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Original Article

Sensitivity and specificity of C3d staining for the diagnosis of discoid lupus erythematosus

Apasee Sooksamran[1*], Jade Wititsuwannakul[2], Penvadee Pattanaprichakul[3], and Nutpacha Chotikawichean[4]

[1] *Department of Pathology, Faculty of Medicine, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[2] *Division of Dermatology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Pathum Wan, Bangkok, 10330 Thailand*

[3] *Division of Dermatopathology, Department of Dermatology, Faculty of Medicine, Siriraj Hospital Bangkok, Mahidol University, Bangkok Noi, Bangkok, 10700 Thailand*

[4] *Department of Anatomical Pathology, Faculty of Medicine, Rangsit University, Ratchathewi, Bangkok, 14000 Thailand*

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Abstract

Discoid lupus erythematosus (DLE) can be clinically and histologically challenging to diagnose. Some laboratories provide immunofluorescence testing, but this requires fresh tissue. C3d staining for the diagnosis of DLE in formalin-fixed paraffin-embedded specimens was used to determine the sensitivity and specificity of detection of this complement protein. We tested 59 cases of DLE and a control set composed of 59 cases of lichen planus (LP), 9 cases of LP-like keratosis, 5 cases of cheilitis, and 3 cases of mucositis. Blinded assessment of the C3d staining was carried out by three pathologists. Fisher's exact test, the independent *t*-test, and kappa analysis were used for analyzing the sensitivity and specificity of C3d, which were 44% and 97%, respectively. C3d staining in dermoepithelial junctions confirmed DLE and excluded LP. C3d staining in DEJ is specific for DLE but has low sensitivity.

Keywords: C3d, discoid lupus erythematosus, formalin-fixed paraffin-embedded specimens, specificity, sensitivity

1. Introduction

Discoid lupus erythematosus (DLE) is a type of chronic cutaneous lupus erythematosus that has been reported to develop into systemic lupus erythematosus (SLE) in about 20% of cases. Moreover, 68% of patients with SLE present with DLE (Gronhagen, Fored, Granath, & Nyberg, 2011). DLE typically presents as an annular erythematous violaceous plaque, skin atrophies, follicular plugging, and scarring (Okon & Werth, 2013). However, paraffin-embedded tissues are needed to confirm diagnoses of atypical clinical presentations using histopathology, with additional fresh tissue required for direct immunofluorescence (DIF) (Bharti *et al*., 2015). The most common differential diagnoses for DLE are lichen planus (LP), psoriasis, lymphocytoma cutis or benign reactive lymphoid hyperplasia, cutaneous T cell lymphoma, and sarcoidosis, which are generally distinguished using histopathology (Okon, & Werth, 2013). However, LP sometimes has pathological features that overlap with DLE features. In these cases, diagnosis can be confirmed by immunofluorescence (Hussein, Aboulhagag, Atta, & Atta, 2008). Currently, the immunohistochemistry (IHC) protocol

*Corresponding author
Email address: dr.apasee@gmail.com

used to distinguish between DLE and LP consists of staining for the proteins CD20, CD34, and CD3 on the surface of leukocytes and antigens of other cells, including endothelial cells (Hussein *et al*.,2008; Ramezani *et al*., 2017). The counting of stained cells can be subjective, with inter-rater variability between pathologists and between sections of tissues used for examination. In this study, we aimed to find a simple procedure to diagnose DLE using paraffin-embedded, formalin-fixed tissues (FFPE), which might be useful when additional immunofluorescence is not possible. Magro *et al*. tested staining for C3d, a degradation product of the classic and the alternative complement pathway, in multiple inflammatory skin disease specimens and found positive staining at the dermo-epidermal junction (DEJ) in samples from patients with DLE. In contrast, the staining was negative in patients with LP. Unfortunately, their sample size was too low to draw a robust conclusion (Magro & Dyrsen, 2008). Thus, in this study we stained for C3d with a larger sample size to determine the sensitivity and specificity of immunohistochemistry-based C3d staining for distinguishing between DLE and LP.

2. Materials and Methods

2.1. Patients and samples

We retrospectively collected cases with both clinically and histopathologically confirmed diagnosis of DLE with or without systemic lupus erythematosus (SLE), LP, lichen planus-like keratosis (LPLK), cheilitis, and mucositis between January 2015 and July 2019 by searching the electronic medical records of the Department of Dermatology, Chulalongkorn University Hospital. SLE was defined according to the American College of Rheumatology (ACR)-97 classification criteria (Bakula Čikeš, & Anić, 2019). The criteria for histopathological diagnosis of DLE were hyperkeratosis, basement membrane thickening, follicular damage, and perivascular leukocyte infiltration involving the deep dermis (Haber, Merola, & Werth, 2016). We then performed the diagnostic test on the selected cases using an antibody against C3d on paraffin-embedded, formalin-fixed tissue. Moreover, we collected the available DIF data required. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2.2. DIF study

Additional fresh tissues from DLE and LP biopsies were embedded in freezing medium and sectioned in a cryostat (1). After cryosectioning, tissues were stained with fluorescent antibodies against C3d, IgG, IgM, IgA, and fibrinogen (dilution 1:30, all antibodies rabbit anti-human fluorescein-conjugated monoclonal antibodies, Dako A/S, Glostrup, Denmark) (Shen *et al.*, 2012). We retrospectively retrieved this information from the electronic medical record database of our hospital.

2.3. IHC

The C3d antibody (Polyclonal Rabbit Anti-Human C3d Complement, Dako-A0063) was diluted 1:500 and incubated with the samples for 32 minutes using the Ventana Benchmark XT automatic system, following the manufacturer's protocol, with the following parameters: mild antigen retrieval and ultra-view brown detection, antigen retrieval CC1, and high pH buffer. We used acute rejected kidney transplant tissues as positive controls and universal negative control - rabbit antibodies (N1699; DAKO) as negative controls to detect processing errors that could lead to false-negative or false-positive staining, respectively. The IHC results for C3d deposition along the DEJ were submitted for independent, blinded evaluation to three pathologists (two dermatopathologists and a general pathologist). The inter-rater agreement was very high (0.87, 95% CI = 0.83-0.90). The patterns of deposition were classified as linear or granular located entirely or focally at the DEJ. The grading of immunoreactive deposition along the DEJ was subjectively assessed with the following scale: absent (0), mild (1), moderate (2), and marked (3). Staining at adnexal structures was also recorded. Otherwise, they were evaluated as background staining.

2.4. Statistical analysis

We analyzed the sensitivity and specificity of C3d staining for the diagnosis of DLE using a 2 × 2 table, defining DLE as the test group and other cases (59 LP, 9 LPLK, 5 cheilitis, and 3 mucositis) as the control. Sensitivity referred to the proportion of DLE cases that were C3d-positive. Specificity referred to the proportion of other cases that were C3d-negative. The independent *t*-test and Fisher's exact tests were used to compare continuous and categorical data, respectively. We measured inter-rater agreement by kappa analysis. SPSS version 22 software was used for all analyses. A P-value less than 0.05 was considered as statistically significant.

3. Results

From a total of 160 cases, we excluded the inadequate, duplicate, and diagnostic unclear specimens (clinical-pathological discrepancy). We ended up with 135 samples, including 59 DLE and 76 other cases (59 LP, 9 LPLK 5 cheilitis, and 3 mucositis). The demographic data for the cases included in this study are shown in Table 1.

Of 59 DLE cases, 26 cases (44%) were C3d positive at the DEJ with mild (18.6%) to moderate (23.7%) intensity when compared to the control group ($P = 0.001$ and $P < 0.001$, respectively). The most common staining pattern was a homogeneous brownish line along the entire DEJ (68%) (Figure 1). Other observed staining patterns were granular dots along the DEJ (4.5%) and a focal deposit in a granular (4.5%) and linear (4.5%) pattern along the DEJ. Statistically significant periadnexal and perieccrine staining was also observed ($P = 0.033$ and $P < 0.001$, respectively). In total, 43 of 59 DLE cases had complete histories recorded, whether the patient had systemic involvement or not. Furthermore, of 43 DLE cases, 14 were C3d-positive and 29 were C3d-negative. Moreover, 4 of 14 C3d-positive DLE cases had a recorded history of systemic involvement, whereas 20 out of 29 (68.96%) negative cases had no systemic involvement (sensitivity 30.8%, specificity 66.7%, $P = 1$). The most common systemic involvement was lupus nephritis (46.2%).

Table 1. Demographic data

Parameter	Total (n = 135)	DLE (n = 59)	Others[#] (n = 76)	P-value
Age, mean ± SD.	48.82 ± 17.76	43.28 ± 15.14	53.05 ± 18.53	0.001*
Sex, n (%)				
Female	101 (74.8%)	49 (83.1%)	52 (68.4%)	0.072
Male	34 (25.2%)	10 (16.9%)	24 (31.6%)	0.072
Location, n (%)				
sun-protected area	12 (11.5%)	4 (8%)	8 (14.8%)	0.5503
sun-exposed area	21 (20.2%)	19 (38%)	2 (3.7%)	<0.001*
mucosal area	3 (2.9%)	0 (0%)	3 (5.6%)	0.256
scalp area	26 (25%)	13 (26%)	13 (24.1%)	0.5142
lip area	22 (21.2%)	3 (6%)	19 (35.2%)	0.002*
unidentified and other areas	20 (19.2%)	11 (22%)	9 (16.7%)	0.331

Control set including lichen planus (LP), lichen planus-like keratosis (LPLK), cheilitis, and mucositis cases.
*Statistically significant P-value (< 0.05) for comparison of values between the groups, DLE and Others

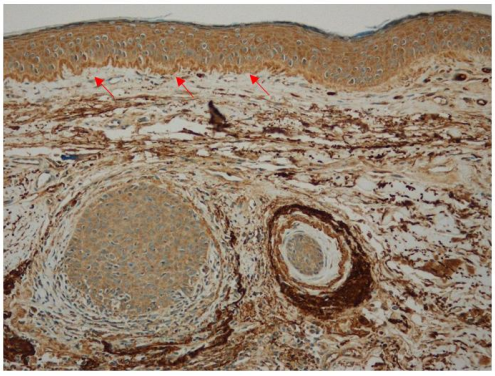

Figure 1. Immunohistochemistry of a C3d-positive discoid lupus erythematosus (DLE) sample showing a deposit in a homogeneous linear brownish line entirely along the dermo-epidermal junction. Magnification: 200×

Other organ involvements observed were the hematologic system (30.8%), the musculoskeletal system (7.7%), and unidentified organ systems (15.4%). The C3d positive and negative cases had a mean antinuclear antibody titer (ANA) of 1:1900.6 and 1:1209.4, respectively (P = 0.407). The numbers of DLE-positive and -negative cases in different areas are shown in Table 2.

Table 2. Location of C3d-positive and –negative discoid lupus erythematous (DLE) samples within the body

Location, n (%)	C3d Positive DLE (n = 26)	C3d Negative DLE (n = 33)	P-value
sun-protected area	2 (50%)	2 (50%)	1
sun-exposed area	6 (31.6%)	13 (68.4%)	0.263
mucosal area	0 (0%)	0 (0%)	N/A
scalp area	8 (61.5%)	5 (38.5%)	0.209
lip area	1 (33.3%)	2 (66.7%)	1
unidentified areas and others	9 (45%)	11 (55%)	1

In the 76 control cases, 74 (97.36%) were negative for C3d staining, while only 2 (2.6%) were C3d positive, with mild intensity (Figure 2). The staining pattern of the two C3d-positive non-DLE cases was a non-specific linear brownish line above the DEJ, as shown in Figures 3 and 4.

The positive control for acute rejected kidney transplant tissues exhibited peritubular capillary staining as shown in Figure 5. The universal negative control rabbit antibodies were negative, as shown in Figure 6.

The overall diagnostic performance of C3d staining was compared to that of traditional diagnosis using Hematoxylin and Eosin staining (135 cases) and is summarized in Table 3. The sensitivity, specificity, positive predicting value (PPV), negative predictive value (NPV), and accuracy of C3d detection were 44%, 97%, 93%, 69%, and 74%, respectively. Compared with DIF (19 cases), the sensitivity, specificity, PPV, and NPV values of C3d staining were 33.3, 85.7, 80.0, 42.9, and 52.6%, respectively, (P = 0.18).

4. Discussion

DLE has a complex overlap with SLE that includes genetic, environmental, and immune factors (Kulik *et al*., 2019). Abnormal nucleic acid metabolism in cutaneous lupus erythematosus is one of the mechanisms that cause the autoantibody-mediated formation of immune complexes along the basement membrane zone (BMZ), triggering activation of the complement cascade (Günther, 2019; Achtman & Werth, 2015). By immunofluorescence, it is possible to observe the third component of complement (C3) at BMZ *in vitro* (Gammon, 1983). However, this technique cannot be used on FFPE (Nasr, Fidler, & Said, 2018). C3 is a component of the classic and the alternative pathway which is converted into the active forms C3a and C3b, leading to the formation of the membrane attack complex. C3d is the final form that permanently binds to the tissue (Stowell *et al*., 2012). C3d is the ligand for co-receptors in the B cell activation pathway that can prolong inflammation in DLE patients (Kulik *et al*., 2019).

In contrast, other pathologies analyzed in this study have different etiologies. LP is caused by a $CD8^+$ T cell-mediated basal cell keratinocyte injury. In contrast to DLE, complement deposition along DEJ is not a typical feature of this disease, whereas fibrinogen deposits along DEJ and IgM, as well as C3 and C4 deposits at the colloid body are usually

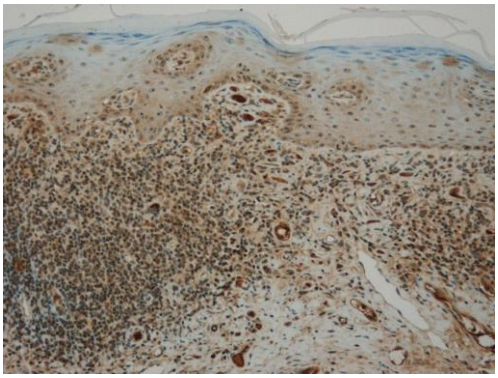

Figure 2. Immunohistochemistry of a C3d-negative lichen planus (LP) sample at the dermo-epidermal junction. Magnification: 200×

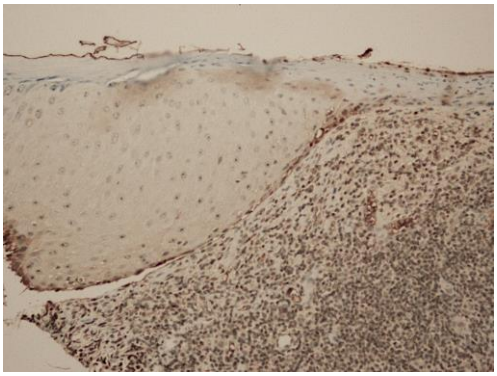

Figure 3. Immunohistochemistry of the false-positive lichen planus showing C3d staining above the dermo-epidermal junction, not at the dermo-epidermal junction. Magnification: 200×

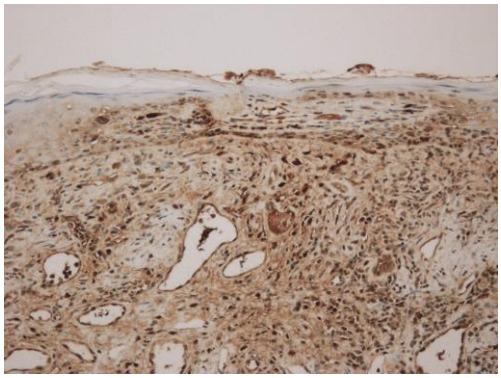

Figure 4. Immunohistochemistry of a C3d-positive mucositis sample above the dermo-epidermal junction. Magnification: 200×

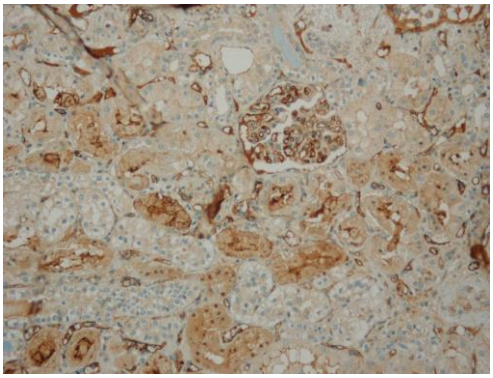

Figure 5. Positive control for acute rejected kidney transplant tissues shows peritubular capillary staining. Magnification: 200×

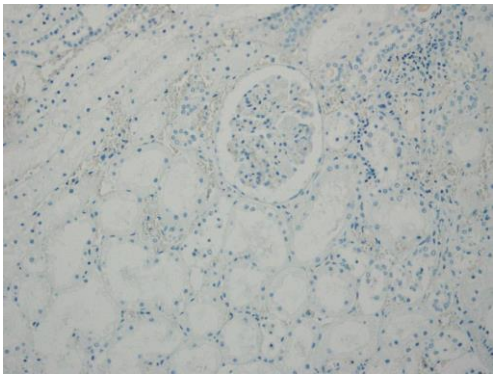

Figure 6. Universal negative control from rabbit antibodies demonstrates empty staining. Magnification: 200×

observed (Gupta & Jawanda, 2015). As in LP, cytotoxic T cells also play a pivotal role in LPLK (Bayer-Garner, Ivan, Schwartz, & Tschen, 2004). In mucositis, one of differential diagnoses of DLE from the oral mucosa, a recent study suggested the involvement of bacterial translocation and endotoxin in the initiation of the innate immune response (Bowen *et al*., 2019).

Our study stems from the observation that C3d is present in the basement membrane zone (BMZ) in DLE but should be absent in LP, LPLK, and mucositis. Indeed, C3d staining showed high specificity and accuracy for distinguishing DLE samples from most of the control samples. Since C3d is a marker of complement activation (Thurman *et al*., 2013), it is very likely that the complement pathway plays a more significant role in DLE than it does in the other diseases. The performance of immunohistochemical staining may change depending on the type of antibody used (Thurman *et al*., 2013). Previous data showed that a rabbit IgG

Table 3. C3d staining performance compared to that of traditional hematoxylin and eosin staining (135 cases)

Performance C3d	Diagnosis		Statistics						P-value
	DLE	Others#	n	Sensitivity	Specificity	PPV	NPV	Accuracy	
Positive Negative	26 33	2 74	135	44.1%	97.4%	92.9%	69.2%	74.1%	< 0.001*

*****Statistically significant P-value (<0.05).
Control set including lichen planus (LP), lichen planus-like keratosis (LPLK), cheilitis, and mucositis cases

polyclonal anti-C3d antibody yielded better results than did a mouse IgM and three mouse IgG monoclonal anti-C3d antibodies (Chaplin & Monroe, 2009). This discrepancy could be due to the use of different peptide cross-linking designs, resulting in different antigen-antibody matching that affected sensitivity and specificity (Namiki, Valencia, Hall, & Hearing, 2008). The challenge for staining-based diagnostic methods is to improve signal and reduce noise (non-specific binding) at the same time. In our study, staining for C3d as a marker had a low sensitivity. Perhaps because specimens had been in storage since January 2015 and had been stained starting from August 2019, storage could have caused a loss of antigen which affected the sensitivity of the antibody staining.

Erythrocyte-bound C3d (E-C3d) is a complement-activated feature associated with disease activity and disease severity in SLE, as recorded by the Systemic Lupus Activity Measure (SLAM) index (Kao *et al*., 2010; Mikdashi & Nived, 2015). Owing to the retrospective design of our study, we could not assess the correlation between the presence of C3d in the skin with the SLAM index, but we found that C3d staining at DEJ was not significantly associated with systemic involvement. It has been reported that ANA titer-specific PPV for SLE increases with the antibody level (Willems *et al*., 2019). Our C3d-positive DLE samples had a higher mean ANA titer level than did the negative samples; however, this difference was not statistically significant. Interestingly, this is the first study reporting that the skin area from which the biopsy was taken, such as sun-protected, sun-exposed, scalp, and lip, does not significantly impact the C3d staining.

In conclusion, the presence of C3d at DEJ is correlated with DLE with high specificity and accuracy, relative to other diseases such as LP and LPLK. The result does not depend on the biopsy site. We found that 28% of patients with DLE showing C3d-positive staining had systemic involvement, mostly lupus nephritis, and were associated with a high level of ANA titer, although the latter is not statistically significant. The limitations of this study are related to the retrospective study design and incomplete data on DIF, disease severity, and disease activity. Additionally, the long-term storage of FFPE specimens may have had an impact on the antibody performance, which explains the low sensitivity. Future prospective controlled studies could more accurately validate the use of C3d staining as a diagnostic marker. Recently, an SLE murine model showed that the disruption of the critical C3d–ligand-receptor binding step is sufficient to block autoimmunity and ameliorate renal disease (Kulik *et al*., 2019), thus suggesting that C3d is a promising marker for remediation. Our study confirms the presence of C3d at DEJ in DLE and supports the use of this marker both for diagnosis and as a potential therapeutic target.

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