Direct Immunofluorescence Study in Thai Patients with Scleroderma

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Objective: *Previous reports of direct immunofluorescence (DIF) studies of the skin biopsies in scleroderma were either negative or positive at various percentages and patterns. The present study was designed to evaluate the positive yield and pattern of DIF in Thai patients with scleroderma and its possible clinical correlation.*

Material and Method: *Twenty-two patients with localized or systemic sclerosis, who attended the Department of Dermatology, Siriraj Hospital, from 1996 to 2002, were enrolled in the present study. Skin biopsy was performed for DIF studies.*

Results: *Nine out of 22 patients were diagnosed with systemic sclerosis (SS), eleven with morphea, and two with overlapping syndrome. Fifteen of 22 patients (68%) had positive DIF findings; seven of nine (78%) patients with SS, six of eleven (55%) patients with morphea and two of two (100%) with overlapping syndrome. The common sites of deposit in SS, morphea and overlapping syndrome were dermo-epidermal junction and epidermal nuclei. The common immunoreactant deposit in all groups was IgM. There was no significant difference in the comparison of DIF findings with duration of biopsy lesion, clinical correlation, and the positive result of serum antinuclear antibody (ANA) in the three groups of patients.*

Conclusion: *Positive DIF yield in the present study was higher than previous reports from Western countries. Similar to the study reported from Western country, there was no statistical significant difference in comparison of DIF findings with the duration of lesion, clinical correlation, and the positive result of serum ANA in our three groups of patients. However, patients with SS had a tendency to give more frequently positive ENS and DEJ deposits than those with morphea.*

Keywords: *Scleroderma, Immunofluorescence*

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Scleroderma is a chronic disease of unknown etiology that affects the microvasculature and loose connective tissue. It is characterized by fibrous deposition and obliteration of vessels in the skin, lung, gastrointestinal tract, kidney, and heart[1]. There are two clinical forms of scleroderma: the diffuse thickening and induration of the skin accompanied by fibrosis and vascular obliteration of the skin and internal organs is the systemic form or systemic sclerosis (SS) or systemic scleroderma, which is often progressive and fatal; localized scleroderma is not a life threatening disease but can cause disfigurement. The most common type of localized scleroderma is morphea, which is few in number and does not involve systemic organs[2].

Tests have been positive in some of these patients such as antinuclear antibody (ANA), anti Scl-70 etc. Biopsy of skin for direct immunofluorescence (DIF) study appears to be such a test. The DIF studies of skin biopsy specimens from patients with systemic scleroderma have yielded controversial results and the significance of these immunoreactant deposits remains unclear.

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Winkelmann et al[3] reported a negative DIF study in 10 patients with SS. However, nine patients with mixed connective tissue disease (scleroderma and myositis or lupus erythematosus) had an immunoglobulin (Ig) and complement (C) deposition at the dermo-epidermal junction (DEJ) or within the blood vessel walls (bl.vv.). Reimer et al[4] found granular IgM deposits at the basement membrane zone in 60% of 30 patients with SS, and speckled epidermal nuclear deposition in 23%. Chen et al[5] reported Ig deposits at the DEJ in normal skin of the forearm in 40% (6/15) of SS patients. Miike et al[6] found IgM fluorescence in blood vessels and along the DEJ in biopsied skin from a 5-year-old girl with linear scleroderma.

Shibeshi et al[7] reported from Poland that there was no correlation between positive DIF findings and clinical picture (acrosclerosis, diffuse scleroderma, and transitional form acrosclerosis-diffuse scleroderma).

The authors' objective was to study DIF findings in Thai patients with scleroderma and its possible clinical correlation.

Material and Method

From 1996 to 2002, the authors collected data of 22 patients with localized or systemic sclerosis on the basis of clinical and histological criteria at the Department of Dermaotology, Siriraj Hospital, Mahidol University, Bangkok, Thailand. The data were analyzed according to groups of the disease, age of lesion on the day the skin biopsy was performed, and DIF findings. Direct immunofluorescence studies of skin lesions were performed as previously described[8]. Briefly, skin biopsy specimens were embedded in the Cryomatrix embedding medium (SHANDON, USA) and snap frozen at -70°C until sectioned.The cryostat tissue section of 4 m each were air-dried and then washed twice with a phosphate-buffered saline (PBS), pH 7.4 for 10 minutes before being overlaid for 30 minutes with fluorescein isothiocyanate-conjugated rabbit anti-human IgG, IgA, IgM, and C3 (DAKKO patt, Denmark). Thereafter, section slides were incubated in a humidified chamber at room temperature, then washed twice with PBS for 10 minutes and mounted with a medium before viewing with a fluorescent microscope. Direct immunofluorescent findings were interpreted according to standard criteria[9]. A specimen was considered positive if it was stained at any of the following sites: DEJ, epidermal nuclear staining (ENS), colloid bodies, blood vessel, or appendage. Statistical analyses were conducted with SPSS for windows version 11.5.

Data were presented as number and percentage, mean $\pm$ SD. Chi-square tests or Fishers' Exact tests were used appropriately for category data. A p-value was set at 0.05 for significant level.

Results

Twenty-two patients were enrolled in the present study. Fourteen were female and eight were male, with a mean age of 42.8 years (range 8-72 years, SD $\pm$ 17.9). Nine were diagnosed as systemic sclerosis, 11 as morphea, and 2 as overlapping syndrome (both were scleroderma and SLE). The duration of the lesions at enrollment ranged from 1 month to 8 years (mean 1 year, SD $\pm$ 20.2). Males had more frequent positive DIF findings than females (Chi square test; $p = 0.02$). Table 1 shows clinical presentation of the patients with positive DIF findings.

Table 2 shows the details of immunoreactant deposits in the lesions of positive cases. The common sites of deposits in SS, morphea and overlapping syndrome were DEJ and ENS.

Table 3 shows details of immunoreactant deposits in lesions. Seven of nine patients with SS (78%) had positive DIF findings. Immunoreactant deposits were IgM at the DEJ (3/9; 33%), C3 at the DEJ (1/9; 11%), IgM at the colloid bodies (1/9; 11%), and IgG ENS (5/9; 56%). In mophea, six of eleven patients (55%) had positive DIF findings. These immunoreactant deposits were IgM (1/11; 9%), C3 (2/11; 18%) at the DEJ, IgM at the colloid bodies (1/11; 9%), C3 at skin appendage (1/11; 9%), one each (1/11; 9%) of IgG and IgA at the blood vessel wall and IgG ENS (1/11; 9%). In overlapping syndrome, two patients had positive DIF findings; the immunoreactant deposits were IgM at the DEJ (2/2,100%), and IgG ENS (1/2, 50%).

SS was slightly more positive in the percentage of immunoreactant deposits than morphea ($p = 0.05$). There was no statistically significant difference in the percentage of IgM deposits at the DEJ among the groups of patients with SS and morphea ($p = 0.28$). The common immunoreactant deposit at the DEJ in all groups was IgM.

The age of biopsy lesions had no influence on positive or negative DIF findings (unpaired t-test; $p = 0.33$). Positive serum ANA was 46% (10/22), i.e., 55% in SS, 27% in morphea, and both in the overlapping syndrome (100%). ENS and serum ANA patterns were commonly speckled (57% and 70%, respectively). There was no significant difference in the percentage of positive ENS between the SS and morphea groups (Fisher's Exact test; $p = 0.05$). No significant difference in the

Table 1. Clinical presentation of patients with a positive DIF study

Age (years)	Sex	Age of lesions: month	Site of lesions	DIF Finding						Clinical finding
				DEJ	Colloid	Blood vessel	Appendage	ENS	ANA	
38	M	2	Extensor forearm	-	IgA, IgM	-	-	+	+	Diffuse systemic sclerosis
60	M	4	Extensor forearm	C3	-	-	-	+	+	Diffuse systemic sclerosis
49	M	3	Volar forearm	IgM	-	-	-	-	-	Diffuse systemic sclerosis
48	M	3	Volar forearm	IgM	-	-	-	+	-	Diffuse systemic sclerosis
39	F	4	Volar forearm	IgM	-	-	-	-	-	Diffuse systemic sclerosis
68	M	5	Chest	-	IgM	-	-	+	+	Diffuse systemic sclerosis
34	F	5	Chest	IgM, C3	-	-	-	+	+	Diffuse systemic sclerosis
69	M	24	Hand	IgG, IgA	IgA	IgA	-	+	-	Generalized morphea
21	M	3	Extensor forearm	-	IgM	-	-	-	-	Localized morphea
27	F	3	Palm	IgM	-	IgM	-	-	-	Localized morphea
19	F	3	Leg	C3	-	-	-	-	-	Generalized morphea
8	M	48	Buttock	C3	-	-	C3	-	-	Localized morphea
42	F	12	Scalp	-	-	-	-	-	+	Localized morphea
31	F	12	Volar forearm	IgM	-	-	-	-	+	Overlapping syndrome (diffuse systemic sclerosis and SLE)
44	F	2	Arm	IgM	-	-	-	+	+	Overlapping syndrome (diffuse systemic sclerosis and SLE)

DIF = Direct immunofluorescent, DEJ = Dermoepidermal junction, ENS = Epidermal nuclear staining, ANA = Antinuclear antibody

percentage of positive serum ANA between the SS and morphea groups was detected (Fisher's exact test; p = 0.36). There was a weak correlation between ENS and with positive serum ANA (k = 0.34, p = 0.09).

Table 4 shows previous DIF studies in scleroderma compared with the present study. The authors' positive DIF yield was slightly higher than previous reports from Western countries. The frequency of positive DIF at the DEJ and/or ANA fixed in vivo reported from Western countries differs from 0 to 60%.

Discussion

The authors reviewed the clinical and DIF finding of 22 patients, nine with systemic sclerosis (SS), eleven with morphea, and two with overlapping syndrome (scleroderma and systemic lupus erythematosus; SLE). There were positive DIF findings in fifteen of twenty-two patients (68%).

Chen et al [5] reported an immunoglobulin (Ig) deposit at the DEJ in normal skin of the forearm in 40% (6/15) of SS patients. Reimer et al[4] reported granular IgM at the DEJ in sun-exposed skin in 60% (18/30) of their SS patients and in all patients with mixed connective tissue disease. They found granular IgM at the DEJ in the sun-protected uninvolved skin in six of eight mixed connective tissue disease patients, but did not observe any scleroderma. Jablonska et al[10] reported a weak IgM deposit at the DEJ in light-exposed skin of patients with systemic scleroderma and regarded this as a non specific finding. Shibeshi et al[7] reported positive DIF findings in 15.3% of 26 patients with acrosclerosis, 42.3% of 26 cases with diffuse scleroderma, and

Table 2. Details of immunoreactant deposits in lesions in positive cases (n = 15)

Location	Positive cases (%)		
	Systemic sclerosis	Morphea	Overlapping syndrome
Dermoepidermal junction			
DEJ	5/7 (71%)	4/6 (67%)	2/2 (100%)
DEJ and colloid bodies	-	-	-
DEJ and blood vessels	-	-	-
DEJ, colloids and blood vessels	-	1/6 (17%)	-
Colloid bodies	2/7 (29%)	1/6 (17%)	-
Blood vessels	-	-	-
Appendage	-	1/6 (17%)	-
IgG Epidermal nuclear staining			
Only ENS	-	-	-
ENS* and DEJ**	3/7 (43%)	1/6 (17%)	1/2 (50%)
ENS and colloid bodies	2/7 (29%)	-	-

ENS* = epidermal nuclear staining, DEJ** = DEJ with or without immunoreactant deposit at colloid bodies, blood vessels or appendages

Table 3. Type of immunoreactants at various locations

Diagnosis	Immunoreactants/cases (%)				
	IgG	IgA	IgM	C3	Mixed type
Systemic sclerosis (n = 9)					
DEJ (5/9)	-*	-	3/9 (33%)	1/9 (11%)	1/9 (11%) (IgM+C3)
Colloid bodies	-	-	1/9 (11%)	-	1/9 (11%) (IgM+IgA)
Blood vessels	-	-	-	-	-
Appendage	-	-	-	-	-
ENS	5/9 (56%)	-	-	-	-
Morphea (n = 11)					
DEJ (4/11)	-	-	1/11 (9%)	2/11 (18%)	1/11 (9%) (IgG+IgA)
Colloid bodies	-	-	1/11 (9%)	-	-
Blood vessels	-	1/11 (9%)	1/11 (9%)	-	-
Appendage	-	-	-	1/11 (9%)	-
ENS	1/11 (9%)	-	-	-	-
Overlapping syndrome (n = 2)					
DEJ (2/2)	-	-	2/2 (100%)	-	-
Colloid bodies	-	-	-	-	-
Blood vessels	-	-	-	-	-
Appendage	-	-	-	-	-
ENS	1/2 (50%)	-	-	-	-

-* = negative; C = complement; DEJ = Dermoepidermal junction; Mixed type = any combinations of IgG, IgA, IgM and C3

10% of 10 cases with a transitional form of acrosclerosis-diffuse scleroderma. In the present study, the lupus band test was positive in 13.5% of patients with acrosclerosis and diffuse scleroderma but negative in patients with the transitional form. The Ig deposit at the DEJ consisted mainly of IgM or a combination of

Table 4. Previous direct immunofluorescence studies in scleroderma as compared with our study

Authors	Site of biopsy	Positive DIF No. (%)	Positive site
Winkelman et al[3]	not defined	0/10 (0%)	-
Reimer et al[4]	sun-exposed area	18/30 (60%)	DEJ
Chen et al[5]	normal skin (forearm)	6/15 (40%)	DEJ
Shibeshi et al[7]	not defined		DEJ and/or ENS
Acrosclerosis		4/26 (15.3%)	
Diffuse scleroderma		11/26 (42.3%)	
Transitional form		1/10 (10%)	
Our study	sun-exposed and non sun-exposed area	7/9 (78%)	DEJ and/or ENS

IgG and IgM. However, Winkelman et al[3] reported negative immunofluorescence at the DEJ in SS, but positive IgM and a complement deposit on BMZ or blood vessel wall in MCTD. In the present study, seven of nine patients with SS (77%) had a positive DIF study, i.e., IgM at the DEJ (33%), C3 at the DEJ (11%), IgM at the colloid bodies (11%) and IgG ENS (55%).

Positive DIF yield in the present study was higher than previous reports from Western countries (0-60%)[3-5,7]. There were some reports[11,12] of immunogenetic differences between Oriental and Caucasian populations, resulting in differences in clinical presentation and frequency of autoantibodies detected in some autoimmune diseases. This probably helped the authors to explain the differences among the positive DIF yield and others.

Chen et al[13] performed DIF study from three different sites in 18 patients with systemic sclerosis. Immunoglobulin deposits were detected at the DEJ in 8/14 nail folds, 6/15 forearms, and in none of the buttock specimens. ENS was present in 6/14 nail folds, and in 6/15 forearms and buttocks. Moreover, Fabre et al[14] reported positive immunofluorescence in 20% of biopsy specimens (10 in 50 specimens) taken from sun-exposed skin of normal young adults. Five specimens revealed a continuous, granular IgM band at the DEJ in combination with other Igs. Leibold et al[15] reported weak, interrupted, linear, and granular IgM deposits at the DEJ in the sun-exposed skin of 10 out of 41 healthy adults. These data suggest that weak, interrupted, linear or granular IgM deposits alone at the DEJ can be detected in normal sun-exposed skin. Two of our seven SS patients had lesional skin biopsies from the extensor forearms. The other three sections were from the volar forearms. Difference in biopsy sites was also probably another reason to explain the differences among the positive DIF yield and others.

Six of the presented eleven patients with morphea (54%) had positive DIF findings. The immunoreactant deposits were IgM (9%), C3 (18%) at the DEJ, IgM at the colloid bodies (9%), C3 at the appendage area (9%), one case each (9%) of IgG and IgA at the blood vessel wall, and IgG ENS (9%). Fontan et al[16] reported positive DIF findings in four of six children with linear scleroderma.Three of the four patients had IgM fixation on the DEJ, and one had speckled fixation of IgG on the epidermal nuclei. Vincent et al[17] described IgM and C3 at the BMZ and in the dermal blood vessel of linear scleroderma. In contrast, Jablonska et al[18] reported a rare deposition of IgM along the DEJ.

In the overlapping syndrome, two of the presented patients had an IgM deposit at the DEJ (100%) and one patient (50%) had IgG ENS. In the present study, ENS and serum ANA seemed to be more frequently positive in SS and overlapping syndrome than in morphea. It should be noted that the authors had a small number of patients. The common pattern of ENS and ANA in the presented patients was the speckled type. Reimer et al[4] reported the speckled type of ENS in SS and patients with mixed connective tissue disease. Serum ANA was demonstrated in 96% and 100% of SS and mixed connective tissue disease, respectively. In mixed connective tissue disease, Gilliam et al[19] reported DIF of normal unexposed skin in 15 patients, with subepidermal immunoglobulin deposition in five of 15 patients and the speckled ENS pattern in all 15 patients. Fontan et al[16] demonstrated ANA in nine of eleven cases with linear scleroderma; the immunofluorescence staining pattern was homogeneous in all nine with a low titer. Burrows et al[20] described 22 patients with ENS; seven of them had negative serum ANA at the time of biopsy. They suggested that ENS does not occur because of tissue contamination during processing. However, Morel et al[21] reported that ENS on DIF

of covered normal skin was found in two patients with scleroderma and the patients had high serum concentrations of the antibody to nucleolar antigen.They suggested that ENS in a speckled, nucleolar, homogeneous or peripheral pattern appears to correlate with high titer serum antinuclear antibody giving immunofluorescence the same staining pattern. In the present study, there was no correlation of ENS with positive serum ANA.

There was no statistical significant difference in comparison of DIF findings with the duration of lesion, clinical correlation, and the positive result of serum ANA in all three groups of patients. Patients with SS had a tendency to give more frequently positive ENS and DEJ deposits than those with morphea. These were similar to the study of Shibeshi et al[7] who reported no correlation between positive DIF findings and clinical picture, course of the disease, titer, and specificity of ANA. However, they observed that positive results of DIF were more frequent in diffuse scleroderma compared with acrosclerosis and transitional form.

In conclusion, positive DIF yield in the present study was higher than previous reports from Western countries. Similar to the study reported from Western country, there was no statistically significant difference in comparison of DIF findings with the duration of lesion, clinical correlation, and the positive result of serum ANA in the three presented groups of patients. However, patients with SS had a tendency to give more frequently positive ENS and DEJ deposits than those with morphea.

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การศึกษา Direct immunofluorescence ของผู้ป่วยโรคหนังแข็ง

กนกวลัย กุลทนันทน์, สุขุม เจียมตน, ชัญญา ต่ายใหญ่เที่ยง, สำรวย ปิ่นแก้ว, ป่วน สุทธิพินิจธรรม

วัตถุประสงค์: *การศึกษา Direct immunofluorescence (DIF) ของชิ้นเนื้อจากผิวหนังของผู้ป่วยโรคหนังแข็งที่รายงานจากต่างประเทศ ให้ผลบวกในเปอร์เซ็นต์และรูปแบบของ DIF ที่แตกต่างกัน วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาการให้ผลบวก และรูปแบบของ DIF ในคนไทยที่เป็นโรคหนังแข็งชนิดต่าง ๆ และดูความสัมพันธ์ทางคลินิก*

วัสดุและวิธีการ: *ใช้ผู้ป่วยโรคหนังแข็งชนิดต่าง ๆ จำนวน 22 รายของภาควิชาตจวิทยา โรงพยาบาลศิริราชระหว่างปี พ.ศ. 2539 – พ.ศ. 2545 ผู้ป่วยได้รับการตัดชิ้นเนื้อจากรอยโรคผิวหนังเพื่อศึกษา DIF*

ผลการศึกษา: *ผู้ป่วยทั้งหมด 22 ราย, 9 รายเป็น systemic sclerosis (SS), 11 ราย เป็นโรคหนังแข็งเฉพาะที่ (morphea) และ 2 รายเป็น overlapping syndrome ผู้ป่วย 15 ราย (68%) มี DIF ให้ผลบวก ได้แก่ 7 ใน 9 ราย (78%) ของ SS, 6 ใน 11 ราย (55%) ของ morphea และ 2 ใน 2 ราย (100%) ของ overlapping syndrome ตำแหน่งที่มีการติดของสารอิมมูนเรืองแสงที่พบบ่อย คือ ที่รอยต่อของชั้นหนังกำพร้ากับชั้นหนังแท้ และที่นิวเคลียสของเซลล์ในชั้นหนังกำพร้า พบการติดของ IgM บ่อยที่สุด ไม่พบความแตกต่างของลักษณะทาง DIF เมื่อดูจากอายุของรอยโรค ความสัมพันธ์ทางคลินิก และการมีผลบวกของ antinuclear antibody ในซีรัม ในผู้ป่วยทั้ง 3 กลุ่ม*

สรุป: *การศึกษา DIF ของชิ้นเนื้อจากผิวหนังของผู้ป่วยโรคหนังแข็งชาวไทย ให้ผลบวกในเปอร์เซ็นต์ที่สูงกว่าที่รายงานจากต่างประเทศ เช่นเดียวกับรายงานจากต่างประเทศการศึกษานี้ไม่พบความแตกต่างอย่างมีนัยสำคัญของ DIF ที่พบเมื่อเปรียบเทียบกับระยะเวลาของรอยโรค อาการทางคลินิก และผลบวกของซีรัม antinuclear antibody ในผู้ป่วยทั้ง 3 กลุ่ม ผู้ป่วย SS มีแนวโน้มที่จะมีผลบวก DIF ที่รอยต่อของชั้นหนังกำพร้ากับชั้นหนังแท้ และที่นิวเคลียสของเซลล์ในชั้นหนังกำพร้ามากกว่าผู้ป่วย morphea*