

C3d immunohistochemistry on formalin-fixed tissue is a valuable tool in the diagnosis of bullous pemphigoid of the skin

Background: Direct immunofluorescence (DIF) testing is an important procedure in the diagnosis of autoimmune bullous dermatoses. We investigated the expression of C3d in formalin-fixed, paraffin-embedded tissue of autoimmune bullous dermatoses.

Methods: The immunohistochemical expression of C3d in bullous pemphigoid (BP) (n = 32), pemphigoid gestationis (PG) (n = 3), pemphigus (n = 14), dermatitis herpetiformis duhring (DHD) (n = 10), linear immunoglobulin A (IgA) dermatosis (n = 4), mixed forms of BP and linear IgA dermatosis (n = 2), and 44 controls was analyzed on formalin-fixed tissue.

Results: Thirty-one of 32 cases (97%) of BP and 3 out of 3 cases (100%) of PG showed a linear positivity of C3d along the basement membrane. Only 3 out of 14 (21%) cases of pemphigus showed an intraepidermal intercellular expression of C3d. The two mixed forms of linear IgA dermatosis and BP showed a linear positivity of C3d along the basement membrane. All cases of DHD, linear IgA dermatosis and all of the controls were negative for C3d.

Conclusions: C3d immunohistochemistry is a valuable tool in the diagnosis of BP and PG of the skin with a sensitivity of at least 97%. Mixed forms of linear IgA dermatosis, and BP, DHD and linear IgA dermatosis can only be identified by DIF. A positive result may prompt serologic confirmation of BP without further need for DIF.

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**Katrin Pfaltz¹, Kirsten Mertz¹,
Christian Rose², Paul
Scheidegger³, Madeleine
Pfaltz¹ and Werner Kempf¹**

¹Kempf & Pfaltz Histological Diagnostics,
Zürich, Switzerland,

²Department of Dermatology, University of
Lübeck, Lübeck, Germany, and

³Dermatologische Praxis, Brugg, Switzerland

Werner Kempf, Kempf & Pfaltz Histological
Diagnostics, Seminarstrasse 1, P.O., CH-8042
Zürich, Switzerland
Tel: +41 44 233 3377
Fax: +41 44 233 3378
e-mail: kempf@kempf-pfaltz.ch

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Autoimmune bullous skin diseases are characterized by the presence of autoantibodies in specific adhesion antigens of the epidermis or the dermoepidermal junction zone. Binding of these antibodies to their target antigens causes loss of adhesion between epidermal keratinocytes or at the basement membrane zone, which results in blister formation. The diagnosis of autoimmune bullous skin diseases

is based on clinical findings, histopathology, indirect and direct immunofluorescence, and immunoblot investigations. Tissue-bound antibodies can be detected by direct immunofluorescence (DIF) microscopy in perilesional skin. Characteristic fluorescence patterns allow a further classification of autoimmune bullous skin disorders, but DIF requires fresh frozen tissue. So far, there are only

sparse data on the use of immunohistochemistry on paraffin-embedded tissue to show complement and immunoglobulin deposition in the skin.

Complement protein C3 plays an important role in the activation of the complement system in the classical and alternate pathway. C3-convertase activates C3 by cleavage of C3 into C3a and C3b. C3b reacts with other components of the complement cascade leading to formation of the membrane attack complex, the end product of the complement cascade. Membrane attack complex causes lysis of the target cell. C3d is formed as a result of deactivation of C3b. After repair of cell injury, C3 and other components of the complement system disappear and are no longer bound to the tissue, while C3d remains attached to the target cell and may therefore be used as a marker of complement activation.^{1–3}

Immunohistochemical demonstration of complement components is a useful diagnostic procedure in the assessment of renal biopsies,⁴ where demonstration of C3d and C4d has shown to be a marker of humoral rejection.^{5,6} Only a few studies have been published so far^{1,7} concerning immunohistochemical deposits of C3d and C4d in inflammatory skin diseases.

Materials and methods

Case selection

Thirty-two cases of bullous pemphigoid (BP), 14 cases of pemphigus, 10 cases of dermatitis herpetiformis (DHD), 4 cases of linear immunoglobulin A (IgA) dermatosis, 2 cases of a mixed form of BP and linear IgA dermatosis, and 40 controls were selected from our institute, diagnosed between 2006 and 2009. The diagnosis of the autoimmune bullous diseases was based on histopathologic findings and a positive DIF. In case of BP, immunohistochemical staining showed collagen type IV at the ground of the blister excluding epidermolysis bullosa acquisita (EBA). The pemphigus group included 11 cases of pemphigus vulgaris, 2 cases of pemphigus foliaceus and 1 case of IgA pemphigus. Two cases represented mixed forms of BP and linear IgA dermatosis, demonstrating a linear positivity of C3, immunoglobulin G (IgG) and IgA by DIF. Controls of bullous diseases with subepidermal and intraepidermal blistering with a negative result by DIF were analyzed, including 12 cases of bullous drug reaction, 2 cases of bullous diabetorum, 6 cases of bullous arthropod bite reaction, 4 cases of mechanical bullae, 1 case of bullous Sweet syndrome, 9 cases of Grover's and Darier's disease, 5 cases of impetigo contagiosa and 1 case of Hailey–Hailey's disease. In addition, we analyzed three cases of pemphigoid gestationis (PG) and four cases of pruritic urticarial papules

and plaques of pregnancy (PUPPP) from the Department of Dermatology of the University of Lübeck.

Direct immunofluorescence

Cryostat-cut sections of frozen tissue mounted on superfrost slides were immersed in acetone for 10 min, air-dried and washed in Tris buffer for 10 min. Antibodies against fibrinogen, C3, IgA, IgG and immunoglobulin M (IgM) (Dako, Glostrup, Denmark: F0111, F0201, F0204, F0202, F0203) were diluted with Tris buffer (fibrinogen 1:40, C3 1:30, IgA 1:30, IgG 1:50, IgM 1:30). After incubation for 30 min, the slides were rinsed in Tris buffer for 10 min and cover slipped.

Immunohistochemical studies

Immunohistochemistry was performed on sections from formalin-fixed paraffin-embedded tissue. The slides were pretreated with enzyme 1 (Vision BioSystem bond pretreatment kit) for 5 min. The primary antibody, rabbit antihuman polyclonal antibody against C3d (Abcam, Cambridge, UK: 15981), was diluted 1:600 and incubated for 20 min. These staining conditions turned out to give optimal staining in several test series using different antibody concentrations and pretreatment protocols. The Vision BioSystems Bond polymer alkaline phosphatase Red Detection kit was used. All cases of pemphigus and all negative controls with intraepidermal blistering were additionally stained with a dilution of 1:450.

Determination of C3d positivity

Immunohistochemistry was blindly reviewed by two pathologists. The deposition of C3d along the basement membrane was scored: 0 = no reactivity, 1+ = weak reactivity, 2+ = moderate reactivity and 3+ = strong reactivity. Cases with a score of 1+, 2+ and 3+ were defined as positive cases. Cases with focal deposits were interpreted as positive. Deposition of C3d only within the blister cavity or at the roof or base of the blister without concomitant deposition at the basement membrane of perilesional skin was interpreted as a negative result.

Results

Bullous pemphigoid

Thirty-one of 32 cases (97%) of BP including 6 cases of (clinically and histologically) prebullous pemphigoid showed linear deposits of C3d along the basement membrane (Table 1 and Fig. 1). Histologically, a subepidermal blister was present in

Table 1. C3d immunohistochemistry of autoimmune bullous dermatoses on formalin-fixed tissue

Diagnosis	C3d positivity
Bullous pemphigoid (32)	31/32 (97%) Linear dermoepidermal junction
Pemphigoid gestationis (3)	3/3 (100%) Linear dermoepidermal junction
Pemphigus	3/11 (27%) Intercellular
• Pemphigus vulgaris (11)	0/2 (0%)
• Pemphigus foliaceus (2)	0/1 (0%)
• IgA pemphigus (1)	0/4 (0%)
Linear IgA dermatosis (4)	0/10 (0%)
Dermatitis herpetiformis (10)	2/2 (100%) Linear dermoepidermal junction
Mixed form bullous pemphigoid/IgA dermatosis (2)	0/44 (0%)
Negative controls (44)	

IgA, immunoglobulin A.

20 cases. Six cases of BP did not histologically exhibit blister formation, but clinical data reported the presence of bullae. Thus, these cases were classified as BP. Twelve cases showed a linear C3d staining along the base of the blister, five along the base and roof of the blister and three were only positive in the perilesional skin without any positivity along the base or roof of the blister. Seven out of the 31 positive cases showed a weak (1+), 14 cases a moderate (2+) and 10 cases a strong (3+) reactivity. Twelve cases showed focal deposits. One case was negative for C3d.

Pemphigoid gestationis

All three cases (100%) showed a linear positivity of C3d along the basement membrane. In two of the cases, a subepidermal blister was present. One case

showed a linear positivity along the base of the blister and the other case was only positive in the perilesional skin without any staining along the base or roof of the blister. Two cases showed a moderate (2+) focal reactivity and one case a strong (3+) continuous positivity.

Pemphigus

Three of the 14 cases (21%) showed an intraepidermal intercellular expression of C3d. One case of pemphigus vulgaris revealed a strong (3+) and two cases of pemphigus vulgaris a focal weak (1+) reactivity for C3d (Fig. 2).

DHD and linear IgA dermatosis

All cases were negative for C3d (0/14).

Mixed form of linear IgA dermatosis and BP

The two cases showed linear C3d along the basement membrane. One was scored as weak (1+) and one as moderate (2+) positive. C3d deposition was found along the base of the blister in one case and at the base as well as the roof of the blister in the other case.

Vesiculobullous controls and PUPPP

Deposition of C3d along the dermoepidermal junction or intercellular was not found in any of the cases (0/44).

Discussion

DIF testing is an important procedure in the diagnosis of autoimmune bullous dermatoses. Disadvantages of this procedure include the requirement of fresh frozen tissue submitted in 0.9% NaCl or Michel's solution, the need for a laboratory equipped to perform the procedure, the storage of frozen tissue which may result in loss of antigen reactivity

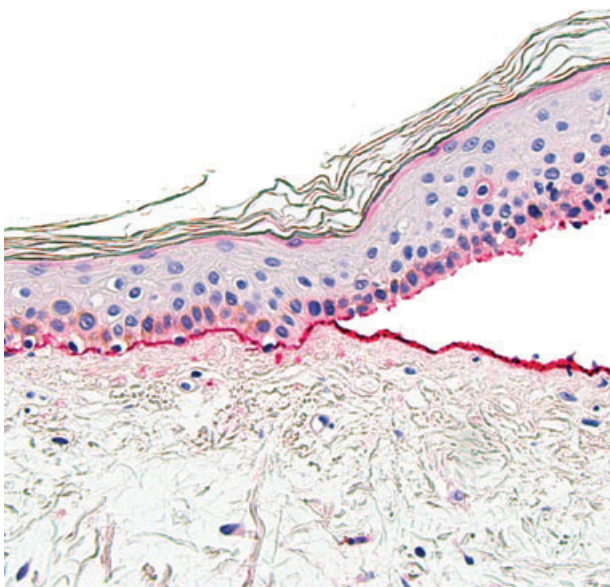


Fig. 1. Linear C3d staining along the dermoepidermal junction on formalin-fixed paraffin-embedded tissue from a case of bullous pemphigoid.

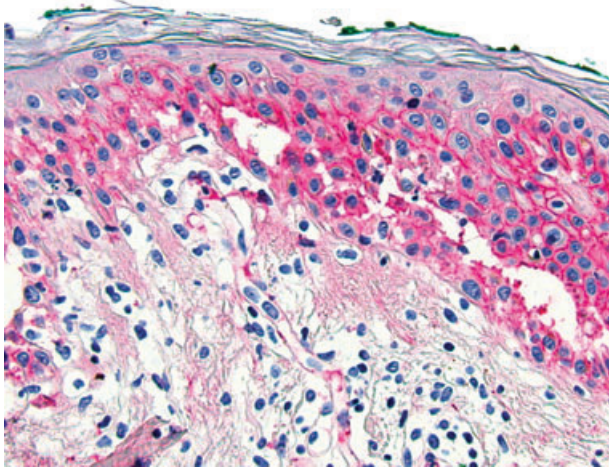


Fig. 2. Intercellular C3d staining on formalin-fixed paraffin-embedded tissue from a case of pemphigus vulgaris.

and the comparatively high costs. Sometimes only formalin-fixed tissue is available for examination of bullous skin diseases, particularly in cases in which an autoimmune bullous disease is not clinically suspected. The application of DIF on formalin-fixed paraffin-embedded tissue is associated with a lower sensitivity compared to frozen tissue.^{8,9} On the other hand, the use of immunohistochemistry on paraffin-embedded tissue to show complement and immunoglobulin deposition in the skin is not well established. Only a few studies report the use of immunohistochemistry on formalin-fixed tissue in the assessment of inflammatory skin diseases^{10–12} demonstrating a comparatively high number of false-negative results. Immunohistochemical detection of C3d and C4d deposition in inflammatory skin diseases has recently been described as a valuable diagnostic tool by Magro et al.¹ We analyzed the expression of C3d in formalin-fixed tissue in autoimmune bullous disorders as a possible substitute for DIF. We did not perform a staining for C4d as a lower sensitivity of C4d compared to C3d has been reported.^{1,2}

BP represents the most frequent autoimmune bullous skin disease in adults. It is characterized by linear deposits of IgG and C3 in the dermoepidermal junction zone in DIF. C3 is almost invariably present in BP, whereas the presence of IgG in the absence of C3 is rare.¹³ Patients with EBA are more likely to have linear IgG staining along the basement membrane zone without deposition of C3 than patients with BP.¹⁴ Our results of C3d expression in all cases of BP except for one case are consistent with the results of Magro et al.,¹ who described an immunohistochemical linear positivity of C3d along the basement membrane in 17 out of 17

cases of BP. The only case of BP that did not show deposits of C3d in our series showed a linear positivity of only IgG in DIF without concomitant expression of C3, whereas all other cases showed linear deposits of C3 in DIF. Collagen IV was located at the ground of the blister in that case, suggesting the diagnosis of BP and not EBA. As none of the negative controls displayed deposits of C3d, a positive result can confirm the diagnosis of BP. Detecting BP by immunohistochemical demonstration of C3d has very high sensitivity (97%), a positive predictive value of 100% and a negative predictive value of 98%. A negative result of C3d makes the presence of BP unlikely, but cannot rule out the diagnosis of BP in all cases. As no case of EBA was available for inclusion in our study, no conclusion could be drawn on differences between BP and EBA. In case of blister formation, immunohistochemical demonstration of collagen type IV could be a useful marker for differentiation for BP and EBA.¹⁵ Furthermore one has to be aware that a negative result for C3d cannot rule out other autoimmune bullous diseases, such as linear IgA dermatosis or DHD.

PG is characterized by linear deposition of C3 along the basement membrane zone in DIF in all cases, while IgG deposits are only present in about 30% of the cases. All three cases of PG showed a linear staining of C3d along the basement membrane. As IgG is not detected in about 70% cases of PG, demonstration of C3 deposits along with the typical clinical and histologic findings allows to establish the diagnosis of PG.¹⁶ Therefore our results indicate that staining of C3d is also a useful tool in the diagnostic work-up of PG.

For the pemphigus group, which is characterized by intercellular deposits of IgG and C3 in DIF, the immunohistochemical demonstration of C3d has a low sensitivity and is therefore not of diagnostic value. Only 3 out of 12 of our cases of pemphigus vulgaris revealed an intercellular positivity. A strong positivity was observed only in one case, whereas the other cases only showed a weak focal positivity. All the other acantholytic diseases, such as Grover's disease, Darier's disease and impetigo contagiosa, were negative for C3d. Magro et al.¹ also reported a higher number of negative results of C3d immunohistochemistry in pemphigus compared to BP; however, they were able to show intercellular deposits in 82% of their cases. Our data indicate that immunohistochemical detection of C3d is not of diagnostic value for the diagnosis of pemphigus vulgaris, pemphigus foliaceus or IgA pemphigus.

All cases of DHD, linear IgA dermatosis and IgA pemphigus, which are characterized by the deposition of IgA, were negative for C3d. A negative result was expected in these cases, because IgA does

not activate the complement pathway.¹⁷ This implies that additional deposits of IgA such as in mixed forms of linear IgA dermatosis and BP will be incompletely identified by demonstration of C3d like in our two cases of mixed form. The histologic findings of one case of mixed form were similar to those of linear IgA dermatosis, whereas the other case rather resembled BP. DIF revealed linear depositions of IgA, IgG and C3 and C3d immunohistochemistry a linear pattern along the basement membrane zone in both cases. Similar cases have been reported as a subgroup of patients with subepidermal blistering diseases characterized by the presence of both IgA and IgG antibasement membrane antibodies.¹⁸⁻²¹ The clinical findings and the histology of these patients mostly reflect the characteristics of linear IgA dermatosis.¹⁸⁻²¹

In conclusion, C3d immunohistochemistry is a helpful adjunct in the diagnosis of BP, especially in the cases in which only formalin-fixed and paraffin-embedded tissue is available for analysis. A linear positivity of C3d is highly suggestive of the presence of BP, while a negative result makes the diagnosis of a BP unlikely. Indeed a positive result of C3d immunohistochemistry could replace DIF in almost all of our cases, except the mixed forms of BP and linear IgA dermatosis. In those rare cases of mixed form, a positive result of C3d would lead to the diagnosis of BP and the co-existent deposits of IgA would not be noticed without performing a DIF. Immunohistochemical demonstration of C3d can, however, not replace DIF in all autoimmune bullous disorders, because a negative result does not exclude disorders with IgA deposits such as linear IgA dermatosis and DHD. Before DIF can be replaced completely by immunohistochemistry on formalin-fixed tissue in the diagnosis of subepidermal blistering diseases, reliable immunohistochemical detection of IgA and IgG on formalin-fixed tissue has to be established.

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