Laboratory diagnostics of bullous autoimmune diseases

Ph.D. thesis

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Budapest
2019

I. Introduction

The diagnostics of bullous autoimmune diseases is usually not complicated; however, one often finds cases with borderline fluorescence in the microscope and with atypical clinical picture. It is not easy to diagnose such patients; one might have to repeat the diagnostics that leads to delayed diagnosis and treatment.

There are several groups within the autoimmune bullous diseases, but for my theses only the pemphigus and pemphigoid groups are relevant. Bullous pemphigoid (BP) belongs to the pemphigoid group, it is caused by antibodies against the hemidesmosomes and characterized by subepidermal blistering. The pemphigoid group also includes gestational pemphigoid, mucous membrane pemphigoid, linear IgA bullous disease and epidermolysis bullosa acquisita (EBA).

The most important pemphigoid antigens are BP180 (collagenXVII, BPAG2) and BP230 (dystonin-e, BPAG1). The gestational pemphigoid is a bullous disease which only appears during pregnancy or shortly after delivery. The most important autoantigen of gestational pemphigoid is BP180. Epidermolysis bullosa acquisita is a very rare disease, its autoantigen is collagen VII.

The two most important members of the pemphigus group are pemphigus vulgaris (PV) and pemphigus foliaceus (PF). In the cutaneous pemphigus forms, desmoglein-1 antibodies are present, while in the mucosal dominant forms desmoglein 3 antibodies play the most important role.

The main autoantigen of pemphigus foliaceus is desmoglein 1. Two dominant subtypes of the disease exist: the endemic fogo selvagem, and the in Europe most common form, the idiopathic pemphigus foliaceus.

We use different diagnostic tools to identify bullous autoimmune diseases.

One of the most important diagnostic tools is the direct immunofluorescence microscopy. In certain cases, patients can be diagnosed solely by direct immunofluorescence microscopy if the clinical picture is typical and blisters are present, even without serology. However; it will not give us any information about the exact antigen.

The technique is based on the fact that tissue-bound antibodies can be visualized by fluorescein marked antibodies. We expect to see a linear fluorescence intercellularly in the epidermis or along the basement membrane. This fluorescence consists mostly of IgG, but other Ig subclasses such as IgA can be present as well.

The fluorescence pattern of indirect immunofluorescence is similar to that of direct immunofluorescence. The indirect immunofluorescence is used to examine the patients' sera, not the skin. The sera can be incubated with monkey, rabbit, guinea pig or human esophagus (pemphigus and pemphigoid), rat and monkey bladder (paraneoplastic pemphigus), or amnion epithelia (pemphigus and pemphigoid).

The enzyme-linked immunosorbent assay (ELISA) is a specific and sensitive diagnostic tool for autoimmune bullous dermatoses. We can detect specific antibodies from the patients' sera. The antibodies are directed against the following antigens: BP180, BP230, collagen VII (col7), desmoglein 1 (dsg1) and desmoglein 3 (dsg3). There are different products on the market; thus, it differs from laboratory to laboratory which ones are in use.

II. Aims

The main goal of our work was to improve the diagnostics of bullous autoimmune diseases and increase the sensitivity.

Answers to the following questions were looked for:

1. Does the "Anti-SKIN profile test" have a better performance than other commercially used, previously available ELISAs? Which advantages and disadvantages does it have?
2. Can the complement fixation test be used to diagnose bullous pemphigoid? Does it improve the serological diagnostic performance? Is it able to diagnose cases with ambiguous results?
3. If the indirect immunofluorescence microscopy on monkey esophagus is negative, can the IgG subclasses be used to make a diagnosis? Can the sensitivity of the indirect immunofluorescence microscopy be increased by detecting IgG subclasses?
4. If the fluorescence of the dermo-epidermal junction is negative or questionable, can the fluorescence of sweat gland ducts be used to make a diagnosis?

III. Materials and methods

1. AntiSKIN profile test (ASPT)

We conducted a retrospective analysis of serum samples from 178 patients with the new ELISA kit, the AntiSKIN profile test (ASPT). 138 patients' sera were included who had been diagnosed with autoimmune blistering diseases. In every case the diagnosis was based on the compatible clinical picture, traditional histology and confirmatory results in at least two of three distinct diagnostic methods, including direct immunofluorescence (DIF) microscopy, indirect immunofluorescence (IIF) microscopy (on monkey esophagus, rabbit esophagus and salt-split skin) and specific ELISAs (BP180, BP230, dsg1, dsg3, or col7, as appropriate). Each of these ELISA systems was used according to the manufacturer's (MBL) instructions. The cut-off value was chosen as determined by MBL. Results in the grey zone were regarded as negative in this study. All sera having false positivity or negativity were double or triple checked. DIF and IIF microscopy were performed using standard laboratory methods.

ASPT was performed according to the instructions of MBL. The cut-off was 15 U/mL for every sub-assay in the ASPT. A total of 25 PF, 40 PV, 52 BP, 21 EBA, and 40 control serum samples were tested in the study.

From 313 available BP sera which had been pre-tested using individual ELISA kits (MBL) for detection of BP180 and BP230 autoantibodies, 52 serum samples were selected so that the specificities and sensitivities of the results approximately corresponded to those obtained in a previous study performed on these 313 sera, but we also deliberately included some samples with borderline results so that differences in the performance were better detected.

Control sera were chosen randomly from patients in whom bullous autoimmune diseases had been clearly excluded.

2. Analysis of the complement fixation test (CFT)

This study was a single-center, retrospective, serological case-control study with 300 patients with BP and 136 control patients. The diagnosis was based on clinical features together with at least 2 positive results of traditional histology, DIF, IIF, BP180 or BP230 ELISA. IIF was regarded as positive if IIF either monkey esophagus, or rabbit esophagus or salt-split human skin was positive. For traditional histology, DIF, IIF, BP180 and BP230 ELISA (MBL), standard clinical laboratory methods were used.

All BP and control sera (including a negative and a positive internal control for each test run) were diluted 1:2 with phosphate-buffered saline (PBS), pH 7.4, and incubated on unfixed, salt-split, frozen cut, healthy human skin samples for 30 min at 37°C. After washing for 3 × 10 min with PBS containing 0.005% Tween-20, a mixture of freshly taken sera from 3 donors without autoimmune disease was prepared as a complement source. This serum mixture was incubated on the sections at a dilution of 1:5 with barbital buffer for 30 min. After washing, polyclonal rabbit anti-human C_3 complement antibody labelled with fluorescein isothiocyanate was incubated at a dilution of 1:100 in PBS for 30 min at 37°C in a dark, humid chamber. After the last wash, sections were mounted in 2.5% 1,4-diazabicyclooctane, 0.1% sodium azide, and 10% PBS in glycerin for visualization. Positivity was defined as linear deposition of C_3 at the epidermal side of the basement membrane of the salt-split skin.

3. IIF examination of the monkey esophagus with IgG subclasses

We performed a retrospective analysis of serum samples from 64 patients with BP and 43 control patients. All tests were performed as described before. BP sera were negative for IIF on monkey and rabbit esophagus. Control patients had various blistering diseases, autoimmune diseases or inflammatory skin conditions that had to be distinguished from BP. In all controls, BP could be clearly excluded.

We conducted all indirect immunofluorescences using our standard method. Sera (diluted 1 : 20) were incubated on rabbit and monkey esophagus, and bound autoantibodies were visualized by subsequent incubation with fluorescein isothiocyanate (FITC)-labelled secondary antibodies directed against human IgG.

For the indirect immunofluorescence using IgG subclass antibodies, to prevent nonspecific binding, a neutralizing agent for human isoagglutinins was added for preincubation of all sera, used at a dilution of 1 : 1 for 30 min according to the manufacturer's instructions. All BP and control sera were then diluted 1 : 10 with PBS (final dilution 1 : 20) and incubated on unfixed, frozen-cut monkey and rabbit esophagus for 30 min at 37 °C, after which the tissue was washed with PBS containing 0.005% Tween-20 (PBS-T) three times for 10 min each wash. Subsequently, blocking was performed for 30 min at 37 °C using normal mouse serum at a dilution of 1 : 100 in PBST. After repeat washing, secondary antibodies (mouse monoclonal anti-human FITC-labelled, IgG_1, IgG_3, and IgG_4 antibodies were prepared at a dilution of 1 : 64 with PBS-T and incubated on the sections in a dark, humid chamber for 30 min. The sera were also tested with an IgG antibody cocktail (i.e. a mixture of IgG_1, IgG_3 and IgG_4 in a

dilution of 1 : 64). After the final wash, sections were mounted in 2.5% diazabicyclooctane, 0.1% sodium azide and 10% PBS-T in glycerin for visualization. Positivity was defined as linear deposition of an IgG subtype along at least one-third of the total area of esophageal basement membrane in both epithelial and mucosal papillae.

For ELISAs detecting BP180 and BP230, commercial assays were used following the manufacturer's (MBL) instructions.

4. Analysis of the sweat gland ducts using direct immunofluorescence

A single-center, case-control study was conducted on biopsy samples from 64 BP and 82 control patients.

The diagnosis was based on the integration of clinical, histological, immunopathological and serological findings. Inclusion criteria were a clearly visible, continuous, linear IgG fluorescence along the epidermal basement membrane, the presence of SGDs in the sections, and the evident diagnosis of BP. Control DIF sections were obtained from 82 patients with various other skin disorders diagnosed in 2016, in which BP could be clearly ruled out. Patients lacking a definite diagnosis or SGDs in the sections were excluded.

The *in vivo* bound IgG autoantibodies were detected by DIF in the patients' skin. All sections were stained with fluorescein isothiocyanate (FITC)-labelled goat anti-human IgG (diluted 1:30 in phosphate-buffered saline for 30 min at room temperature in a dark, humid chamber. No blocking was used. The intensity of the fluorescence was semi-quantitatively graded between 0 and 4 (0, no fluorescence; 4, strongest fluorescence).

5. Statistics

Sensitivities and specificities were calculated with 95% CIs.

Mann-Whitney's nonparametric, unpaired, two-tailed test was performed for statistical comparison of autoantibody titers of the ASPT, the complement fixation test and for examination of the sweat gland ducts. For comparison of (positive and negative) test results from the ASPT, Fisher's exact test was performed. For comparison of sensitivities or specificities of the different assays, McNemar's test was performed.

GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, California, USA, or calculators on the GraphPad homepage (http://www.graphpad.com/ quickcalcs/) were used for statistical calculations. SPSS version 21.0, SPSS Inc., Chicago, IL, USA was used for ROC analysis.

IV. Results

1. The "MESACUP anti-Skin profile TEST" is a fast and reliable diagnostic tool

The ASPT could be performed significantly more rapidly than previous ELISA systems, because the incubation times were only 50% of those required in previous specific MBL kits, and all five tests could be performed simultaneously in one single strip of assay. Thus, the time needed between pipetting the serum into the wells until the printing of the results was approximately 1.8 hours. The ASPT could be performed economically even with one single serum as each strip of assay contained eight ELISA wells; five for the autoantigens and three for positive and negative controls. Therefore, one strip of assay was used for each single serum sample, and any number of strips could be used at the same time.

Comparison of data obtained from the distinct, previously performed MBL ELISAs with those from the ASPT showed that results from the ASPT were concordant with those from previous ELISAs in 88.2%. The antibody titers of the ASPT and the results obtained from previous ELISAs did not differ significantly when comparing dsg1, dsg3, BP180, BP230, and col7 tests (P = 0.49, 0.62, 1.0, 0.32, and 0.34, respectively).

The specificities of ASPT were 100% for dsg1 in PF, dsg3 in PV, BP230 in BP, and col7 in EBA. The specificity of ASPT for BP180 in BP was 97.5%. Specificities were not calculated for our previous ELISAs because of no scientific relevance, since literature data shows a nearly 100% specificity value. In the ASPT, the sensitivities were 92.5% for dsg3 in PV, 92% for dsg1 in PF, 59.62% for BP180 and 61.54% for BP230 antibodies, and 80.95% for col7 in EBA. Using the previous MBL ELISAs, the sensitivities for dsg1, dsg3, BP180, BP230 and col7 ELISAs were 100%, 97.5%, 59.62%, 50.0% and 95.24%, respectively. The sensitivities of BP180 and BP230 tests evaluated simultaneously in the ASPT and in previous MBL ELISAs were 80.77% and 75%, respectively (specificities: 97.5% and 100%, respectively).

The performance of the ASPT could be further optimized by ROC curve analysis. The sensitivity for dsg1 antibodies in PF could be increased from 92% to 96% by decreasing the cut-off from 15 to 8.8 U/mL, while the specificity remained 100%. The area under the curve (AUC) was 0.962 (95%CI, 0.889-1.0). The dsg3 test performed even better than the dsg1. Keeping 100% specificity, the sensitivity could be increased up to 95% by decreasing the cut-off value from 15 to 10.2 U/mL; the AUC was 0.992 (95% CI, 0.978-1.0). The cut-off value of 15 U/mL was optimal for both the BP180 and the BP230 tests in the ASPT. The AUC values

for BP180 and BP230 antibodies were 0.870 (95% CI, 0.796-0.943) and 0.838 (95% CI, 0.756-0.919), respectively. The performance of col7 ASPT could also be improved by setting the cut-off to 11.6 U/mL, which resulted in an increase of sensitivity to 85.7% without affecting the specificity; the AUC was 0.994 (95% CI, 0.983-1.0). By decreasing the cut-off further to 9 U/mL, the sensitivity could be increased to 90.5% (specificity: 97.5%).

2. The complement fixation test is a good tool for the diagnosis of bullous pemphigoid

Sera from 300 patients with were tested with CFT and compared with 136 controls. CFT was positive in 215 patients with BP, thus the sensitivity of the CFT was 71.7%; negative results were obtained in 85 patients with BP (28.3%). The sensitivities of DIF, BP180, BP230, IIF monkey, IIF rabbit, IIF monkey and rabbit esophagus together, and IIF salt-split skin were 91.8%, 71%, 56.4%, 73.7%, 76.3%, 78% and 72.9%, respectively. The difference between the sensitivities of CFT and BP230 ELISA was significant (p<0.0001); however, there was no significant difference between the sensitivities of CFT and any other serological test.

The titers of BP180 and BP230 autoantibodies detected by ELISA were significantly different between patients with BP and controls ($p < 0.0001$).

All control samples were negative for the CFT; thus, specificity was 100%. Specificities for DIF, BP180, BP230, IIF monkey, IIF rabbit, IIF rabbit and monkey esophagus together, and IIF salt-split skin were 98.6%, 97.5%, 98.3%, 100%, 98.5%, 98.5% and 100%, respectively.

Although the sensitivity of each serological method was lower than 80%, the combined sensitivity of BP180, BP230 and CFT was 90.7% because the CFT detected 20 of 46 patients with BP (43.5%) who were serologically negative for both ELISAs. Upon combination of CFT with IIF monkey and rabbit together, sensitivity increased to 88.7%, since the CFT identified 31 of 66 patients with BP (47.0%) who were negative for IIF on esophagus. The combination of CFT with all serological assays resulted in a sensitivity of 95.3%, because 5 of 14 patients with BP (35.7%) were positive for CFT who were serologically negative for all other serological assays. In addition, CFT diagnosed 7 of 18 patients with BP (38.9%) in whom DIF was negative.

3. **Indirect immunofluorescence with IgG subclasses**

All 64 BP sera on monkey esophagus had negative findings using standard IIF microscopy methods to detect total serum IgG. All controls were negative for IIF on any substrate. Similarly, all controls were negative in both BP180 and BP230 ELISAs, and most

BP sera (57 of 64; 89%) were also negative in BP230 ELISA, but 34 of 64 (53%) of the BP sera were positive in BP180 ELISA.

The DIF microscopy gave positive results for 59 (92.2%) patients with BP and negative results for 1 (1.5%); the remaining 4 (6.3%) were not assessable. DIF microscopy was performed for only 9 controls, as it would be unethical to take biopsies unnecessarily.

Borderline results were relatively common in the IIF microscopy assay of IgG subclasses. Of the 64 BP serum samples, 9 (14.1%), 5 (7.8%), 1 (1.6%) and 11 (17.2%) samples were borderline for IgG_1, IgG_3, IgG_4 and the antibody cocktail (mixture of all these three antibodies), respectively. For calculation of sensitivities and specificities, each serum sample was designated either positive or negative.

Using IgG_1, IgG_3, IgG_4 and the triple antibody cocktail, 29 (45.3%), 12 (18.8%), 21 (32.8%) and 31 (48.4%) of the 64 BP sera were positive, respectively; thus, the sensitivities of our IIF-negative test sera were 45.3%, 18.8%, 32.8% and 48.4%, respectively. Only one false-positive result occurred in the control group, which was when the antibody cocktail was used; thus, the specificity was 100% for each of the IgG_1, IgG_3 and IgG_4 antibodies, and 97.7% for the antibody cocktail.

The results with the antibody cocktail did not correlate exactly with those of the distinct IgG subclass antibodies. Interestingly, 12 of 64 (18.8%) BP samples were negative with the antibody cocktail but positive with at least one of the three separate IgG subclass antibodies. This phenomenon might be explained by the higher background produced by the antibody cocktail. By contrast, 4 of 64 sera (6.3%) were reactive only with the antibody cocktail. For this reason, a simple addition of the results of the IgG_1, IgG_3 and IgG_4 antibodies was not identical to the results of the antibody cocktail. When all results of all four assays were merged (positivity defined as positivity in any assay, and negativity defined as negativity in all assays), 44 of 64 BP sera (68.8%) were positive, resulting in a total sensitivity and specificity of 68.8% and 97.7%, respectively.

The predominating circulating antibody was IgG_1, which was detectable in 29 of 44 (65.9%) subclass-positive sera, followed by IgG_4 (21 of 44; 47.7%); however, the difference between subclasses was not significant ($P = 0.14$). The IgG_3 IIF was less useful than the others as it detected only 27.3% (12 of 44) subclass-positive BP sera, and only 4 (9.1%) of these 44 sera were positive solely for IgG_3. By contrast, IgG_1 and IgG_4 antibodies recognized 9 and 7 (20.5% and 15.9%), respectively, of 44 sera, when no other subclass antibody was positive.

Therefore, excluding the antibody cocktail-positive sera, 20 of 40 subclass-positive sera (50%) were positive for > 1 subclass antibody.

4. The fluorescence of sweat gland ducts (SGDs) alone cannot be used for the diagnosis of BP

Sections from 64 patients with BP were examined and compared with 82 control patients. Linear IgG fluorescence along the BM of SGDs and other adnexa was assessed in both groups. Fifty-eight (90.6%) patients with BP and 44 (53.7%) controls showed linear deposition of IgG along the BM of the SGDs. Fluorescence intensity of SGDs in DIF microscopy was significantly higher in patients with BP than in controls ($p < 0.0001$). The sensitivity and specificity of positive SGDs for BP was 90.6% and 46.3%, respectively. With increasing intensity of the fluorescence, the sensitivity decreased, whereas the specificity increased. Positive SGDs with intensity grades 3 and 4 showed the lowest sensitivity (31.2%) and the highest specificity (97.5%). Fluorescence intensity of SGDs did not correlate significantly with the patients' circulating BP180 and BP230 autoantibody titers.

V. Conclusions

We could evaluate the ASPT in a real clinical setting. The ASPT has a similar clinical performance to each previous, specific ELISA alone, so it is suitable for routine diagnostics. The BP230 test of the ASPT has been considerably improved compared to the previous BP230 ELISA. The incubation time of the ASPT is just half of that of the previous specific tests and the setup of the assay makes the testing of one single serum sample economic. In addition, the ASPT is an excellent tool to examine problematic sera, overlap syndromes, and it can also indicate the appearance of epitope spreading during follow-up.

CFT is useful for the diagnosis of not only gestational pemphigoid but also BP, and we recommend its use as a secondary test for patients who are either negative or borderline-positive for other serological assays or DIF. Given its high specificity, a positive result has a very high positive predictive value; false positive results are not expected. Thus, it is especially useful for confirming borderline positive results and for the diagnosis of serologically challenging cases.

The sensitivity of indirect immunofluorescence on monkey esophagus can be significantly increased with the use of IgG subclasses. We showed that detection of mainly IgG_1 and IgG_4, but also IgG_3 anti-subclass antibodies by IIF on monkey esophagus significantly improved sensitivity and maintained the high specificity of the standard IIF, therefore their detection can be recommended for analysis of BP sera that are negative for traditional IIF microscopy. We recommend further studies to determine which secondary antibody cocktail (e.g. a mixture of anti-total-IgG with anti- IgG_1 and anti- IgG_4 subclass secondary antibodies) is most suitable for a cost-effective and efficient IIF assay for the diagnosis of BP.

Linear deposits of IgG along the BM of sweat gland ducts (SGDs) are highly sensitive for BP in each level of dermis; however, only strong fluorescence has acceptable specificity. SGD positivity in the upper dermis was found to be more BP-specific than in the lower dermis. Further controlled studies are necessary to recommend SGD fluorescence as the basis for diagnosis of BP (also in case of fragmented samples), even with strong signal intensity.

VI. Literature

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