

Real-time, non-invasive microscopic confirmation of clinical diagnosis of bullous pemphigoid using *in vivo* reflectance confocal microscopy

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Background: Bullous pemphigoid is an autoimmune disease affecting prevalently the elder. *In vivo* reflectance confocal microscopy is a non-invasive technique for real-time imaging of the skin with cellular-level resolution. No previous data has been reported about confocal microscopy of bullous pemphigoid. Aim of this preliminary study is the evaluation of the potential of *in vivo* reflectance confocal microscopy for real-time, microscopical confirmation of clinical bullous pemphigoid diagnosis.

Methods: A total of nine lesions from patients affected by pemphigoid underwent *in vivo* reflectance confocal microscopy before histological examination.

Results: In our preliminary study, confocal microscopy showed high grade of correspondence to histopathology. In particular, presence of sub-epidermal cleft and variable amount of oedema of the upper dermis associated with inflammatory cells infiltration were seen as prevalent confocal features in the bul-

lous lesions considered. Differently, in urticarial lesions, no specific features could be appreciated at confocal analysis beside the presence of signs of spongiosis and perivascular inflammation.

Conclusion: Confocal microscopy seems to be useful for *in vivo*, microscopical confirmation of the clinical suspect of bullous pemphigoid and for biopsy site selection in urticarial lesions to obtain a more significant specimen for histopathological examination.

Key words: bullous pemphigoid – autoimmune bullous diseases – *in vivo* reflectance confocal microscopy – non-invasive diagnosis – real-time microscopy – sub-epidermal cleft

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BULLOUS PEMPHIGOID (BP) is an autoimmune skin disease predominantly affecting the elderly (1). It is characterized by chronic, recurrent tense blisters arising on erythematous skin and generally preceded by itching and eruption of urticarial lesions (2, 3). In addition to clinic, histopathology and direct immunofluorescence (DIF) are generally required for diagnosis confirmation; however, these techniques are invasive, need time to be achieved and may delay the diagnosis and start of appropriate treatment (4, 5).

Histology of urticarial lesions discloses presence of variable degree of eosinophilic spongiosis and perivascular inflammation with the prevalence of eosinophils. Differently, bullous lesions are characterized by sub-epidermal clefting associated with variable degree of exocytosis and dermal infiltration of inflammatory cells, mainly composed by eosinophils (1–3).

DIF reveals the presence of deposits of IgG and C3 at the basement membrane; indirect immunofluorescence examination shows circulating autoantibodies (BP-180, BP-230) in 60% to 70% of patient's sera (4, 5).

In vivo reflectance confocal microscopy (RCM) is a non-invasive technique for real-time imaging of the superficial layers of the skin down to the upper dermis with cellular-level resolution similar to conventional histopathology (6). RCM has been already used for the evaluation of several inflammatory skin conditions such as psoriasis, lupus erythematosus, Darier's disease and acute contact dermatitis, demonstrating a good agreement with conventional histopathology (7–13).

To our knowledge, no previous RCM data have been reported concerning bullous pemphigoid. Description of cases of pemphigus foliaceus and pemphigus vulgaris evaluated with RCM had

been reported in literature disclosing the potential efficacy of this technology for bullous diseases evaluation (14, 15).

In this study, we describe our preliminary experience on BP *in vivo* confocal features and their correspondence to optical histology to test its potential use in the clinical setting for clinical diagnosis, real-time, microscopical confirmation and patient management.

Patients and Methods

Patients

Seven patients, two male and five females, with age ranging from 47 to 85 years old, presenting eruption of urticarial and/or bullous lesions suspected for BP were recruited from the outpatient service of San Gallicano Dermatological Institute after signed informed consent. IRB approval for the study has been obtained. All the patients presented variable diffusion of tense blisters on erythematous skin and in three to seven patients also urticarial plaques were seen. A total of nine lesions (six bullous and three urticarial lesions) underwent RCM before punch biopsies for histopathology and DIF.

Methods

In vivo reflectance confocal microscopy

Vivascope 1500[®] (Lucid Technologies, Henrietta, New York, USA), a commercially available RCM for clinical *in vivo* imaging was used. Technical characteristics have been already described elsewhere (9). RCM was performed at the margin of a bullous lesion at the interface with perilesional skin. RCM acquisition protocol consisted of series of 4 by 4 mm blocks taken at the level of the spinous layer, dermoepidermal junction (DEJ) and the upper dermis plus 1 vertical stack of five microns step, taken from the centre of the lesion and starting from the cornified layer down to the dermis.

Histological examination

Punch biopsy (4 mm) was taken from the same site of RCM examination. Specimens were fixed in phosphate-buffered neutral formalin, embedded in paraffin and sectioned vertically according to standard protocols. Five micrometre thick sections were stained with haematoxylin and eosin.

A second biopsy was performed on perilesional skin to perform DIF on fresh tissue.

Results

In all the six bullous lesions considered (100%), RCM showed substantially normal superficial layers of the epidermis (stratum corneum and spinosum) with a preserved honeycombed pattern as generally seen in normal skin. In only 3–6 bullous lesions (50%), features of mild spongiosis were seen at RCM as darker areas in comparison to the surrounding epithelium with increased intercellular spaces, and in which bright, small cellular structures were present but in different amount. Those features were seen to correspond on histology to the presence of variable amount of inflammatory cells, prevalently eosinophils, between epidermal keratinocytes determining what is named 'eosinophilic spongiosis'.

Reflectance confocal microscopy mosaics taken at the level of the dermoepidermal junction (DEJ) disclosed, in all the bullous lesions considered (100%), the presence of a well-delimited dark area filled by bright, pinpoint structures and greyish material separated by mildly bright septa (Figs 1c and 2c) corresponding to the bulla clinically and dermoscopically visible. At the close-up (RCM single images), the dark area was delimited by 80 µm median thick epithelial structure (ranging from 70 µm to 110 µm) with RCM features of the full layers normal epidermis (Fig. 1d and Fig. 2d) and corresponding on histology to the sub-epidermal cleft. Moreover, this epidermal wall was seen to cover a dark-greyish area filled by different amount of mildly bright, round to oval cellular structures corresponding on histopathology to dermal oedema with inflammatory cells (mostly eosinophils) located at the level of the papillary dermis and filling the bulla. Furthermore, inside of the dark longitudinal spaces, mildly refractive homogeneous material was present in different degree and corresponded to fibrin deposition visible on histology (Fig. 1d).

In all the three urticarial lesions considered, RCM examination lacked to show any specific features, but showed signs of spongiosis with aggregates of inflammatory cells (Fig. 3c). Normal dermal papillae and sparse inflammatory cells in the upper dermis were also visible (Fig. 1 3d). Histology of urticarial lesions

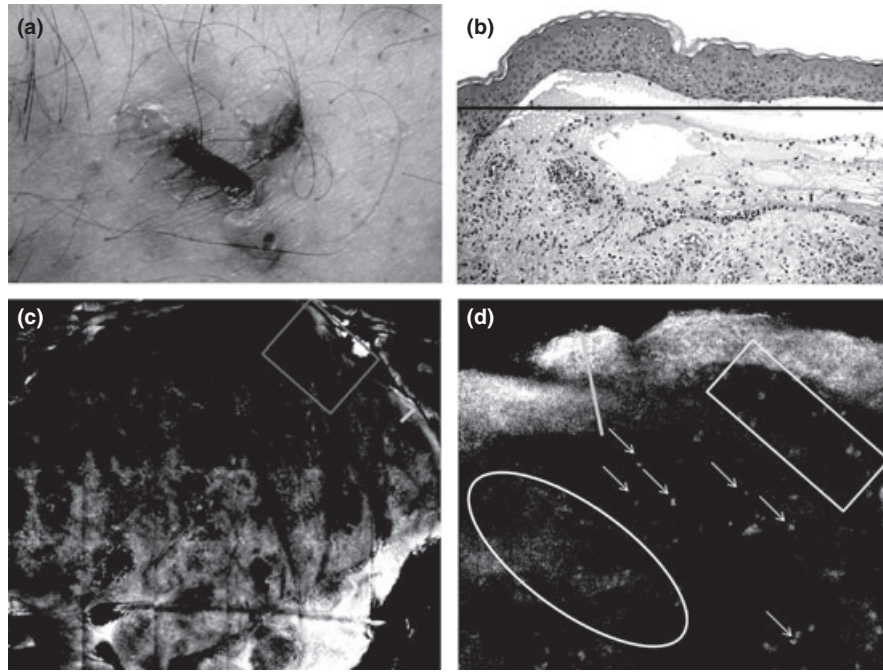


Fig. 1. (a) Case 1. Blister from the trunk of a 47-year-old man; (b) histology (H&E $\times$ 400): sub-epidermal blister with eosinophils and fibrin associated with superficial dermal oedema. Level of RCM examination (black line); (c) RCM mosaic taken at the level of the DEJ: well-defined area with diffuse dark spaces separated by mildly bright septa (bullae); (d) close-up: peripheral wall made by full-layer epidermis (green line) well separated from the upper dermis. Just below the peripheral wall, dark longitudinal space (yellow square) associated with bright round cellular structures, corresponding to leucocytes (arrows). Mildly refractive homogeneous material corresponding to deposit of fibrin just below the epithelial wall (white circle).

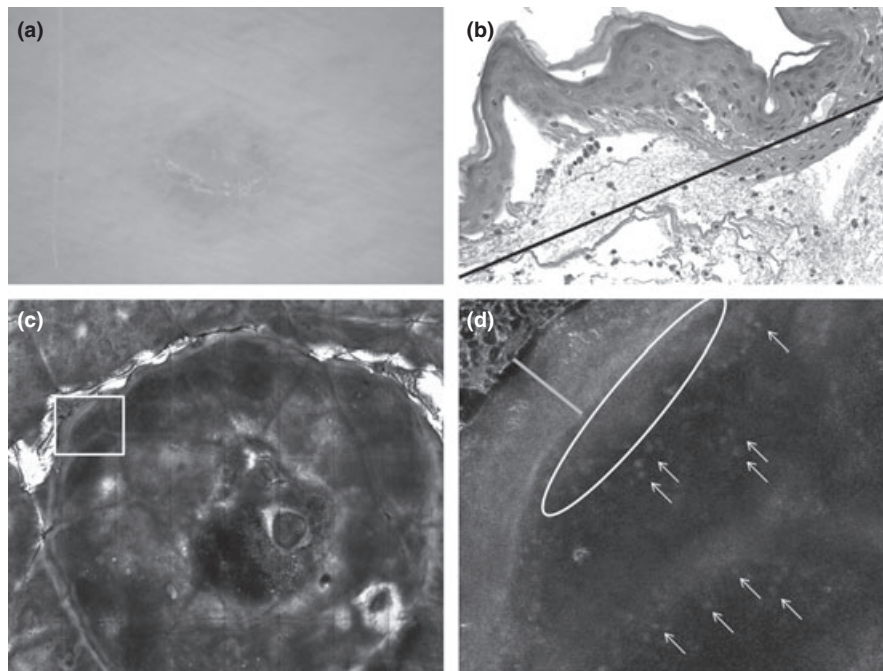


Fig. 2. (a) Case 3. Bullous lesion from the left arm; (b) histology (H&E $\times$ 400): sub-epidermal blister with prominent deposit of fibrin associated with inflammatory infiltration of the superficial dermis composed mainly by eosinophils; (c) RCM mosaic at the level of the DEJ: clear-cut blister delimited by a thick epithelial wall; (d) RCM close-up: thickness of the border (green line) associated with inflammatory cells (arrows) and deposit of fibrin (white circle).

disclosed the presence of eosinophilic spongiosis associated with eosinophils around dermal vessels (Fig. 3b).

In vivo confocal microscopy/histopathology correlations are summarized in Table 1. Diagnosis of pemphigoid (Figs. 1b, 2b and 3b) was later

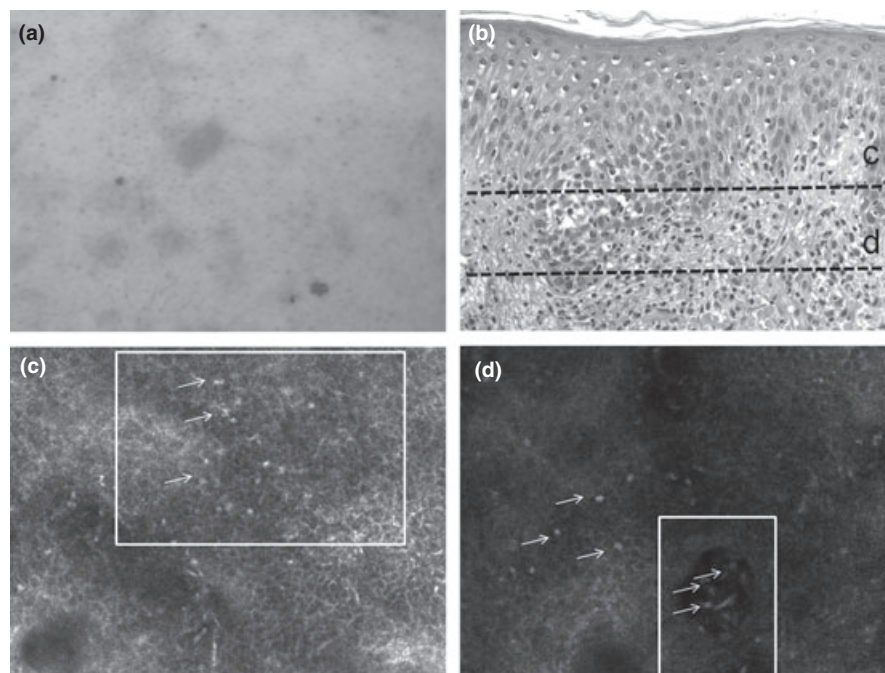


Fig. 3. (a) Case 7. Urticarial lesion from the trunk of a 78-year-old man; (b) histology (H&E $\times 400$): eosinophilic spongiosis and perivascular inflammatory infiltration with eosinophils; (c) RCM single image from the spinous layer showing spongiosis and epidermal infiltration of inflammatory cells, respectively, as darker areas in comparison to the surrounding epithelium with increased intercellular spaces (white square), and small cellular structures were present in different amount (arrows); (d) RCM single image from the DEJ with inflammatory cells in the lower epidermal layers (arrows) and inflammatory cells in the upper dermis visible is throughout the DP (yellow arrows in the white square).

TABLE 1. RCM features correlation with histology

Histopathology	RCM
Spongiosis with exocytosis of inflammatory cells	Darker area in the stratum spinosum of the epidermis, in comparison to the surrounding epithelium, showing increased intercellular spaces in which bright, small cellular structures were present in different amount.
Blister roof	Peripheral wall of the dark area made by a 80 μm median thick epithelium (ranging from 70 μm to 110 μm) with RCM features of the full epidermis layers.
Sub-epidermal cleft/Oedema of the upper dermis with inflammatory cells	Well-delimited dark, longitudinal area just below the epithelial wall (epidermis) filled by mildly bright, round cellular structures with no visible nucleus.
Fibrin deposition	Mildly refractive homogeneous material inside of the longitudinal, dark area just below the full epidermal layer epithelial wall.
Dermal/perivascular inflammation	Bright, small cellular structures with no visible nucleus located in the papillary dermis and around dermal vessels.

confirmed for all the lesions considered by histology at first and by DIF positivity for IgG and complement deposit at the DEJ. Moreover, detection in the serum of antibodies against basal membrane proteins BP180 or BP230 (16) was also positive.

Discussion

Clinical presentation of BP is generally highly suggestive; however, histology, immunofluorescence and laboratory data are generally required

to confirm the diagnosis (4, 5, 17). Sometimes the biopsy can provide a non-diagnostic specimen or the sampling can be inadequate and the immunofluorescence can provide false-negative results for sampling or technical errors (16). The limits of those integrative diagnostic exams can affect the final diagnosis as well as the onset of an appropriate treatment. The possibility of real-time, microscopical evaluation of the lesion could support the first clinical diagnosis and better orient clinician in the early and appropriate onset of treatment and patient management.

RCM, a harmless, non-invasive, high-resolution imaging technique, that already showed advantages in the identification of descriptive microscopical aspects of other bullous diseases as pemphigus vulgaris and foliaceus in which acantholytic keratinocytes, as sign of intra-epidermal blisters, have been identified (14, 15). When clinical confocal correlation lacks to confirm the diagnosis of BP, RCM lets, at least, the correct biopsy site selection for a more significant tissue sampling for histopathology and immunological tests.

In general, in skin inflammatory diseases examination, the major limit for RCM is represented by the depth of visualization (max 300 μ m below the skin surface) (6); however, in BP, the inflammatory process is entirely located within the upper skin layers. Considering, the most common bullous diseases differential diagnosis, as already tested in literature and confirmed by our experience, one of the main limit of the use of RCM in pemphigus is represented by the skin stripping (Nikolsky sign) due to the adhesive windows applied on the lesional and surrounding areas needed for confocal microscope connection to the skin. Moreover, in pemphigus, because of the horizontal approach to the tissue of RCM, an accurate identification of the location of the epidermal cleft can be extremely not easy and sometimes impossible.

On the counterpart, the horizontal approach to an intact bulla of pemphigoid lets an easier identification of the level of clefting as shown in Figures 1c and 2c with no risk of Nikolsky.

In our preliminary experience with RCM in BP, the examination can be easily performed providing in real-time, microscopical information useful for clinical diagnosis confirmation. Confocal features highly corresponded to optical histology. Moreover, RCM identified repeatable microscopic features in all the six bullous and the three non-bullous lesions examined. In particular, presence of sub-epithelial cleft, upper dermal oedema associated with inflammatory cells infiltration and sub-epithelial vari-

able degree of deposit of fibrin were seen. Urticarial lesions did not show specific features at RCM, but moderate-to-severe spongiosis and upper dermal inflammation were constantly seen in the cases observed.

In our view, the major limit of RCM in BP is represented by the impossibility of leucocytes subtype identification, where eosinophils are a key histological feature for BP diagnosis.

In our preliminary experience and according to the developing literature, RCM in BP seems to be useful for the real-time, microscopical examination of the skin sample for diagnosis confirmation and/or follow-up of the microscopical changes during therapy or at least to drive the biopsy, especially in urticarial lesions, to obtain a more sensitive histopathology. Furthermore, RCM can support – microscopically, non-invasively and in real time – the clinical differential diagnosis with other bullous diseases, thanks to the immediate and direct clinical-microscopical correlation. In specific, BP can be easily differentiated from pemphigus vulgaris according to the layer of epidermal “blister” location. Moreover, in BP, the presence of the sub-epidermal cleft, a more significant amount of spongiosis and/or inflammatory process distribution in the different skin layers can be easily detected. RCM seems to be more indicative in BP than in other bullous diseases like pemphigus.

For a better definition of the real potential of RCM, further studies with larger number of cases – comparing pemphigoid with pemphigus and other bullous diseases – are needed to confirm our preliminary findings and ideas, and to evaluate the real potential role of RCM in this field on inflammatory skin diseases.

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