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Diagnostic Utility of C4d by Direct Immunofluorescence in Bullous Pemphigoid

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INTRODUCTION

Bullous pemphigoid (BP) is the most prevalent autoimmune blistering disease that commonly affects the elderly. Direct immunofluorescence (DIF) on skin biopsies demonstrates specific linear immunoglobulin G (IgG) and/or C3c deposition along the basement membrane (BM). Deposition of complement fragments such as C3c and C1q is routinely used for diagnostic reasons.¹ Our group has demonstrated that in 11.7% of patients with BP, DIF is double negative for IgG and C3c, whereas in 15% only IgG without C3c is demonstrated.^{2,3} In these situations, there is a need for a more stable marker of BP, such as C4d. C4d is a split product of C4 and thereby reflects previous classical and/or lectin pathway activation of complement. C4d is biologically inactive, but has a long half-life, rendering it a long-lived marker for antibody-mediated complement activation.⁴ The detection of C4d has already been extensively used as a marker of antibody-mediated rejection in kidney transplantation.⁵ In skin biopsies of patient with BP, several reports have demonstrated linear C4d deposition along the BM in formalin-fixed paraffin-embedded (FFPE) tissue. This was suggested to be diagnostically useful with a sensitivity varying from 24% to 90% for C4d and could therefore be a potential substitute for DIF in the near future.⁶⁻⁹ We hypothesized that C4d detected by DIF could also be a promising diagnostic marker for BP, particularly in IgG/C3c DIF-negative cases. Although one case report already showed C4d deposition in an IgG/C3c negative case, our study is the first larger case series to investigate the use of C4d detected by DIF in BP.¹⁰

MATERIAL AND METHODS

This single-center retrospective study was performed at the national referral center for autoimmune bullous diseases in the Netherlands (Groningen Center for Blistering Diseases). The study population consisted of patients suspected for BP. All patients included had a skin biopsy and a blood sample taken. Patients were divided into 4 groups as follows: (1) patients with a biopsy positive for IgG but negative for C3c (n = 26), (2) patients with a biopsy positive for IgG and C3c (positive controls, n = 10), (3) patients with a biopsy negative for IgG and C3c but with positive serological testing, that is, a positive salt-split skin, and clinical features compatible with BP (n = 33), and (4) a negative control group, that is, a negative skin biopsy for IgG, IgM, IgA, and C3c and serology and no clinical diagnosis of BP (n = 39). According to national regulations in the Netherlands, this type of retrospective noninterventional study does not require approval from the local medical ethical committee.

Abstract: Bullous pemphigoid (BP) is an autoimmune blistering disease that commonly affects elderly patients. Direct immunofluorescence (DIF) for immunoglobulin G (IgG) and C3c on frozen skin biopsies is the gold standard for the diagnosis of BP. In a minority of cases, IgG and/or C3c are found negative, and in these situations, there is a need for a more stable diagnostic marker of BP. C4d is biologically inactive, but has a long half-life, rendering it a long-lived marker for antibody-mediated complement activation. Previous studies already demonstrated that C4d was diagnostically useful in formalin-fixed paraffin-embedded skin biopsies of patients with BP. We hypothesized that C4d detected by DIF could also be a promising diagnostic marker for BP, particularly in IgG and/or C3c DIF-negative cases. In this single-center retrospective study, 69 cases of BP were analyzed for linear deposition of C4d; of the 69 cases, n = 26 were IgG+/C3c-, n = 10 IgG+/C3c+, and n = 33 IgG-/C3c-. Results were compared with n = 39 negative controls. Seven of the 26 (27%) IgG+/C3c- and 3 of the 33 (9%) IgG-/C3c- BP cases were positive for C4d. All 10 IgG+/C3c+ cases were also C4d positive. In the negative control group, 2 of the 39 (5%) were found positive for C4d. In conclusion, the current study shows that C4d is a more sensitive but not a 100% specific marker of BP. We conclude that C4d by DIF could be an interesting diagnostic adjunct for BP, particularly in IgG-/C3c- double negative cases.

Key Words: bullous pemphigoid, complement, C4d, immunofluorescence

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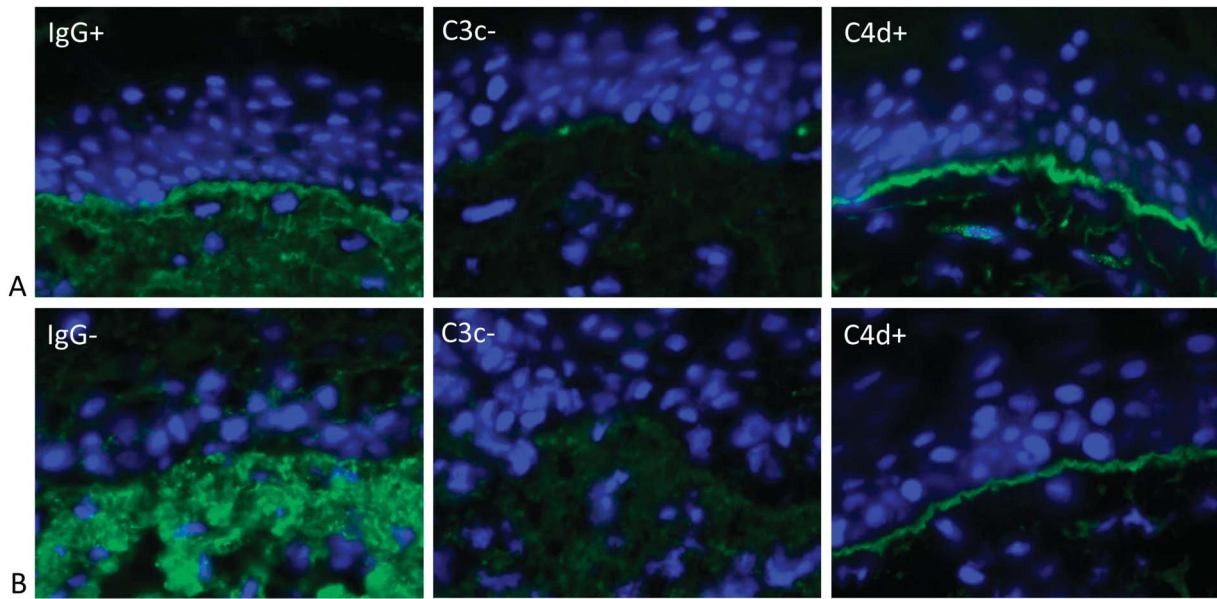


FIGURE 1. A, Shows the DIF results of a case from group 1. Weak but specific linear deposition of IgG along the BM. C3c is negative, but C4d is positive. B, Shows the DIF results of a case from group 3. IgG and C3c are negative, but C4d is positive.

Biopsies were transported and stored mainly in saline solution (0.9% NaCl, overnight), liquid nitrogen, or Michel medium. The following components were stained: IgG (1:80, rabbit polyclonal, DAKO, Glostrup, Denmark), C3c (1:100, rabbit polyclonal, DAKO, Glostrup, Denmark), and C4d (1:30, rabbit polyclonal, Biomedica, Wien, Austria). The staining process has been previously described.¹¹ DIF was considered positive when linear deposits of IgG and/or C3c and/or C4d along the BM were observed varying in intensity from moderate to strong (Fig. 1). The routine multistep serological test procedure included human salt-split skin substrate, monkey esophagus substrate, immunoblot with keratinocyte extract and tested for IgG and IgA autoantibodies against BP180 and BP230, and commercially available BP180 NC16A (≥ 2007) and BP230 (≥ 2009) ELISAs (Medical and Biological Laboratories Co Ltd, Nagoya, Japan) according to the manufacturer’s protocol and a positivity cutoff value $\geq 9 \mu\text{m/L}$.

RESULTS

Table 1 summarizes the observed C4d positivity in the different groups. In group 1, 7 of the 26 scored positive for C4d (27%). All 10 positive controls from group 2 were also positive

for C4d. In group 3, 3 of the 33 (9%) were positive for C4d. In the negative control group 4, 2 of the 39 (5%) were found positive for C4d. The first false-positive case concerned an 86-year-old man with pruritus without blisters. A total of 5 biopsies were taken from this patient in a period of 3 years. All biopsies remained negative as well as all serological testing. The final diagnosis was a cutaneous drug reaction. The second false-positive case concerned a 61-year-old woman also with pruritus without blisters. The biopsy and serology were both negative, and the final diagnosis was pruritus sine materia.

DISCUSSION

DIF for IgG and C3c on frozen skin biopsies is the gold standard for the diagnosis of BP. Recently, we reported that in 11.7% of patients with BP, DIF is negative for IgG and C3c. There are several reasons that could explain a negative DIF in patients with BP, including loss of immunoglobulins and complement in lesional skin biopsies, improper transport and delay, and (in case of IgG+/C3c- cases) complement independent mechanisms. In case of a negative DIF, additional analyses including detection of circulating antibodies by ELISA and indirect immunofluorescence on a salt-split skin

Table 1. Results of C4d Staining

	Group 1 IgG+, C3c-	Group 2 IgG+, C3c+	Group 3 IgG-, C3c-	Group 4 IgG-, C3c-
Positive for C4d	7 (27%)	10 (100%)	3 (9%)	2 (5%)
Negative for C4d	19 (73%)	0 (0%)	30 (91%)	37 (95%)
Total	26	10	33	39

Group 1, biopsy positive for IgG, but negative for C3c; Group 2, biopsy positive for IgG and C3c; Group 3, biopsy negative for IgG and C3c, but positive serology; Group 4, biopsy negative and serology negative.

are required.² However, in many diagnostic laboratories, no such techniques are available and therefore there is need for more sensitive and specific markers for BP by DIF. C4d is a split product of C4 and thereby reflects previous classical and/or lectin pathway activation of complement. C4d binds tissue covalently through a thio-ester bond, resistant to shedding. C4d has been widely been accepted in kidney transplantation as a stable marker (“foot print”) of antibody-mediated rejection.⁵ In BP, linear C4d deposition has been demonstrated on FFPE as a rescue method, when no frozen material is available.^{6–9} The aim of our study was to investigate the use of C4d on DIF as an diagnostic adjunct on frozen sections for routine clinical practice, particularly in IgG/C3c double negative biopsies. An important finding of our study is that 9% of IgG/C3c double negative and 27% of IgG+/C3c– cases showed C4d positivity, whereas all IgG+/C3c+ positive biopsies were also C4d positive. These results are in line with a case report from Kassaby et al¹⁰ on frozen section. Although C4d showed a high sensitivity, 2 of the 39 control biopsies also showed linear C4d positivity, seen previously by Villani et al⁸ on FFPE who found 1 false-positive (dermatitis herpetiformis) staining in 44 controls. These results indicate that C4d is not a 100% specific marker but should prompt the dermatopathologist to suggest a diagnosis of BP. Interpretation of data should always be carefully integrated with other findings such as hematoxylin and eosin morphology and additional analysis of serum markers. We conclude that C4d by DIF could be an interesting diagnostic adjunct for BP in C3c-negative cases and particularly in IgG/C3c double negative cases suspected for BP.

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