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Study of direct immunofluorescence, immunofluorescence mapping and light microscopy in porphyria cutanea tarda

Estudo da imunofluorescência direta, imunomapeamento e microscopia ótica na porfiria cutânea tardia

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Abstract: BACKGROUND: Even though porphyria cutanea tarda is the most frequent type of porphyria, there are few studies about its cutaneous physiopathology.
OBJECTIVE: To evaluate skin changes in porphyria cutanea tarda using light microscopy and direct immunofluorescence before and after treatment with chloroquine. To perform antigen immunomapping of bullae to study their level of cleavage.
METHODS: Light microscopy and direct immunofluorescence of 28 patients are reported in three different phases: 23 patients with active porphyria before treatment (Phase A), 7 patients with clinical remission during treatment (Phase B), and 8 patients with biochemical remission (Phase C). Immunomapping was performed on 7 patients.
RESULTS: In active porphyria, direct immunofluorescence showed homogenous and intense fluorescence on the inside and on the walls of blood vessels as well as in the dermal-epidermal junction. In clinical remission (Phase B) and biochemical remission (Phase C), the deposit of immunoglobulins was maintained, but the deposit of complement was reduced in most cases. Immunomapping revealed no standard cleavage plane.
CONCLUSION: No correlation was observed between clinical response and immunoglobulin deposits. The reduction of complement favors the hypothesis that activation of the complement cascade represents an additional pathway that leads to endothelial damage.
Keywords: Fluorescent antibody technique; Microscopy; Porphyria cutanea tarda; Porphyria cutanea tarda/physiopathology

Resumo: FUNDAMENTO: Apesar de a porfiria cutânea tardia ser a mais frequente das porfirias, há poucos estudos que abordam sua fisiopatologia cutânea.
OBJETIVO: Avaliar as alterações cutâneas na porfiria cutânea tardia utilizando a microscopia ótica e a imunofluorescência direta, antes e depois do tratamento com cloroquina. Realizar o imunomapeamento antigênico da bolha para estudo do seu nível de clivagem.
MÉTODOS: Relata-se a microscopia ótica e imunofluorescência direta de 28 pacientes em três fases diferentes: 23 pacientes com porfiria ativa antes do tratamento (Fase A), sete pacientes com remissão clínica durante o tratamento (Fase B) e oito pacientes com remissão bioquímica (Fase C). O imunomapeamento foi realizado em sete pacientes.
RESULTADOS: Na porfiria ativa, a imunofluorescência direta demonstrou fluorescência homogênea e intensa no interior e na parede dos vasos e na junção dermoepidérmica. Na remissão clínica (Fase B) e na remissão bioquímica (Fase C), o depósito de imunoglobulinas se manteve, mas o depósito de complemento apresentou diminuição na maioria. O imunomapeamento não demonstrou plano de clivagem fixo.
CONCLUSÃO: Não houve correlação entre a resposta clínica e os depósitos de imunoglobulinas. A diminuição do complemento favorece a hipótese de que a ativação da cascata do complemento representa uma via adicional que leva à lesão endotelial.
Palavras-chave: Imunofluorescência; Microscopia; Porfiria cutânea tardia; Porfiria cutânea tardia/fisiopatologia

INTRODUCTION

Porphyria cutanea tarda (PCT), the most common form of porphyria, is characterized by partial deficiency of uroporphyrinogen decarboxylase. Direct immunofluorescence (DIF) of injured skin shows deposition of immunoglobulins (Ig) G, M and A and / or complement (C3) with characteristic fluorescence that extends from the wall to the lumen of the blood vessels and in the dermal-epidermal junction (DEJ). [1,2] The skin was evaluated using light microscopy and DIF before and after treatment with chloroquine in an attempt to investigate possible correlations between treatment and skin changes. In order to better understand the physiopathology of lesions in PCT, subepidermal blisters were subjected to antigen immunomapping, indirect immunofluorescence which uses specific labeled antibodies directed against known antigens of the DEJ, such as the bullous pemphigoid antigen, laminin, type IV collagen and type VII collagen, to determine the level of cleavage in the DEJ. [3] The bullous pemphigoid antigen is located above the lamina lucida in epidermal cells of the basal layer. Laminin, a non-collagen glycoprotein present in the basement membrane, has been implicated in the adhesion of epidermal cells to the basement membrane and it is the main component of the lamina lucida. Type IV collagen is the main element of the lamina densa of the basement membrane. Type VII collagen composes the anchoring fibrils located in the sublamina densa. As the location of the antigens in the basement membrane of the blister is known, it is possible to establish the exact level of subepidermal cleavage. This method can help with differential diagnosis of several bullous diseases.

MATERIALS AND METHODS

The study design was non-directional and ambispective. At the beginning of the study, we obtained the data of the patients who had already presented themselves to the outpatient clinic by means of a cross-sectional analysis, and for 3 years we included all new cases and the data of patients who presented clinical and/or biochemical remission after treatment. We used Excel ® spreadsheet (Microsoft ®) to describe the data and applied measures for the analysis of the frequency of some factors in the study population. We described the proportions of each finding and used "Fisher's Exact Test" for the comparative analysis of some data, with a significance level of 0.05. For the statistical analysis, we used SPSS for Windows, version 10.0.

Patients

After signing the consent form, the patients answered questions related to the research object and had their material collected as described in the following item. Twenty-eight patients with PCT were selected for this study, with their ages varying from 16 to 66. The diagnosis of PCT was based on: 1 - Typical clinical manifestations; 2 - *Screening* test using Wood's lamp (Rimington-Doyle and Rimington Method) [5] to detect porphyrins in urine, feces, and erythrocytes; 3 - 24-hour urine collection with increased concentration of uroporphyrin (URO) at least three times the level of coproporphyrin (Copro), that is, ratio URO/COPRO greater than 3:1; [6] and 4 - Histology and direct immunofluorescence. The quantification of urinary porphyrins in the 24-hour urine sample was determined by the method of high performance liquid chromatography (HPLC). [7] The pattern of excretion of urinary porphyrins is diagnostic for PCT. [6] Anuric patients or patients who did not present the excretion pattern of porphyrins (ratio URO/COPRO greater than 3:1) were excluded from the study. Disappearance of blisters and improvement of skin fragility were the criteria used for clinical remission, and reduction of total porphyrins in the 24-hour urine sample to normal parameters (females <159 μg and males <199 μg) was considered biochemical remission.

Skin biopsies

Biopsies for histopathology and direct immunofluorescence (DIF) were obtained as described in the chart (Figure 1). A total of 28 patients were studied. In 23 patients (Phase A), the first skin biopsy was obtained from skin lesions, regardless of their location. Five patients had already started treatment and had no skin biopsies prior to it. In 7 patients (Phase B), the biopsy was performed on clinically normal skin of the dorsum of the hand (exposed skin) during treatment with chloroquine and in clinical remission, but without biochemical remission. In 8 patients (Phase C), biopsy was performed on clinically normal skin of the dorsum of the hand during clinical and biochemical remission (inactive porphyria). In 7 patients with active PCT (Phase A), a biopsy of the perilesional region, independent of the others, was performed for antigen immunomapping of the DEJ.

Histopathology

For the histopathological examination, skin fragments were fixed in 10% formalin, embedded in paraffin and submitted to routine histological technique using hematoxylin-eosin, *periodic acid-Schiff* (PAS) and Perls' stain (to identify hemosiderin).

Direct immunofluorescence (DIF)

The skin fragment was transported in gauze moistened with saline solution 0.9% and taken for
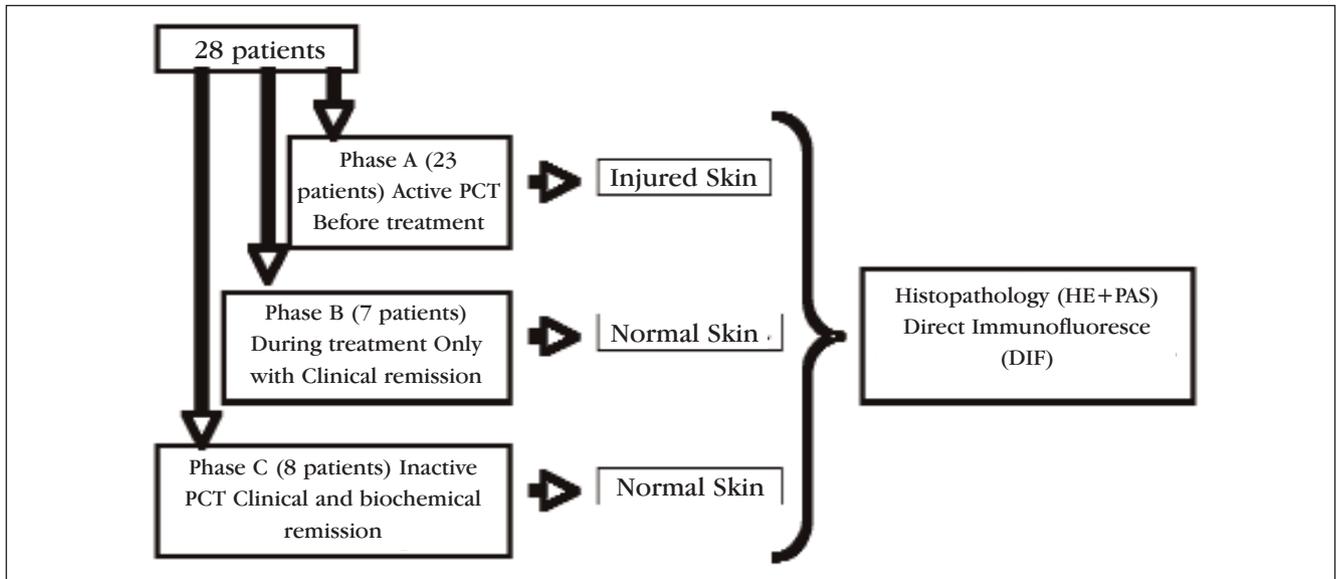
FIGURE 1: Chart describing how biopsies were obtained in different phases (A, B and C)

immediate cryopreservation in tissue freezing medium (Leica manufacturer) and stored at -20 ° C until cryomicrotomy. After cryosection, three 4-micrometer-thick cuts were placed on albuminized slides. The slides were placed in a humidity chamber at room temperature; and the conjugates (anti-human immunoglobulins produced in immunized animals and labeled with fluorescein isothiocyanate) were placed on the cuts. The conjugates were diluted in TBS pH 7.5 (tris-buffered saline - calcium acetate buffer) containing 3 mg% of Evans blue dye (Interlab manufacturer). We used anti-human IgA, SIGMA (1:20 dilution), anti-human IgM, SIGMA (1:20 dilution), anti-human IgG, SIGMA (1:130 dilution) and anti-human C3, Dako (1:40 dilution). A conjugate was used for each slide. After 30 minutes of incubation, the slides were washed in TBS pH 7.5 for two periods of ten minutes each. To mount the slides, we used buffered glycerin (pH 9 / 0.5 M) and glass coverslip. The reading was done using an epiluminescence microscope (Zeiss, model Axiolab).

Immunomapping

The skin fragment was transported in gauze moistened with saline solution 0.9% and taken for immediate cryopreservation in tissue freezing medium (Leica manufacturer) and stored at -20 ° C until cryomicrotomy. Cryosection was performed in a cryostat and four cuts (4µ) were placed on albuminized slides. One of the monoclonal antibodies (produced in mice), namely, anti-human laminin (clone LAM-89, 1:20 dilution), anti-human type IV collagen (clone col-94, 1:25 dilution) and anti-human type VII collagen (clone LH7-2, 1:25 dilution), all from Sigma

Aldrich and commercially purchased, were placed on each cut. As a source of antibodies against bullous pemphigoid antigen, the serum of a patient previously diagnosed with the disease was used (1:20 dilution). The dilutions were performed in TBS pH 7.5. Each of the diluted antibodies was placed on a cut and incubated for a period of 30 minutes in a humidity chamber at room temperature. After that, the slides were washed in TBS for two periods of 10 minutes each. Then, to reveal the reaction, we used the anti-IgG antibody conjugated with fluorescein isothiocyanate. For binding to the monoclonal antibodies, we used an anti-mouse IgG (1:30 dilution) produced in rabbit *[mouse immunoglobulins / FITC-rabbit F* (ab') 2, Dako], and for binding to the antibody of the pemphigoid antigen, we used an anti-human IgG (1:130 dilution) also produced in rabbits *(anti-Human IgG-Whole molecule-FITC conjugate*, Sigma Aldrich).The dilutions were performed in TBS buffer containing 3mg% of Evans blue dye. After 30 minutes of incubation (in a humidity chamber and at room temperature), the slides were washed again in TBS for two periods of 10 minutes each. When the slides were slightly dry, they were mounted with buffered glycerin and glass coverslip, and the reading was done in an epiluminescence microscope.

RESULTS

We selected 28 patients with porphyria cutanea tarda before and after treatment with chloroquine. Seven patients were female (25%) and 21 were male (75%). The mean age was 30.3 years (median 29.0 years) for women and 44.5 years (median 49 years) for men. Alcohol intake was the predominant triggering

factor in men, while estrogen therapy was the predominant factor in women (contraception and hormone replacement). Hepatitis C was present in 57.1% of all patients (71.4% men and 14.3% women). There were no reports of exposure to porphyrinogenic polychlorinated hydrocarbons. Other diseases present in the patients were hepatitis B (39.3%), diabetes *mellitus* (17.9%), infection with human immunodeficiency virus - HIV (7.1%), chronic renal failure (3.6%), hepatocellular carcinoma (3.6%), multiple myeloma (3.6%) and myelofibrosis (3.6%). All patients were treated with chloroquine diphosphate (250mg, twice a week), except for one patient treated with phlebotomy.

Biopsies were performed in three different phases: Phase A, prior to the beginning of treatment; phase B, during treatment with chloroquine and in clinical remission but without biochemical remission; and phase C, in clinical and biochemical remission, that is, inactive porphyria (Figure 1).

Light microscopy

In patients with active PCT (Phase A), light microscopy with hematoxylin-eosin stain revealed subepidermal bullae in 86.9% (20 of 23 patients), and 47.8% (11 of 23) presented dermal papillae irregularly protruding from the base of the bullae into the cavity with a festooning aspect (Figure 2). Perivascular lymphomononuclear inflammatory infiltrate was occasionally found. No fibrosis was observed, except in a patient's sclerodermatous lesion where the dermis showed thick collagen fibers arranged in a more compact form.

Staining with periodic acid-Schiff (PAS) of the skin lesions of 95.6% of the patients (22 of 23) (Phase A) revealed homogeneous thickening of upper dermal vessel walls by diastase-resistant, PAS-positive hyaline material (Figure 2). This thickening of the vessel walls remained in 92.9% of patients (13 out of 14) with clin-

ically normal skin (Phases B and C). Vascular changes were more accentuated in the papillary dermis and the amount of hyaline material around the vessels varied in different biopsies. It was possible to compare the thickening of vessel walls in five patients before treatment (Phase A) and in biochemical remission (Phase C). They showed thickening of the vessel for PAS before treatment, and four of them presented slighter thickening in biochemical remission while one presented no wall thickening (Table 1).We do not know how long two of the patients (cases no. 20 and 21) had already been in biochemical remission when their second biopsy was performed (after 10 and 6 years, respectively); as for the other three patients, they had their second biopsy performed as soon as they went into biochemical remission.

All samples (Phase A, B and C) were subjected to Perls' stain, but no hemosiderin deposit in the dermis was identified.

Direct immunofluorescence (DIF)

In active porphyria (phase A), DIF demonstrated intense and homogeneous characteristic fluorescence on the inside and on the walls of blood vessels and in the dermal-epidermal junction (Figure 3). Table 2 presents the results of DIF for each patient in the different phases (A, B and C). In Phase A, four patients showed negative DIF and 19 showed immunofluorescence with deposits of IgG and C3 in the vessels (65.2% and 52.2%, respectively) and in the DEJ (47.8% and 39.1%, respectively) (Table 3). Fluorescence in the vessels for IgG was moderate in four (17.4%) and intense in nine (39.1%) while fluorescence for C3 was moderate in four (17.4%) and intense in six (26.1%). Deposition was more prominent in the papillary vessels, but vessels of the reticular dermis were also frequently involved. Fluorescence was focal or continuous and showed
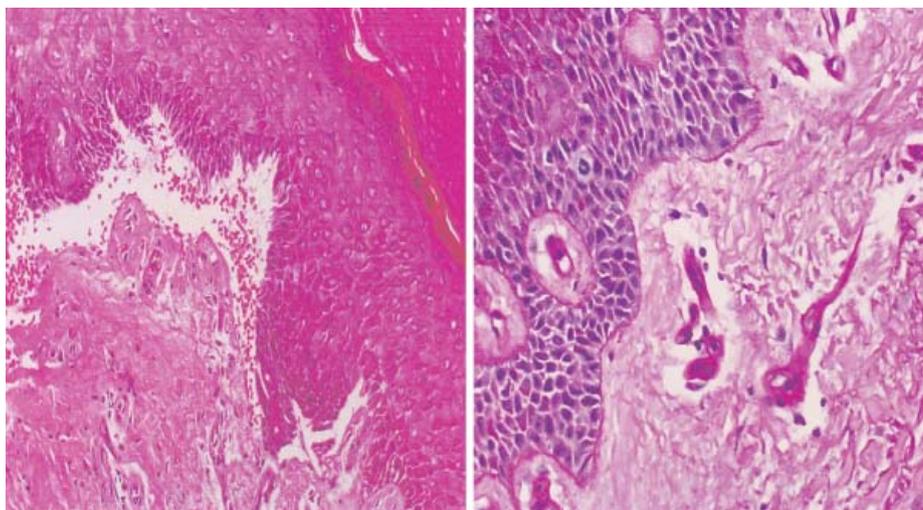

FIGURE 2: Porphyria cutanea tarda: 1. Histopathology with hematoxylin-eosin stain showing a subepidermal bulla with protruding dermal papillae and no inflammatory infiltrate (HE, 40x). 2. Staining with periodic acid-Schiff (PAS) showing diastase-resistant, PAS-positive hyaline material thickening dermal vessel walls (PAS, 40x)

TABLE 1: Intensity of thickening of vessel walls by diastase-resistant, PAS-positive hyaline material (intense, moderate or slight) before treatment (active porphyria) and after biochemical remission, in addition to duration of treatment after the second biopsy

Case No.	Biopsy Before treatment	Biopsy with Biochemical Remission	Duration of Treatment
Case No. 4	Moderate	Slight	18 months
Case No. 13	Intense	Slight	28 months
Case No. 16	Intense	Slight	16 months
Case No. 20	Intense	Slight	10 years
Case No. 21	Moderate to slight	No thickening	6 years

NOTE: The number (No.) of the cases described in this table is the same used to describe these patients in Table 2.

granular or homogenous pattern in the DEJ.

In phase B, deposition of IgG on the vessel walls occurred in 85.7% of the seven patients tested, three of them (42.9%) showed moderate fluorescence and two (28.6%) showed intense fluorescence. Deposition of C3 in the vessels was present in only one case (14.3%), but with slight fluorescence.

In inactive porphyria (Phase C), immunofluorescence was negative in one case and deposition of IgG on the vessel wall occurred in 87.5% of the eight patients tested, three of them (37.5%) showed moderate fluorescence and four (50.0%) showed intense fluorescence. In this phase, deposition of C3 was present in 37.5% (3 out of 8) and fluorescence intensity was slight, moderate and intense in each of the three cases (12.5% each).

Comparing patients with active disease (Phase A) with patients in clinical remission (phases B and C), the number of cases with deposition of IgG on the vessel walls increased (from 65.2% (95% CI [42.7, 83.6]) in phase A to 85.7% (95% CI [42.1, 99.6]) and 87.5% (95% CI [47.3, 99.7]) in phases B and C, respectively) and there was no decrease in fluorescence intensity. The number of cases with deposition of complement (C3) in the vessels decreased (from 52.2% (95% CI [30.6, 73.2]) in phase A to 14.3% (95% CI [0.4, 57.9]) and 37.5% (95% CI [8.5, 75.5]) in phases B and C, respectively) and presented a decrease in fluorescence intensity. This reduction of C3 was important, and it was more significant for patients in phase B (p = 0.089) than for patients in phase C (p = 0.382). When analyzing whether that decrease was significantly different between phases B and C, nothing was found (p = 0.338).

Considering the patients who presented positive DIF (Table 2), in phase A, 57.9% of the patients (11 out of 19) showed intensity of fluorescence on the vessel walls as noticeable as that in the DEJ and 31.6% (6 out of 19) showed more intense fluorescence on the vessel walls than in the DEJ. As for the patients with clinical remission, phase B, 42.9% of the patients (3 out of 7) showed intensity of fluorescence on the vessel walls as noticeable as that in the DEJ, and 57.1% of the patients (4 out of 7) showed more intense fluorescence on the vessel walls than in the DEJ. As for the patients with inactive PCT, phase C, 28.6% (2 out of 7) showed intensity of fluorescence of the vessels similar to that of the DEJ, and 71.4% of the patients (5 out of 7) showed more intense fluorescence on the vessel walls than in the DEJ. It was observed that fluores-
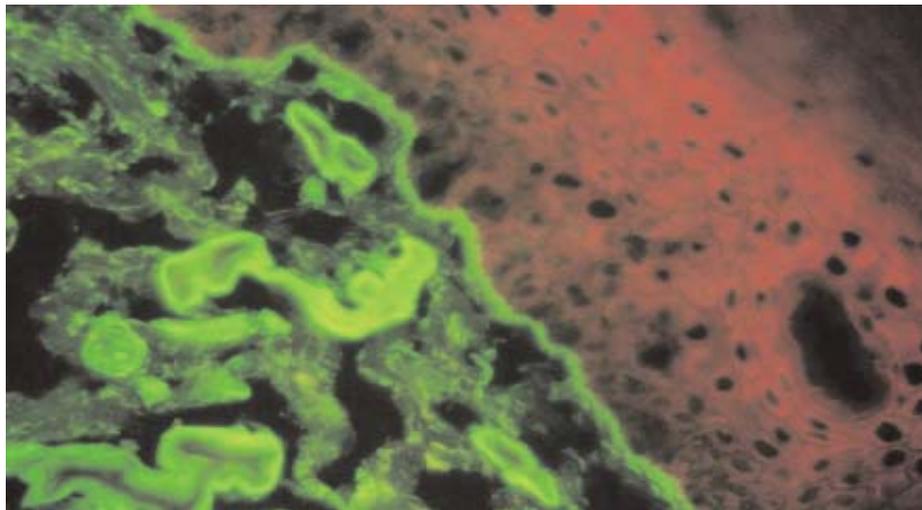

FIGURE 3: Porphyria cutanea tarda: Direct immunofluorescence showing intense and homogeneous characteristic fluorescence on the inside and on the walls of blood vessels as well as in the dermal-epidermal junction for anti-IgG (40x)

CHART 2: Study of direct immunofluorescence (DIF) of the patients - number, intensity of fluorescence of each immunoglobulin (Ig) and of C3 fraction of the complement (Intensity: 0 = negative, 1 = slight, 2 = moderate, 3 = intense) and their location in the dermal-epidermal junction (DEJ) and / or vessel wall, period of time (in years) after diagnosis when biopsy was performed in phases B and C and determination of urinary porphyrins when immunofluorescence was performed in phase C

DIF without treatment (Phase A)

Cases No.	DEJ IgG	DEJ IgM	DEJ IgA	DEJ C3	Vessels IgG	Vessels IgM	Vessels IgA	Vessels C3
1	0	0	0	0	3	3	3	0
2	0	0	0	0	3	0	0	3
3	0	0	0	0	0	0	0	0
4	3	0	3	3	3	0	0	3
5	—	3	—	3	3	3	0	3
6	3	3	2	3	—	—	—	3
7	2	0	2	2	2	0	2	2
8	0	1	1	1	1	1	1	1
9	2	2	2	2	3	3	3	3
10	1	0	0	0	0	0	0	0
11	0	0	0	0	3	0	0	0
12	3	0	0	0	3	0	0	0
13	3	3	3	3	3	3	3	3
14	2	2	—	2	2	2	—	2
15	2	2	2	2	2	2	2	2
16	0	2	0	2	2	0	0	0
17	—	—	—	—	—	—	—	—
18	0	—	0	—	0	0	—	0
19	—	—	—	—	—	—	—	—
20	0	0	0	0	0	0	0	2
21	0	0	0	2	0	0	0	2
22	—	—	—	—	—	—	—	—
23	3	3	3	3	3	0	0	0
24	1	1	1	1	1	1	1	1
25	0	0	0	0	2	2	2	2
26	0	0	0	0	0	3	3	3
27	1	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0

DIF with clinical remission (Phase B)

Cases No.	DEJ IgG	DEJ IgM	DEJ IgA	DEJ C3	Vessels IgG	Vessels IgM	Vessels IgA	Vessels C3	Period of time after diagnosis (years)
1	0	1	0	0	0	0	0	0	5
2	2	0	2	0	2	2	2	0	3
3	2	2	0	0	2	2	2	0	4
10	0	0	0	0	2	2	2	0	24
14	—0	—0	—0	1	—1	—0	—1	1	2
22	—0	1	—0	0	3	3	3	0	2,5
26	0	0	0	0	3	0	0	0	6

DIF with clinical and biochemical remission (Phase C)

Cases No.	DEJ IgG	DEJ IgM	DEJ IgA	DEJ C3	Vessels IgG	Vessels IgM	Vessels IgA	Vessels C3	Total porphyrins (μg/24hs)	Period of time after diagnosis (years)
4	2	0	2	0	2	0	2	0	199	1,5
5	2	0	2	2	3	3	3	3	121	22
13	3	1	1	1	3	1	1	1	45,9	2,3
16	0	2	0	0	3	0	0	0	<2,5	1,3
17	0	0	0	0	2	0	2	2	103	4
19	0	3	0	0	3	0	0	0	114	16
20	0	0	0	0	2	0	0	0	104	10
21	0	0	0	0	0	0	0	0	72	6

CHART 3: Direct immunofluorescence findings: Number of cases with deposition of IgG, IgM and IgA and complement (C3) at the dermal-epidermal junction (DEJ) and in blood vessels

Clinical Diagnosis	No. of cases	IgG		IgM		IgA		C3	
		DEJ %	Vessels %	DEJ %	Vessels %	DEJ %	Vessels %	DEJ %	Vessels %
Phase A:Active PCT before treatment	23	11 (47,8)	15 (65,2)	7 (30,4)	9 (39,1)	6 (26,1)	9 (39,1)	9 (39,1)	12 (52,2)
Phase B: PCT with clinical remission and no biochemical remission	7	2 (28,6)	6 (85,7)	3 (42,9)	2 (28,6)	1 (14,3)	4 (57,1)	1 (14,3)	1 (14,3)
Phase C: PCT with clinical and biochemical remission	8	3 (37,5)	7 (87,5)	3 (37,5)	1 (12,5)	3 (37,5)	4 (50,0)	2 (25,0)	3 (37,5)

Note: DEJ - dermal-epidermal junction

cence becomes more prominent on the vessel walls than in the DEJ as the patient moves from active porphyria (Phase A) to clinical remission (Phase B) and then to biochemical remission (Phase C).

Antigen Immunomapping

Antigen immunomapping to determine level of cleavage was performed on seven patients with active PCT (Table 4). In three cases, all of the antigens (bullous pemphigoid antigen, laminin, type IV collagen and type VII collagen) were found on both sides of the bullae and did not show defined level of cleavage. Two cases presented all of the antigens at the base of the bullae; therefore, cleavage was intra-epidermal. In one case, type IV collagen was found on the roof and type VII collagen on both sides of the bulla, and thus cleavage was at the level of the sublamina densa; in another case, all of the antigens were found on the roof of the blister, thus cleavage occurred below the sublamina densa (Figure 4). Consequently, there was no characteristic pattern in terms of cleavage level.

DISCUSSION

The clinical characteristics of the patients in this study are similar to those found in the literature, [8] which emphasize predominance in men and alcohol intake as the main triggering factor. The association with HCV, hepatitis B, diabetes *mellitus,* HIV, chronic renal failure, hepatocellular carcinoma, multiple myeloma and myelofibrosis was consistent with previous studies.[8-12] We found a high incidence of HCV, but its pathogenic role in triggering PCT has not been clarified yet. Chloroquine in low doses was the treatment chosen, and its benefits have been stressed in several reports. [13,14]

Staining with PAS revealed deposits of diastase-resistant and PAS-positive material on the vessel walls of the upper dermis in 95.6% of the cases, as shown in previous studies. [15] Electron microscopy studies showed that this hyaline material is composed of multiple layers of basal lamina and enlargement of perivascular spaces with thin collagen fibers scattered by a small amount of filamentous and amorphous material.[2,16] The immunohistochemical reaction for type IV collagen and the immunofluorescence technique, using anti-laminin and type IV anti-collagen serum, revealed that this perivascular material results from excess synthesis of the basement membrane.[17] The hyaline deposits would be a response resulting from repeated injury to the vessel wall and consequent leakiness of its contents.[18] Histochemical studies demonstrated that the hyaline deposits contain tryptophan, which is derived from blood and is not found in collagen or the elastic tissue. [19] Therefore, these studies suggest that the amorphous hyaline material is derived from the vessel wall and contents. The DEJ presents structural changes identical to those found in the vessels.[2,16] The vessels of non-exposed skin present normal thickness. [15,16]

In phases B and C, the PAS-positive hyaline material on vessel walls remained in 92.9% of the patients. Timonen *et al.* [15] believed that thickening of vessel walls was a chronic and irreversible change. However, Epstein *et al.* [16] showed that vascular hyalinization was more pronounced in the injured skin of patients with active porphyria than in the exposed skin of patients with inactive porphyria, but these authors did not compare biopsies before and after treatment in the same patient, and this decrease in hyalinization of vessel walls could be because patients
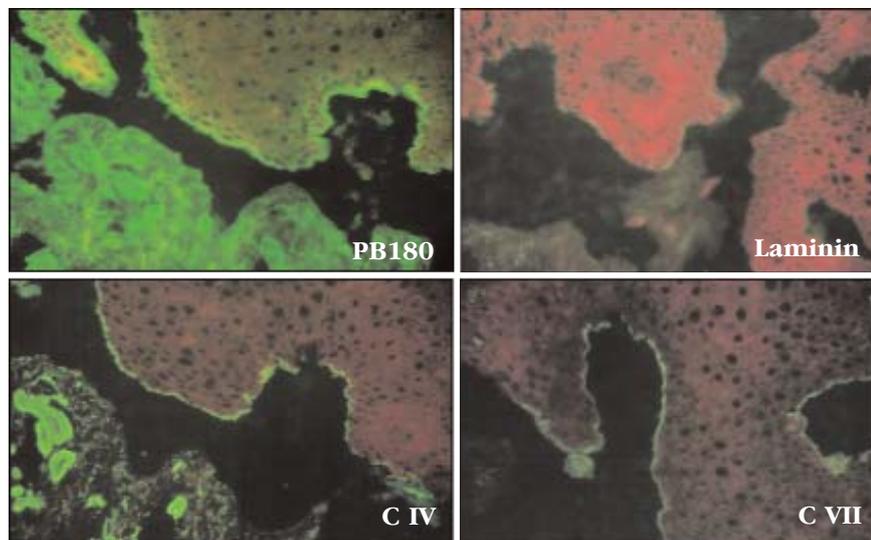
FIGURE 4: Porphyria cutanea tarda - Antigen immunomapping of basement membrane with all antigens - the antigen of bullous pemphigoid (PB180), laminin, type IV collagen and type VII collagen - on the epidermal side, and thus with level of cleavage in the upper dermis below the sublamina densa (40x)

with inactive PCT presented milder vascular changes in the active phase or because the remission period was long and vascular changes improved during that period. There is a reduction of this deposition after biochemical remission among the five patients in our study (Table 1), but a larger sample and a longer follow-up period after biochemical remission is needed to reach a definite conclusion.

The study of immunofluorescence in PCT was first carried out by Baart de la Faille-Kuyper and Cormane in 1968, using polyspecific conjugates without immunological specificity. [20] In 1971, Cormane *et al.* [1], with the improvement of microscopic equipment (decreased autofluorescence of collagen fibers) detected immunoglobulins (IgG, IgA and IgM) and complement deposits on the inside and on the walls of blood vessels, as well as in the DEJ; they suggest the existence of an alternate pathway that activates the complement sequence, induced by photodynamic reaction and independent of immunological reactions. In 1973, Epstein *et al*, [16] showed IgG and complement in a sporadic way on the vessel walls and in the DEJ of injured skin of patients with active PCT. As for the patients with inactive PCT, fluorescence was less intense on exposed skin and absent on skin protected from light, indicating that light and porphyrins were essential to generate injury. Timonen *et al.*, [15] in 1991, studied DIF before and after treatment with chloroquine in four patients: IgG was present on the vessel walls of three patients and complement was present on the vessel walls of one patient before treatment. After remission, deposition of IgG was less intense (two), increased (one) or negative (one). From six to twelve months after biochemical remission, intense deposition of IgG was found in two patients, including one who did not present deposition of IgG before treatment.

The results confirm previous studies on DIF, for they revealed deposits of IgG and C3, in addition to IgA and IgM, producing characteristic fluorescence that extends from the vessel wall to the lumen and at the DEJ of injured skin of 18 patients with active PCT (five presented negative DIF). In most patients with active PCT, fluorescence was homogeneous and very intense. The patients with clinical remission (Phase B) and biochemical remission (Phase C) maintained, on exposed skin, deposition of IgG on the vessel wall and at the DEJ, and there was no decrease in fluorescence intensity in relation to that observed in active PCT. After treatment, deposition of Ig was maintained even though porphyrin levels were normal and, therefore, there was no absorption of light to cause vessel injury. Several authors do not consider these deposits a result of an immunologic phenomenon, since circulating auto-antibodies against DEJ, vascular or perivascular antigens were not identified. [2,16,21] It is assumed that the deposits of immunoglobulins (Igs) result from the diffusion of circulating Igs by the vessel wall and their imprisonment in perivascular hyaline material. [2,21] This did not occur with deposition of C3, since phases B and C showed a decrease in the number of cases with deposition of C3 in the vessels (from 52.2% in phase A to 14.3% and 37.5% in phases B and C, respectively) and a decrease in fluorescence intensity. This decrease was important, but not statistically significant $(p = 0.089$ and $p = 0.382$, respectively). The complement is possibly involved in the pathogenesis of the lesion. There is extensive evidence that the complement is involved: (1). Radiation *in vitro* of the serum of patients with PCT results in activation of the complement. [22,23] (2). Photosensitivity induced by porphyrins in animal models is associated with complement activation and is suppressed in animals with complement depletion or congenital deficiency of C5. [24] (3). C5-

CHART 4: Antigen immunomapping of the dermal-epidermal junction and cleavage level of bullae

NO. OF CASES	ANTIGENS	CLEAVAGE LEVEL OF BULLAE
3	All antigens on the epidermal and dermal side	No defined level of cleavage (Bullae in regeneration)
1	Antigens of bullous pemphigoid and laminin were negative; Type IV collagen was found on the epidermal side and type VII collagen on the epidermal and dermal side	Sublamina densa
1	All antigens on the epidermal side	Upper dermis (below the sublamina densa)
1	All antigens on the dermal side of the bulla	Intraepidermal (basal cells)
1	All antigens on the dermal side of the bulla	Intraepidermal (Above the basal cells)

induced chemotactic activity was observed after exposing the skin of patients with PCT to Soret band irradiation. [25] It is believed that porphyrins and light activate the alternate pathway of the complement cascade, independent of immune responses, leading to endothelial injury. [1] It is assumed that complement activation, mediated by porphyrins after light radiation, results from the generation of reactive oxygen species, most likely *singlet* oxygen. [25] It has not been clarified whether endothelial damage follows activation of the complement cascade or whether these two processes occur independently. [2]

As for deposition at the DEJ, it probably has the same origin as deposition on the vessel wall, since no circulating antibodies against the DEJ were found and immunoglobulins are equivalent to those found in blood vessels. [2] As the patient progresses from active porphyria to clinical remission and subsequent biochemical remission, fluorescence becomes more predominant on the vessel wall than at the DEJ. It can be concluded from these data that porphyrins leading to endothelial injury cause the leakiness of immunoglobulins (Igs) and complement (C3) to be greater in the active phase (Phase A) and, therefore, fluorescence at the DEJ is more frequent. In clinical remission (Phase B) and biochemical remission (Phase C), there is a decrease in fluorescence at the DEJ, probably because the leakiness of Igs and C3 is slighter and more restricted to the perivascular area.

Even though histopathologic changes and immunofluorescence suggest that the primary focus of the cutaneous lesion is the vessel wall, the pathogenesis of skin fragility and blister formation has not been totally elucidated yet. Blisters can be induced by friction, [2] but not so often by phototest. [26] The immunoglobulins deposited at the DEJ cannot be considered responsible for skin fragility, for they also occur in EPP, which does not present blisters. [2] Further evidence that immunoglobulins are not responsible

for the formation of blisters and fragility is their presence at the DEJ of normal skin of patients with inactive PCT. Changes in electron microscopy of the DEJ are found only in patients with PCT and VP; [16] this difference may be related to the concentration and solubility of the porphyrins involved. In EPP, protoporphyrins are present in large amounts in the blood vessels and, since they are not water soluble, they do not diffuse easily out of the vessels. This explains why vascular injury is more pronounced than changes at the DEJ in this condition. [16] In PCT, the blisters result from variable cleavage, sometimes in the lamina lucida, [3,27,28] sometimes in the papillary dermis [2,15,29] or at the level of basal keratinocytes that show degenerative changes. [30] In some patients, the three types of cleavage can be observed in different biopsies, or even in the same biopsy. [30] Some authors believe that the blister initially originates in the junction zone and then, with additional stimulus, it quickly becomes a dermal-cleavage blister; this would explain the occurrence of scars. [28] Other authors have hypothesized that patients with PCT have exposed skin so rigid that it does not tolerate friction or trauma. [2] Reinforcing this hypothesis, it was experimentally observed in patients with active PCT that exposed skin with normal clinical appearance presented cleavage formation in electron microscopy. This cleavage is formed beneath the basal lamina in the superficial layers of the dermis, and basement membrane reduplications probably result from multiple episodes of microscopic cleavage and its subsequent regeneration. [2] Another study on the morphological event of blister formation with electron microscopy showed that the phenomenon is conditioned by formation of vacuoles limited by membranes, and these are observed in the superficial dermis around the vessels and immediately below the basal lamina. [29] It is assumed that irradiation of porphyrins concentrated in lysosomes [19] leads to photodynamic damage of lysosomal membranes, causing

enzymes to escape into the cytoplasm and cells to suffer apoptosis. Vacuoles formed by cytolysis of dermal cells and rupture of vacuolar membranes cause dermal-epidermal cleavage. Lysosomal injury may also affect endothelial cells and keratinocytes in the basal layer. Another suggestion of these authors, which would explain the development of bullae, is the formation of pseudopodia of cells in the basal layer spreading through spaces in the basement membrane into the dermis.[29] This degenerative phenomenon in the basal cells would explain why some authors claim that bullae can occur above the basal membrane.[3,27,28,30] They conclude that cleavage is a consequence of photodynamic injury of lysosomes, affecting endothelial cells, keratinocytes and dermal cells of the basal layer.[29]

In our study, we used antigen immunomapping to determine the level of cleavage of bullae in seven patients. It was not possible to identify the level of cleavage in three patients, since they presented all of the antigens on the epidermal and dermal side of the bullae, which means that they were probably in regeneration; in two patients, cleavage was intraepidermal; in one patient, it was in the sublamina densa; and in another patient, cleavage occurred below the sublamina densa. Therefore, a single level of cleavage was not found, which is in agreement with the findings of several authors who found variable levels of cleavage in electron microscopy. Only two studies used immunomapping of the DEJ to determine the level of cleavage of the bullae: a study of five large bullae, in which four were junctional and one was dermal cleavage,[27] and another study of five cases of PCT and two of pseudoporphyria, where cleavage was observed in the lamina lucida (junctional) in the seven cases.[3] Other authors[2,15] believe that the difference between

level of cleavage found in electron microscopy and that found in immunomapping is due to a sampling problem, that is, to the size of the bullae. In electron microscopy, cleavage was described in the upper dermis, since recent and small vesicles are needed for this one. Small vesicles would be related to dermal cleavage, while large blisters would be junctional. The advantage of immunomapping in comparison with electron microscopy is that it allows for an analysis of a larger area of cleavage, that is, a whole larger bulla.[27] A junctional bulla cannot be considered a specific morphological characteristic resulting from a specific pathological mechanism, as it may be a secondary phenomenon resulting from formation of dermal blister, whose fluid leaks and separates the basal lamina at the level of lamina lucida, since this area acts as a *locus minoris resistentiae.*[27] Unlike other studies that used immunomapping of the DEJ to study the cleavage level of bullae, we did not find any cases of cleavage at the level of lamina lucida. Our findings are more in line with electron microscopy, which showed variable levels of cleavage.

CONCLUSION

Our study contributes to understanding the physiopathology of skin lesions in PCT. We found that immunoglobulin deposition is not related to fragility and blistering, but reduced deposition of complement in biochemical remission supports the hypothesis that activation of the complement cascade represents an additional pathway that leads to endothelial injury. As for the mechanism that defines the different levels of cleavage at the DEJ, it is, most likely, due to the fact that photodynamic damage of lysosomes affects different target cells, the basal keratinocytes and/or dermal cells.   ❑

REFERENCES

1. Cormane RH, Szabò E, Hoo TT. Histopathology of skin in acquired and hereditary porphyria cutanea tarda. Br J Dermatol. 1971;85:531-9.
2. Wolff K, Hönigsmann H, Rauschmeier W, Schuler G, Pechlaner R. Microscopic and fine structural aspects of porphyrias. Acta Derm Venereol Suppl (Stockh). 1982;100:17-28.
3. Dabski C, Beutner EH. Studies of laminin and type IV collagen in blisters of porphyria cutanea tarda and drug-induced pseudoporphyria. J Am Acad Dermatol. 1991;25(1 Pt 1):28-32.
4. Burgeson RE, Christiano AM. The Dermal-epidermal Junction. Curr Opin Cell Biol. 1997;9:651-8.
5. Magnus IA. Dermatological Photobiology. Clinical and

Experimental Aspects. Appendix II - Clinical Screening tests for excess porphyrins. London: Blackwell Scientific Publications; 1976. p.253-5.

6. Bickers DR, Pathak MA. The porphyrias. In: Fitzpatrick TB, Eisen AZ, Wolff K, Freedberg IM, Austen KF, eds. Dermatology in General medicine. 4th ed. New York: McGraw-Hill; 1993. p.1854-93.

7. Lim CK, Peters TJ. Urine and faecal porphyrin profiles by reversed-phase high-performance liquid chromatography in the porphyrias. Clin Chim Acta. 1984;139:55-63.

8. Vieira FMJ, Martins JEC. Porfiria cutânea tardia. An Bras Dermatol. 2006;81:573-84.

9. Roengk HH Jr, Gottlob ME. Estrogen-induced porphyria cutanea tarda. Arch Dermatol. 1970;102:260-6.

10. Chuang TY, Brashear R, Lewis C. Porphyria cutanea tarda and hepatitis C virus: A case-control study and meta-analysis of the literature. J Am Acad Dermatol. 1999;41:31-6.

11. Mansourati FF, Stone VE, Mayer KH. Porphyria cutanea tarda and HIV/AIDS: a review of pathogenesis, clinical manifestations and management. Int J STD AIDS. 1999;10:51-6.

12. Fracanzani AL, Taioli E, Sampietro M, Fatta E, Bertelli C, Fiorelli G, et al. Liver cancer risk is increased in patients with porphyria cutanea tarda in comparison to matched control patients with chronic liver disease. J Hepatol. 2001;35:498-503.

13. Kordac V, Papezová R, Semrádová M. Chloroquine in the treatment of porphyria cutanea tarda. N Eng J Med. 1977;296:949.

14. Freesemann A, Frank M, Sieg I, Doss MO. Treatment of porphyria cutanea tarda by the effect of chloroquine on the liver. Skin Pharmacol. 1995;8:156-61.

15. Timonen K, Niemi KM, Mustajoki P. Skin morphology in porphyria cutanea tarda does not improve despite clinical remission. Clin Exp Dermatol. 1991;16:355-8.

16. Epstein JH, Tuffanelli DL, Epstein WL. Cutaneous changes in the porphyrias: A microscopic study. Arch Dermatol. 1973;107:689-98.

17. Wick G, Hönigsmann H, Timpl R. Immunofluorescence demonstration of type IV collagen and a noncollagenous glycoprotein in thickened vascular basal membranes in protoporphyria. J Invest Dermatol. 1979;73:335-8.

18. Hönigsmann H, Gschnaiat F, Konrad K, Stingl G, Wolff K. Mouse model for protoporphyria. III. Experimental production of chronic erythropoietic protoporphyria-like skin lesions. J Invest Dermatol. 1976;66:188-95.

19. Ryan EA. Histochemistry of the skin in erythropoietic protoporphyria. Br J Dermatol. 1966;78:501-18.

20. Baart de la Faille-Kuyp, Cormane RH. The occurrence of certain serum factors in the dermal-epidermal junction and vessel walls of the skin in lupus eythematosus and other (skin) diseases. Acta Derm Venereol. 1968;48:578-88.

21. Krajnc I, Vizjak A, Hvala A, Jurcic V, Rozman B. The significance of histologic analysis of skin lesions in porphyria cutanea tarda. Light microscopy, electron microscopy, immunohistochemical and immunofluorescence analysis. Wien Klin Wochenschr. 1998;110:651-4.

22. Lim HW, Perez HD, Poh-Fitzpatrick M, Goldstein IM, Gigli I. Generation of chemotactic activity in serum from patients with erythropoietic protoporphyria and porphyria cutanea tarda. N Engl J Med. 1981;304:212-6.

23. Torinuki W, Miura T, Tagami H. Activation of complement by 405-nm light in serum from porphyria cutanea tarda. Arch Dermatol Res. 1985;277:174-8.

24. Lim HW, Gigli I. Role of complement in porphyrin-induced photosensitivity. J Invest Dermatol. 1981;76:4-9.

25. Lim HW, Poh-Fitzpatrick MB, Gigli I. Activation of the complement system in patients with porphyrias after irradiation in vivo. J Clin Invest. 1984;74:1961-5.

26. Rimington C, Magnus IA, Ryan EA, Cripps DJ. Porphyria and photosensitivity. Q J Med. 1967;36:29-57.

27. Klein GF, Hintner H, Schuler G, Fritsch P. Junctional blisters in acquired bullous disorders of the dermal-epidermal junction zone: role of the lamina lucida as the mechanical locus minoris resistentiae. Br J Dermatol. 1983;109:499-508.

28. Nagato N, Nonaka S, Ohgami T, Murayama F, Yamashita K, Irifune H, et al. Mechanism of blister formation in porphyria cutanea tarda. I. Histopathological observation of blisters in three cases of porphyria cutanea tarda.. J Dermatol. 1987;14:551-5.

29. Caputo R, Berti E, Gasparini G, Monti M. The morphologic events of blister formation in porphyria cutanea tarda. Int J Dermatol. 1983;22:467-72.

30. Perrot H, Schmitt D, Thivolet J, Leung J, Germain DU. Étude ultrastructurale de la bulle dans les porphyries cutanées hépatiques. Bull Soc Fr Derm Syph. 1972;79:12-8.

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