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

Damaris G. Ortolan, Danielle P. G. Souza, Valéria Aoki, Claudia G. Santi, Tatiana V. B. Gabbi, Ligia M. F. Ichimura, Celina W. Maruta

Faculdade de Medicina da Universidade de São Paulo, Department of Dermatology, São Paulo, Brazil.

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.

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 reaction2 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 cellular antigens.14 This may suggest that the urinary bladder epithelium is an alternative substrate for indirect immunofluorescence in patients with PNP.
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. **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 |

**RESULTS**

**Mucosal pemphigus vulgaris (MPV)**

Seventeen of 20 MCPV patients were classified as 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 Dsg1, and 1 patient also had a positive ELISA result for Dsg3.

**Mucocutaneous pemphigus vulgaris (MCPV)**

Of 20 MCPV patients, 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). The mean titer of...
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. Five (25%) had positive IIF-RBE. Four of nine PF patients were classified as in remission on minimal therapy, and two (10%) 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.

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. The specificity of the RBE substrate for PNP was reported to be high, varying from 83% to 95%. However, Cozzani et al. 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. In our study, all PV patients with positive IIF-RBE belonged to the mucocu-
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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 | 4/25/M | 5 y | 1:1280 | - | + | + |
| 1 | 40/M | 8 m | 1:1280 | - | - | + |
| 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 | 1:1280 | - | - | + |
| 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 foliaceous; F: female; M: male; m: months; y: years; Ind.: indeterminate; (-): negative; (+): positive.

It is unlikely that anti-DP antibodies in 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 PNP 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

Oortolan 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., Santig C. 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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