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C4d immunohistochemical stain is a sensitive method to confirm immunoreactant deposition in formalin-fixed paraffin-embedded tissue in bullous pemphigoid

Background: Bullous pemphigoid (BP) is characterized clinically by the onset of pruritic urticarial plaques, vesicles and bullae in a predominantly elderly population. While the diagnosis may be suspected on routine hematoxylin and eosin histology of formalin-fixed paraffin-embedded tissue, fresh-frozen tissue must be used to show the immunologic nature of the bullous process by direct immunofluorescence (DIF). The diagnosis is further confirmed and separated from epidermolysis bullosa acquisita (EBA) by subsequent serologic studies to detect antibodies directed against BP180 and BP230 antigens and characteristic antibody deposition on salt-split skin.
Methods: Using a polyclonal complement fragment 4d (C4d) antibody, we stained formalin-fixed paraffin-embedded skin biopsy specimens from cases of BP and controls.
Results: We showed characteristic linear basement membrane deposition of C4d in formalin-fixed paraffin-embedded tissue in seven of nine cases diagnosed as BP vs. EBA by DIF on fresh-frozen tissue. None of the four controls for which we had adequate tissue were positive.
Conclusion: These results indicate that formalin-fixed paraffin-embedded tissue can be stained for the immunoreactant C4d to show characteristic immunoreactant deposition, potentially obviating the need for repeat biopsy for DIF and allowing clinicians to proceed to serologic confirmation of BP.

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Bullous pemphigoid (BP) is characterized clinically by the onset of pruritic urticarial plaques, vesicles and bullae in a predominantly elderly population. The classic histopathology is a subepidermal split at the basement membrane with a dermal infiltrate of eosinophils. The disease is characterized on direct immunofluorescence (DIF) by deposition of immunoglobulin (Ig)G and complement at the basement membrane in the lamina lucida, which can be confirmed by indirect immunofluorescence on salt-split skin.

The diagnosis is frequently considered on routine histology of formalin-fixed paraffin-embedded tissue. Demonstration of the immunologic process currently requires a second biopsy for DIF of fresh-frozen sections stained with fluorescein-conjugated antibodies.

Current methods are not sensitive enough to allow effective staining of formalin-fixed paraffin-embedded tissue to detect the diagnostic antibody and complement findings.[1,2] The diagnosis of BP is confirmed and separated from epidermolysis bullosa acquisita (EBA) with serologic testing including indirect immunofluorescence on salt-split skin and detection of antibodies directed against BP180 and BP230 antigens.

Complement fragment 4d (C4d), an index of complement activation by the classical pathway, is well established as a marker for antibody deposition in renal allograft biopsies.[3,4] It is reliably detected by immunohistochemistry on formalin-fixed paraffin-embedded kidney biopsies and correlates with serologic studies that confirm the presence of donor-specific renal allograft antibodies.[5–7]

We hypothesized that C4d antibody could be used in formalin-fixed paraffin-embedded skin biopsy sections to detect antibody-mediated complement deposition at the basement membrane in BP patients.[8–10] This would allow immunopathologic confirmation of the bullous process without necessitating repeat biopsy and processing of fresh-frozen tissue for DIF.

Methods

Specimens

We retrospectively studied 10 cases diagnosed as BP vs. EBA based upon linear staining of the basement membrane with C3 and/or IgG by DIF of cryostat sections. We selected consecutive cases who had concurrent specimens submitted for both fresh-frozen DIF as well as routine histology to our laboratory (Immunodermatology Laboratory, Department of Dermatology, University of Utah, Salt Lake City, UT, USA) from 2006 to 2007. Subsequently, laboratory records were searched to identify confirmatory serologic testing results, which were available for five cases.

Five patients with skin biopsies who had been submitted for DIF and concurrent routine histology, with a clinical impression of BP, but were negative by DIF, were used as controls.

Immunostaining

We processed the 15 paraffin-embedded specimens (10 cases and 5 controls) for C4d on the Ventana Benchmark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) at 42°C with a rabbit anti-human polyclonal C4d antibody (catalog no. 04-BI-RC4D; ALPCO Diagnostics, Salem, NH, USA).

The sections were first mounted on plus slides, then deparaffinized on the autostainer with EZ Prep solution (Ventana Medical Systems). They were treated with CCl for 60 min followed by Protease 3 (Ventana Medical Systems) for 4 min. The primary antibody, rabbit anti-human polyclonal C4d antibody, was applied at a 1 : 100 dilution for 60 min. The Amplification Kit (Ventana Medical Systems) was then applied followed by the AB Blocking Kit (Ventana Medical Systems). Detection was accomplished with the IView DAB Detection Kit (Ventana Medical Systems), then counterstained with hematoxylin for 4 min. The slides were rinsed with dH_2O, placed in iodine for 30 s (to remove metal precipitates from fixation) and then sodium thiosulfate for 30 s (to remove the iodine). They were dehydrated in successive alcohol gradients, xylene, coverslipped and allowed to air-dry.

A kidney biopsy from a patient with known antibody-mediated allograft rejection was used as a positive control. The C4d antibody was not added to the negative control.

We compared the results of the C4d immunostaining with the results of the DIF of fresh-frozen cryostat sections.

Results

Of the 10 cases diagnosed as BP vs. EBA by fresh-frozen DIF, 7 had linear deposition of immunoreactant C4d at the basement membrane of formalin-fixed paraffin-embedded tissue (Figs. 1 and 2). Two specimens were negative for C4d, and one specimen was inadequate (Table 1). Of the seven specimens positive for C4d, three showed a definite dermal-epidermal separation. In all three of these cases, the linear C4d staining was present on the floor of the blister (Fig. 2). In one case with blister-floor C4d staining, serologic testing had been performed, which showed low positive antibody against BP180 (10 units, negative < 9) and dermal staining of human salt-split skin (1 : 40, negative $< 1 : 10$). This was interpreted as most consistent with EBA. In the four other cases for which serologic testing results were available, the results were most consistent with the diagnosis of BP (Table 1).

Four controls who were negative for linear basement membrane complement and immune globulin deposition by fresh-frozen DIF were also negative for linear C4d deposition at the basement membrane on formalin-fixed paraffin-embedded tissue. One represented an inadequate specimen (Table 2).

Discussion

Our results indicate that staining of formalin-fixed paraffin-embedded tissue for C4d can be used for confirmation of linear basement membrane immunoreactant deposition in cases of BP. This proved to be a reliable method for confirmation in seven of the nine cases in which we had an adequate specimen. With this staining, we could potentially obviate the need for

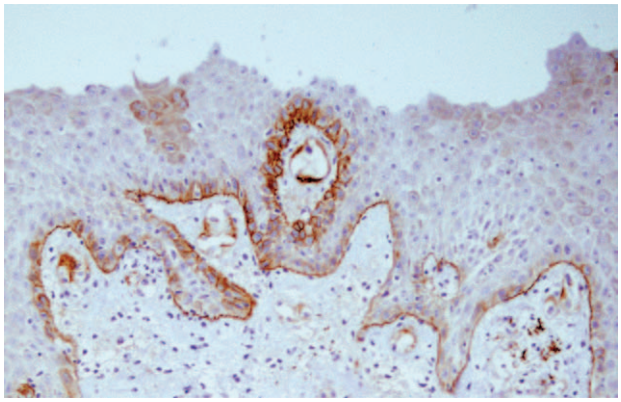


Fig. 1. Linear staining of the basement membrane with C4d on formalin-fixed paraffin-embedded tissue from a case of bullous pemphigoid.

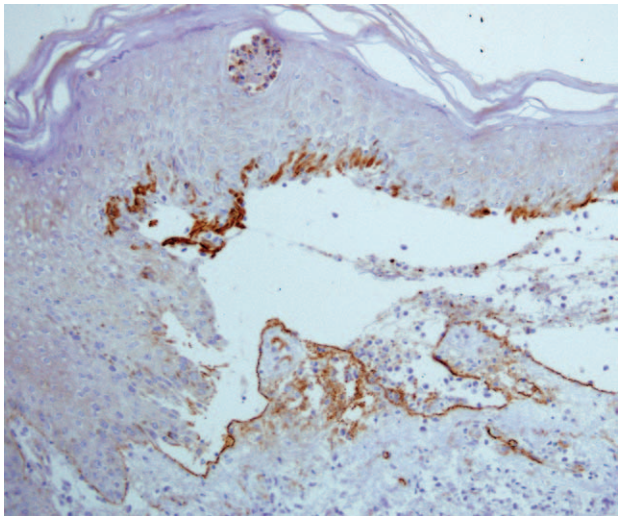


Fig. 2. Linear staining of the blister floor with C4d on formalin-fixed paraffin-embedded tissue from a case of bullous pemphigoid.

rebiopsy and subsequent DIF of fresh-frozen sections in the diagnosis of our patients and proceed to serologic confirmation.

The specificity of this method in a small sample of four adequate controls showed no evidence of false positivity. Other investigators recently showed granular deposition of C4d at the basement membrane on formalin-fixed skin from cases of systemic lupus erythematosus and discoid lupus erythematosus.[11] However, these deposits were granular, not continuous and linear, as they were in our positive cases.

Historically, investigators have not been able to use formalin-fixed paraffin-embedded tissue to effectively show immunoreactant deposition in cases of BP.[1,2] A previous study evaluating antigen retrieval and DIF on formalin-fixed paraffin-embedded skin biopsies in patients with BP had a sensitivity of 50–60%.[1] Contemporary investigators have recently reported success in identifying C3d deposition in formalin-fixed tissue from cases of BP but not C4d.[11] Our small study indicates that in 70% of cases of BP, the immunologic process can be confirmed with automated immunohistochemical staining for C4d on formalin-fixed paraffin-embedded tissue.

While our controls did not include any immunobullous diseases, we expect that the C4d staining pattern will allow separation of immunobullous diseases to a similar extent that DIF on fresh tissue for IgG does. Therefore, BP, epidermolysis bullosa and bullous lupus erythematosus may all look similar with linear basement membrane C4d staining and would

Table 1. Characteristics of cases*

Status	IgG	IgM	IgA	C3	C4d	Serology
Case	−	−	−	++ linear BM	+	SSS: D 1 : 40 180: 10 units 230: 3 units
Case	+++ linear BM	−	−	+++ linear BM	+	SSS: E 1 : 1280 180: 69 units 230: 8 units
Case	++ linear BM	−	−	+++ linear BM	INAD	NP
Case	+ discont BM	−	−	+++ linear BM	+	NP
Case	+++ linear BM	−	−	+++ linear BM	−	NP
Case	++ linear BM	−	−	+++ linear BM	−	SSS: E 1 : 320 180: 10 units 230: 31 units
Case	++ linear BM	−	−	+++ linear BM	+	NP
Case	+++ linear BM	−	−	+++ linear BM	+	NP
Case	++ linear BM	−	+ linear BM	+++ linear BM	+	SSS: E 1 : 1280 180: 67 units 230: 3 units
Case	+++ linear BM	+ gran BM	−	+++ linear BM	+	SSS: E 1 : 640 180: 58 units 230: 19 units

BM, basement membrane; C4d, complement fragment 4d; discont, discontinuous staining; gran, granular staining; Ig, immunoglobulin; INAD, inadequate specimen; SSS, human salt-split skin; D and E, dermal and epidermal pattern, respectively, with positive titer > 1 : 10; 180 and 230, serologic enzyme-linked immunosorbent assay for IgG against BP180 and BP230 antigens with positive result > 9 units; NP, not performed.

*Staining pattern of cases used in this study: IgG, IgM, IgA and C3 stains were performed on fresh-frozen tissue with fluorescein-conjugated antibodies. C4d was performed on formalin-fixed paraffin-embedded tissue.

Table 2. Characteristics of controls

Status	Clinical impression	Microscopic impression	C4d
Control	Urticarial BP	Urticarial hypersensitivity	INAD
Control	BP	Dermatitis and eczema	—
Control	BP vs. EBA vs. DH vs. arthropod	Drug eruption	—
Control	BP vs. other blistering	Dermatitis and eczema	—
Control	BP vs. DH	Cutaneous hypersensitivity	—

BP, bullous pemphigoid; C4d, complement fragment 4d; EBA, epidermolysis bullosa acquisita; DH, dermatitis herpetiformis; INAD, inadequate specimen; -, negative for linear C4d staining.

still require serologic studies for separation. The IgG autoantibodies that characterize herpes gestationis are typically sparse; however, C3 deposition at the basement membrane is usually intense. Studies have suggested that the IgG antibodies of herpes gestationis are particularly active catalysts for the generation of complement via the classical pathway.[12–14] We therefore expect there to be an intense C4d staining of the basement membrane in cases of herpes gestationis. In contrast, we expect that linear IgA disease would be largely negative for C4d basement membrane staining. This is because IgA, which is deposited at the basement membrane in cases of linear IgA disease, does not activate the classical complement pathway with nearly the efficiency of IgG or IgM.[15] In cases of pemphigus vulgaris and pemphigus foliaceous, C4d staining could be useful in supporting the diagnosis with formalin-fixed paraffin-embedded tissue.

There are several possible explanations for why linear C4d staining was not found at the basement membrane in the two cases of BP for which we had adequate tissue (Table 1). One immunologic explanation is that the particular IgG autoantibody that was depositing at the basement membrane in our negative cases was not an efficient activator of the classical complement pathway. Therefore, little C4d was generated. Multiple studies have shown that IgG4, which does not activate the classical complement pathway, is the most consistent IgG subclass deposited at the basement membrane in BP.[16–20] One or more of the complement-fixing subclasses of IgG (IgG1, IgG2 and IgG3) is deposited at the basement membrane in a majority of, but not all, cases of BP. Lesional skin biopsies where significant deposition of IgG4 is present can have linear C3 deposition at the basement membrane without identifiable IgG by standard DIF.[21] The absence of IgG by DIF, despite the consistent presence of IgG4 by more sensitive techniques, is explained by a lack of sensitivity of commercial anti-human IgG preparations for IgG4.[21] The presence of C3 can be because of deposits of IgG1–3 below the threshold of standard DIF detection or may be further evidence of alternative pathway activity in BP.[21,22] Interestingly, in this study, the two cases who were negative for C4d did have linear deposition of IgG by DIF (Table 1). Also, the single case negative for IgG deposition at the basement membrane by DIF was positive for C4d. This latter finding could have resulted from IgG1–3 deposition below the threshold of detection, yet still sufficient to catalyze detectable levels of C4d via the classical pathway.

At this time, we have no clear explanation for why linear C4d deposition occurs consistently on the floor of the blister (Fig. 2). Serologic results were available for only one of these three cases, which showed positive dermal staining with indirect immunofluorescence on salt-split skin, consistent with the diagnosis of EBA. This raises the possibility that blister separation occurred at the lamina lucida and the C4d pattern reflects antibody deposition in the lamina densa, characteristic of EBA.

It is our opinion that in the future, formalin-fixed paraffin-embedded tissue can be evaluated for the presence of C4d, and in positive cases, it allows clinicians to proceed directly to serologic confirmation of the immunobullous process, without rebiopsy for DIF.

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