β4. Some of the proteins, such as BP230 and BP180,

Dsc = desmocollin
 Dp = desmoplakin
 Env = envoplakin
 Pkg = plakoglobin

LC-MS/MS = liquid chromatography mass spectrometry

were detected at threshold levels, and the BMZ proteins Lam γ 1, Lam γ 2, Lam β 3, Lam α 3 and Col7 were absent altogether. In contrast, the HDR extract contained all the epidermal and dermal antigens targeted in autoimmune bullous skin diseases in a sufficient amount, except for BP180 which was identified at threshold levels.

IMMUNOBLOTTING

The results are presented in Tables 2 and 3. Human amniotic membrane and HEP protein extracts were used for detection of circulating anti-Dsg1 and/or Dsg3 autoantibodies in 25 pemphigus patients. The bands that were recognized by patients' sera were confirmed by immunoblotting with anti-human Dsg1 and Dsg3 polyclonal IgG antibodies. Immunoblotting performed on HEP protein extract demonstrated anti-Dsg1 and/or anti-Dsg3 antibodies in 19 (76.0%) of the 25 pemphigus patients.

Six healthy individuals (16.6%) and 2 pemphigoid patients (4.8%) had detectable levels of anti-Dsg1 and/or anti-Dsg3 autoantibodies when using HEP immunoblotting ($P < 0.001$). No specific bands corresponding to Dsg1 and/or Dsg3 antigens were detected in the sera from pemphigus patients and the control group when using HAM immunoblotting.

HAM, HEP and HDR protein extracts were used as the antigenic substrates for the detection of anti-BP180 and BP230 autoantibodies in pemphigoid patients. The bands recognized by patients' sera were confirmed by immunoblotting with anti-human BP180 and BP230 IgG polyclonal and monoclonal antibodies, respectively. Anti-BP180 (180 kDa) antibodies were found in 21 of the 41 pemphigoid patients (51.2%) using HEP extract and in 22 of the 41 pemphigoid patients (53.7%) when using HDR or HAM protein extracts. Three (8.3%) of the 36 healthy individuals and none of the 25 pemphigus patients had anti-BP180 autoantibodies when using HDR protein extract, and one healthy individual (2.7%) and none of the pemphigus patients showed anti-BP180 autoantibodies when utilizing HAM and HEP protein extracts ($P < 0.001$). Two bands corresponding to BP230 (230 kDa) with molecular weights of ~280 and ~230 kDa [6] were detected when sera from 11 of the 41 pemphigoid patients (26.8%) were tested with immunoblotting using HEP or HDR protein extracts, but not when using HAM protein extract. Identical bands were obtained following incubation of the HEP and HDR blots with a monoclonal anti-BP230 IgG antibody, but not when applying pemphigoid sera or specific monoclonal anti-BP230 IgG antibody to HAM immunoblotting. One healthy individual (2.7%) and none of the pemphigus patients had anti-BP230 antibodies when using HEP and HDR immunoblotting.

Col7 = collagen 7
Lam = laminin
Int = integrin
Per = periplakin

INDIRECT IMMUNOFLUORESCENCE

The results are presented in Tables 2 and 3. Of the 25 pemphigus patients, 18 (72.0%) and 24 (96.0%) had anti-ICS autoantibodies using HAM and monkey esophagus as substrates, respectively. One of the 36 healthy individuals (2.7%) and 3 of the 41 pemphigoid patients (7.3%) revealed ICS staining when indirect immunofluorescence analysis was performed on HAM ($P < 0.001$). Four healthy individuals (11.1%) and none of the pemphigoid patients demonstrated ICS staining when using monkey esophagus as a substrate ($P < 0.001$).

Among the 41 pemphigoid patients, 20 (48.7%) and 24 (58.5%) had anti-BMZ autoantibodies using HAM and monkey esophagus as substrates, respectively. One of the 25 pemphigus patients (4.0%) and none of the 36 healthy individuals had anti-BMZ autoantibodies when indirect immunofluorescence was performed on HAM ($P < 0.001$), and none of the healthy individuals or pemphigoid patients demonstrated BMZ staining when using monkey esophagus as a substrate ($P < 0.001$).

ELISA

The results are presented in Tables 2 and 3. Circulating anti-Dsg1 and anti-Dsg3 antibodies were assayed using ELISA in 25 pemphigus patients. Seventeen of them (68.0%) had both anti-Dsg1 and anti-Dsg3 antibodies, and 5 (20.0%) and 1 (4.0%) had anti-Dsg3 and anti-Dsg1 antibodies only, respectively. The remaining two pemphigus patients (8.0%) were negative for both. Altogether, 92.0% of the patients with pemphigus had at least one of the two antibodies. None of the 41 pemphigoid patients or the 36 healthy individuals had anti-Dsg1 and/or anti-Dsg3 antibodies ($P < 0.001$).

Circulating anti-BP180NC16A and anti-BP230-C terminal domain antibodies were assayed in 41 patients with pemphigoid. Fifteen (36.6%) had both anti-BP180NC16A and anti-BP230-C terminal domain antibodies. Anti-BP180NC16A or BP230-C terminal domain antibodies were found in 20 patients (48.8%) and 1 patient (2.4%) respectively. Five pemphigoid patients (12.2%) were negative for both antibodies. Altogether, 87.8% pemphigoid patients had at least one of the two antibodies. Two of the 36 healthy individuals (5.5%) and one of the 25 pemphigus patients (4.0%) had anti-BP180NC16A and/or anti-BP230-C terminal domain antibodies, respectively ($P < 0.001$).

COMPARATIVE ANALYSIS OF IMMUNOBLOTTING, INDIRECT IMMUNOFLUORESCENCE AND ELISA

The results are presented in Tables 2 and 3. In assessing the diagnostic performance of HAM as an antigenic source and a substrate for immunoblotting and indirect immunofluorescence analyses, a statistical comparison was performed with regard to the other studied methods. In the evaluation of HAM applicability as an antigenic source for immunoblotting

ICS = intercellular space

analysis, a comparison performed on HEP and/or HDR protein extracts was performed. No specific bands corresponding to Dsg1, Dsg3 or BP230 antigens were observed using HAM immunoblotting in the diagnosis of pemphigus and pemphigoid patients, respectively. Consequently, a statistical comparison between HAM immunoblot and HEP and HDR immunoblots was performed for the detection of anti-BP180 antibodies only. HAM, HEP and HDR immunoblots were found to display close sensitivities (53.7%, 51.2% and 53.7%, respectively) and very close specificities (98.4%, 98.4% and 95.1%, respectively) in detecting anti-BP180 antibodies (not significant).

Similar sensitivities and specificities to anti-BMZ antibodies were obtained for HAM indirect immunofluorescence (sensitivity 48.8%, specificity 98.4%) and monkey esophagus indirect immunofluorescence (sensitivity 58.5%, specificity 100.0%) in the diagnosis of pemphigoid (not significant). A significant difference ($P = 0.031$) between the sensitivity, but not specificity of HAM indirect immunofluorescence (sensitivity 72.0%, specificity 94.8%) and monkey esophagus indirect immunofluorescence (sensitivity 96.0%, specificity 94.8%) was found for the diagnosis of pemphigus.

The sensitivity of ELISA against the NC16A domain of the BP180 protein was significantly higher ($P < 0.005$) than the sensitivities of both immunoblotting and indirect immunofluorescence using HAM in diagnosing pemphigoid. ELISA was also a sensitive method in detecting anti-BP230 antibodies in patients with titers < 25 RU/ml, raising the ELISA sensitivity of detecting anti-BP180 NC16A and/or anti-BP230 C terminus domain antibodies in pemphigoid to 87.8%. ELISA against NC16A domain and/or BP230 C terminus domain

Table 2. Pemphigus vulgaris antibodies: results and statistical significance of the serological analyses

Method	Indirect immunofluorescence		Immunoblotting		ELISA
	Monkey esophagus	HAM	HEP	HAM	
Antibodies to	ICS	ICS	Dsg1 and/or Dsg3	Dsg1 and/or Dsg3	Dsg1 and/or Dsg3
Pemphigus vulgaris	24/25 (96.0%)	18/25 (72.0%)	19/25 (76.0%)		23/25 (92.0%)
Bullous pemphigoid	-/41 (-)	3/41 (7.3%)	2/41 (4.8%)		-/41 (-)
Controls	4/36 (11.1%)	1/36 (2.7%)	6/36 (16.6%)		-/36 (-)
Sensitivity (%) CI (%)	96.0 (78.6–100)	72.0 (52.1–85.8)	76.0 (56.1–88.7)		92.0 (73.7–98.8)
Specificity (%) CI (%)	94.8 (86.9–98.3)	94.8 (86.9–98.3)	89.6 (80.5–94.8)	Negative*	100.0 (94.2–100.0)
PPV (%) CI (%)	85.7 (72.8–98.7)	81.8 (65.7–97.9)	70.4 (53.1–87.6)		100.0 (100.0–100.0)
NPV (%) CI (%)	98.6 (96.0–100.0)	91.3 (85.1–97.4)	92.0 (85.9–98.1)		97.5 (94.0–100.0)
Accuracy (%) CI (%)	95.1 (90.9–99.3)	89.2 (83.2–95.2)	86.3 (79.6–93.0)		98.0 (95.3–100.0)
Pvalue	< 0.001	< 0.001	< 0.001		< 0.001

* No specific bands corresponding to Dsg1 and/or Dsg3 antigens were detected

Pvalue for Fischer exact test

CI = 95% confidence interval, - = not identified, BP = bullous pemphigoid, ICS = intercellular space, ELISA = enzyme linked immunosorbent assay, HAM = human amnion membrane, HEP = human epidermis, PPV = positive predictive value, NPV = negative predictive value

was the most accurate test (92.2%) for the diagnosis of pemphigoid with equally high estimated positive predictive value (92.3%) and negative predictive value (92.1%).

Table 3. Bullous pemphigoid antibodies: results and statistical significance of the serological analyses

Method	Indirect immunofluorescence		Immunoblotting			ELISA	
	Monkey esophagus	HAM	HEP	HDR	HAM	NC16A BP180	NC16A BP180 and/or BP230C
Antibodies to	BMZ	BMZ	BP180	BP180	BP180		
Bullous pemphigoid	24/41 (58.5%)	20/41 (48.7%)	21/41 (51.2%)	22/41 (53.7%)	22/41 (53.7%)	35/41 (85.3%)	36/41 (87.8%)
Pemphigus vulgaris	-/25 (-)	1/25 (4.0%)	-/25 (-)	-/25 (-)	-/25 (-)	-/25 (-)	1/25 (4.0%)
Controls	-/36 (-)	-/36 (-)	1/36 (2.7%)	3/36 (8.3%)	1/36 (2.7%)	2/36 (5.5%)	2/36 (5.5%)
Sensitivity (%) CI (%)	58.5 (43.3–72.2)	48.8 (34.3–63.5)	51.2 (36.5–65.7)	53.7 (38.8–67.9)	53.7 (38.8–67.9)	85.4 (71.1–93.4)	87.8 (73.9–95.0)
Specificity (%) CI (%)	100.0 (92.7–100.0)	98.4 (90.3–100.0)	98.4 (90.3–100.0)	95.1 (85.8–98.8)	98.4 (90.3–100.0)	96.7 (88.0–99.7)	95.1 (85.8–98.8)
PPV (%) CI (%)	100.0 (100.0–100.0)	95.2 (86.1–100.0)	95.5 (86.8–100.0)	88.0 (75.3–100.0)	95.7 (87.3–100.0)	94.6 (87.3–100.0)	92.3 (83.9–100.0)
NPV (%) CI (%)	78.2 (69.0–87.4)	74.1 (64.5–83.6)	75.0 (65.5–84.5)	75.3 (65.7–85.0)	75.9 (66.5–85.4)	90.8 (83.7–97.8)	92.1 (85.4–98.7)
Accuracy (%) CI (%)	83.3 (76.1–90.6)	78.4 (70.4–86.4)	79.4 (71.6–87.3)	78.4 (70.4–86.4)	80.4 (72.7–88.1)	92.2 (86.9–97.4)	92.2 (86.9–97.4)
Pvalue**	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Pvalue reported for Fischer exact test

BMZ = basement membrane zone, CI = 95% confidence interval, - = not identified, BP = bullous pemphigoid, PV = pemphigus vulgaris, HAM = human amnion membrane, HEP = human epidermis, HDR = human dermis, PPV = positive predictive value, NPV = negative predictive value

ELISA and indirect immunofluorescence performed on monkey esophagus as a substrate were the most sensitive and accurate techniques in detection of anti-Dsg1 and Dsg3 autoantibodies among the pemphigus patients (92.0% and 96.0% sensitivities and 98.0% and 95.1% accuracies, respectively).

DISCUSSION

The overall sensitivities and specificities of indirect immunofluorescence and immunoblotting in detecting anti-desmosomal or BMZ autoantibodies in patients with autoimmune bullous skin diseases are dependent on the type of the substrate used [7,8]. A previous study by Oyama et al. [3] proposed HAM to be a more reliable source for immunoblotting since it does not require epidermal-dermal separation and therefore yields a sufficient amount of desmosomal and hemidesmosomal proteins. In the present study immunophenotyping of HAM demonstrated all the antigens known to be involved in autoimmune bullous skin diseases, except for BP230. The absence of BP230 in the protein extract was also confirmed by the lack of a specific band corresponding to BP230 with the subsequent use of monoclonal and polyclonal anti-human BP230 IgG antibodies. In addition, BP230 was also not detected by the LC-MS/MS analysis. This contrasts with two previous studies in which anti-B230 bands were identified in HAM immunoblotting [3,4].

The LC-MS/MS study detected all other antigens that were located by immunophenotyping in HAM, although Dsg1, Dsg3 and Dsc3 were detected only at threshold levels. These low threshold levels of Dsg1 and Dsg3 in HAM were apparently enough to be detected by immunophenotyping but not enough to be detected by the pemphigus autoantibodies when using HAM as a substrate for immunoblotting and indirect immunofluorescence studies. These results contrast with the results of the HAM immunoblotting study by Oyama et al. [3], who demonstrated anti-Dsg1 and anti-Dsg3 antibodies in 100% of their pemphigus patients using HAM immunoblotting, and with the findings of Grootenboer-Mignot et al. [4] who detected a lower rate of 64% of these autoantibodies in their pemphigus patients. When performing immunoblotting using HEP extract, shown to be rich in Dsg1 and Dsg3 antigens by the LC-MS/MS analysis, we detected the presence of anti-Dsg3 and/or Dsg1 antibodies in 76.0% of the pemphigus patients. Our results suggest that low threshold levels of Dsg1 and Dsg3 in HAM make it an inferior source and substrate of antigens for immunoblotting and indirect immunofluorescence in pemphigus.

The LC-MS/MS analysis revealed that HAM is rich in other desmosomal plaque proteins such as plakoglobin, desmoplakin, envoplakin and periplakin, which make it a potential substrate for detecting autoantibodies in paraneoplastic pemphigus [9]. A recent study by Taille and co-authors [10] successfully used HAM as an antigenic source for immuno-

blotting to identify autoantibodies against periplakin in the sera of patients with idiopathic pulmonary fibrosis.

Immunoblotting using HAM as the antigenic source demonstrated comparative results to immunoblotting using HEP and HDR extracts in detecting anti-BP180 antibodies but not in detecting anti-BP230 antibodies. In the studies by Oyama et al. [3] and Grootenboer-Mignot et al. [4], anti-BP230 antibodies were detected by HAM immunoblotting in 70% and 50% of their bullous pemphigoid patients, respectively. In our study, BP230 was not detected in HAM by LC-MS/MS or immunophenotyping. Therefore, immunoblotting using HAM as an antigenic source for the diagnosis of pemphigoid is deficient according to our findings. It might be useful, however, in the diagnosis of more rare subtypes of pemphigoid where it may detect autoantibodies to other basement membrane components such as laminin 5 subunits, $\beta 4$ integrin subunit, and collagen VII [11].

All the studied desmosomal and basement membrane proteins were found in the HDR extract using LC-MS/MS. These overlaps are due most likely to an impure epidermal-dermal separation by the keratome that we used. The dermo-epidermal junction undulates in the normal skin while the keratome cuts straight. This may lead to epidermal remnants attached to the dermis and an impure protein extract [1].

Indirect immunofluorescence using HAM as a substrate demonstrated inferior accuracy in detecting anti-ICS autoantibodies in pemphigus patients compared to indirect immunofluorescence using monkey esophagus as a substrate, and to ELISA in detecting anti-Dsg1 and Dsg3 antibodies. It has been suggested that indirect immunofluorescence is a more sensitive method than immunoblotting most probably due to the presence of autoantibodies in pemphigus that are directed against conformational epitopes which are denatured by the process used for immunoblotting [2]. Our results suggest that the low concentrations of these antigens in HAM may also play a substantial role.

In the pemphigoid group, both the indirect immunofluorescent and immunoblot analyses using HAM were as accurate as monkey esophagus indirect immunofluorescence and normal human skin immunoblotting, respectively, but all of them were significantly inferior in their sensitivities to the ELISA method in detecting anti-BP180 autoantibodies. ELISA to anti-BP230-C autoantibodies is a sensitive tool for the detection of even low titers of BP230. However, its sensitivity-added value compared with BP180 ELISA alone was relatively low, as was previously found [12].

In summary, HAM did not offer advantages as a substrate over monkey esophagus indirect immunofluorescence and normal healthy skin immunoblotting. ELISA showed the best overall accuracy in diagnosing bullous pemphigoid, and both indirect immunofluorescence using monkey esophagus and ELISA showed the best accuracies in diagnosing pemphigus vulgaris compared to all the other studied methods.

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Capsule

Structure-based programming of lymph-node targeting in molecular vaccines

In cancer patients, visual identification of sentinel lymph nodes (LNs) is achieved by the injection of dyes that bind avidly to endogenous albumin, targeting these compounds to LNs, where they are efficiently filtered by resident phagocytes. Liu et al. translate this “albumin hitchhiking” approach to molecular vaccines, through the synthesis of amphiphiles (amph-vaccines) comprising an antigen or adjuvant cargo linked to a lipophilic albumin-binding tail by a solubility-promoting polar polymer chain. Administration of structurally

optimized CpG-DNA/peptide amph-vaccines in mice resulted in marked increases in LN accumulation and decreased systemic dissemination relative to their parent compounds, leading to 30-fold increases in T cell priming and enhanced anti-tumor efficacy while greatly reducing systemic toxicity. Amph-vaccines provide a simple, broadly applicable strategy to simultaneously increase the potency and safety of subunit vaccines.

Nature 2014; 507:5 19

Eitan Israeli

Capsule

Sessile alveolar macrophages communicate with alveolar epithelium to modulate immunity

The tissue-resident macrophages of barrier organs constitute the first line of defense against pathogens at the systemic interface with the ambient environment. In the lung, resident alveolar macrophages (AMs) provide a sentinel function against inhaled pathogens. Bacterial constituents ligate Toll-like receptors (TLRs) on AMs, causing AMs to secrete pro-inflammatory cytokines that activate alveolar epithelial receptors, leading to recruitment of neutrophils that engulf pathogens. Because the AM-induced response could itself cause tissue injury, it is unclear how AMs modulate the response to prevent injury. Using real-time alveolar imaging in situ, Westphalen et al. show that a subset of AMs attached to the alveolar wall form connexin 43 (Cx43)-containing gap junction channels with the epithelium.

During lipopolysaccharide-induced inflammation, the AMs remained sessile and attached to the alveoli, and they established intercommunication through synchronized Ca²⁺ waves, using the epithelium as the conducting pathway. The intercommunication was immunosuppressive, involving Ca²⁺-dependent activation of Akt, because AM-specific knockout of Cx43 enhanced alveolar neutrophil recruitment and secretion of pro-inflammatory cytokines in the bronchoalveolar lavage. A picture emerges of a novel immunomodulatory process in which a subset of alveolus-attached AMs intercommunicates immunosuppressive signals to reduce endotoxin-induced lung inflammation.

Nature 2014; 506: 503

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