Diagnostic value of immunohistochemistry on formalin-fixed paraffin-embedded skin biopsy specimens for bullous pemphigoid

Running title

Immunohistochemistry studies for the diagnosis of bullous pemphigoid

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What's already known about this topic?

- Direct immunofluorescence microscopy (DIF) studies represent the gold standard for the diagnosis of bullous pemphigoid (BP).
- Recent studies have reported that immunohistochemistry techniques using formalin-fixed, paraffin-embedded skin biopsy specimens are able to detect tissue-bound immunoreactants in BP with high sensitivity and thus constitute an alternative and practical diagnostic tool.

What does this study add?

- Immunohistochemical analyses are not sensitive enough to recommend their use for the diagnosis of BP, since less than 50% of 51 consecutive paraffin-embedded skin biopsy specimens obtained from patients with newly diagnosed BP were positive.
- If DIF cannot be performed, immunohistochemistry represents a useful diagnostic tool only in presence of histologically subepidermal blistering and/or with positive immunoserological tests , which are associated with a better sensitivity.

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Abstract

Background: Direct immunofluorescence microscopy (DIF) studies constitute the gold standard for the diagnosis of bullous pemphigoid (BP), but depend on the availability of specialized laboratories and often on an additional skin biopsy specimen.

Objective: To assess the value of immunohistochemical analyses (IHCA) in the diagnosis of BP using formalin-fixed, paraffin-embedded skin biopsy specimens as alternative to DIF. Furthermore, to study the correlation between the results of IHCA and the presence of histological subepidermal blister formation and of circulating autoantibodies by indirect immunofluorescence (IIF) studies using split-skin and or by ELISAs.

Methods: We included all newly diagnosed BP patients evaluated between 2008 and 2010. There were 51 consecutive skin biopsy specimens obtained from 38 BP patients with positive DIF.

Results: By IHCA diagnostic deposits of immunoreactants were found in 45.1% of all tested cases. Deposits of C3d, IgG, IgM, IgE and IgA were found in 37.3%, 23.5%, 2%, 0%, and 0% of cases, respectively. Deposits of C3d and/or IgG were found in 79.2% of the 24 cases with a blister and in 83% of the 12 cases with subepidermal blistering and positive immunoserological analyses, respectively.

Conclusion: In contrast to previous studies, our findings in an unselected patient cohort, indicate that IHCA are not sufficiently sensitive to replace DIF studies for confirming the diagnosis of BP. IHCA sensitivity significantly increases in the presence of histological blistering and/or of circulating autoantibodies. IHCA represents a potential rescue diagnostic technique only if specialized laboratories and/or a second biopsy specimen for DIF are unavailable.

Key words: bullous pemphigoid, direct immunofluorescence, immunohistochemistry, C3d, IgG

Introduction

Bullous pemphigoid (BP) is the most frequent autoimmune subepidermal blistering disease of the skin and mucosae. It is characterized by the presence of IgG autoantibodies directed against the BP antigen 230 (BP230, epithelial isoform of BPAG1) and the BP antigen 180 (BP180, BPAG2 or type XVII collagen), two components of hemidesmosomes in stratified epithelia.[1,2] Ample evidence exists indicating that IgG anti-BP180 autoantibodies are pathogenic by triggering an inflammatory cascade that leads to tissue damage and subepidermal blister formation.[1-3]

BP predominantly affects elderly patients and typically presents with widespread vesicles and bullae, together with urticarial plaques and eczematous lesions. Nevertheless, excoriations, prurigo-like, eczematous, and/or urticarial lesions may constitute the only signs of the disease.[4]

Diagnosis of BP is based on a combination of criteria, including compatible clinical features, histological, and direct immunofluorescence (DIF) microscopy findings.[6] Since clinical and histopathological features may be nonspecific, a positive DIF of a perilesional skin biopsy specimen is required for diagnosis.[6] The latter shows linear deposits of IgG, C3, and, more rarely, IgA and/or IgE immune deposits along the epidermal basement membrane zone (BMZ).[1,2,7,8] Proper classification of BP often requires the detection of circulating autoantibodies by either indirect immunofluorescence (IIF) microscopy using NaCl-separated normal human skin or by ELISA-BP180 and/or ELISA-BP230.[6]

Although DIF studies represent the golden standard for the diagnosis of BP, they often pose a number of disadvantages, such as the necessity of obtaining a second biopsy, rapid transport of the sample, specialized technical processing, and, finally, a relatively high cost.[9] For these reasons, a few studies have recently assessed the value of immunohistochemistry analyses (IHCA) on paraffin-embedded tissue as alternative for the diagnosis of autoimmune bullous skin diseases.[10-14] In other immune-mediated diseases, such as renal diseases, IHCA already constitute a well-established diagnostic procedure.[15-17]

In this retrospective study, we have systematically assessed the value of IHCA using paraffin-embedded skin biopsy specimens obtained from all newly diagnosed cases of BP evaluated over three years.

Material and Methods

Patients and diagnostic criteria. In this monocentric retrospective study we systematically included all biopsy specimens obtained from patients with a new diagnosis of BP, evaluated in our tertiary referral centre between January 2008 and December 2010. Diagnosis of BP was based on the following widely accepted criteria: (i) compatible clinical features (excoriated, eczematous, urticarial and/or bullous lesions) associated with itch; (ii) deposits of IgG and/or C3c along the epidermal side of the basement membrane by DIF microscopy studies; (iii) presence of circulating autoantibodies binding to the epidermal side or both the epidermal and dermal side of NaCl-separated normal human skin by IIF microscopy; alternatively, presence of anti-BP180 and/or anti-BP230 IgG autoantibodies by ELISA (MBL, Nagoya, Japan); and (iv) in case that criterion (iii) was either negative or if patient's serum was not available, diagnosis was based on the presence of 3 of 4 validated clinical criteria for BP.[5]

Skin biopsy specimens obtained from patients diagnosed with Grover's disease, Hailey-Hailey disease, Darier's disease, cutaneous lupus erythematosus, lichen planus, toxic epidermal necrolysis, drug reactions, erythema multiforme and Sweet syndrome were used as controls (total n=28).

Immunohistochemical staining. Immunohistochemical stainings were carried out on formalin-fixed paraffin-embedded skin tissue samples processed using the Leica Bond Max autostainer (Leica, Germany). For this purpose, 4µm-thick sections on star frost slides (Knittel Glass, Germany) were used. Pretreatment: C3d and IgE: Bond Pretreatment Kit (AR9551) followed by Bond Epitope Retrieval Solution 2 (AR9640). IgG and IgM, first unmasked in EDTA solution. IgA: citrate solution. The used primary antibodies included: rabbit polyclonal anti-human C3d antibody (Abcam, No. ab15981), dilution 1:600; rabbit polyclonal anti-IgG antibody (Cell Marque, CMC26911021), dilution 1:1500; mouse monoclonal anti-human IgA antibody (Novocastra, NCL- L- IgA), dilution 1:600; mouse monoclonal anti-human IgM antibody (Novocastra NCL-L-IgM), dilution 1:800; rabbit polyclonal anti-human IgE antibody (Diagnostic BioSystems, No RP-022-05), dilution 1:100.

In analogy to the approach used for the evaluation of DIF, presence of continuous linear deposits of immunoreactants along the epidermal BMZ, was required to consider the results of IHCA as positive, whereas the presence of discontinuous granular immune deposits along the epidermal BMZ was considered not diagnostic. IHCA were independently evaluated by three investigators (SG, HB and IH). Tissue specimens obtained from tonsils or B-cell lymphoma containing germinal centres and plasma cells were also tested by IHCA and used as positive controls (positivity of C3d in germinal centres and of immunoglobulin in plasma cells).

For direct IF microscopy studies, the method and reagents used are described elsewhere[8].

Statistical analysis. Data are presented as either means with standard deviations (SD) or as numbers with percentages for continuous and categorical variables, respectively. The impact on IHCA results of demographical variables, histological blistering, IIF and ELISAs was assessed by using univariate generalized linear models with repeated measure design with appropriate and logit link function. The association was expressed in terms of odds ratios (OR) along with their 95% confidence interval (CI) and p-values. In case of zero frequency, a continuity correction was used to compute OR and the Pearson's X^2 test was used to compute p-value. All tests were considered significant at $p < 0.05$. Analyses were performed with SPSS ver. 20 (IBM Corp).

Results

A total of 51 skin biopsy specimens obtained from 38 patients with newly diagnosed BP over a three-year period were included. The mean age of the patients was 76.5 years (range 48-94 years) and there were 18 males and 20 females. In 29, 7, and 2 patients, there were 1, 2 and 4 formalin-fixed skin biopsy specimens available for testing, respectively.

All patients had positive DIF microscopy studies with detection along the epidermal BMZ of linear deposits of C3c, IgG, IgM, IgE, and/or IgA in 88.2%, 52.9%, 21.6%, 17.6% or 11.8% of cases, respectively (Table 1).

By IHCA linear deposits of C3d, IgA, IgE, IgG, and or IgM were found along the epidermal BMZ in 23 of 51 (45.1%) specimens (Table 1).

Specifically, positive IHCA with C3d were observed in 19 of 51 (37.3%) specimens (Figure 1). Furthermore, in the 6 biopsy specimens obtained from patients, in which DIF studies for C3c were negative, IHCA was also systematically negative.

Diagnostic linear deposits of IgG were observed in 12 of the 51 (23.5%) skin specimens by IHCA. Noteworthy, when only the cases, which were positive for IgG deposits by DIF were analysed by IHCA, only 7 of 27 (25.9%) were also positive. Furthermore, in the 24 biopsy specimens obtained from patients, in which DIF studies for IgG were negative, IHCA was positive in 5 cases.

With regard to other immunoreactants (IgM, IgA, IgE), the results were almost invariably negative by IHCA. Linear IgM deposits were found only in one out of 51 (2%) tested skin specimens, whereas search for IgA and IgE deposits was always negative. In the one case in which linear deposits of IgM were found by IHCA , DIF was also positive.

As controls, we tested 18 formalin-fixed, paraffin-embedded skin biopsy specimens obtained from patients presenting with different types of inflammatory cutaneous reactions. In all these cases, IHCA did not disclose any immune deposit. Furthermore, IHCA of tissue specimens obtained from tonsils or B-cell lymphoma with germinal centres and plasma cells showed positivity of C3d in germinal centres and of immunoglobulin in plasma cells.

Next, we assessed whether the IHCA results were affected by the presence of distinct parameters, such as age and gender of affected patients, presence of histological blistering, positive IIF microscopy findings and/or positive ELISA-BP180 and/or ELISA-BP230.

First, age older than 75 years or gender did not affect the IHCA results (Table 2). Next, when the 24 skin biopsy specimens in which histologically subepidermal blister formation was observed were tested, there was a statistically significant increase (p-value: <0.001) in the detection rate of immune deposits by IHCA. In fact, among the latter, 19 of 24 cases (79.2%) showed positive results with either C3d and/or IgG when compared to only 4 (14.8%) of the 27 tissue specimens without histological blistering. Specifically, C3d deposits were found in 15 (62.5%) of 24 cases with histological blistering, while IgG in 12 (50%) of them (Table 2).

Nineteen of the 27 (70.4%) tested BP sera were positive by IIF microscopy studies using salt-split normal human skin. When the corresponding biopsy specimens were tested by IHCA, the positive rate of IHCA also significantly increased (p-value: 0.03). In fact, 14 (73.7%) of these 19 cases were positive. Specifically, 12 of these 14 cases were positive for C3d, 7 cases positive for IgG and 5 cases positive for both IgG and C3d. In contrast, only 2 (25%) of the 8 biopsy specimens obtained from BP patients with negative IIF were positive by IHCA. Search for circulating autoantibodies by ELISA-BP180 and/or ELISA-BP230 was performed in 32 cases, 21 (65.6%) of which were positive (Table 2). Fourteen (66,6%) of these 21 cases showed positive findings by IHCA with either C3d (12 of 14 cases), IgG (8 cases), or both IgG and C3d deposits (6 cases). When the biopsy specimens obtained from the 16 BP cases in which both IIF and ELISAs were positive, 12 cases (75%) were positive by IHCA with either C3d (10 cases), IgG (7 cases) or both IgG and C3d deposits (5 cases). The rate of positive IHCA was thus significantly increased (p-value: 0.03) when compared to the 12 probes obtained from BP patients with both negative IIF and ELISAs, which were positive in only 4 cases

(33%). Finally, the rate of IHCA positivity was the highest when skin specimens obtained from patients with histological blistering and with both positive IIF and ELISAs were tested. In fact in the latter cases IHCA was positive in 83.3% of the 12 tissue samples, whereas only 7 of 31 (22.6%) cases lacking these characteristics were positive. The difference was again statistically significant (p-value: 0.002) (Table 2). Noteworthy, presence of histological blistering, positive IIF microscopy findings and/or positive ELISA-BP180 and/or ELISA-BP230 significantly affected the positivity of IHCA even when reactivity with either IgG or C3d was analysed separately (data not shown).

Discussion

We have assessed the value of IHCA in the diagnosis of BP using formalin-fixed paraffin-embedded skin biopsy specimens obtained from all BP cases diagnosed consecutively over three years in our centre. The ability of IHCA to detect the presence of linear deposits of immunoreactants along the epidermal BMZ was retrospectively compared to that of DIF microscopy studies, the positivity of which is essential and required for the diagnosis of BP.[6]

Our study, which represents the largest series available so far, indicates that the sensitivity of IHCA is poor when compared to that of DIF. In fact, by IHCA only 45.1% of 51 skin biopsy specimens showed positive diagnostic findings, whereas all included cases (100%) were positive by DIF as obligatory inclusion criterion (Table 1). Specifically, IHCA demonstrated the presence of C3d, IgG and IgM in only 37.3%, 23.5% and 2% of cases, respectively, whereas deposits of either IgA or IgE were not observed in any of 51 tested biopsy specimens. Hence, the number of positive samples by IHCA was invariably lower compared to that obtained by DIF for all tested immunoreactants (Table 1). Furthermore, IHCA were never able to detect the presence of distinct specific immunoreactants such as IgG or C3d in samples obtained from subjects, in which the corresponding DIF microscopy studies were negative for those reactants.

We further assessed factors potentially affecting the results of IHCA (Table 2). While age of BP patients and gender did not have an impact on IHCA positivity, the latter significantly increased using skin biopsies showing histologically subepidermal blister formation. In fact, almost 80% of the 24 tissue samples with subepidermal blistering were positive for either C3d and/or IgG by IHCA. Furthermore, in skin biopsy specimens obtained from patients with circulating IgG autoantibodies as detected by either ELISA (ELISA-BP180 and/or ELISA-BP230) and/or by IIF, the sensitivity of IHCA also increased, since diagnostic deposits of immunoreactants were found in up to 75% of tissue specimens (Table 2). These rates were thus significantly higher compared to those obtained using tissue samples from seronegative patients (Table 2). Finally, in the cases, in which there were both histological subepidermal blistering and positive immunoserological findings by either ELISAs and/or IIF, the percentage of positive IHCA results were the highest, since 83.3% of 12 cases showed positive IHCA with at least C3d and/or IgG deposits (Table 2).

Another recent IHC study similarly reports the detection of diagnostic linear deposits of C4d in 86% of 29 skin biopsies obtained from BP patients. Again the majority of the included cases (>70%) showed histologically subepidermal blister formation. [18]

The results obtained in our series with an unselected group of newly diagnosed BP patients over a three-year period are hence at variance with those from 4 previous studies.[10-13] The latter reported

sensitivities of 70% to 100% of IHCA when compared to DIF as well as a high concordance of the results between IHCA and DIF. For example, Magro et al. found linear deposits of C3d and of C4d staining in 100% and in 23.5% of the formalin-fixed, paraffin-embedded tissue samples obtained from BP patients, respectively.[10] Pfalz et al. found diagnostic linear deposits of C3d in 97% of the 32 studied cases of BP.[11] Chandler et al. analysed 10 biopsy specimens obtained from either BP or epidermolysis bullosa acquisita patients.[12] In 7 (70%) of these 10 cases, they detected C4d deposits along the epidermal BMZ. Finally, in a retrospective series, Kwon et al. also found the presence of diagnostic deposits of C4d in 10 (83%) of 12 patients with BP as well as in 8 (100%) out of 8 patients with gestational pemphigoid.[13] At least two factors may account for the low positive rate of IHCA in our study when compared to previous reports. First, the latter have most likely encompassed selected BP patients with cutaneous blistering and high disease activity. In the study of Pfalz et al.[11], at least 75% of the included patients showed clinically and/or histological blistering. In the study of Chandler et al.[12] three of the 7 positive patients also had subepidermal blister formation. In this study, diagnosis of BP was not confirmed by immunoserological criteria in 5 cases. In the remaining two studies details about clinicopathological features were not provided.[10,13] Two recent studies have shown that at least 20% of patients with BP do not present clinically with obvious skin blistering at time of diagnosis. [4,19] These patients are reasonably expected to exhibit a reduced amount of tissue-bound autoantibodies, since levels of circulating IgG autoantibodies to BP180 usually correlate with disease severity.[1,2,6] Hence, the inclusion of BP patients with clinically blistering and high disease activity is likely to represent a selection bias accounting for the previously described high sensitivity of IHCA in BP. In contrast, we assessed tissue samples obtained from an unselected cohort of patients with newly diagnosed BP. The inclusion of selected samples in previous immunopathological studies of BP, such as for testing the diagnostic value of the ELISA-BP180, has invariably resulted in exaggerated high sensitivity, which could not be later confirmed when consecutive serum samples were tested.[1]

Another factor potentially explaining the lower positivity rate of our IHCA is the use of a different protocol with other second-step antibodies and reagents. It is well possible that our protocol for IHCA can be improved to increase its sensitivity. Nevertheless, it is unlikely that a technical problem accounts for the low detection rate found in our study. In fact, the positivity of IHCA in patients with histological blistering and/or circulating autoantibodies was similarly high as that described in previous reports. The range of sensitivity is comparable to the lower end of the previously published results.

In our study DIF was able to detect the presence of linear deposits of IgA along the epidermal BMZ in up to 12% of the tested biopsy specimens. In contrast, all cases studied by IHCA were negative. Since the presence of tissue-bound IgA deposits is a critical diagnostic criterion for both linear IgA bullous dermatosis and dermatitis herpetiformis, the diagnostic value of IHCA in these two diseases with features potentially mimicking BP is particularly questionable and limited. The inability to detect linear deposits of IgE by IHCA was also somehow unexpected. Evidence exists indicating that the detection of tissue-bound IgE is of diagnostic utility in BP and that anti-BP180 IgE autoantibodies contribute to tissue damage.[8,20,21]

In conclusion, our results in an unselected cohort of BP patients indicate that IHCA is not sufficiently sensitive to replace DIF microscopy studies for the immunopathological diagnosis of BP. The sensitivity of IHCA for detection of either IgG and/or C3d deposits is less than 50% when compared to

DIF microscopy studies. Hence, IHCA cannot be proposed as standard procedure for confirming the diagnosis of BP patients in the daily routine. Nevertheless, if either specialized immunopathological laboratories or appropriate biopsy specimens for DIF are not available, IHCA may be a useful rescue method, particularly in cases showing histologically subepidermal blistering and/or with positive immunoserological tests.

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Tables

Table 1. Survey of the results observed by both direct immunofluorescence microscopy (DIF) studies and immunohistochemical analysis (IHCA) for the various immunoreactants.

Methods	Positive results / all cases (%)	C3	IgG	IgA	IgM	IgE
DIF	51/51 * (100%)	45/51 (88.2%)	27/51 (52.9%)	6/51 (11.8%)	11/51 (21.6%)	9/51 (17.6%)
IHCA	23/51 (45.1%)	19/51 (37.3%)	12/51 (23.5%)	0/51 (0.0%)	1/51 (2%)	0/51 (0%)

* As for inclusion, all studied tissue samples were obtained from patients showing positive findings with at least one immunoreactant by DIF studies

Table 2. Summary of the results obtained by IHCA according to a number of different parameters with statistical assessment of their impact on the detection rate of either C3d and or IgG deposits along the epidermal basement membrane zone.

		N	Any IHC +		OR (95% CI)	p	C3d+		OR (95% CI)	p	IgG+		OR (95% CI)	p
			n	%			n	%			n	%		
Age (yrs)	< 75	25	10	40.0%	1		9	36.0%	1		3	12.0%	1	
	75+	26	13	50.0%	1.50 (0.50 - 4.55)	0.47	10	38.5%	1.11 (0.36 - 3.46)	0.86	9	34.6%	3.88 (0.91 - 16.58)	0.07
Gender	M	25	14	56.0%	2.40 (0.78 - 7.44)	0.13	11	44.0%	1.77 (0.56 - 5.57)	0.33	9	36.0%	4.31 (1.01 - 18.46)	0.05
	W	26	9	34.6%	1		8	30.8%	1		3	11.5%	1	
Histology	-	27	4	14.8%	1		4	14.8%	1		0	0.0%	1	
	+	24	19	79.2%	21.85 (5.13 - 93.00)	<0.001	15	62.5%	9.58 (2.45 - 36.8)	0.001	12	50.0%	55.00 (3.01 - 1004.28)*	<0.001
IIF	-	8	2	25.0%	1		1	12.5%	1		1	12.5%	1	
	+	19	14	73.7%	8.40 (1.26 - 56.07)	0.03	12	63.2%	12.00 (1.21 - 118.89)	0.03	7	36.8%	4.08 (0.41 - 40.45)	0.23
ELISA	-	11	3	27.3%	1		2	18.2%	1		1	9.1%	1	
	+	21	14	66.7%	5.33 (1.07 - 26.61)	0.04	12	57.1%	6.00 (1.03 - 34.84)	0.04	8	38.1%	6.15 (0.66 - 57.59)	0.11
IIF + ELISA	-	12	4	33.3%	1		3	25.0%	1		1	8.3%	1	
	+	16	12	75.0%	6.00 (1.15 - 31.23)	0.03	10	62.5%	5.00 (0.96 - 26.11)	0.06	7	43.8%	8.56 (0.88 - 83.06)	0.06
Histology + IIF + ELISA	-	31	7	22.6%	1		6	19.4%	1		1	3.2%	1	
	+	12	10	83.3%	17.14 (2.91 - 100.82)	0.002	8	66.7%	8.33 (1.79 - 38.71)	0.007	7	58.3%	42.00 (4.20 - 419.4)	0.001

N, total number of samples per category; IHC, immunohistochemical analyses; n, number of positive samples; OR, odds ratio; CI, confidence interval; p, p-value; Histology, presence of histological blistering; -, negative results; +, positive results; ELISA, either ELISA-BP180 and or ELISA-BP230; IIF, indirect immunofluorescence microscopy studies using NaCl-separated normal human skin; OR and p-values were computed,

unless otherwise specified, by using univariate generalized linear models with repeated measure design where appropriate and logit link function; * Continuity corrected OR. Pearson's X^2 test was used to assess significance.