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Reaction pattern to histamine and codeine in a human intradermal skin test model

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Clinical and Experimental Allergy

Summary

Background Skin prick and intradermal skin tests (IDT) are useful tools in evaluating IgE-mediated allergic disorders. In the literature, many variations of the techniques used are described. No general agreement exists on test procedures and reading of test results.

Objective To analyse test conditions for IDT to facilitate comparability between different study protocols.

Methods We tested 24 healthy volunteers with three concentrations of histamine and codeine each on the upper back, lateral upper arm and volar forearm, with/without addition of ethylene diamine tetra-acetic acid. Reading of the resulting weal was performed by taking a digital image of the weal, later outlining the weal perimeter in triplicate and calculating the weal area using the NIH Image J software version 1.3.

Results Weal size was dose dependent for both substances, generally larger on the upper back than on the forearm and upper arm, and larger after codeine than after histamine. Addition of the Ca^{2+} -chelator ethylene diamine tetra-acetic acid did not significantly affect weal size.

Weal size induced by histamine showed better consistency than that induced by codeine.

Conclusions The results and our technique provide valuable tools for the daily routine as well as for the ability to compare information of intradermal tests from different studies or clinical reports. When assessing skin reactivity, we recommend the use of 1 mg/mL codeine as well as 0.1 mg/mL histamine to reflect aspects of mast-cell releasability and of vascular reactivity. The involvement of local factors influencing the vascular reactivity or differences in opiate receptor density on mast cells surfaces needs to be addressed in future studies.

Keywords codeine, histamine, intradermal skin tests, weal size

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Introduction

Skin prick and intradermal skin tests (IDTs) are valuable tools for the evaluation of IgE-mediated allergic disorders. IDT are the method of choice for testing with low-potency extracts and substances such as venoms and drugs [1].

In the literature, a variety of techniques used for IDT are described and there is no general agreement on standard procedures of performing the tests and reading the test results.

Typically, tests are performed using a disposable sterile tuberculin syringe (0.5–1 mL) with a fine needle (26–30 gauge). 0.02–0.05 mL of a sterile solution is injected intradermally to produce a fluid depot. After 15–30 min,

the resultant reaction is analysed for formation and size of weal (WS) and flare.

In the literature, specification of this procedure varies greatly regarding the injected volume, localization of the testing area (volar forearm [2, 3], lateral upper arm [1, 4], upper back [3]) and concentration and preparation of the substance tested [1, 5]. Some authors suggest injection of a volume sufficient to result in a fluid depot 3 mm [2] to 4–6 mm [6] in diameter; others suggest a larger depot.

A further point of discussion is the time-point and method of reading the resultant weal and flare and the interpretation of the result. The different standpoints of various groups are shown in Table 1.

Especially in publications from North America, the sum of perpendicular erythema diameters is proclaimed as a

Table 1. Different definitions in the literature of what is considered a positive intradermal skin test reaction and the times of reading after injection

Author	Time of reading (min)	Definition of positive reaction in IDT
Barbaud [4, 6]	30	Weal > 10 mm
Brockow [2]		
Warrington [7]	15–20	Size of initial weal increases ≥ 3 mm and association with flare
Van Hage-Hamsten [8]	15	Negative, if weal smaller than 5 mm
Gupta [9]	15	Weal diameter twice that of bovine saline solution and at least 6 mm, associated with flare ≥ 15 mm
Laxenaire [10]	15	Weal ≥ 8 mm
Dib [11]	15	Weal $\geq 50\%$ of positive histamine control
Berg [12]	15	Weal size doubled from time zero
Workgroup on practice parameters for Allergy diagnostic tests [3]		Any reaction larger than the negative control indicates the presence of specific IgE; however, small positive reactions may not be clinically relevant

IDT, intradermal skin tests.

measure of positivity [13, 14], which is supported by the Federal Drug Administration (FDA) [15]. However, in some patients, especially those with dark skin, the reading of the flare is difficult and is therefore not recommended by all [16].

The means applied to determine the WS or flare also vary greatly. Generally, it is preferred to outline the injection area and the WS on a translucent cellophane tape or paper. Then, the mean diameter is determined by measuring and summing up the largest diameter and the smallest perpendicular to the largest and dividing the sum by 2 [2]. Others take the sum of the largest diameter of weal and flare, respectively, and add the largest one perpendicular to the first and divide it by 2 [12]. Sussman [17] multiplies the largest by the smallest diameter. Other methods described are either planimetric or computerized or laser-scanning techniques [18]. All these techniques are rather time-consuming, and inter-examiner reproducibility is often not very high. There is a need to standardize not only the technique of performing the test and the interpretation of the result but also the reading of the resultant weal. Although outlining the weal seems to be a good approach, any variation of sum or product of any diameters is a rather crude way to calculate the size, yielding the fact that the resultant weals are mostly not circular. In this study, we will introduce a simple, digital way of calculating the WS based on the circumference of the weal.

Histamine, in varying concentrations, is mostly used as a positive control [3, 11, 19]. Some groups, however, prefer codeine, a marker for histamine releasability [20, 21]. Because of the different mode of action, the clinical reaction to the two might not be comparable. Problems with comparison of test results may arise, especially if the positivity of IDT is defined in relation to the size of the positive control.

Currently, there is no generally accepted definition of a positive result in IDT. To improve comparability between

different centres, a clear definition of test procedures, reading and interpretation is required, especially when topics such as the evaluation of threshold concentrations for intradermal drug testing are concerned.

The aims of this study were to identify the optimal body site for IDT, to assess differences in reactivity between histamine and codeine at three different concentrations, to determine the influence of the calcium-chelating substance ethylene diamine tetra-acetic acid (EDTA) on reactivity to codeine and histamine and to apply a simple digital technique for the measurement and analysis of the weal size.

Materials and methods

Twenty-four healthy volunteers, 18 males (28 ± 5 years, range 23–43 years) and six females (28 ± 6 years, range 23–36 years) who were not taking any medication were recruited. The study was approved by the local ethical committee of the University Hospital Basel, and written informed consent was obtained from the volunteers. The study solutions were injected intradermally on both sides of the upper back, lateral upper arms and volar forearms of each participant. The substances tested were histamine hydrochloride (0.1, 0.01, 0.001 mg/mL) and codeine phosphate (1, 0.1, 0.01 mg/mL), each with and without addition of Na-EDTA (0.2 mg/mL). EDTA is typically added as a stabilizer in many drug vials. All solutions were prepared according to current GMP guidelines by the Institute of Hospital Pharmacy, University Hospital Basel. 0.9% saline solution was used as a negative control on each site. The 12 different solutions were injected in groups of six (codeine with and without EDTA, histamine with and without EDTA) plus the negative control according to a randomized scheme, keeping the different factors (localization, body side, substance, concentration gradient and additive) balanced, granting that in each patient each combination of parameters was applied. Special attention

was given to sufficient distances of ≥ 2 cm between sites of injection.

0.1 mL of each of the solutions was drawn using a new needle and a new syringe each time. A new needle was used for the injection, and 0.05 mL was injected. Recording of the resulting weal was carried out after 15 min. For this purpose, a digital image of each weal was taken using a Nikon Coolpix 5000 digital camera (Nikon Inc., Melville, NY, USA) equipped with an optical device for direct skin contact with an integrated scale. This has been originally developed to document pigmented lesions of the skin photographically (Optical Devices Neuhaus, Schaffhausen, Switzerland).

The digital images were then transferred to a computer and analysed using the NIH Image J software version 1.3 (freeware). After standardization to the integrated scale, the circumference of the weal was determined in triplicate and the area was calculated.

To perform comparisons of WS according to different factors, areas were first averaged for the respective factor categories within subjects. The paired *t*-test was used for simple comparisons of means of WS between factors with two categories. If means of factors with more than two categories were compared, a two-way analysis of variance with the respective factor and the factor for subject was used. Use of these parametric tests is justified as the residuals of the predicted areas were close to being normally distributed. Alternative non-parametric tests, i.e. Wilcoxon's signed rank test (for two categories' factors) and Friedman's test (more than two categories' factors), gave similar results for all comparisons.

Results

Figure 1 shows examples of typical images taken from the upper back. The respective mean WS and the standard

deviation, resulting from the three different measurements, are given in the lower left-hand corner.

As expected and shown in other studies, the WS decreased with decreasing concentration of codeine [22] and histamine. The lowest concentrations (0.01 mg/mL codeine, 0.001 mg/mL histamine), which equal 1% of the most commonly used concentrations, still induced sufficient weals (mean $\pm$ SD: 0.59 ± 0.22 cm² for histamine, 0.76 ± 0.30 cm² for codeine) in all individuals (Fig. 2). The geometric means ratio between the highest and the lowest concentration was 2.41 [95% confidence interval (CI): 2.30–2.51, *P*-value: < 0.0001], and 1.54 (95% CI: 1.47–1.60, *P*-value: < 0.0001) between the intermediate and the lowest concentration.

Histamine and codeine were tested in equimolar concentrations; however, codeine produced overall statistically significant larger WS than histamine (mean $\pm$ SD for histamine: 1.01 ± 0.51 cm², and for codeine: 1.19 ± 0.61 cm², *P*-value: < 0.0001), as shown in Table 2.

WS were the largest on the upper back (mean $\pm$ SD: 1.29 ± 0.66 cm²), followed by the forearm (1.06 ± 0.50 cm²) and upper arm (0.93 ± 0.47 cm²), *P*-value: < 0.0001 (Table 2). This applies not only for the mean WS of all concentrations but also for each single concentration of both substances (codeine: upper back: 1.45 ± 0.71 cm², forearm: 1.14 ± 0.48 cm², upper arm: 0.97 ± 0.51 cm², *P*-value: < 0.0001 ; histamine: upper back: 1.14 ± 0.56 cm², forearm 0.99 ± 0.51 cm², upper arm: 0.90 ± 0.41 cm², *P*-value: < 0.0001) (Fig. 2). There was a considerable inter-individual variability, regardless of localization, substance or concentration, with some patients developing WS about three times as large as others (Fig. 3).

Figure 4 finally shows histamine to be overall more consistent with respect to the induced weal than codeine,

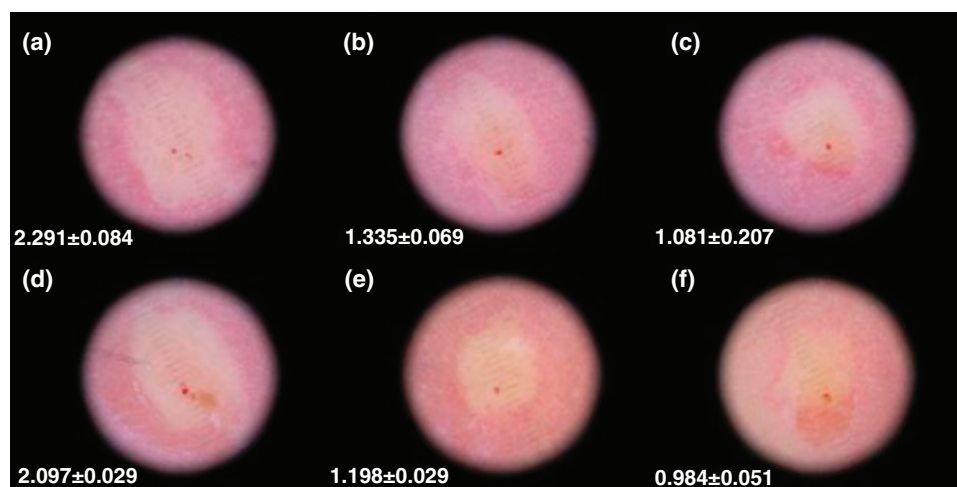


Fig. 1. Digital images of typical weals in a patient 15 min after injection of three different concentrations of codeine with and without ethylene diamine tetra-acetic acid (EDTA) in the upper back. On the left lower corner, the mean weal area size (cm²) and the standard deviation (cm²) as derived from three different measurements are stated. (a) 1 mg/mL, (b) 0.1 mg/mL, (c) 0.01 mg/mL. (d) 1 mg/mL + EDTA, (e) 0.1 mg/mL + EDTA, (f) 0.01 mg/mL + EDTA.

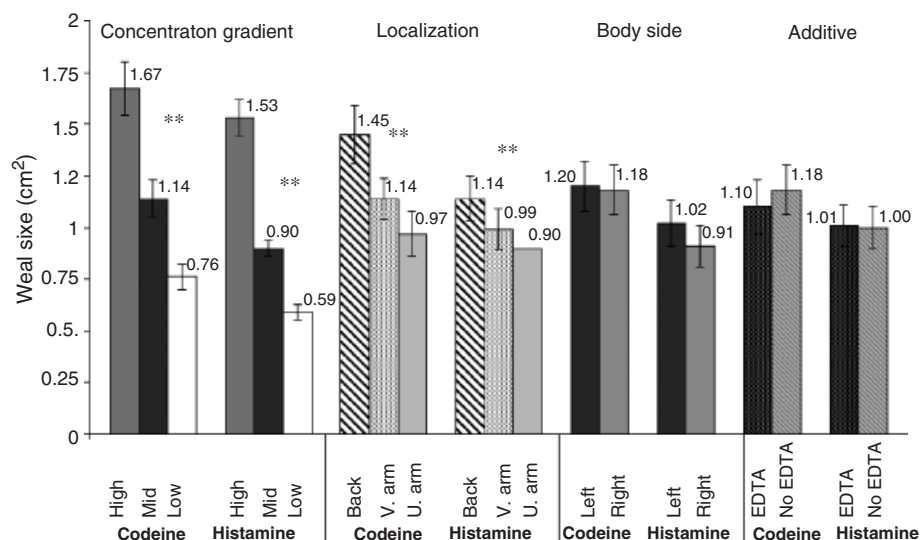


Fig. 2. Mean size of weal within subjects produced by different factor categories stratified by codeine or histamine. Whiskers indicate standard errors. Concentration gradients for codeine: 1.0, 0.1, 0.01 mg/mL, for histamine: 0.1, 0.01, 0.001 mg/mL (** P -value < 0.0001).

Table 2. Mean size of weal within subjects produced by the factors considered

Factor	Category	Weal size (cm ²): Mean $\pm$ SD*	Within-subject variability: SD	P -value [†]
Substance	Codeine	1.19 $\pm$ 0.61	0.21	< 0.0001
	Histamine	1.01 $\pm$ 0.51	0.16	
Concentration gradient	High	1.60 $\pm$ 0.56	0.27	< 0.0001
	Middle	1.02 $\pm$ 0.37	0.15	
	Low	0.68 $\pm$ 0.28	0.12	
Localization	Upper back	1.29 $\pm$ 0.66	0.27	< 0.0001
	Volar forearm	1.06 $\pm$ 0.50	0.14	
	Lateral upper arm	0.93 $\pm$ 0.47	0.16	
Body side	Left	1.11 $\pm$ 0.41	0.18	0.4904
	Right	1.09 $\pm$ 0.57	0.18	
Additive	EDTA	1.10 $\pm$ 0.63	0.17	0.6876
	No-EDTA	1.09 $\pm$ 0.60	0.19	

*Standard deviation of individual measurements.

[†]Paired t -test or two-way ANOVA.

indicated in the relatively small variation in the box plot and the narrower 25–75% range.

Body side did not influence the result with either substance (mean WS left: 1.11 ± 0.57 cm², right: 1.09 ± 0.57 cm², P -value: 0.4904).

Table 3 shows that Ca²⁺-chelating substance EDTA did not influence the WS significantly in any concentration with either substance on any localization. On average, addition of EDTA increased WS by a factor of 1.01-fold as compared with without EDTA (95% CI: 0.97–1.04, P -value: 0.7070). However, in some volunteers (Fig. 1), the WS did decrease with addition of EDTA.

All volunteers reported that the injection of the solutions containing EDTA elicited a transient burning, which is probably due to the acid pH of the substance. Also, injection

of codeine induced more stinging than histamine. However, none of the volunteers withdrew from the trial.

The method used to register the weal area was fast and easy to handle. In addition, the images were digitally stored and could be re-evaluated if necessary. The time to draw the circumference has to be taken into account just as for the conventional method with the cellophane tape; however, this can be done independently from the typical reading time at 15–20 min.

Determination of the coefficient of variation (data not shown) for the three measurements showed a 'learning' process, meaning that there was a difference between the first and the second measurement (P -value < 0.0001) but not between the second and the third measurement (P -value: 0.3581).

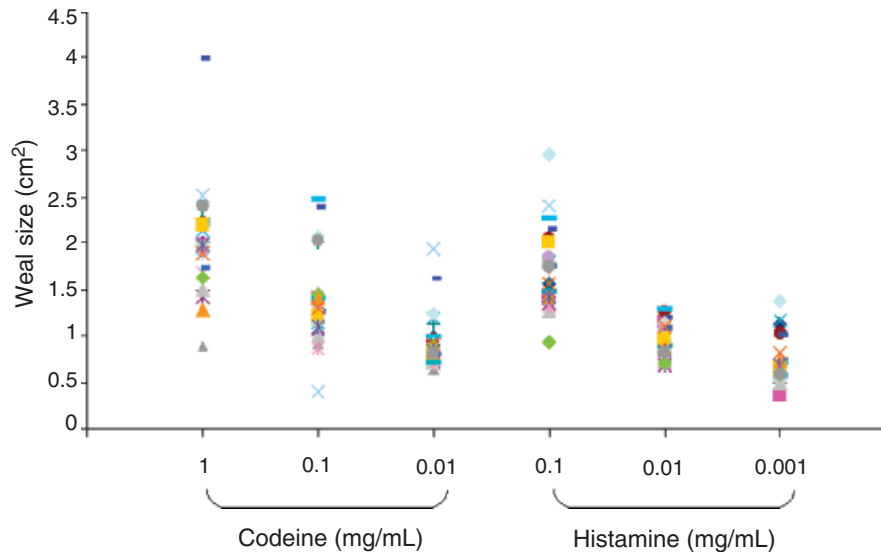


Fig. 3. Mean weal sizes of all patients on the upper back after injection of codeine (1, 0.1, 0.01 mg/mL) and of histamine (0.1, 0.01, 0.001 mg/mL).

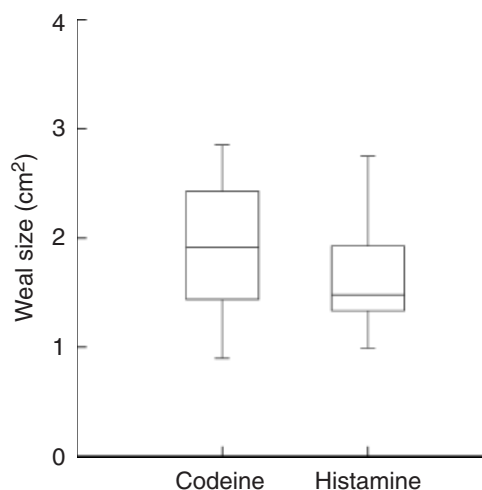


Fig. 4. Box plot of size of weal induced by codeine (1 mg/mL) and histamine (0.1 mg/mL) on the upper back: histamine shows greater consistency than codeine.

Discussion

This study addresses the necessity of consistent preconditions in IDT if comparability of test results between different studies or test centres is to be achieved.

Skin reactivity was assessed with histamine and codeine, two substances that induce obligatory weal and flare reactions, and are therefore commonly used as positive controls in IDT. Most authors used various allergens to assess skin reactivity or worked with skin prick tests (SPTs) [13, 23, 24]. The degree of sensitization to allergens, however, may vary among tested individuals, and SPTs with drugs are considerably less sensitive than IDT. For this reason, we chose an approach that is more reproducible.

Nelson et al. [23] showed with SPTs that WS on the back are generally larger than WS on the forearm. Olson et al. [16] retrospectively compared reactivity with intradermal tests with non-equimolar concentrations of codeine (1 mg/mL) and histamine (0.1 mg/mL) on the upper arm. He observed that codeine WS were generally slightly larger (13.6 ± 0.3 mm) than histamine WS (11 ± 0.2 mm) and that patients treated with systemic corticosteroids for prolonged periods of time had significantly diminished IDT responses to codeine phosphate, but not histamine.

In our study, reactivity was most pronounced on the upper back, followed by the volar forearm and the lateral upper arm for both substances (Fig. 2). WS increased with increasing doses of substance. With histamine, WS were generally smaller than with codeine (Fig. 2).

Weber et al. [25] investigated the mast cell (MC) density by total body surface mapping using toluidine blue for MC staining. They distinguished 15 body sites and seven skin layers. According to their observation, MC density was considerably higher on the forearm (total of 211 MC/mm² in all skin layers, 120 MC/mm² in the papillary dermis) than on the upper arm (168; 80 MC/mm²) and on the upper back (172; 70 MC/mm²), which were rather similar in MC density. On the basis of the assumption that skin reactivity to codeine could be a function of MC density, these findings cannot explain why WS are about 20% larger on the upper back than on the forearm. However, MC density may not be the only crucial factor for skin reactivity. The larger WS from histamine on the upper back could originate from an increased density of H1 receptors or other, e.g. vascular factors on the upper back; however, to our knowledge, this has not been investigated in a human model so far.

Table 3. Mean size of weal within subjects produced by the presence or absence of ethylene diamine tetra-acetic acid in solutions of codeine or histamine at different concentration gradients

Substance and concentration gradient	Additive	Mean WS (cm ²): Mean $\pm$ SD*	Within-subject variability: SD	P-value [†]
Codeine (1.0 mg/mL)	EDTA	1.65 $\pm$ 0.64	0.41	0.7757
	No EDTA	1.68 $\pm$ 0.65	0.46	
Codeine (0.1 mg/mL)	EDTA	1.18 $\pm$ 0.54	0.28	0.1276
	No EDTA	1.09 $\pm$ 0.35	0.23	
Codeine (0.01 mg/mL)	EDTA	0.74 $\pm$ 0.26	0.16	0.3940
	No EDTA	0.78 $\pm$ 0.34	0.21	
Histamine (0.1 mg/mL)	EDTA	1.56 $\pm$ 0.45	0.35	0.4515
	No EDTA	1.50 $\pm$ 0.47	0.28	
Histamine (0.01 mg/mL)	EDTA	0.88 $\pm$ 0.21	0.13	0.2320
	No EDTA	0.92 $\pm$ 0.23	0.16	
Histamine (0.001 mg/mL)	EDTA	0.60 $\pm$ 0.22	0.11	0.2646
	No EDTA	0.58 $\pm$ 0.23	0.15	

*Standard deviation of individual measurements.

[†]Paired *t*-test or two-way ANOVA.

WS, size of weal; EDTA, ethylene diamine tetra-acetic acid.

Casale et al. [26] observed that IDT on volar forearms with codeine and morphine did induce MC degranulation by direct μ -receptor agonistic effects that can be blocked by the non-specific opiate-receptor antagonist naloxone. The question of whether μ -receptors density on the MC surface is constant on all sites of the body or whether other local factors influence the amount of histamine released from MC granules has not been addressed so far. The fact that not only codeine- but also histamine-induced weals were larger on the back than on the other locations could suggest other, non-MC-related factors with an influence on the vascular reactivity.

As expected, WS increases with increasing concentration of both substances as shown in Fig. 2. We found that even 1% of the commonly used dose of codeine or histamine (0.01 mg/mL codeine, 0.001 mg/mL histamine) yields distinct weals (0.59 $\pm$ 0.22 cm² for histamine, 0.76 $\pm$ 0.30 cm² for codeine) in all individuals and can therefore be used as positive controls in a routine setting. However, for greater clarity of the data, we suggest the use of 1 mg/mL of codeine or 0.1 mg/mL of histamine, respectively.

Especially in Fig. 4, the massive inter-individual variability in reactivity can be seen, with some individuals reacting on an up to threefold higher level than others. This also applies for the histamine data. When investigating skin reactivity with allergens instead of obligatory weal-inducing substances such as codeine or histamine, this inter-individual variation has to be taken into account and could be misinterpreted as different degrees of sensitivity to the respective allergens.

Codeine, the methylether of morphine, reflects the receptor-mediated releasability of mediators from MCs and therefore also monitors the influence of certain drugs (e.g. corticosteroids). Histamine, on the contrary, directly

acts on the histamine receptors and increases the permeability of skin capillaries. The codeine reaction mimics the pathophysiological situation on the MC in the presence of an allergen closer than histamine. As Petersen et al. [22] were able to show, the codeine-induced histamine release directly correlates with the weal size and has a short half-life time of only a few minutes. This suggests that reading of the positive control should rather be performed at 15 min than at 30 min. Peterson et al. also demonstrated a steep histamine gradient from the centre of the weal to the periphery after administration of codeine, whereas in the flare response no histamine release could be detected.

In our study, the weal response to codeine was 10–20% larger than to histamine in contrast to Berther's result [27]. In his subjects, the WS after 0.01% histamine were about 25% larger than after 0.1% codeine (+0.5% phenol). Goldberg et al. [28] tested 13 healthy control patients on the forearm with equimolar concentrations of histamine and codeine and reported equal WS.

Histamine-induced skin reactions reflect vascular reactivity and are useful to determine whether histamine antagonists are present. Codeine skin reactions are a function of both MC reactivity and vascular responsiveness. In this, the reaction pathway to codeine is comparable to allergens, which bind to specific IgE on high-affinity receptors on the MC surface and stimulate mediator release, among others, also histamine. The use of both substances therefore provides a more accurate impression of the reactivity of the individual than one substance alone. Codeine WS, as it depicts a more complex reaction than histamine, is more susceptible to potentially influencing factors. This is reflected by the larger spread of the box (25–75% of all WS) in Fig. 4, indicating a lower variation of histamine- as compared with codeine-induced weals. The

concentration and localization leading to the largest weal sizes are given in Fig. 4.

Upon MC stimulation, intracellular Ca^{2+} levels increase and eventually histamine is released from the granules. The intracellular Ca^{2+} increase is due to a strong Ca^{2+} influx from the extracellular space in the first 2 min after stimulation and directly correlates with the amount of Ca^{2+} present extracellularly [29]. The ionized calcium concentration in the extracellular fluid is maintained at a relatively constant level of approximately 1 mM [30] and is in equilibrium with ionized calcium in storage pools such as bone, proteins in the circulation and within the intracellular fluid. The intracellular fluid concentration of calcium is more than 10 000-fold lower than in the ECF.

EDTA, a calcium-ion chelating substance, was shown *in vitro* to block efficiently the Ca^{2+} influx and thereby MC degranulation [31]. To investigate whether this effect can also be seen clinically, meaning a reduction in WS, we performed all tests with and without 0.2 mg/mL EDTA (approximately 0.68 mM) at all concentrations of histamine and codeine. Overall, no statistically significant difference could be seen between WS with and without EDTA (Table 3, Fig. 1). No difference was present between histamine and codeine, although EDTA should only influence the codeine WS, if at all. The slightly smaller WS with EDTA in Fig. 1 are incidental. The findings suggest that possibly the mode of administration and amount of EDTA are crucial. Possibly, the EDTA has to be applied before the fast-acting codeine to allow the blockage of calcium. Another reason for the lack of response to EDTA might be that EDTA could be washed out to the tissue too quickly to allow a recognizable reaction. A third factor might be the concentration, as the amount of ionized calcium in the extracellular fluid ($\sim 1\text{--}1.5$ mM) is slightly higher than the applied amount of EDTA (~ 0.7 mM). However, already at a concentration of 0.2 mg/mL, all test persons reported a burning sensation during injection. A higher concentration of EDTA may therefore not be tolerable.

Documentation with our method was easily and quickly performed and, in contrast to the different variations of measurement hitherto used, the weal area is more precisely assessed particularly if pseudopods are present. In addition, the accuracy of determining the perimeter in triplicate is high as exemplified by the standard deviation shown in Fig. 1. We therefore consider our method superior to the commonly used methods and recommend its use especially in clinical studies where accurate information on WS is crucial. However, due to the restricted size of the device, a measurement of the erythema area is not possible.

Conclusions

Both 1 mg/mL codeine and 0.1 mg/mL histamine should be used as positive controls in IDT to assess both MC

releasability and vascular reactivity if precise information on skin reactivity is required. In addition, the allergen-induced WS could be compared with the histamine- or codeine-induced size, and for better comparison and reproduction a quotient could be calculated to define positive IDT results.

Codeine yields larger WS than histamine and WS on the back are generally larger than those on the forearm or upper arm for reasons so far unknown. We therefore recommend the upper back for IDT.

Both our results and the documentation technique provide valuable tools not only for the daily routine but particularly for the conduction of multi-centre studies, allowing for better comparison among different studies or clinical reports.

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