CLINICAL SCIENCE

Analysis of the reactivity of indirect immunofluorescence in patients with pemphigus foliaceus and pemphigus vulgaris using rat bladder epithelium as a substrate

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OBJECTIVES: To evaluate the reactivity of indirect immunofluorescence using rat bladder epithelium as a substrate in patients with pemphigus foliaceus and pemphigus vulgaris from the Department of Dermatology, University of São Paulo Medical School, Brazil.

METHODS: Thirty-two patients (8 male and 24 female) from the Department of Dermatology, University of São Paulo Medical School, were selected. Three had mucosal pemphigus vulgaris, 20 had mucocutaneous pemphigus vulgaris, and 9 had pemphigus foliaceus. Patients' sera were tested by indirect immunofluorescence performed on human foreskin and rat bladder epithelium and by ELISA assays utilizing baculovirus-expressed recombinant desmoglein 3 and desmoglein 1.

RESULTS: No patients with mucosal pemphigus vulgaris, 5 of 20 patients with mucocutaneous pemphigus vulgaris (25%) and 4 of 9 patients with pemphigus foliaceus (44%) had positive indirect immunofluorescence using rat bladder epithelium as a substrate.

CONCLUSION: Indirect immunofluorescence using rat bladder epithelium as a substrate is recommended whenever a diagnosis of paraneoplastic pemphigus is considered. The identification of a subset of pemphigus foliaceus and pemphigus vulgaris patients that recognizes desmoplakins by this laboratory tool is critical to avoid the misdiagnosis of paraneoplastic pemphigus.

KEYWORDS: Pemphigus vulgaris; Paraneoplastic pemphigus; Indirect immunofluorescence; Rat bladder epithelium; Pemphigus foliaceus.

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INTRODUCTION

Desmoplakin I (DP I) and desmoplakin II (DP II) are constitutive desmosomal plaque proteins that provide a link between the desmosomal cadherin and the intermediate filament cytoskeleton, thereby contributing to the functional integrity of the desmosome-keratin filament complex.[1] DP autoantibodies are present in paraneoplastic pemphigus (PNP) as a component of a complex humoral immune reaction[2] and were once considered to be a sensitive and specific feature in the diagnosis of PNP.[3] However, these autoantibodies have also been found in other diseases, including pemphigus foliaceus (PF), pemphigus vulgaris (PV), bullous pemphigoid (BP), and erythema multiforme major.[4-12] A possible mechanism for the development of autoantibodies to DP in those dermatoses is explained by the epitope-spreading phenomenon.[5,6] This phenomenon includes an initial autoimmune response against a specific antigen that may lead to the recognition of other antigens that are not necessarily related by homology but are physically linked or share proximal locations.[13]

The presence of anti-DP antibodies in IgG-mediated pemphigus does not seem to characterize a particular subgroup,[7] and it is unlikely that these antibodies could be solely responsible for acantholysis. It is possible that anti-DP antibodies could potentiate the disruption in cell-cell adhesion originally initiated by anti-desmoglein antibodies.[6]

The urinary bladder epithelium has desmosomes that contain DP I and/or DP II but do not express PF or PV

No potential conflict of interest was reported.

Table 1 - Classification of disease activity.

Complete remission	Off therapy	Absence of new or established lesions while the patient is off all systemic therapy for at least 2 months
	On therapy	
Partial remission	Off therapy	Presence of transient new lesions that heal within 1 week without treatment and while the patient is off all systemic therapy for at least 2 months
	On therapy	The presence of transient new lesions that heal within 1 week while the patient is receiving systemic therapy
	On minimal therapy	The presence of transient new lesions that heal within 1 week while the patient is receiving minimal therapy, including topical steroids
Relapse/Flare		Appearance of at least three lesions/month that do not heal spontaneously within 1 week, or by the extension of established lesions, in a patient who has achieved disease control

antigens.[14] Therefore, the reactivity of indirect immunofluorescence using rat bladder epithelium (IIF-RBE) as a substrate in patients with PF or PV suggests the presence of anti-DP autoantibodies.

OBJECTIVES

The aim of this study was to analyze the reactivity of IIF-RBE in patients with PF and PV from the Department of Dermatology, University of São Paulo Medical School to evaluate whether this diagnostic tool could lead to a misdiagnosis of PNP for PF and PV patients.

MATERIALS AND METHODS

Upon approval by the Ethics Committee, 32 patients (8 male and 24 female, with a mean age of 45 years) followed up by the Department of Dermatology, University of São Paulo Medical School between 1994 and 2009 were selected for the study. Three of 32 patients had mucosal pemphigus vulgaris (MPV), 20 had mucocutaneous pemphigus vulgaris (MCPV), and 9 had pemphigus foliaceus (PF). All diagnoses were confirmed by clinical, histopathological, and direct immunofluorescence evaluations. No patients were diagnosed with PNP until the completion of this study. The disease activity was classified according to the criteria adapted from the consensus statement on definitions of the disease, end points and the therapeutic response for pemphigus (Table 1).[15]

Patients' sera were tested by indirect immunofluorescence and an enzyme-linked immunosorbent assay (ELISA). IIF analysis of the patients' sera was performed on human foreskin and rat bladder epithelium. ELISA tests utilized baculovirus-expressed recombinant desmoglein 3 (Dsg3) and desmoglein 1 (Dsg1).

1. Indirect immunofluorescence using human foreskin (IIF-HFS) or rat bladder epithelium (IIF-RBE) as a substrate:

Four micrometer cryostat sections of HFS and RBE were incubated for 60 minutes with sera dilutions starting at 1:20. The slides were washed in Tris-buffered saline (TBS) twice (20 minutes each) and then covered with fluorescein isothiocyanate-conjugated (FITC) goat anti-human IgG at a dilution of 1:30 (Sigma, USA) for 30 minutes. After two additional 20-minute washes (TBS), the slides were mounted in buffered glycerol and examined under an epiluminescent microscope (Zeiss, Germany).

1. ELISA

Sera samples (1:100 dilution) were added to microwell plates coated with baculovirus-expressed desmogleins for 60 minutes. After washing, horseradish peroxidase-conjugated IgG was added and allowed to incubate for 60 minutes. Following another wash, the peroxidase substrate was added and allowed to incubate for an additional 30 minutes. Then, a 1.0-N sulfuric acid solution was added to each well to terminate the enzyme reaction and stabilize the color development. The absorbance was measured at 450 nm by an ELISA plate reader (MBL, Japan). A single PF serum sample and a single PV serum sample were selected as positive control serum samples for the Dsg1 and Dsg3 ELISAs, respectively. The index was calculated as follows: index = (optical density (OD) of the tested serum - OD of the negative control)/(OD of the positive control serum - OD of the negative control) ×100. The interpretation of results was conducted according to the following parameters.

RESULTS

Mucosal pemphigus vulgaris (MPV)

All MPV patients (n = 3) were in partial remission on therapy, and the mean follow-up time was 4.6 months. All patients had negative IIF-RBE and positive IIF-HFS, with titers ranging from 1:40 to 1:320 (mean titer of 1:160). All had positive ELISA results for Dsg3, and 1 patient also had a positive ELISA result for Dsg1.

Mucocutaneous pemphigus vulgaris (MCPV)

Seventeen of 20 MCPV patients were classified as in partial remission on therapy, and 4 (23%) had positive IIF-RBE. Three of 20 MCPV patients were classified as in partial remission on minimal therapy, and 1 (33%) had positive IIF-RBE. Therefore, 5 of 20 (25%) PV sera showed reactivity in IIF-RBE, with titers ranging from 1:40 to 1:160 (mean titer of 1:80) (Figure 1).

Positive IIF-HFS was in 18 of 20 MCPV sera, with titers ranging from 1:80 to 1:5,120 (mean titer of 1:1,280). The 2 PV patients with negative IIF-HFS were in partial remission on therapy.

The mean IIF-HFS titer among patients with MCPV in partial remission on therapy was 1:1,280 (mean titer of 1:1,280 among patients with positive IIF-RBE and 1:1,280 among patients with negative IIF-RBE). The mean titer of

1. Dsg1

Less than 14	Negative
14 to 20	Indeterminate
Greater than 20	Positive

1. Dsg3

Less than 9	Negative
9 to 20	Indeterminate
Greater than 20	Positive

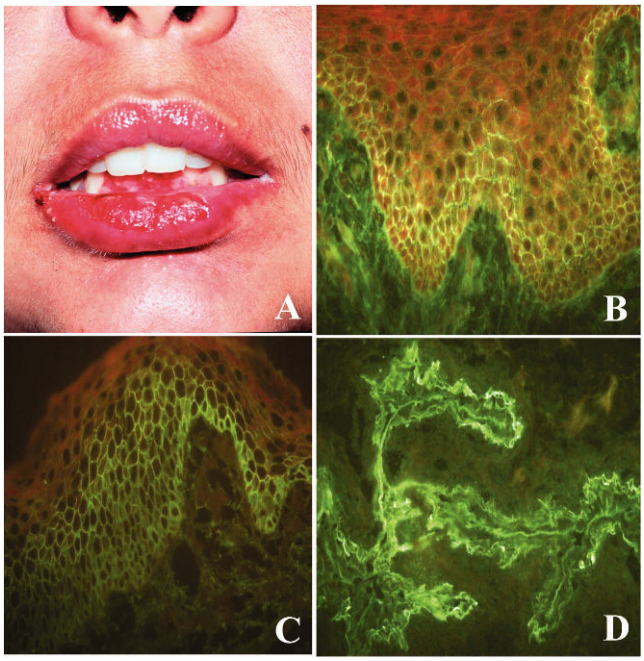

Figure 1 - (A) Clinical aspects of the lesions of a PV patient and (B) the results of direct immunofluorescence demonstrating intercellular IgG at the basal layers of the epidermis (×400); (C) indirect immunofluorescence using human foreskin as a substrate showing intercellular intraepidermal IgG deposits (×400); and (D) indirect immunofluorescence using rat bladder epithelium as a substrate demonstrating intercellular intraepithelial IgG deposits (×400).

IIF-HFS among the MCPV patients in partial remission on minimal therapy was 1:1,280 (mean titer of 1:2,560 among patients with positive IIF-RBE and 1:160 among patients with negative IIF-RBE).

Seventeen of 20 MCPV patients had positive ELISA results for Dsg3, and 10 were anti-Dsg1 positive.

The mean follow-up time of the MCPV patients was five years. MCPV patients in partial remission on therapy had a mean follow-up of five years (three years among patients with positive IIF-RBE and six years among patients with negative IIF-RBE). MCPV patients in partial remission on minimal therapy had a mean follow-up of four years (four years among patients with positive IIF-RBE and three years among patients with negative IIF-RBE).

The overall reactivity of IIF-RBE in all 23 PV patients was 22% (5/23).

Pemphigus foliaceus (PF)

Interestingly, four out of nine PF patients had a positive IIF-RBE with titers ranging from 1:20 to 1:80 (mean titer of 1:40). All were in partial remission. The overall reactivity of IIF-RBE in all nine PF patients was 44% (4/9) (Figure 2).

Five of nine PF patients were classified as in partial remission on therapy, and two (40%) had positive IIF-RBE. Four of nine PF patients were classified as in remission on minimal therapy, and two (50%) had positive IIF-RBE. All nine PF sera showed positive results for IIF-HFS, with titers ranging from 1:80 to 1:5,120 (mean titer of 1:1,280).

All nine PF sera showed negative results for Dsg3 ELISA, and one showed negative Dsg1 ELISA results. This patient was in partial remission on minimal therapy.

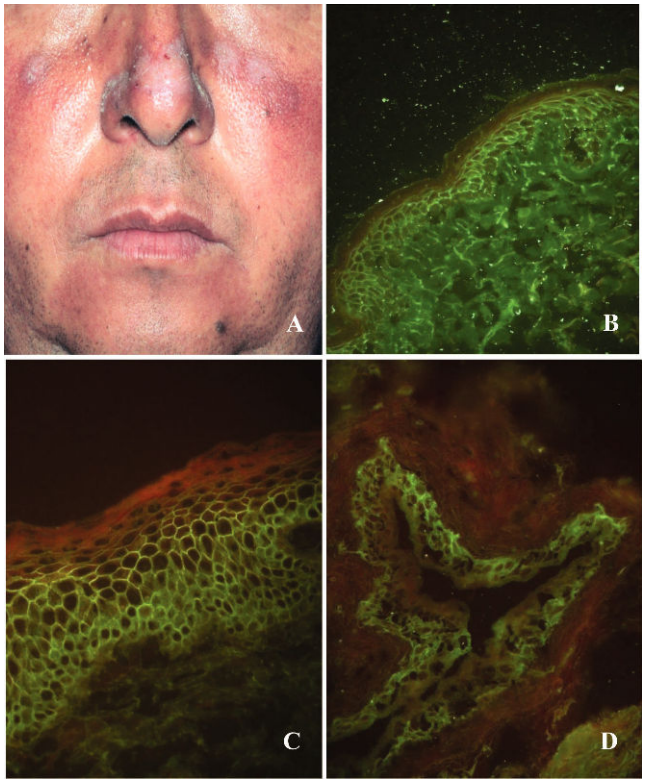

Figure 2 - (A) Clinical aspects of the lesions of a PV patient and (B) the results of direct immunofluorescence demonstrating intercellular intraepidermal IgG deposits (×400); (C) indirect immunofluorescence using human foreskin as a substrate showing intercellular intraepidermal IgG deposits (×400); and (D) indirect immunofluorescence using rat bladder epithelium as a substrate demonstrating intercellular intraepithelial IgG deposits (×400).

The mean IIF-HFS titer among patients with PF in partial remission on therapy was 1:1,280 (mean titer of 1:2,560 among patients with positive IIF-RBE and 1:1,280 among patients with negative IIF-RBE). The mean IIF-HFS titer among patients with PF in partial remission on minimal therapy was 1:1,280 (mean titer of 1:1,280 among patients with positive IIF-RBE and negative IIF-RBE).

PF patients with positive IIF-RBE had a long-term disease (4-15 years). The mean follow-up of the PF patients was five years. Patients with PF in partial remission on therapy had a mean follow-up of six years (ten years among patients with positive IIF-RBE and three years among patients with negative IIF-RBE). Patients with PF in partial remission on minimal therapy had a mean follow-up of six years (four years among patients with positive IIF-RBE and eight years among patients with negative IIF-RBE).

The demographic data from the patients are shown in Table 2.

DISCUSSION

The use of RBE as a substrate for IIF in PNP started in 1990 with Anhalt et al.[16] The specificity of the RBE substrate for PNP was reported to be high, varying from 83% to 95%.[3,14,17] However, Cozzani et al.[7] found 21% positive IIF-RBE in patients with PV, which is in accord with our data (22% in PV patients). These authors suggested a role for anti-DP in determining disease severity.[6,7] In our study, all PV patients with positive IIF-RBE belonged to the mucocu-

Table 2 - Clinical and immunological profile of pemphigus patients.

Patient	Age/Sex	Follow-up	IgG IIF-HFS	IgG IIF-RBE	ELISA Dsg1	ELISA Dsg3
MPV – partial remission on therapy						
1	40/M	8 m	1:40	-	-	+
2	25/M	4 m	1:320	-	+	+
3	27/M	2 m	1:320	-	-	+
MCPV – partial remission on therapy						
4	28/F	5 y	1:640	-	+	+
5	39/F	1 y	1:5120	-	+	+
6	44/M	5 y	1:80	-	-	+
7	47/F	2 y	1:1280	1:80	+	+
8	64/F	5 y	1:160	-	-	+
9	71/F	2 y	-	-	+	+
10	25/F	11 y	1:1280	-	-	+
11	72/F	15 y	1:640	-	+	+
12	31/F	6 y	1:320	1:160	-	+
13	61/M	7 y	1:1280	-	+	-
14	40/F	7 m	1:320	-	Ind.	+
15	55/F	2 y	1:640	1:40	Ind.	+
16	57/F	12 y	-	-	-	+
17	41/F	3 y	1:2560	-	+	+
18	38/F	4 y	1:1280	-	+	-
19	60/F	6 y	1:1280	-	-	+
20	40/F	1 y	1:5120	1:40	+	+
MCPV – partial remission on minimal therapy						
21	35/F	4 y	1:2560	1:40	+	-
22	32/F	2 y	1:160	-	-	+
23	48/F	5 y	1:320	-	-	+
PF – partial remission on therapy						
24	62/M	5 y	1:1280	-	+	-
25	55/M	6 y	1:1280	1:20	+	-
26	56/F	15 y	1:5120	1:40	+	-
27	62/F	3 y	1:1280	-	+	-
28	29/F	2 y	1:320	-	+	-
PF – partial remission on minimal therapy						
29	58/F	4 y	1:80	1:80	-	-
30	42/M	5 y	1:2560	1:40	+	-
31	41/F	12 y	1:320	-	+	-
32	51/F	4 y	1:2560	-	+	-

IIF-HFS: indirect immunofluorescence using human foreskin; *IIF-RBE*: indirect immunofluorescence using rat bladder epithelium; *MPV*: mucosal pemphigus vulgaris; *MCPV*: mucocutaneous pemphigus vulgaris; *PF*: pemphigus foliaceus; *F*: female; *M*: male; *m*: months, *y*: years; *Ind.*: indeterminate; (–): negative; (+): positive.

taneous variant, and this observation supports that suggestion; however, those patients were in partial remission. In our study, there was not a clear correlation between the IIF-RBE results and disease activity. Furthermore, the results of IIF-HFS and the follow-up time did not correlate with the reactivity of IIF-RBE.

Anti-desmoglein 1 and/or 3 autoantibodies were detected in all patients except for one who had PF in clinical remission on minimal therapy. The presence of these autoantibodies reinforced the diagnosis of PF or PV. The sensitivity and specificity of ELISA with recombinant Dsg1 and 3 is reported to be approximately 95-98%.[18]

Anti-DP antibodies have previously been described in one PF patient,[4] which is in contrast to our IIF-RBE results showing reactivity in 4 of 9 PF patients (44%). All PF patients had a long-term follow-up (4-15 years).

The long-term follow-up of our positive IIF-RBE patients (three years for MCPV and six years for PF) may indicate that these patients will not develop PNP.

A possible explanation for the presence of anti-DP antibodies in PF and PV is the epitope spreading phenomenon. However, it is unlikely that anti-DP antibodies in our PF and PV patients played a role in the loss of keratinocyte adhesion, leading to acantholysis and blister formation. Moreover, the possible presence of anti-DP autoantibodies in these patients could be explained by long-term chronic autoimmune disease.

IIF-RBE is a relevant tool when considering patients with PNP. The anti-desmoplakin response of IIF-RBE is not a routine technique employed to investigate PF or PV patients and should only be performed in patients with suspected PNP. The identification of a subset of PF and PV patients with positive IIF-RBE is relevant to avoid a misdiagnosis of PNP in doubtful cases. Therefore, the correlation of clinical features, histopathology, direct immunofluorescence, and indirect immunofluorescence is necessary to achieve correct diagnoses.

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AUTHOR CONTRIBUTIONS

Ortolan DG was responsible for the data acquisition, data analysis and interpretation, and drafting of the manuscript. Souza DPG was responsible for the data acquisition, and drafting of the manuscript. Aoki V, Santi CG and Maruta CW conceived and designed the study, and were also responsible for the acquisition, analysis and interpretation of data, drafting and critical revision of the manuscript for important intellectual content and study supervision. Gabbi TVB was responsible for the data acquisition.

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