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Original Article

Use of C3d and C4d Immunohistological Markers to Differentiate Various Inflammatory Dermatosis

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Abstract

Introduction: The application of C3d and C4d as a diagnostic adjunct in the cases of inflammatory skin disease has been rarely reported in the literature. The purpose of our study is to define the significance of C3d and C4d deposition by an immunohistochemistry (IHC) on paraffin-embedded, formalin-fixed tissue as a correlative diagnostic adjunct in the assessment of inflammatory skin disease.

Objective: To evaluate use of C3d and C4d by immunohistochemistry (IHC) on formalin-fixed tissue as diagnostic adjunct in the various inflammatory skin diseases.

Method: 1) Diagnosed cases of inflammatory skin diseases on histology were selected for the study. 2) H&E slides were studied for morphological assessment. 3) IHC makers for C3d and C4d were performed on the paraffin blocks using C3d and C4d- antibody based detection kits.

Result: 100% cases of DLE & SLE were positive for deposition of C3d & C4d at dermo-epidermal junction. 100% cases of Bullous Pemphigoid & 57% of cases of Bullous Pemphigoid were positive for C3d and C4d deposition respectively at dermo-epidermal junction. 100% cases of Behçet's disease & 42% cases ofLeukocytoclastic Vasculitis were positive for deposition of C3d & C4d in blood vessel.

Conclusion: The C3d and C4d assays define an important diagnostic adjunct in the evaluation of collagen vascular disease, cutaneous vascular disease, and autoimmune vesiculobullous disease. The assays may prompt further DIF testing or, in some instances, may even define a reasonable substitute for DIF and/or add to the morphologic assessment of a biopsy specimen submitted for routine light microscopic assessment.

Introduction

Inflammatory Skin Diseases are broadly classified into 3 subgroups; Collagen Vascular Disease, Autoimmune Vesiculobullous Disease & Vasculopathic Conditions. Histopathology (H&E based Histology) has remained mainstay investigation in order to diagnose the diseases under these subgroups. On H&E based histology, there are many diseases which share common overlapping features causing a great difficultly to diagnose the case. For that sake, there are very few ancillary techniques like indirect and direct immunofluorescence and immunoblot investigation etc., which can aid in the diagnosis of inflammatory conditions in skin. However, availability of these investigation is not so wide and the cost is high. Hence, any alternative test to these ancillary techniques which is of low cost and widely available will be readily accepted.

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Immunohistochemistry (IHC) methodology on paraffin-embedded, formalin-fixed tissue is now a well-accepted technique & is available in many setups. It is cost effective as compared to other ancillary techniques mentioned earlier. Hence any IHC marker in the realm of inflammatory skin disease which is cost effective, sensitive & diagnostic adjunct to these old ancillary techniques will be readily accepted.

The skin represents an active immune organ with complex interactions between cellular components and various mediators.[1] Complement involvement has been associated with several skin diseases, such as psoriasis, lupus erythematosus, cutaneous vasculitis, urticaria, and bullous dermatoses[1] & these complement can be used in forming diagnosis of disease.

The purpose of our study was to define and confirm the significance of complements C3d and C4d deposition by an IHC methodology on paraffin-embedded, formalin-fixed tissue as a diagnostic adjunct in the assessment of inflammatory skin disease.

The categories of inflammatory skin disease considered here includes Collagen vascular disease mainly Lupus Erythematosus, Autoimmune vesiculobullous disease mainly Pemphigus Vulgaris, Pemphigus Vegetans and Bullous Pemphigoid and Leukocytoclastic type of Vasculitis.

Aims and Objective

- To evaluate use of C3d and C4d by immunohistochemistry (IHC) on formalin-fixed tissue as a diagnostic adjunct in the various inflammatory skin diseases.
- To reveal the significance of C3d and C4d in the various inflammatory skin diseases.

Material and Methods

- *Nature of study***:** Cross sectional (Observational) study.
- *Sample size:* 41 patients attending tertiary health care center with clinical and histopathological diagnosis.
- *Method:*
  A. Diagnosed cases of inflammatory skin diseases on histology were selected for the study.
  B. H&E slides were studied for morphological assessment.
  C. IHC makers for C3d and C4d were performed on the paraffin blocks using C3d and C4d- antibody based detection kits.
  D. These slides stained for IHC C3d and C4d were then studied in details
  E. Detailed history of the patients was taken retrospectively in their follow up of the treatment.
  F. Regional Ethical Committee has granted permission to carry out the study.

Selection of Cases

Inclusion Criteria

A. Patients of all age group were taken into the study
B. Biopsy of skin which is positive for inflammatory disease of skin on histology will be included in the study.

Exclusion Criteria

A. Biopsy negative or suspicious for inflammatory disease of skin on histology will be excluded from the study.
B. Patients who were not willing to give consent
C. Patients who were not fulfilling the clinical criteria

Immunohistochemistry (IHC)

Immunohistochemistry was carried out using rabbit polyclonal & monoclonal antibodies against C3d & C4d respectively.

Materials Used in our Study

Apparatus / Equipment

- Decloaking Chamber (For antigen retrieval, BioCare company)
- Pipettes & Pipette tips
- Silane coated slides
- Coverslip
- Microtome (Leica Company)
- Glass beakers
- Graduated cylinders
- Glass bottle
- Weighing Machine
- pH meter
- Dako Real Envision polymer kit

- Thermo Scientific Shandon Sequenza Immunostainer
- Absorbent wipes
- Wash bottles
- Incubator
- Diamond Pencil etc.

Immunohistochemistry Interpretation

The grading of immunoreactant deposition along the dermo-epidermal junction (DEJ) was one based on subjective quantitative assessment of deposition intensity with the 4 standard assigned grades being absent (0), mild (1), moderate (2), and marked (3).

Positive blood vessel (BV) deposition was defined as mural (wall) deposition; endoluminal serum and/or endothelial cell localization will not beconsidered as positive staining. Those cases showing homogenous BV staining will only be deemed positive if the BV staining exceeded background staining. Elastic fiber staining within the dermis including the vessel walls will be considered nonspecific staining.

Statistic & Software

The data analysis was done on Microsoft Excel & Word 2016. Qualitative data variables expressed by using frequency and percentage. Quantitative data variables expressed by using mean +- SD. Sensitivity (%) of IHC markers C3d and C4d was calculated with HPE. For referencing; we have used Mendeley Desktop.

Result

Table 1: Distribution of Study Population.

Sr. No.	Disease / Disorder Group	No. of Cases	Percentage
1	Collagen Vascular Disease	5	12.19%
2	Autoimmune Vesiculobullous Diseases	11	26.82%
3	Vasculopathic Condition	8	19.51%
4	Control	17	41.46%
		Total=41	100%

In our study, we included 41 cases in total which contain 5 cases of Collagen Vascular Disease, 11cases of Autoimmune Vesiculobullous Diseases, 8 cases of Vasculopathic conditions & 17 cases as control.

Table 2: Collagen Vascular Disease

Disorder	Cases	Dermo-epidermal Junction		Blood Vessel	
		C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)	C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)
DLE	1	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/1)
SCLE	2	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)
SLE	2	100% (2/2)	100% (2/2)	100% (2/2)	50% (1/2)
Lichen Planus (Control)	7	0% (0/7)	0% (0/7)	14% (1/7)	0% (0/7)
Lichenoid Drug Eruption (Control)	1	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)
Chronic Lichenified Eczema (Control)	1	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)

Collagen Vascular Diseases

Inflammatory Control for Interface Dermatitis of Collagen Vascular Diseases (9 Cases)

We evaluated 7 cases of lichen planus, 1 case of chronic lichenified eczema & 1 case of lichenoid drug eruption. Deposition of C3d or C4d along dermo-epidermal junction was not identified in any of these cases. However, 1 case of Lichen Planus (1/7) showed granular positivity of C3d around blood vessel. C4d for deposition around blood vessel were negative all cases of lichen

planus, chronic lichenified eczema & of lichenoid drug eruption.

LE (5 Cases)

All cases (1 case) of Discoid Lupus Erythematosus (DLE) showed granular C3d as well as C4d positivity along the dermo-epidermal junction especially basal cell layer. The staining intensity was mild to moderate. This case of DLE did not show any granular blood vessel staining for C3d as well as C4d.

In addition, all 2 cases of Subacute Cutaneous Lupus Erythematosus (SCLE) (0/2) were negative for C3d & C4d along dermo-epidermal junction. Also, C3d & C4d were negative for deposition around blood vessels in these cases (0/2). In all 2 cases there were fine granular nuclear deposits of C4d within epidermal keratinocytes appreciable only under oil immersion, which corresponded to the presence of anti-Ro antibodies.

Like DLE, all cases of Systemic LE (SLE) (2/2) demonstrated prominent focal granular C3d as well as C4d along dermo-epidermal junction (Image 1 & 2). In contrast with DLE; all cases of SLE (2/2) showed granular deposition of C3d around blood vessel & 1 out 2 cases showed granular deposition of C4d around blood vessel.

Before including the cases of SLE, we checked the anti-Sm and anti-RNP status of the patients & all those cases of SLE were positive for both of them.

Tabl e 3: Vesiculobullous Disorder

Disorder	Cases	Dermo-epidermal Junction		Blood Vessel	
		C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)	C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)
Bullous Pemphigoid	7	100% (7/7)	57% (4/7)	28% (2/7)	28% (2/7)
Pemphigus Vulgaris	3*	0% (0/3)	0% (0/3)	33% (1/3)	0% (0/3)
Pemphigus Vegetans	1*	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)

*100% cases of Pemphigus Vulgaris (3/3) & Pemphigus Vegetans (1/1) showed intercellular positivity for C3d.

Vesiculobullous Disorder

Bullous Pemphigoid (7 cases)

Homogenous linear C3d positivity along the dermo-epidermal junction in all 7 cases (7/7) (Image 3). In 4 out of 7 cases there was homogenous linear C4d deposition along the dermo-epidermal junction (Image 4). 2 out of 7 cases were positive for both C3d and C4d deposition around blood vessels. In all these cases, both C3d & C4d were observed & we noted that the intensity of staining for C3d deposition exceeds than that of intensity of C4d deposition.

Pemphigus Vulgaris (3 cases)

All 3 cases (3/3) of pemphigus vulgaris exhibited intercellular C3d and C4d deposition. 1 out of 3 cases showed mural mild granular positivity for C3d around blood vessels however there was no deposition of C3d at dermo-epidermal junction. There was no case (0/3) which showed positivity for C4d deposition at dermo-epidermal junction or around blood vessels.

Pemphigus Vegetans (1 cases)

In all cases (1/1) of pemphigus Vegetans showed intercellular C3d & C4d deposition. In this case, there was no deposition of C3d & C4d in dermo-epidermal region or around blood vessels.

Table 4: Vasculopathic Conditions

Disorder	Cases	Dermo-epidermal Junction		Blood Vessel	
		C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)	C3d Positivity (Sensitivity)	C4d Positivity (Sensitivity)
Leukocytoclastic Vasculitis	7	0% (0/7)	0% (0/7)	42% (3/7)	42% (3/7)
Behçet's disease	1	0% (0/1)	0% (0/1)	100% (1/1)	100% (1/1)
Psoriasis (Control)	8	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/8)

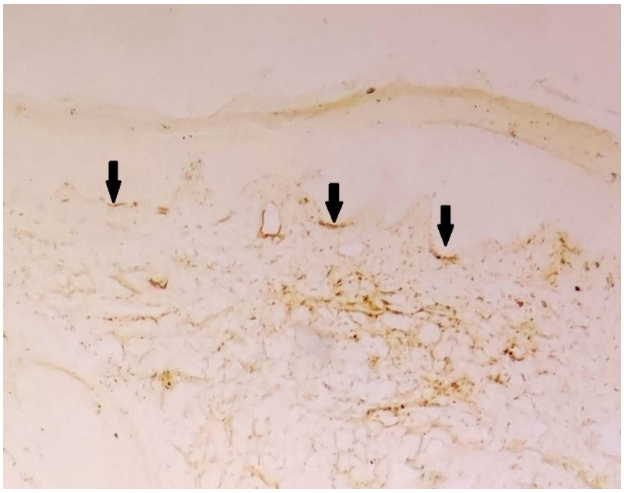

Fig. 1: Systemic Lupus Erythematosus; C3d Stain; 10X; Biopsy specimen of Systemic Lupus Erythematosus demonstrates focal moderate amount of granular deposition of C3d at dermo-epidermal junction (Black Down Arrows) of the same case.

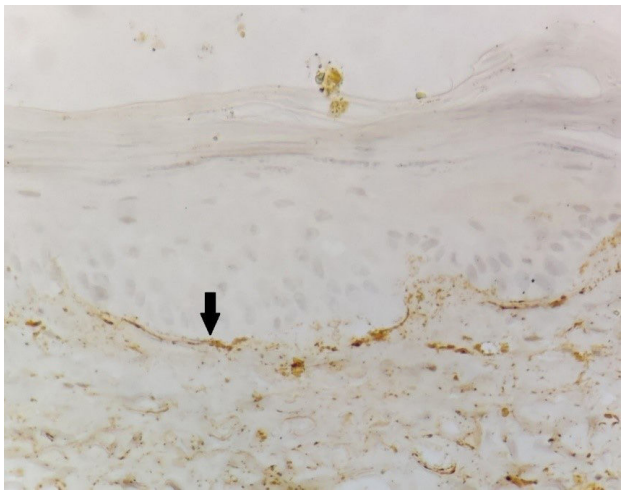

Fig. 2: Systemic Lupus Erythematosus; C4d Stain; 40X; Biopsy specimen of Systemic Lupus Erythematosus demonstrates linear moderate amount of granular deposition of C4d at dermo-epidermal junction (black down arrows) of the same patient.

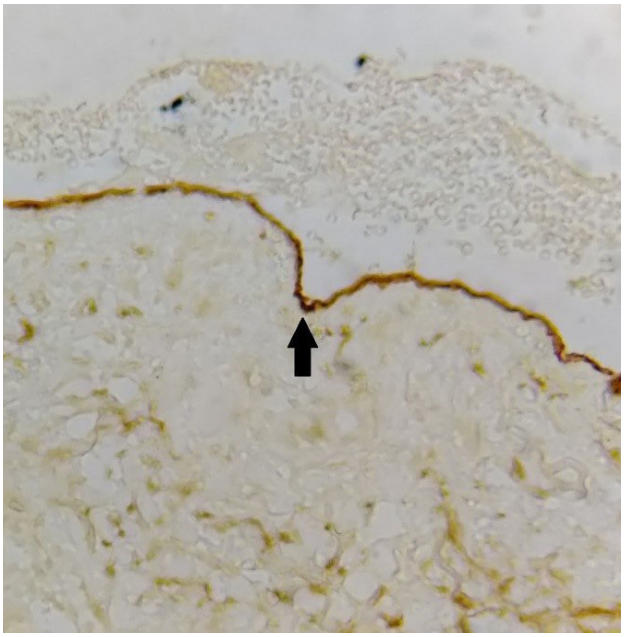

Fig. 3: Bullous Pemphigoid; C3d Stain; 40X; Biopsy specimen of Bullous Pemphigoid showing homogeneous linear deposition of C3d at dermo-epidermal junction of the same patient (Black Up Arrow).

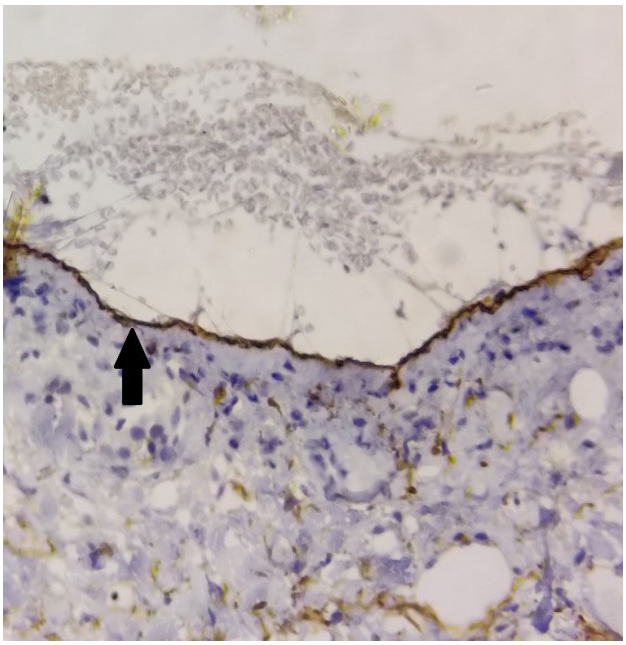

Fig. 4: Bullous Pemphigoid; C4d Stain; 40X; Biopsy specimen of Bullous Pemphigoid showing homogeneous linear deposition of C4d at dermo-epidermal junction of the same patient (Black Up Arrow).

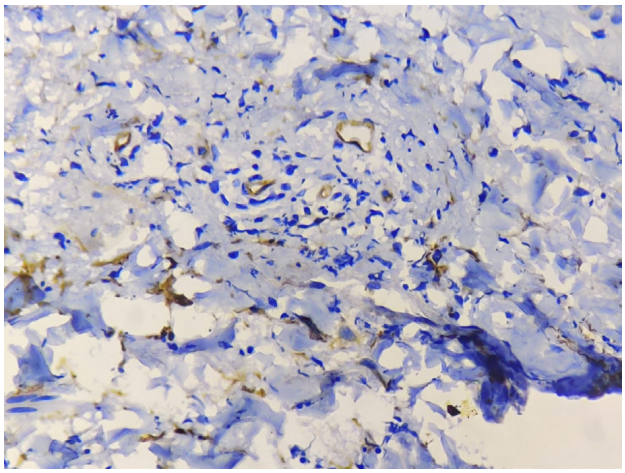

Fig. 5: Leukocytoclastic Vasculitis; C3d Stain; 40X; Biopsy specimen of Leukocytoclastic Vasculitis showing mural deposition of C3d in blood vessel without any staining at dermo-epidermal region..

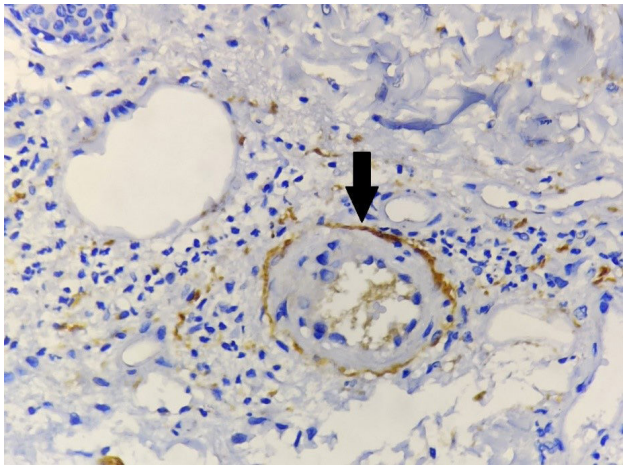

Fig. 6: Leukocytoclastic Vasculitis; C4d Stain; 40X; Biopsy specimen of Leukocytoclastic Vasculitis showing mural deposition of C4d in blood vessel (Black Down Arrow) without any staining at dermo-epidermal region of the same case.

Vasculopathic Conditions

Leukocytoclastic Vasculitis (7 cases)

3 cases out of 7 of Leukocytoclastic Vasculitis showed granular C3d as well as C4d deposition within blood vessel wall (3/7) (Image 5 & 6). Out of C3d and C4d which were observed for deposition around blood vessels in these cases; the deposition of C4d was stronger than that of C3d deposition when assessed by these IHC staining. However, no case (0/7) showed positivity for C3d and C4d deposition at dermo-epidermal junction.

Behçet's disease (1 case)

All the cases i.e. (1/1) of Behçet's disease showed granular and homogeneous staining of blood vessel with C3d as well as C4d. The dermo-epidermal junction was negative for C3d as well as C4d deposition. Like Leukocytoclastic Vasculitis the staining intensity for C4d deposition was more as compared to C3d deposition around blood vessel. Like Leukocytoclastic Vasculitis, the case showed no positivity for C3d and C4d deposition at dermo-epidermal junction.

Discussion

We have assessed the potential application of C3d and C4d as a diagnostic adjunct in the evaluation of cutaneous inflammatory disease.

C3d and C4d are stable components of complement activation and have achieved significant diagnostic use in the setting of solid organ transplantation.[2,3,4] There is very little literature reporting C3d or C4d in skin biopsy material.

The initial pilot study on the C3d and C4d assessment by IHC on formalin-fixed tissue defining a diagnostic adjunctivity in the evaluation of inflammatory skin diseases was carried out by Mangro et al.[5]

Two more separate studies were carried out to check utility of IHC C3d and C4d deposition in cases of Bullous Pemphigoid by Pfaltz et al[6] & Chandler et al.[7]

With respect to cases of LE, the pattern of C3d and C4d deposition varied according to the subtype of LE. 100% DLE cases showed prominent focal deposition of C3d & C4d along the DEJ. Also 100% SLE cases showed deposition of C3d and C4d along the DEJ with C3d and/or C4d deposition in blood vessels. The different pattern of C3d and C4d deposition for DLE and SLE is interesting and could imply pathogenetic differences in regard to the mechanisms by which immunoglobulins and complement are trapped within the basement membrane.[5] Previous studies have shown a greater role of alternative pathway complement activation in patients with DLE relative to SLE.[8] Another study found that the immunoreactant associated with the greatest degree of specificity for SLE was C4, indicative perhaps of the importance of classic complement activation in the pathogenesis.[9]

As expected in the setting of SCLE, C3d and C4d were not observed along the DEJ. In the study conducted by Mangro et al[5]; there were also negative results for C3d & C4d deposition along DEJ in all

cases (0/15) of SCLE which were tested against & reflecting negative lupus band test. All cases of LE associated with anti-Ro antibodiesshowed nuclear localization of C4d, which assumed a granular pattern discernible under oil immersion.

We examined other cases of interface dermatitis unrelated to collagen vascular disease specifically in the context of lichen planus and lichenoid drug eruption and in no case, there were deposits of C3d and or C4d observed at DEJ. One case of lichen planus was showing deposition of C3d in blood vessel. However, there is considerable literature stating that leukocytoclastic vasculitis can be present in the setting of lichen planus.[10]

In leukocytoclastic vasculitis, there were granular deposits of both C3d (42% cases) and C4d (42% cases) although with greater intensity of C4d. It has been established that the classic pathways are activated in the forms of leukocytoclastic vasculitis and vascular injury syndromes mediated by anti-endothelial cell antibodies. In the setting of microvascular injury syndromes associated with anti-endothelial cell antibodies, prominent mural deposits of C4d were observed. However, our study showed lesser sensitivity for both C3d and C4d deposition in cases of leukocytoclastic vasculitis when compared to the study conducted by Mangro et al.[5] In their study the C3d and C4d depositions were identified in 100% of the cases i.e., in all 6 cases included by them. Also, to note a case of Behçet's disease included showed granular and homogenous deposition of C3d and C4d around blood vessel.

We observed prominent linear deposits of C3d (100% i.e., 7/7) and/or C4d (57% i.e., 4/7) along the DEJ were characteristic for Bullous Pemphigoid. C3d was observed with greater intensity relative to C4d. Other studies have also shown enhanced sensitivity of C3d relative to C4d (128). Pfaltz et al (6) reported 97% (31/32) of cases of Bullous Pemphigoid were showing deposition of C3d at dermo-epidermal junction which is matching with our results. Chandler et al(7) reported that 70% (7/10) of the cases of Bullous Pemphigoid showed positivity for C4d deposition at dermo-epidermal junction. We evaluated 7 cases of Bullous Pemphigoid & reported that all of cases i.e., 100% were showing deposition of C3d at dermo-epidermal region & 57% cases were positive for C4d deposition at dermo-epidermal region.

In pemphigus, C3d & C4d were observed in an intercellular array without DEJ positivity which was as expected & this result can be correlated with the study carried out by Magro et al.[5] Also, Pfaltz et al[6] noted that 27% of cases (3/11) of PemphigusVulgaris were showing intercellular positivity & 0% in cases (0/2) of pemphigus foliaceus. Our study showed 100% positivity for intracellular deposition for C3d and C4d in cases of pemphigus vulgaris (3/3) & pemphigus foliaceus (1/1). The deposition was focal and stronger in case of C3d than that of C4d.

Conclusion

C3d and C4d IHC markers are sensitive to diagnose collagen vascular disease, cutaneous vascular disease & autoimmune vesiculobullous diseases. In absence of sophisticated ancillary techniques like DIF & immunoblot; these markers can be useful for morphological assessment of these diseases. Also, these markers will allow clinicians to proceed without any re-biopsies for DIF. This will be also helpful for patients undergoing treatment.

It will be helpful to add these IHC markers to the morphological assessment of biopsies of skin submitted for routine light microscopic examination.

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