Original Article

Direct immunofluorescence of skin biopsy: Perspective of an immunopathologist

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ABSTRACT

Background: By direct immunofluorescence (DIF), presence of immune complexes in the skin biopsy at various locations such as the dermo-epidermal junction, dermal blood vessels, etc. help to arrive at a diagnosis. **Aims:** (1) To study the role of DIF in confirmation or exclusion of diseases involving skin vis-à-vis histopathology and clinical diagnosis, (2) to describe the annual spectrum of dermatologic conditions that present to a tertiary referral center and require DIF examination of skin biopsy for confirmation of diagnosis. **Methods:** A total of 267 biopsies received over a period of 16 months in the Department of Immunopathology were analyzed along with clinical and histopathological details and the correlation between them was studied. **Results:** DIF was positive in 204 skin biopsies. Of these, 127 biopsies showed good clinico-immuno-histopathological correlation. In 10 cases, only DIF could clinch the diagnosis. In another nine cases, immune deposits were noted, which were unexpected in light of clinical and histopathological diagnosis. The most common skin involvement was seen in vasculitides. DIF was, however, non-contributory in lesions like erythema multiformè, post Kala-azar dermal leishmaniasis, sarcoidosis, lupus vulgaris, pyoderma gangrenosum and prurigo nodularis. **Conclusion:** The DIF of skin in conjunction with histopathology gives the best diagnostic yield. It is invaluable in confirming the diagnosis of small vessel vasculitides and bullous lesions of skin and can be used as an additional tool to pinpoint the diagnosis of systemic and localized autoimmune diseases involving the skin.

Key words: Direct immunofluorescence, vasculitis, immune blistering disorders of skin, lupus band test, cytoid body

INTRODUCTION

Skin forms not only a protective covering but is a part of the specialized immune apparatus of the body. Immune perturbations as a part of disease pathogenesis are reflected in the skin and compared with other organ systems of the body. It is easily accessible for biopsy. Apart from well-defined skin lesions that are diagnosed by a biopsy, many systemic conditions such as systemic lupus erythematosus (SLE) and other autoimmune diseases and systemic vasculitis can be diagnosed by a skin biopsy. By direct immunofluorescence (DIF), presence of immune complexes in the skin biopsy at various locations such as the dermo-epidermal junction (DEJ), dermal blood vessels, etc. help to arrive at a diagnosis. The present study is undertaken to present an experience with 267 biopsies studied over a period of 16 months (September 1998–December 1999) at the Department of Immunopathology. The aim of this study is to analyze the contribution of immunofluorescence in diagnosing bullous and non-bullous lesions of the skin in comparison with histopathology and clinical diagnosis. The study is also undertaken to analyze the annual spectrum of lesions in the skin that are amenable to biopsy and are referred to an immunopathologist for definite diagnosis. This would help in understanding the relative prevalence of different skin lesions presenting to a tertiary care center, North of Delhi, where approximately 1% of the patients attending the dermatology division annually require a DIF examination of skin biopsy.

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METHODS

A total of 267 biopsies were received over a period of 16 months in the Department of Immunopathology (September 1998–December 1999). The clinical data were collected from the files of the Department of Dermatology and the same was recorded along with the reports of histopathological diagnosis. All the biopsies were obtained in holding fluid (Michelle's medium) containing a saturated solution of ammonium sulfate in buffer at room temperature and stored at 4˚C until cut. Before cutting, the biopsies were washed thrice in phosphate-buffered saline (PBS) (pH 7.2) for 15 min each time. For the frozen section, the tissue was embedded in OCT medium and 4–5 micron sections were cut (minimum 10 sections). Two sections were layered on each slide and the slides were stored at -20˚C until being stained. For staining, sections were brought to room temperature. Optimally diluted fluorescein isothiocyanate (FITC)-labeled monospecific immunoglobulins (IgG, IgA, IgM, C_3) were layered onto the sections and incubated at 37˚C for 45 min–1 h. Then, the sections were washed in PBS (pH 7.2, 0.1 M) thrice and mounted in buffered glycerin and finally viewed under a Nikon OPTOHOT-2, UV microscope. While reporting, the following parameters were noted:

(1) Nature of immune deposits: IgG, IgA, IgM, C_3
(2) Site of immune deposits: DEJ/intercellular spaces (ICS) in epidermis/blood vessels/hair shaft/cytoid bodies
(3) Semiquantitative grading of strength of fluorescence: + to ++++
(4) Pattern of immune complex deposits: granular or linear

Those biopsies that were dried up/formalin fixed (one case) or were inadequate (no epidermal lining included, seven cases) and those in which either the clinical details or the histopathological findings (55 cases) were unknown were excluded. The study group comprised of 204 biopsies. Fluorescence diagnosis was categorized into the following major diagnostic labels:

(1) **Vasculitis:** Immune complex vasculitis (ICV)
Henoch Schonlein Purpura (HSP)
(2) **Bullous lesions of the skin with immune deposits at the DEJ:**
Bullous pemphigoid (BP)
Pemphigoid gestationis (PG)
Dermatitis herpetiformis (DH)
Linear IgA dermatosis
Chronic bullous disorders of childhood (CBDC)
(3) **Intra-epidermal bullous lesions of the skin with ICS positivity:**
Pemphigus vulgaris
4) **Lupus band test (LBT)*-positive conditions with or without vasculitis:**
Discoid lupus erythematosus (DLE)
SLE
Mixed connective tissue diseases (MCTD)
Scleroderma and progressive systemic sclerosis (PSS)
Rheumatoid arthritis (RA)
Others
(5) **Cytoid body positivity:**
Lichen planus
Other lichenoid lesions
(6) **Immune deposits with hair shaft positivity**
(7) **No significant immune complex deposits**

*To be considered a positive LBT, deposition of IgM in sun-exposed skin should assume a continuous band over at least 50% of the specimen and be at least moderate in intensity.[1]

Finally, fluorescent findings of all cases were compared with the clinical and histopathological diagnosis and the correlation between them was studied.

RESULTS

The study group comprised of 204 skin biopsies. Of these 204 biopsies, 51 belonged to the pediatric population (0–14 years of age). The male to female ratio was 1:1.2. Of 204, DIF showed positive findings in 151 skin biopsies, whereas no significant immune complex deposits were noted in 53 biopsies. These 151 cases were divided into three groups:

Group 1: comprising of 132 cases where DIF diagnosis was consistent with clinical diagnosis [Table 1, Figures 1-4].

Group 2: comprising of 10 cases where definite DIF diagnosis was possible with the reexamination of detailed clinical records, other lab parameters and a relook of histopathology in light of positive DIF findings. In all these cases, the final diagnosis was different from the clinical diagnosis suggested by the clinician [Table 2].

Group 3: comprising of nine cases that showed false-

Table 1: Clinical, immunological and histopathologic findings in 185 skin biopsies (excluding groups 2 [n=10] and 3 [n=9])

Disease Entity	Clinical diagnosis	DIF findings		Consistent histopath diagnosis	
		Positive	Negative	Positive	Negative
ICV	45	41	4	35	10
HSP [IgA +ve DIF rings in vessel wall]	26	23	3	21	5
DLE	22	13	9	18	4
SLE	14	10	4	7	7
MCTD	3	3	0	0	3
SS	2	2	0	2	0
RA	1	1	0	0	1
PV	22	18	4	16	6
BP	13	7	6	10	3
PG	1	1	0	0	1
DH	5	3	2	3	2
LP	7	4	3	3	4
Other lichenoid lesions	6	4	2	2	4
Lupus profundus	2	1	1	2	0
EM	3	0	3	2	1
EN	3	0	3	0	3
PKDL	3	0	3	2	1
BT leprosy	1	0	1	1	0
Sarcoidosis	1	0	1	1	0
Lupus vulgaris	1	0	1	1	0
Pyoderma gangrenosum	1	0	1	1	0
Prurigo nodularis	1	0	1	0	1
Pseudopelade	2	1	1	0	2

EM, erythema multiforme; EN, erythema nodosum; PKDL, post Kalazar dermal leishmaniasis

Table 2: Clinical and histopathological details of cases in Group 2 (n=10)

Definite DIF diagnosis	Suggested clinical diagnosis	Histopathological diagnosis
PV	Mucosal lesion	Descriptive
PV	Mucosal BT leprosy	Descriptive
BP	Reticular necrosis	Descriptive
BP	DH	DH
SLE	Dermatitis	Dermatitis
SLE	DLE	Cicatricial alopecia
SLE	Vasculitis	Descriptive
DLE	Drug-induced vasculitis	Non-specific changes
DLE	Polyarteritis nodosa	Non-specific changes
Linear IgA dermatosis	DLE	DLE

positive DIF findings that were unexpected in light of the clinical and histopathological diagnosis [Table 3].

Table 1 shows that 77% of the clinically suspected cases were found to have the same condition as diagnosed on DIF (clinico-immunological correlation). Seventy percent (70%) were further corroborated on histological examination of formalin-fixed paraffin-embedded skin biopsy (clinico-immunohistological correlation). Thus, it emerges that in 7% of the

Table 3: Details of cases in Group 3 where unexpected findings were noted on DIF (n=9)

Age/sex	Clinical details	Clinical diagnosis	Histopath diagnosis	Fluorescence findings
16 years/F	Treated case of BT leprosy, now presenting with alopecia and anesthesia over the forehead and scalp	Type 1 lepra reaction	Non-specific changes	Band test +ve with IgG and IgM
7 years/M	Treated case of ALL, now developed purpuric eruptions over arms, legs and buttocks with joint pains x 25 days	Vasculitis	LCV	Band test +ve with IgG along with ANF *in vivo* speckled (+)
22 years/M	Bilateral symmetrical erythematous violaceous nodular lesions on the shin	Erythema nodosum	Erythema nodosum	Band test +ve with C_3
40 years/F	Case of Churg-Strauss syndrome	-	LCV	Band test +ve with IgG and IgM along with vasculitis. ANA negative and pANCA ++++
2 years/F	Diffuse stiff arms, legs, trunk and face associated with bending down of skin and contracture of fingers	Pansclerotic morphea of childhood	Non-specific changes	Hair shaft +ve with IgG, IgA and IgM
60 years/F	Multiple hyperpigmented plaque lesions over chest, lower back and scalp with central depigmentation, atrophy and scarring	DLE	DLE	Cytoid bodies at DEJ
26 years/M	Recurrent erythematous papular lesions over acral parts with depigmentation and target lesions	EM	EM	Cytoid bodies at DEJ
12 years/F	Multiple crusted erosions and ulceration over face, photosensitivity with edema of face, hands and feet	SLE	SLE	Cytoid bodies at DEJ with ANF *in vivo*
2 years/M	Multiple well-defined erosions with crusting over face, lower limbs, buttocks, hands and face	Vasculitis	LCV	Cytoid bodies at DEJ

biopsies histopathology could not demonstrate features to support the immunofluorescence findings (immunohistological dyscorrelation). DIF findings in histopathology-consistent cases of cicatricial alopecia helped us to confirm the final diagnosis. Of three cases examined, in two cases the presence of globular cytoid bodies on DIF helped clinch the diagnosis of lichen planus while in the third case full-house LBT with vasculitis confirmed the diagnosis of SLE.

Table 2 enlists the cases where DIF played the principal role in accurately diagnosing the cases. Two more cases of PV could be picked on DIF without clinical and histological proof. Two additional cases of BP were diagnosed on DIF even when there was no clinical or histological proof. Clinically, these two cases of BP were mislabeled as reticular necrosis and DH. Three additional patients were given a diagnosis of SLE by DIF and none of these three cases showed consistent histopathological findings. Two cases could be diagnosed as DLE only on the basis of positive DIF findings when it was not suspected by the clinician and showed non-specific changes on histopathology. One case of linear IgA dermatosis was diagnosed by DIF and had been clinically as well as histopathologically wrongly labeled as DLE.

LBT

DLE: IgM was detected in 14 of 15 specimens (93.3%), IgG in 10 of 15 (67%), IgA in five of 15 (33 %) and C_3 in nine of 15 (60%). Full-house pattern with C3 was seen in two of 15 patients (13%).

SLE: Most commonly detected individual Ig was IgM in 11 (85%) of 13 patients. IgM and IgG was the most common pair expressed with 10 (77%) of 13 patients. The most common triplet was IgM, IgG and C_3 in six patients (46%). Full-house LBT with C_3 was seen in three (23%) of 13 patients. Associated vasculitis was noted in four cases.

MCTD: Full-house LBT with C_3 was seen in one (33%) of three biopsies.

PSS: Full-house LBT with C_3 was seen in one (33%) of three biopsies.

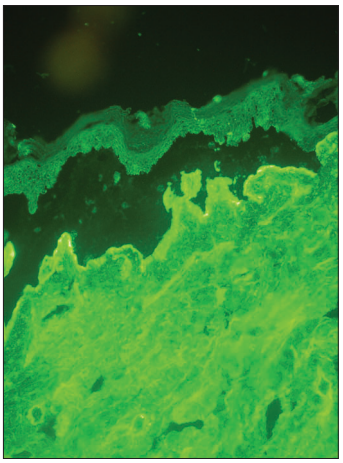

Figure 1: Direct immunofluorescence photomicrograph of a case of systemic lupus erythematosus showing dense granular (++++) deposits of immunoglobulin G at the dermo-epidermal junction of the skin biopsy (x200)

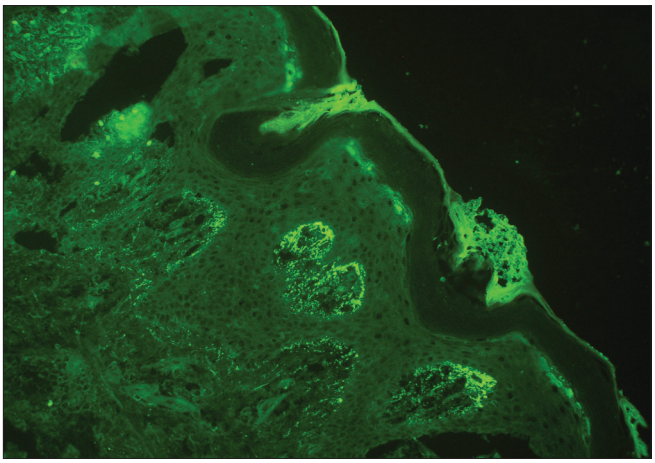

Figure 2: Direct immunofluorescence photomicrograph of a case of dermatitis herpetiformis showing granular (++++) deposits of immunoglobulin A at the dermo-epidermal junction with suprapapillary accentuation (x200)

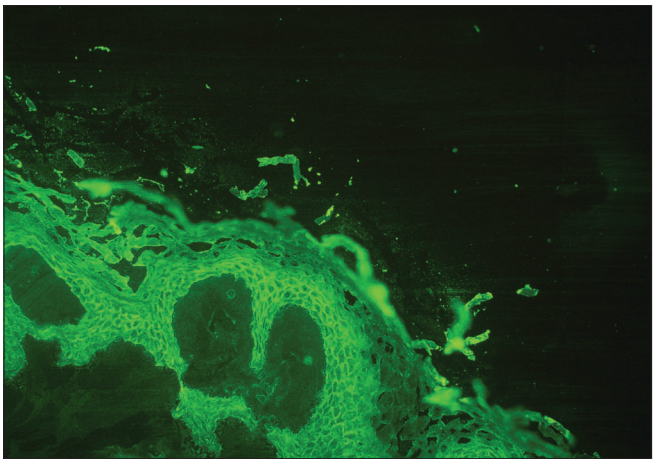

Figure 3: Direct immunofluorescence photomicrograph of a case of PV showing granular (+++) intercellular deposits of immunoglobulin G in the epidermis (x200)

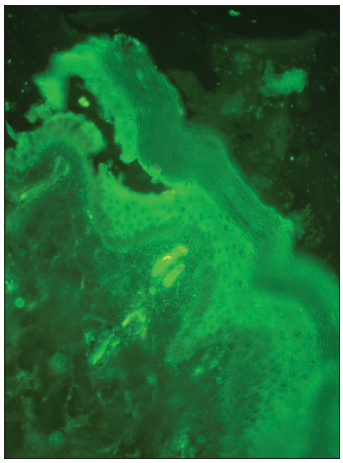

Figure 4: Direct immunofluorescence photomicrograph of a case of Henoch Schönlein purpura showing granular (+++) deposits of immunoglobulin A in the walls of capillaries in the upper dermis (x400)

RA: One case of RA showed band test positivity with IgM and C_3. Vasculitis was also noted.

Band test was seen in five cases of ICV in addition to immunoreactants in blood vessels. False-positive LBT was noted in few of the following cases: BT leprosy (1), treated case of acute lymphoid leukemia (1) and one case each of erythema nodosum and Churg-Strauss syndrome [Table 3].

ANF *in vivo*

ANF *in vivo* was noted in three SLE, one MCTD, one PSS, one DLE, one ICV and one HSP patient.

Hair shaft positivity

One case of cicatricial alopecia showed globular cytoid deposits in the external root sheath of the hair follicle. One case of pemphigus vulgaris also showed hair shaft positivity in addition to positivity in ICS. Hair shaft positivity was also noted in one case of pansclerotic morphea of childhood as a solitary DIF finding [Table 3].

Cytoid body positivity

It was seen in four cases of lichen planus and four lichenoid lesions (four cases of lichenoid drug rash). It was also seen in two cases of ICV, one case of HSP and two cases of DLE in addition to expected DIF findings. One case of SLE, one case of DLE, one case of erythema multiforme and one case of ICV (all clinically suspected and histologically proven) showed only cytoid body positivity, as shown in Table 3.

Table 4: Immunofluorescence patterns in LE patients

Immunofluorescence pattern	SLE (13)	DLE (15)
LBT alone	6	15
LBT with vasculitis	3	-
LBT with ANF *in vivo*	3	-
LBT with vasculitis with ANF *in vivo*	1	-
Immunoreactant positivity		
IgG	10	10
IgM	11	14
IgA	3	5
C3	6	9

Table 4 shows the various immunofluorescence patterns in SLE patients along with the immunoreactant positivity.

DISCUSSION

Analysis of 267 consecutive skin biopsies by a single observer over a period of 16 months shows that the vasculitides form the most common referral to the immunopathologist for confirmation. It is the group of small vessel vasculitis, i.e. HSP and ICV, which can be detected only by DIF examination. Being a sensitive technique, DIF is able to detect 89% of clinically diagnosed HSP whereas ICV, which is found in diverse systemic conditions, could be confirmed by DIF in 91% of clinically suspected vasculitides. This study has demonstrated that the best clinico-immunological correlation is seen in the diagnosis of HSP [Table 1] thus reflecting the expertise and awareness of the referring clinician and the sensitivity and specificity of DIF. HSP is a condition commonly diagnosed among children in our center (22% of the children in this study). It is diagnosed largely by clinical criteria; however, confirmation is provided by DIF of skin biopsy.[2] DIF in all these cases showed IgA-positive DIF rings in the vessel walls of the upper dermis. By histopathology, the changes seen in HSP are indistinguishable from other forms of LCV. The importance of skin biopsy in confirming the diagnosis of HSP is reflected in the ability to effectively treat and follow-up these patients.[3]

Although DIF is a robust tool for studying skin biopsies suspected of vasculitis, concomitant histopathology provides increased sensitivity and specificity of detection. In our study, 6% of the skin biopsies from clinically suspected vasculitis patients were DIF negative and were picked up only on histopathology. In late lesions, or due to improper handling and storage of skin biopsies, the DIF test may be falsely negative.

The systemic connective tissue disorders, most of which have an autoimmune basis, form the next most common category of disorders that involve the skin. This study documents that LBT may be seen in connective tissue disorders other than SLE, DLE like MCTD, PSS and RA.

DLE was diagnosed in 15 patients on DIF whereas clinical suspicion was present in a total of 22 patients. Thus, in only 59% of clinically suspected patients, a band test was demonstrable. The reasons for this negativity may be several, including duration of lesions, their anatomic distribution (sun exposed or unexposed or truncal) and previous treatment[4]. In the present retrospective study, these factors could not be analyzed. It is important to note that in making a diagnosis of DLE, DIF is an essential tool as two cases could be diagnosed as DLE only on the basis of positive DIF findings, when it was not suspected by the clinician and showed non-specific changes on histopathology [Table 2]. On reexamination of detailed clinical records and of histopathology in light of positive DIF findings, the patients were found to be conforming to DLE.

Of the 14 biopsies analyzed from clinically suspected SLE patients, 10 (71%) showed LBT on DIF along with ANA positivity, whereas four patients showed no immune complex deposits on DIF, but histopathology was consistent. Negative DIF in skin biopsies in these four cases is probably due to biopsy of late presenting lesions where immune complexes are not demonstrable or due to some technical problems already mentioned.[4,5] And also, it is the experience of the authors that in treated SLE the skin biopsy may show no immune deposits. However, three additional patients were given a diagnosis of SLE by DIF primarily along with concomitant detection of ANA in our laboratory. None of these three cases showed consistent histopathological findings. Detailed clinical examination along with presence of characteristic "Lupus pattern" on rat liver tissue were used to clinch the diagnosis of SLE in patients where clinical diagnosis of DLE was suspected. The diagnosis of SLE was finally made using the ACR criteria. Clinical records of all these patients were rechecked according to ACR criteria in light of DIF findings.

To the best of our knowledge, this is the first study

from India that demonstrates that a full-house pattern of immune deposits (IgG, IgA and IgM) and C_3 can be seen in MCTD and PSS. In addition, one case of MCTD also showed nuclear keratinocyte decoration with IgG, similar to that observed by Magro *et al*. Magro and Crowson demonstrated nuclear keratinocyte decoration (*in vivo* ANF) with IgG and C_{5b-9} in all cases studied along with positive LBT in two of eight cases.[6]

Two cases of PSS that were included in the study showed positive LBT. A study shows that 13.5% of the patients with systemic sclerosis manifest a positive LBT, a finding that appears to herald a more aggressive course.[7] The present study shows that the DIF findings of deposition of Igs in the skin and other laboratory investigations along with clinical features help to pinpoint the final diagnosis in systemic connective tissue disorders. Histopathology is limited in its ability to detect any specific abnormality in these lesions [Table 1].

The role of DIF in bullous lesions of the skin has been well described in the last two decades. This study corroborates the teaching that DIF is mandatory for a proper diagnostic labeling of all bullous lesions of the skin. Thus, increased detection as well as confirmation of diagnostic labels such as PV, BP, PG and rarer conditions like linear IgA dermatosis, DH and CBDC is possible only on DIF. DIF was able to detect 70% of clinically diagnosed vesiculobullous lesions of the skin in the present study.

Twenty-two cases clinically suspected as PV were found on DIF to be consistent with the clinical diagnosis in 18 cases. In four cases where histopathology demonstrated the lesions, DIF failed to show the same due to technical faults or treatment-induced changes. Because this is a retrospective analysis, this could not be clarified. Two more cases could be picked up on DIF without clinical and histological proof [Table 2]. Thus, DIF is a very reliable diagnostic test for pemphigus. It becomes positive at a very early stage and remains positive for a long period after clinical remission.[8]

A total of 13 cases of clinically suspected BP were analyzed by DIF, of which seven were consistent and two additional ones were picked up on DIF even when there was no clinical or histological proof. Clinically, these two cases were mislabeled as reticular necrosis and DH. Tables 1 and 2 show that histopathology or DIF alone has a poor sensitivity. In clinically suspected cases of BP, combined analysis yields a better diagnosis. False negativity in some cases is attributed to the longer stay of skin biopsies in the transport medium. This observation makes the use of fresh tissue the preferred substrate for DIF studies.[9] In addition, sensitivity of detection of BP can be increased by using more specific enzyme-linked immunosorbent assay to detect BP antigen in the serum.[10,11]

PG and linear IgA dermatosis are rare entities. One case of the former was suspected clinically and confirmed by DIF while the latter could only be diagnosed by DIF and was clinically as well as histopathologically wrongly labeled as DLE [Table 2].

Five clinically suspected cases of DH could be confirmed by both DIF and histopathology in three cases; however, in two cases, neither showed features of DH. The reason for this negativity can be due to obtaining the biopsy from the lesional site. Inflammation in lesional skin degrades the immunoreactants and is usually falsely negative for the diagnostic granular pattern. Because deposits are found throughout normal-appearing skin, the standard practice is to obtain biopsy specimens from normal-appearing perilesional skin for direct immunofluorescent staining. In the absence of the characteristic DIF pattern, one needs the combination of clinical, histologic and immunologic data to support the diagnosis of DH.[12]

In lichen planus and in lichenoid lesions, like lichenoid drug eruptions and lichenoides chronica, the only consistent finding was the presence of cytoid bodies. IgM was the most common immunoreactant found in cytoid bodies. However, cytoid bodies are found in a number of non-specific conditions, as shown in Table 3. Therefore, the diagnosis of LP should be correlated with histopathology. For improving the diagnostic sensitivity of LP by DIF, Kulthanan *et al.* have suggested that a combination of shaggy fibrin deposition at the DEJ and fluorescent cytoid bodies is more characteristic of LP.[13]

Table 3 shows conditions in which unexpected findings were detected by DIF.

The LBT, which was initially thought to be diagnostic only for SLE/DLE, has been found to be positive in a number of conditions, including BT leprosy, treated case of ALL with purpuric eruptions, erythema

nodosum and Churg-Strauss syndrome.[14] In Churg-Strauss syndrome, along with band test, blood vessels in the upper dermis showed immune complex deposits and the serum was 4+ pANCA positive. This is an interesting finding in that a pauci-immune crescentric glomerulonephritis is associated with skin lesions where immune complexes are demonstrable.

It is a known fact that cutaneous vasculitis can develop in association with hematologic malignancy and may follow, accompany or precede the condition. The cause of vasculitis could be attributed to malignancy itself, infection, medication and cryoglobulinemia.[15,16] In our case of ALL, therapy resulted in skin eruptions where band test and *in vivo* ANF (speckled pattern) were demonstrable.

The study proves that presence of abundant cytoid bodies at DEJ is associated with a diagnosis of lichen planus and other lichenoid lesions but can be seen as a non-specific finding in a number of conditions [Table 3]. However, DIF studies may be helpful in disease differentiation for cases with no specific clinical or histologic characteristics or with ambiguous features of other diseases, e.g. SLE.[13]

A brief mention may be made about the three cases who presented with clinical possibility of cicatricial alopecia. On histopathology, all these cases were diagnosed as consistent with cicatricial alopecia with no clue toward the underlying dermatologic disease. DIF was able to correctly clinch the diagnosis in these cases as lichen planus (two cases) and SLE (one case). This assumes importance in view of the fact that, one of the cases diagnosed as LP on DIF had been clinically suspected to be pseudopelade. Thus, DIF is of value in histopathologically inconclusive cases of cicatricial alopecia.[17]

The observation of immune complexes in the hair shaft is a rare finding as seen in Table 3. Here, explanation is difficult. One possibility is that a necrotic hair shaft in the vicinity of inflammation imbibes immune complexes and thereby shows positivity on DIF.

DIF of skin has no role to play in the diagnosis of erythema multiformè, post Kala-azar dermal leishmaniasis, sarcoidosis, lupus vulgaris, pyoderma gangrenosum and prurigo nodularis as evidenced from the immunofluorescence negativity in such cases [Table 1].

It is apparent from the above findings that although DIF is an extremely useful diagnostic tool, it should always be used in conjunction with histopathology and clinical features and the combination of three yields the best results. Changing trends, especially increase in all autoimmune diseases and with them involvement of skin, are increasing in the last decade. We are currently evaluating our decade old data to validate the same.

In the future, the skin that is seen to be involved in the range of conditions can be further studied by the powerful techniques of proteomics to develop new biomolecules that can be used as diagnostic and prognostic markers of diseases.

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