

Noninvasive in vivo confocal laser scanning microscopy is effective in differentiating allergic from nonallergic equivocal patch test reactions

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Abstract Patch testing is the gold standard for the validation of contact dermatitis. It relies on the subjective scoring by an evaluator of the inflammatory reaction induced by an allergen applied to the skin. Equivocal reactions imply faint erythema and could represent allergic, irritant, or negative reactions. They constitute approximately 1 % of the positive reactions encountered in patch test practice. Histological evaluation of the equivocal reaction has proven helpful for the correct interpretation but is however time consuming, and its invasive nature is often unacceptable to the patient. In vivo confocal laser scanning microscopy (CLSM) is a novel, noninvasive imaging technique which permits real-time visualization of skin structures and lesions at a resolution close to that obtained by conventional histology. CLSM has been successfully applied for the differentiation between clinically clear-cut allergic and irritant patch test reactions. The objective of this study is to determine the relevance of CLSM in differentiating between allergic, irritant, and negative equivocal patch test reactions. Fifteen patients who underwent patch testing in our clinic were observed as having 20 equivocal reactions. All 20 reactions were evaluated using in vivo CLSM and compared with adjacent normal skin. In vivo CLSM evaluation revealed that 8 of the 20 equivocal reactions (40 %) showed confocal patterns consistent with the patterns encountered in positive allergic reactions. Anamnestic exposure, i.e., detailed assessment of previous related contact with these allergens,

confirmed high relevance rates. In vivo CLSM is useful in differentiating between allergic, irritant, and negative equivocal patch test reactions, a differentiation that cannot be made by conventional clinical patch test reading.

Keywords Contact dermatitis · Patch test · Confocal laser scanning microscopy · Equivocal reactions

Introduction

Patch testing, the conventional diagnostic procedure for diagnosis of contact dermatitis, is hampered by inter-physician variation when reading the patch test reaction. Thus, Svedman et al. called in a recent study for “calibration” of reading technique in order to improve the outcome of the test [1]. One debatable issue is the interpretation of equivocal patch test reactions. Equivocal reactions imply faint erythema which constitutes approximately 1 % of the positive reactions encountered in patch test practice. These reactions might represent a weak allergic reaction or a nonallergic one (either irritant or negative reaction). Histological evaluation of the equivocal reaction has proven helpful for the correct interpretation but is however time consuming, and its invasive nature is often unacceptable to the patient.

In vivo confocal laser scanning microscopy (CLSM) is a novel, noninvasive imaging technique which permits real-time visualization of skin structures and lesions at a resolution close to that obtained by conventional histology [2]. In vivo CLSM detects single back-scattered photons directly from the illuminated living tissue without prior preparation of the examined skin. Contrast in confocal images is provided by refractive index differences among cellular organelles and

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structures [3]. Current technology enables in vivo CLSM imaging up to a depth of approximately 300 μm below skin surface, which covers the entire epidermis, the papillary dermis, and the upper reticular dermis. Since one of the important reflectance generators in CLSM's images is melanin, CLSM has mainly been useful in the diagnosis of pigmented cutaneous tumors such as melanoma, benign melanocytic nevi [4], and nonmelanoma skin cancers [5, 6]. Further developments in the field have led to the utilization of CLSM in the diagnosis of a variety of inflammatory and infectious skin disorders including folliculitis [7], vitiligo [8], psoriasis [9], cutaneous lupus erythematosus [10, 11], autoimmune bullous dermatoses [12, 13], scabies [14, 15], and demodicidosis [16] among others.

In the field of contact dermatitis in vivo, CLSM was shown to provide insight into the kinetics of allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) [17] and to enable the differentiation between the two reaction types [18–20]. This differentiation is based on the notion that an irritant reaction develops at the outer side of the stratum corneum and progresses inward, whereas an allergic reaction embarks at the dermis and develops toward the outer layers of the skin. Consequently, CLSM features characteristic of ICD that include a greater disruption of the stratum corneum, a heavier inflammatory infiltrate in the upper epidermal layers, and more pronounced parakeratosis than seen in ACD. In contrast, CLSM features characteristic of ACD that include a more severe vesicle formation mainly involving the lower epidermal layers.

Previous publications [17–19] used CLSM to evaluate clinically clear-cut allergic or irritant skin reactions. We here hypothesize that CLSM can be useful in the noninvasive determination of the nature of equivocal patch test reactions. We here demonstrate for the first time that CLSM can differentiate equivocal patch test reactions into allergic, irritant, and true negative reactions, a differentiation that cannot be made by conventional clinical patch test reading.

Patients and methods

Patch tests

All patients were tested to the European standard series and other case-relevant series (Chemotechnique Diagnostics, Malmö, Sweden). The allergens were applied in Finn chambers® on Scanpor® (Epitest, Tuusula, Finland). Readings were taken on days 2 and 4. Forty-one clinically clear-cut (nonequivocal) patch tests were randomly selected from 421 patch test sites occurring on 11 patients. Randomization was performed by choosing every third positive patch test site and every 15th negative patch test site, beginning from top left back and ending at bottom right back. In addition, 20

clinically equivocal patch test reactions appearing in 15 patients were evaluated by CLSM (Table 1). The relevance of a positive patch test result was evaluated in relation to the history of exposure and dermatitis pattern.

CLSM evaluation

CLSM evaluation was based on the following criteria previously encountered in clinically clear-cut allergic or irritant reactions [17–19]: stratum corneum disruption, presence of epidermal blisters, epidermal spongiosis, inflammatory infiltrate, and dermal edema, blisters, and hypervascularity. In addition to these previous publications, each criterion was quantified and graded on a score of 0 to 5 (Table 2) with 0 being findings equivalent to those found at adjacent normal skin and 5 representing findings similar to those found in severe allergic reactions. Based upon these criteria, the nonequivocal (clinically obvious) reactions were categorized as allergic, irritant, or negative reactions. Normal skin adjacent to the patch tests also underwent CLSM evaluation and served as negative control. After clinically determining the existence of equivocal reactions, CLSM was performed both on the equivocal reaction site and on the adjacent normal skin. All equivocal reactions were first categorized either as negative (score <1 for all CLSM criteria) or positive. Since positive reactions could represent allergic and irritant reactions, a further analysis of the positive reaction sites based on the CLSM criteria was then performed to differentiate the two types of reactions (Table 3).

CLSM evaluations were performed by a single evaluator blinded to the clinical interpretation of the reactions as described previously [21] using a commercially available,

Table 1 Equivocal reactions and allergens

Patient (number)	Age/gender	Allergens causing equivocal reaction
1	M/79	Quinoline mix, bacitracin, benzoyl peroxide
2	F/49	Myroxylon pereirae
3	F/24	Cobalt
4	F/38	Bis-GMA
5	M/45	Myroxylon pereirae, fragrance mix, propylene glycol
6	F/39	MCI/MI
7	F/48	Myroxylon pereirae
8	M/21	Dodecyl gallate
9	F/51	Cobalt
10	M/48	<i>P</i> tertbutyl phenol, formaldehyde resin
11	M/55	Methyl methacrylate
12	F/51	Hydroxyethyl methacrylate
13	M/61	MCI/MI
14	M/91	Atenolol
15	M/37	Dodecyl gallate, PCNC

Table 2 CLSM scores of equivocal reactions stratified by allergens and reaction patterns

Allergen (number)/ criteria	Epidermal blisters	Epidermal spongiosis	Stratum corneum disruption	Inflammatory infiltrate	Dermal blisters/ edema	Vascularity	Conclusion
1	0	0	0	0	0	0	Negative
2	0	0.5	0	0	0	0	Negative
3	3	3	0	3	2	2	ACD
4	0	2	3	2	0	0	ICD
5	3	3	0	2	3	3	ACD
6	0	0	0	0	0	0.5	Negative
7	0	0	0.5	0	0	0.5	Negative
8	0	2	3	2	0	2	ICD
9	0	2	0.5	2	3	2	ACD
10	0	0.5	0	0	0	0	Negative
11	2	2	0	2	2	0	ACD
12	3	3	0	3	3	3	ACD
13	0	0	0	0	0	0	Negative
14	3	3	0	2	3	3	ACD
15	0	2	3	0	0	0	ICD
16	0	0	0	0	0	0	Negative
17	0	0	0	0	0	0	Negative
18	0	0.5	0	0	0	0	Negative
19	3	3	0	2	3	0	ACD
20	2	2	0	2	2	0	ACD

ACD allergic contact dermatitis, ICD irritant contact dermatitis

near-infrared, reflectance confocal microscope (Vivascope 1500, Lucid Inc., Rochester, NY, USA). The system uses an 830-nm wavelength diode laser. The laser beam is directed by a dichromatic mirror (beam splitter) toward a pair of mirrors (scanning optics) that perform horizontal scanning of the selected skin area. The laser beam then passes through the microscope objective lens and excites the fluorescence within the

skin. Next, the emitted light goes back through the objective lens and the dichromatic mirror which focuses it onto a pinhole. The light that passes through the pinhole is measured by a Photomultiplier detector. The system provides high optical resolution (horizontal axis 2.0 μm ; vertical axis 5.0 μm) to a penetration depth of 200–300 μm dependent on anatomical site and skin thickness. An ultrasound gel (Aquasonic 100 gel,

Table 3 Mean scores of equivocal reactions stratified by reaction patterns and CLSM diagnosis

Criteria/reaction type	ACD ($n=8$)	ICD ($n=3$)	Negative ($n=9$)	Normal skin ($n=15$)	Statistical significance
Stratum corneum disruption [SD]	0.07 [0.16]	3.00 [0]	0.056 [0.15]	0 [0]	$p<0.001$ ICD compared to all other reactions or normal skin
Epidermal blisters/edema [SD]	2.375 [0.99]	0 [0]	0 [0]	0 [0]	$p<0.001$ ACD compared to all other reactions or normal skin
Epidermal spongiosis [SD]	2.625 [0.48]	2.00 [0]	0.167 [0.23]	0 [0]	$p=0.022$ ACD compared to ICD $p<0.001$ ACD or ICD compared negative reactions and normal skin
Presence of inflammatory infiltrate [SD]	2.25 [0.43]	1.33 [0.94]	0 [0]	0 [0]	$p<0.001$ ACD compared to negative reactions and normal skin. $p=0.009$ ACD compared to ICD.
Dermal blisters/edema [SD]	2.625 [0.48]	0 [0]	0 [0]	0 [0]	$p<0.001$ ACD compared to all other reactions or normal skin
Hypervascularity [SD]	1.625 [1.31]	0.667 [0.94]	0.11 [0.20]	0 [0]	$p<0.002$ ACD compared to negative reactions or normal skin. $p=0.647$ ACD compared to ICD

SD standard deviation, ACD allergic contact dermatitis, ICD irritant contact dermatitis

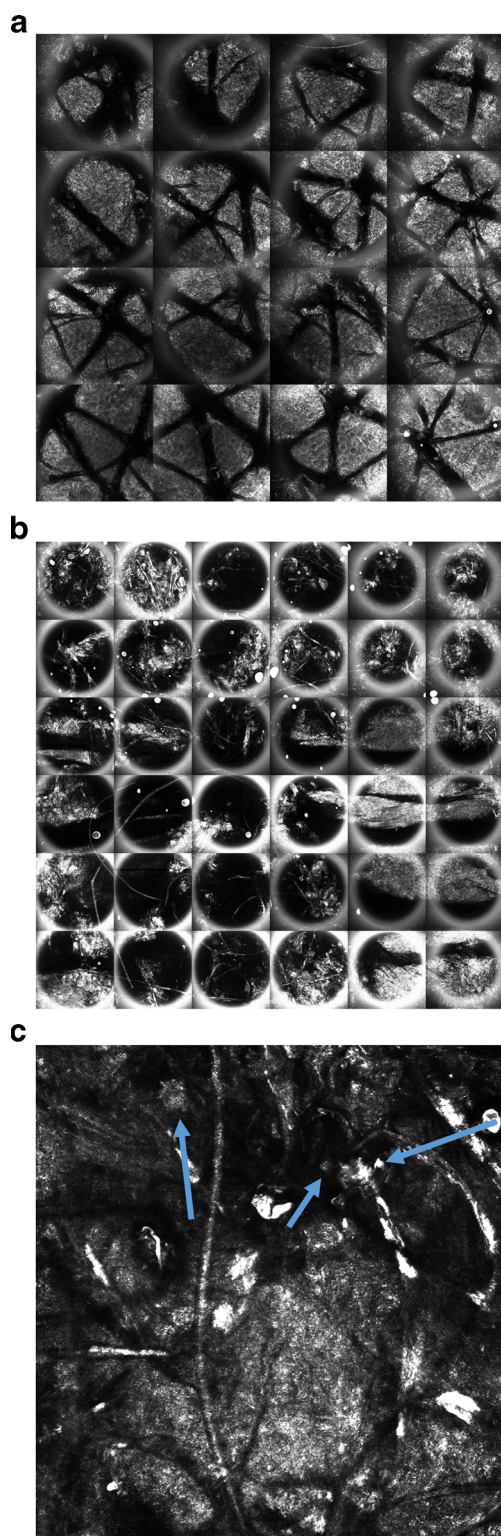


Fig. 1 **a** Confocal capture (*mosaic view*) showing normal stratum corneum in a negative reaction. **b** Confocal capture (*mosaic view*) of a stratum corneum disruption in an irritant reaction. **c** Confocal capture of a stratum corneum disruption in an irritant reaction. *Blue arrows* point to dispersed comeocytes

Fairfield, NJ) was used as immersion medium (refractive index 1.33).

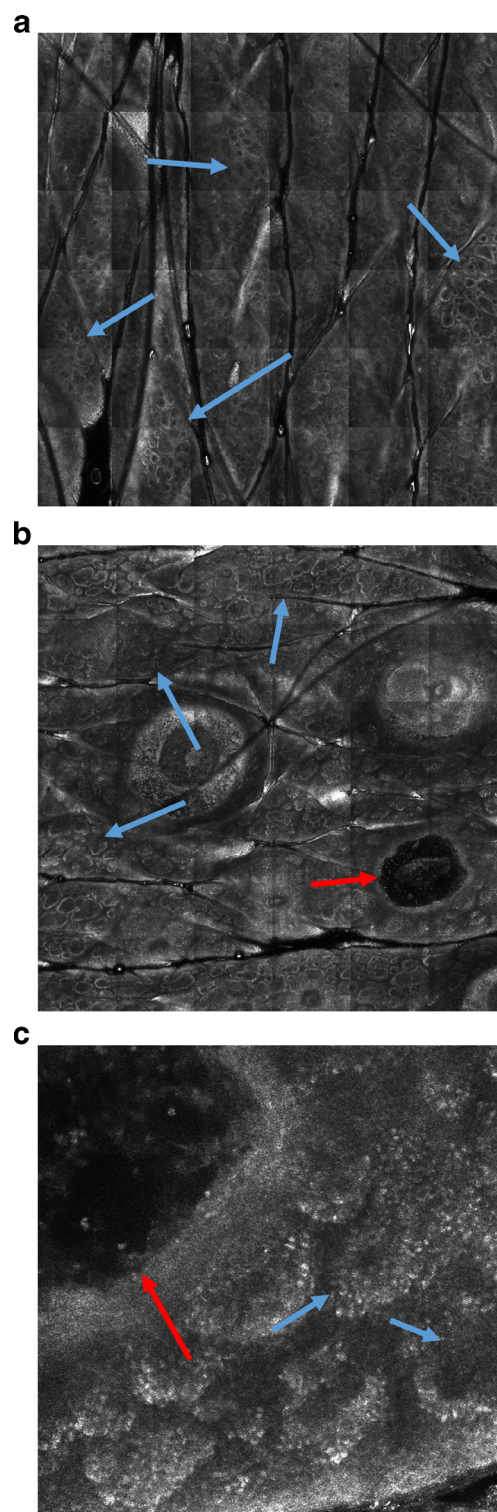


Fig. 2 **a** Confocal capture (*mosaic view*) showing normal dermal-epidermal junction in a negative reaction (*blue arrows* point to normal dermal papillae). **b** Confocal capture (*mosaic view*) of the dermal-epidermal junctional area in an allergic reaction: A *red arrow* points to a blister. *Blue arrows* point to edematous dermal papillae. **c** Confocal capture of the dermal-epidermal junctional area in an allergic reaction: A *red arrow* points to a blister. *Blue arrows* point to edematous dermal papillae

Horizontal sections of the skin spanning from the stratum corneum to the upper dermis were taken using an interval of 30.0 μm between each section. All images were obtained and stored in BMP file format as described earlier [12]. Time required for data acquisition followed by interpretation of the CLSM images ranged between 10 and 15 min per single reaction.

Correlation between CLSM and clinical relevancy

Patients with CLSM assessment displaying allergic reactions were educated to avoid appropriate elements containing the allergens and were followed up 3 months later to determine clinical relevance.

Statistical analysis

The interobserver agreement between clinical and confocal evaluation was performed by Kappa Test using WINPEPI. Significant group differences were identified by analysis of variance (ANOVA). Provided group differences were found, Fisher's protected least significant difference (Fisher PLSD) contrasts were used to test specific pair-wise comparisons. A significance level of $p < 0.05$ was used in all studies.

Results

Out of the 41 nonequivocal (clinically clear-cut) reactions assessed, 17 were evaluated clinically as allergic reactions, and the rest were evaluated as negative reactions. The CLSM

evaluation was found to be consistent with the clinical one (Kappa test results: Kappa=1.00 (0.82, 1.00), $p < 0.0005$).

Having thus verified our CLSM criteria by demonstrating good consistency between clinical and CLSM evaluation, we next applied the CLSM criteria to the equivocal reactions in order to differentiate between allergic, irritant, or "true" negative reactions. Of the 20 equivocal reactions evaluated, 9 were found by CLSM to be negative reactions, i.e., CLSM findings were similar to those obtained from normal adjacent skin (Figs. 1a and 2a). All CLSM scores of equivocal reactions stratified by allergens and reaction patterns are presented in Table 2.

Mean scores of equivocal reactions, their standard deviations and p values, stratified by reaction patterns, and CLSM diagnosis are summarized in Table 3.

Three reactions were categorized to be ICD (Fig. 1b, c), i.e., high grade of stratum corneum disruption (mean value= 3.00 ± 0.0) accompanied by epidermal spongiosis (mean value= 2.0 ± 0.0) and inflammatory infiltrate (mean value= 1.33 ± 0.94). These three criteria were found to be statistically significant in differentiating irritant reactions from allergic and negative ones and from normal skin. Eight reactions were found to be ACD (Fig. 2b, c), i.e., presence of epidermal spongiosis (mean value= 2.62 ± 0.48) and blisters (mean value= 2.37 ± 0.99), marked inflammatory infiltrate (mean value= 2.25 ± 0.48), and dermal edema and blisters (mean value= 2.62 ± 0.48). These four criteria were found to be statistically significant in differentiating allergic reactions from irritant and negative ones and from normal skin.

Hypervascularity was more prominent in allergic (mean value= 1.62 ± 1.31) and irritant (mean value 0.67 ± 0.2) reactions compared to negative reactions (mean value= $0.11 \pm$

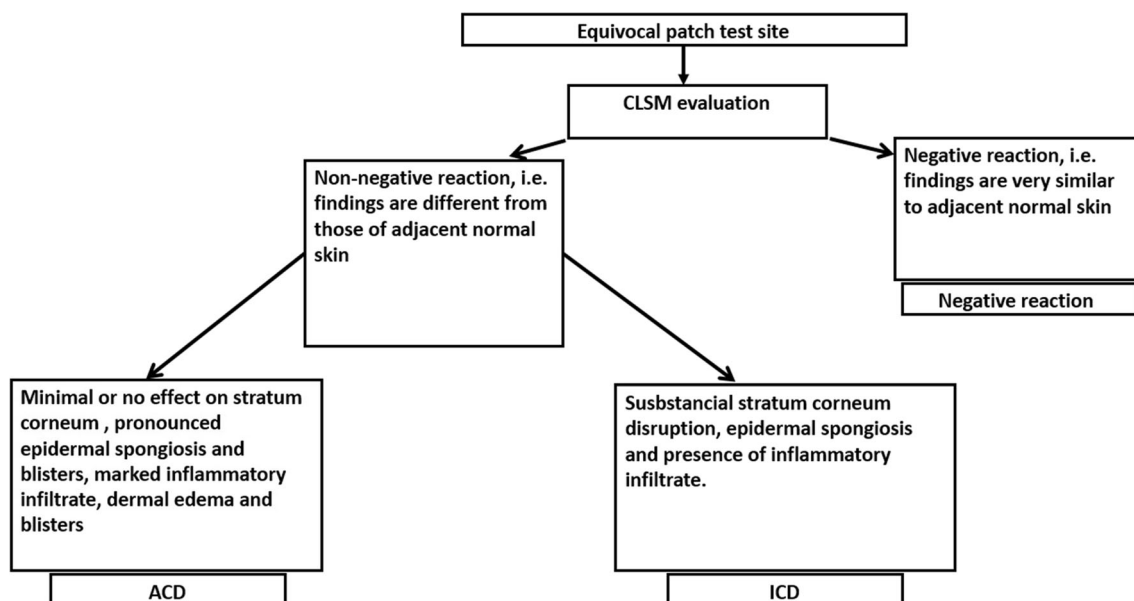


Fig. 3 An algorithm for determining the true nature of equivocal reactions

0.20) and normal skin (mean value=0.0±0.0), yet no statistically significant difference between ICD and ACD was found.

The clinical relevance rate of the eight CLSM-based positive reactions was 100 %. Relevance rates were determined by comparing anamnestic exposure to the potentially offending allergens: detailed anamnestic inquiry into possible past exposure revealed that all eight suspected allergens had previously or currently been encountered by the patients. At the 3-month follow-up, dermatitis had cleared in all patients avoiding the suspected allergens thus reaffirming the 100 % relevance rate.

Discussion

Interpretation of equivocal patch test reactions constitutes a challenge since the reaction can imply allergic, irritant, and true negative reactions. Numerous studies have tried to establish in vitro techniques in order to overcome this obstacle, but they were not validated in large cohort studies and are only defined for selected allergens [22–24]. Our study attempts to tackle this issue using noninvasive, in vivo technology. CLSM has previously been shown to be effective in distinguishing ACD from ICD [18, 19] in clear-cut reactions (either patch test induced or not). Sakanashi et al. [20] have compared routine clinical patch test readings to CLSM evaluation with regard to nickel and cobalt. They found three reactions to cobalt which appeared positive on day 2 and resolved on days 3 or 7, designating these reactions as “doubtful-positive.” As opposed to that study, we here applied CLSM to evaluate 20 truly equivocal reactions, produced by 16 different allergens (and irrelevant of which allergen is producing the reaction), in order to differentiate allergic, irritant, and negative reactions, using a two-step algorithm (Fig. 3). We first determined if the faint erythema represents an actual reaction or rather a negative one, similar to adjacent nonreactive skin. We found 9 out of the 20 equivocal test sites to be true negative reactions, information crucial for excluding the relevant test allergen as cause for clinical contact dermatitis.

We next determined whether the remaining 11 test sites constituted allergic or irritant reactions: Allergic equivocal reactions generally show more pronounced findings including epidermal spongiosis and blisters, marked inflammatory infiltrate, dermal edema and blisters, and hypervascularity, yet they have minimal effect on the stratum corneum. Irritant equivocal reactions, on the other hand, have a striking, disruptive effect on the stratum corneum. Other criteria observed in irritant reactions (yet quantified more mildly as compared to allergic reactions) are epidermal spongiosis and the presence of inflammatory infiltrate. We categorized eight reactions to be allergic and three to be irritant.

In summary, though clinically identical during conventional patch test reading, equivocal patch test reactions designating allergic, irritant, or true negative reactions display

markedly different reaction patterns when evaluated by non-invasive CLSM. Our study adds a relevant tool for the clinician struggling to interpret the significance of an equivocal reaction. Thus, it is the only study so far, which methodically provides insight as to the relevance of these reactions. In accordance in our series, we found 8 out of 20 (40 %) equivocal reactions to be positive reactions, all of which were corroborated clinically with a 100 % relevance rate on a follow-up, consequently leading to clearance of dermatitis in all of these patients. However, though CLSM provides crucial information when evaluating equivocal reactions, the technology is expensive and time consuming. A newly available complementary commercial product, a handheld CLSM device [25], might shorten the time required for data acquisition; however, it does not alter financial cost. Further studies are required to evaluate its cost-effectiveness in daily patch test practice.

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