

Experimental testing of skin reactions to insulin detemir in diabetes patients naïve to insulin detemir

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Background/aims: Sporadic reports on immediate and delayed cutaneous reactions to insulin detemir, a modern insulin analogue, have raised unsupported claims of allergy of type I, III and IV. The purpose of this experimental study using a provocative design was to elucidate the potential mechanisms behind such skin reactions.

Material and methods: A total of 40 patients with type 1 diabetes or insulin-requiring type 2 diabetes, all naïve to insulin detemir, were injected on the thigh with 0.1 mL of insulin detemir (Levemir[®]) administered with an 8 mm needle at three different depths, i.e. intradermal, subdermal and subcutaneously. Saline was injected as control. Any cutaneous reactions were assessed after 10 and 30 min, after 24 and 48 h and after 7 days. Histopathology of positive reactions on day 7 was obtained. The study was randomized, controlled, double-blinded, and conducted in accordance with ICH-GCP guidelines. Blood flow was recorded with the Periflux PF5010, and skin colour (*a**) with the DSMII colorimeter.

Results: Clinical reading, flowmetry and colorimetry consistently showed delayed reactions after intradermal insulin injection (35 of 40 patients reacted with mainly weak reactions, $P < 0.05$), peaking after 48 h, contrasting no special reaction immediately after injection, except for reactions attributed to needle trauma. A total of 22 patients reacted on subdermal injection and 21 on subcutaneous injection. Histopathology on day 7 from 22 reactions in 15 patients showed a consistent pattern of inflammation with eosino-

philia as typically observed in adverse skin reactions to a variety of medicines. Reactions were interpreted as non-specific biologic responses to the insulin different from direct toxic actions and classical allergic reaction patterns. Only one person registered itch/discomfort. A prick test vs. histamine reference excluded insulin detemir to be a pharmacological histamine releaser. Thus, provocative testing with insulin detemir produced delayed skin reaction but no immediate reaction. Measurement of circulating insulin detemir-specific antibodies by RIA before and after 3 months showed no increase.

Conclusion: Non-allergic delayed skin reactions from intradermal and, to a minor degree, subdermal and subcutaneous injections of insulin detemir were frequent in this experimental study and showed a consistent histology pattern of inflammation with eosinophilia. Immediate reactions were not produced. The reactions are unlikely to be specific for insulin detemir, and other insulins should be studied in a similar provocative design.

Key words: insulin – detemir – allergic reaction – cutaneous reaction – tolerability – adverse effect – delayed reaction – immediate reaction – histology – histamin – histamin release – insulin antibodies – laser Doppler flow – skin colour

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LONG-ACTING INSULIN analogues such as insulin detemir and insulin glargine have been alleged to cause local reactions in the skin on the injection site more frequently than traditional insulin products (1). Such reactions may be immediate or delayed, local or generalized, clinically innocent or clinically significant. Allergic, irritant, toxic, idiosyncrasic or local biological effects elicited by the insulin hormone also including pharmacologic histamine release may be considered as the root cause of such reactions.

Reactions may depend on injection technique, on injection depth and on variable reactivity in different skin layers and different individuals. Similarly, a preferred injection field with sub-clinical reaction to previous injections and lower threshold for adverse reaction may burst on re-injection.

Sola-Gazagnes et al. (1) reported in the Lancet 6 cases of immediate and delayed local reactions to insulin detemir and claimed reactions to be type I and IV allergy, despite two patients being insulin

naïve and three patients showing reactions 1 and 2 days from the start of exposure. Dejgaard et al. (2) commented that reactions were more likely to be non-specific as insulin antibodies specific to detemir had not been documented. Case reports have claimed type III allergy in selected patients, i.e. development of initial itch followed by nodular reactions within a few hours after the very first injection (3–6). Indeed, insulin detemir-induced anaphylaxis was claimed in a 79-year-old man with a clinical history of stroke and circulatory insufficiency just after start of insulin detemir; the allergy was not verified through measurement of insulin antibodies (7). Thus, early reports have outlined that some patients surely developed inflammatory reactions directly in the skin after insulin detemir injections, some reactions were associated with immediate symptoms and others were delayed for hours, suggesting two modes of reactions or a biphasic pattern. Claims in the early reports of allergic mechanism behind insulin detemir reactions remain purely speculative and have not been supported by any conclusive data.

Skin reactions to insulin have been reviewed extensively by deShazo et al. (8) back in 1982, including histopathology study. They found insulin reactions to have a delayed tuberculin-like pattern, and in some cases a biphasic pattern with immediate reaction as well. In another review, in 1984 by Sibbald and Schachter (9), immediate, delayed and biphasic reactions to classical insulins were also described. In a recent report, the diversity of local reactions to insulins was confirmed using recombinant human insulins (10). Clarification of the precise mechanism of adverse skin reactions to insulins have been hampered by the general concept that reactions *must* match the traditional classification of allergy into types I–IV with little attention paid to possible non-allergic mechanisms. To the best of our knowledge, no experimental study on skin reactions to modern human insulins has been reported.

It is fundamental that a reaction cannot be concluded as allergic if the patient reacts on the very first exposure and thus did not pass the period of a few weeks of exposure needed for a specific allergic sensitization to occur. For this reason, our study was undertaken in insulin detemir-naïve diabetics, who had no known allergy to insulin.

As local skin reactions to insulin detemir are reported in roughly 1–2% in clinical trials, a

sample of several hundreds of patients would be required to study spontaneous reactions. We decided, as the dermis may be a more reactive compartment that more easily develops inflammation than deeper skin structures and as back-flow into the needle tract may be a risk in local reaction to insulins, to develop a provocative test design with intradermal as well as subcutaneous injections. Moreover, we decided to include very sensitive objective methods additional to clinical reading of reactions by a dermatologist, i.e. laser Doppler flowmetry and colorimetry. In addition, histopathology and measurement of insulin detemir-specific antibodies were included. Thus, we increased the sensitivity to any signal within the study and designed it to detect even weak and doubtful reactions, which might have been innocent or not noticed in clinical routine, albeit relevant in the perspective of research and understanding of the mechanism of reactions. The study was randomized, controlled, double-blinded and conducted in accordance with ICH-GCP guidelines.

Material and Methods

Patients, design and experimental exposures

Patient material and design

The study protocol was approved by the Local Ethics Committee Region H (H-B-2007-107) and by the Danish Drug Agency (EUDRA CT 2007-005677-59), and registered at www.clinicaltrials.gov. GCP monitoring was performed by the GCP unit of RegionH, Copenhagen.

Adult patients ($n = 40$) on insulin treatment for type 1 or type 2 diabetes, however, all naïve to insulin detemir, were included after obtaining their informed consent. Inclusion criteria were a total daily insulin dose of more than 20 IU, BMI 20–35 kg/m², HbA1c below 11% and clinically normal skin on the thighs, where study injections were planned. Patients should not have been treated with medicines, which could influence inflammatory reactions of skin including antihistamines, and they should not have any known clinical allergies. Seven visits were scheduled. Visits included examination for immediate reactions measured 10 and 30 min after injections and examination for delayed reactions after 24, 48 h, and 7 days. Insulin detemir-specific antibodies in blood were measured before study and after 3 months.

Insulin detemir and insulin injections for study of immediate and delayed effects

Insulin detemir (Levemir[®], Novo Nordisk A/S, Bagsværd, Denmark) is a new long-acting basal insulin analogue. Levemir[®] contains 100 IU/mL (14.2 mg/mL) insulin detemir. It contains no protamin. Insulin detemir differs from human insulin in that the amino acid threonine in position B30 has been omitted, and a C14 fatty acid chain has been attached to the amino acid B29. The pharmacology has been reviewed (11).

Injections and injection depths of Levemir[®] and saline control were randomized and given by the study nurse, who was trained and especially experienced in intradermal and subcutaneous injections in patients with diabetes. Seven test sites were marked on the anterior side of the thigh for injection of three insulin samples, three controls and one blank field for measurement only. Distance between test fields was 5 cm. A volume of 0.1 mL of insulin detemir was injected in the thigh at three depths using a standard 0.30 mm (30 G) 8 mm insulin needle, i.e. intradermal (needle parallel to the skin surface), immediately subdermal (needle under an angle of 15–30°) and subcutaneously (needle under an angle of 45–60°), strictly according to manufacturer's recommendation for subcutaneous injection, which requires the injection needle to be maintained *in situ* for 10 s from injection until withdrawal. Total dose of Levemir[®] was 0.3 mL (30 IU). Injections were given in the morning with the patient in the supine position and in the right thigh for study of both immediate and delayed reactions with the same injection sites followed at subsequent visits. Patients avoided their normal long-acting insulin on the day of study.

Control injections

Isotonic saline 9 mg/mL was given similarly as three doses of intradermal, subdermal and subcutaneous injections, in total 0.3 mL. Controls were included in randomization and blinding as mentioned above. An un-blinded reference of untreated skin was also measured.

Prick test with insulin detemir vs. histamine reference to study histamine release of insulin detemir

On day 7, a prick test was performed on the left thigh. Insulin detemir, histamine 10 mg/mL in water, histamine 1 mg/mL in water and isotonic saline 9 mg/mL were pricked into the skin using

a standard allergy test lancet with a 1 mm pointer (Alk-Abello A/S, Hørsholm, Denmark). Reactions were read after 10 and 30 min.

Measuring Methods

Clinical reading by dermatologist of immediate and delayed effects

All clinical evaluations were performed by the same specialist in dermatology, who was blinded for type and depth of injections given by the study nurse. Clinical evaluation occurred before instrumental assessment.

Immediate effects were evaluated 10 and 30 min after injections, and any change simulating a wheal-and-flare reaction was noted, and reactions were concluded as negative, weak, moderate and strong.

Delayed effects were evaluated 24, 48 h and 7 days after injections and graded as follows: 0 (negative, no reaction); 1/2 (doubtful, very weak erythema only); 1 (weak, weak erythema and, possibly, slight oedema); 2 (moderate, moderate degree of erythema with oedema or swelling); 3 (strong, marked erythema and marked oedema or swelling, possibly with vesicles or bullous reaction); and very strong (as 3, with ulceration or necrosis).

Laser Doppler flowmetry with measurement of cutaneous blood flow

Measurement of cutaneous blood flow, in the protocol nominated the primary end point of the study, was measured in all experimental exposures of the study after clinical reading had been concluded and registered in the case report form. The Periflux PF5010[®] (Perimed AB, Stockholm, Sweden) fibre-based system was used. Result was calculated using the manufacturer's software and printed out as raw data. Final result was the mean of three measurements. The recording fibre was positioned 2–3 mm from the needle injection tracts in the skin in order to omit registration of injection trauma. A fibre covers a circular measuring field of about 0.5 mm. Values were taken after a stabilization period of 20 s. The guideline on laser Doppler flowmetry measurement of cutaneous blood flow by the European Society for Contact Dermatitis was followed (12). Units are arbitrary.

Colorimetry with measurement of redness

Skin colour was measured following flowmetry. The DSMII Colorimeter[®] (Cortex Technology,

Hadsund, Denmark) was used. The instrument measures colour in a circular area with diameter of about 3 mm. This instrument can be positioned accurately as the probe tip is transparent. It was applied 1–2 mm from injection needle tracts. One measurement was taken and registered as the result, expressed as redness a^* in the colour space of CIE-lab (see guideline of the European Society for Contact Dermatitis on skin colour measurement, which was followed in this study) (13). Units are arbitrary and referenced to human perception of colour. One division can be distinguished by the human eye.

Histopathology

Clinically doubtful and positive reactions as observed clinically on day 7 after insulin detemir injections were selected at random for histopathology, and a 3 or 4 mm punch biopsy was taken if the patient volunteered after informed consent. Biopsies were processed routinely, stained with haematoxylin–eosin and evaluated blindly by the dermato-histopathologist. Pathologic changes of biopsies were graded as follows: 0, normal skin; 1, scarce inflammation without eosinophilia; 2, scarce inflammation with eosinophilia; 3, inflammation without eosinophilia; and 4, inflammation with eosinophilia.

Insulin detemir-specific antibodies

Blood samples were taken before the study and 3 months after the study. Samples were taken in the morning before insulin had been taken. Serum samples were analysed by MDS Pharma (MDS Pharma Services Switzerland AG, Fehraltorf, Switzerland) by radioimmunoprecipitation (RIP). The analytical laboratory followed the OECD standard for Good Laboratory Practice.

Prick test with insulin detemir

Reactions after 10 and 30 min were read as wheal-and-flare events and graded as 0 (nil), 1 (weak), 2 (moderate) and 3 (strong).

Clinical photos, 20 MHz ultrasound scans of cross-sectional structure of reactions and horizontal imaging of the zone of redness by polarized photography were taken for illustration and supplementary information. Ultrasound imaging showed echolucent band formation preferably in the outer dermis corresponding to inflammation. Polarized photography showed circular redness of the skin in positive reactions typically of diameter 6–10 mm.

The local tolerance and the systemic safety including the diabetic status and general health of the patients during the trial were followed and reported as required in the protocol, referring to ICH GCP.

Clinical symptom scoring

At each examination, patients were asked to score their symptoms of itch, pain and discomfort, registered as nil, weak, moderate or severe.

Statistical analysis

Sample size was calculated based on the assumption that a change in the primary endpoint change in laser Doppler flow (LDF) of 50% at visit 3 between superficial and deep subcutaneous injections is of clinical relevance (corresponding to a difference in log LDF of 0.41). As the variability in LDF is not known, sample size was assessed after 40 patients had been included as part of an adaptive design. On the basis of a variance of 0.16, $\alpha = 0.05$ and $\beta = 0.10$ (power 90%), it was concluded that additional patients were not needed.

An ANCOVA model with injection depth (intradermal, subdermal or subcutaneous) and preparation (saline or insulin detemir) and interaction between depth and preparation as fixed effects as premises, and baseline value of the corresponding variable as covariate, was used for the analyses. Calculations were made using SPSS version 13.0 (Chicago, IL, USA.). A P value < 0.05 was considered significant in two-sided tests.

Results

A total of 40 patients with diabetes (32 males, eight females) and on various insulin preparations but naïve to insulin detemir were enrolled; 16 patients had type 1 diabetes and 24 had type 2 diabetes, with duration of diabetes from 2 to 47 years. Arterial hypertension was present in 35 patients, 21 patients had albuminuria, 28 retinopathy and 22 symptoms of neuropathy. None reported insulin allergy in their history.

Clinical reading by dermatologist of immediate reactions 10 and 30 min after injections with redness and swelling but typically without a flare was observed in about half of intradermal injections, same as with insulin detemir and saline control, as manifestations of intradermal needle

TABLE 1. Clinical grading of inflammatory reactions to insulin detemir (Levemir[®]) 48 h after intradermal (ID), subdermal (SD) and subcutaneous (SC) injection of 0.1 mL as read by a dermatologist.

	Test substance and injection depth					
	Insulin detemir			Saline for control		
	ID	SD	SC	ID	SD	SC
Reaction by grade						
0	5	18	19	35	38	38
1/2	11	18	15	4	2	2
1	22	3	5	1	0	0
2	2	1	1	0	0	0
Total	40	40	40	40	40	40

0 = no reaction; 1/2 = doubtful reaction; 1 = mild reaction; 2 = moderate reaction; 3 = strong reaction.

trauma not related to insulin detemir. Only sporadic reactions were seen from subdermal and subcutaneous injections. Patients did not report itch or pain. Insulin detemir did not elicit wheal-and-flare reactions. Delayed reactions peaked after 48 h, when 35 patients reacted on intradermal insulin detemir, 22 on subdermal insulin and 21 on subcutaneous insulin contrasting sporadic reactions only on saline control (see Table 1). Reactions were significantly ($P < 0.05$) more frequent on intradermal insulin vs. saline and vs. subdermal and subcutaneous insulin, with no difference between the two subcutaneous injections. Thus, clinical reactions from intradermal insulin detemir were frequent and typical of weak grade. Injection depth influenced reactions from intradermal to subdermal but not from subdermal to the subcutaneous injection level. Reactions had become faint at the day 7 reading. Itch, pain or discomfort was only registered in one person after 48 h following intradermal insulin detemir.

Laser Doppler flowmetry: The nominated primary end point of immediate reactions showed no difference between insulin detemir and saline control and was in accordance with clinical observation of needle trauma only. In delayed reactions, the blood flow of intradermal insulin reactions at the 48 h measurement was significantly ($P < 0.05$) increased vs. saline control and subdermal and subcutaneous insulin, which were both over saline control ($P < 0.05$) (see Fig. 1). Flow measured after 24 h and after 7 days showed no statistical difference between insulin and control. Thus, the blood flow of delayed reactions after intradermal injection of insulin detemir was increased after 48 h compared with

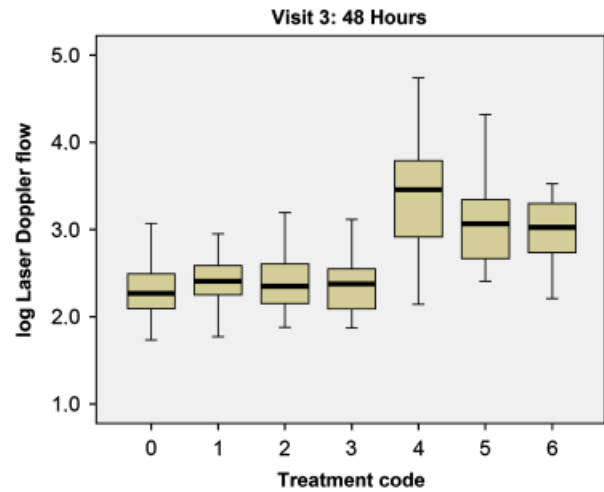


Fig. 1. Cutaneous blood flow (arbitrary units, laser Doppler flowmetry) of test fields 1–6, $n = 40$. 0 = untreated control; 1 = intradermal saline; 2 = subdermal saline; 3 = subcutaneous saline; 4 = intradermal insulin detemir; 5 = subdermal insulin detemir and 6 = subcutaneous insulin detemir.

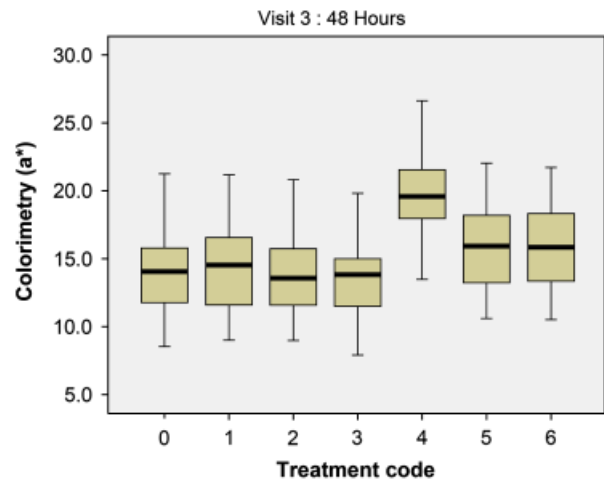


Fig. 2. Colorimetric measurement (a^* , CIE-lab colour space) of redness/hyperaemia of test fields 1–6, $n = 40$. 0 = untreated control; 1 = intradermal saline; 2 = subdermal saline; 3 = subcutaneous saline; 4 = intradermal insulin detemir; 5 = subdermal insulin detemir and 6 = subcutaneous insulin detemir.

both saline and both subcutaneous injections, and also the flow of subdermal and subcutaneous injection of insulin was increased compared with saline, however, independent of subcutaneous level.

Colorimetry of immediate reactions showed no difference between insulin detemir and saline control. In delayed reactions, increase of redness measured as a^* from intradermal insulin peaked after 48 h and was significantly ($P < 0.05$) higher than saline control and the two deeper insulin injections (see Fig. 2). Results overlapped with

TABLE 2. Result of histopathologist's blinded evaluation of histology sections of 22 biopsies from 15 patients, who volunteered for biopsy

Patient #	Substance	Inj. depth	Clinical, 24 h	Clinical, 7days	Pathology grade
06	Levemir	Intradermal	1	1	4
07	Levemir	Intradermal	1	1/2	1
08	Levemir	Intradermal	1	1	0
10	Levemir	Intradermal	1	1	4
11	Levemir	Intradermal	2	1/2	0
14	Levemir	Intradermal	2	1	4
	Levemir	Subdermal	2	1/2	4
17	Levemir	Intradermal	1	1	4
	Levemir	Subdermal	1/2	1/2	0
19	Levemir	Intradermal	2	1	4
	Levemir	Subdermal	2	1/2	0
23	Levemir	Intradermal	1	1	4
24	Levemir	Intradermal	1	1	4
30	Levemir	Intradermal	1	1/2	1
31	Levemir	Intradermal	1	1	4
33	Levemir	Intradermal	1	1	3
	Levemir	Subdermal	1	1/2	0
	Saline	Intradermal	1/2	1/2	1
35	Levemir	Intradermal	3	1/2	2
37	Levemir	Intradermal	2	1	4
	Saline	Intradermal	1	1	1

Biopsies were taken as blinded samples on day 7 from clinically doubtful or positive reactions of any test field with clinically visible reaction.

Pathologic changes of biopsies were graded as follows: 0, normal skin; 1, scarce inflammation without eosinophilia; 2, scarce inflammation with eosinophilia; 3, inflammation without eosinophilia; and 4, inflammation with eosinophilia.

results of flowmetry. Thus, redness from insulin injection was dependent on depth when comparing intradermal injection with subdermal and subcutaneous injection but not when comparing subdermal with subcutaneous injection.

Histopathology was available in 15 patients, who volunteered for 22 punch biopsies of doubtful or positive reaction at day 7 after injections. Biopsies were taken without the investigating dermatologist knowing the type and/or depth of the given injection. It turned out that 20 biopsies originated from insulin detemir injection sites, 15 from intradermal insulin sites, four from immediate subdermal and one from more deep subcutaneous injection sites. Two biopsies of saline-injected sites originated from intradermal injections and were grade 1. The dermato-histopathologist's blinded grading of changes of the biopsy material is shown in Table 2. A total of 11 biopsies out of 14 biopsies from insulin injection sites with changes showed a pattern of inflammation with eosinophilia, which typically was advanced in grade, i.e. grade 4. Thus, inflammation with eosinophilia was dominating, and this abnormality was exclusively observed in the sites injected with insulin detemir. Direct toxic effects on the tissue were not noted, and neither was leucocytoclastic vasculitis a finding. Figure 3 shows an example of the pathology of an insulin detemir-injected site.

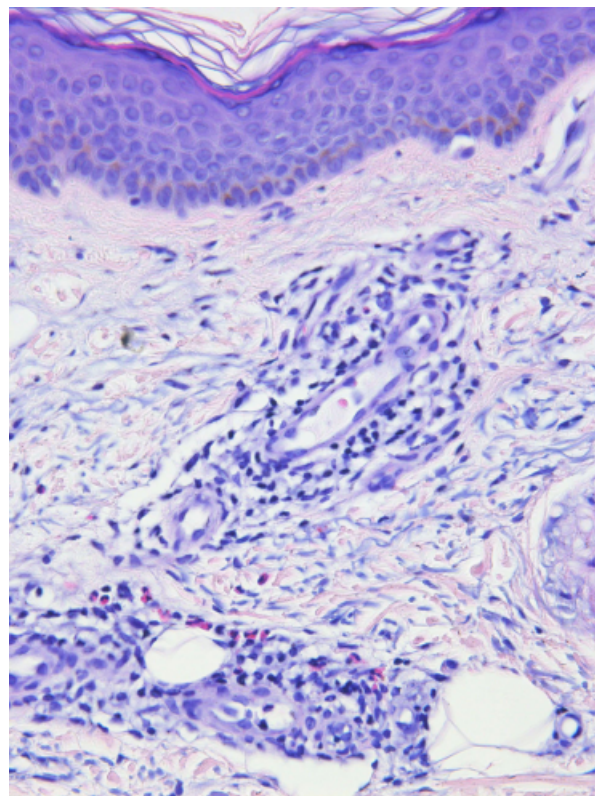


Fig. 3. Histology (HE stain) of test field 5 of the test person shows dense cellular infiltration in the dermis with eosinophilia. Biopsy was taken 7 days after intradermal injection of insulin detemir in the dermis.

Insulin detemir-specific antibodies in serum showed no increase neither in the general level nor in individual cases when follow-up analysis

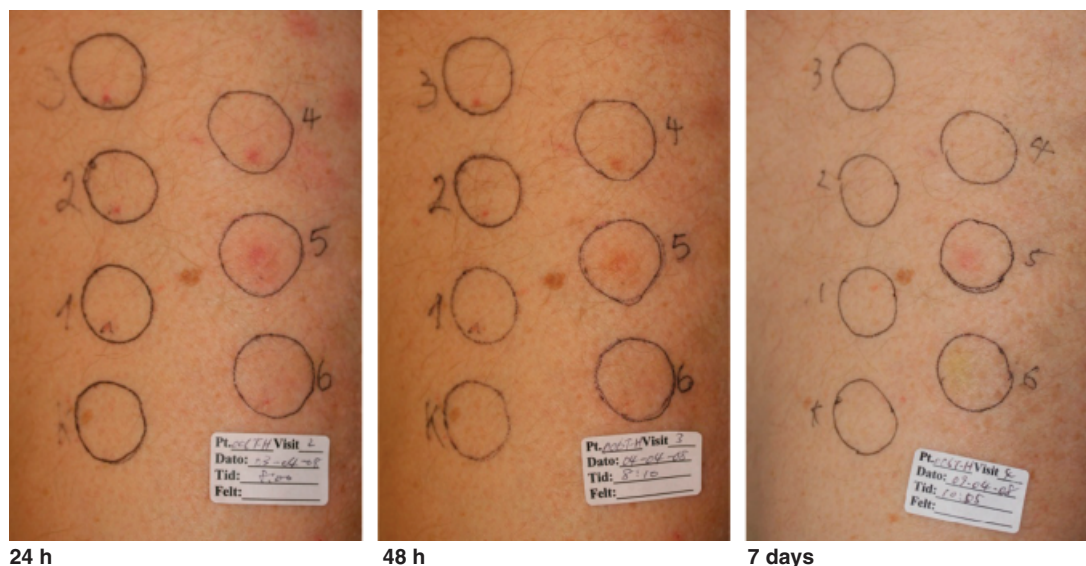


Fig. 4. Clinical photo of a case (06) with inflammatory reaction in test field 5 injected in the dermis with insulin detemir; 1+ (weak) reactions after 24, 48 h and 7 days as well. The site was subject to biopsy on day 7 (see Table 2); the case was further illustrated with non-invasive methods.

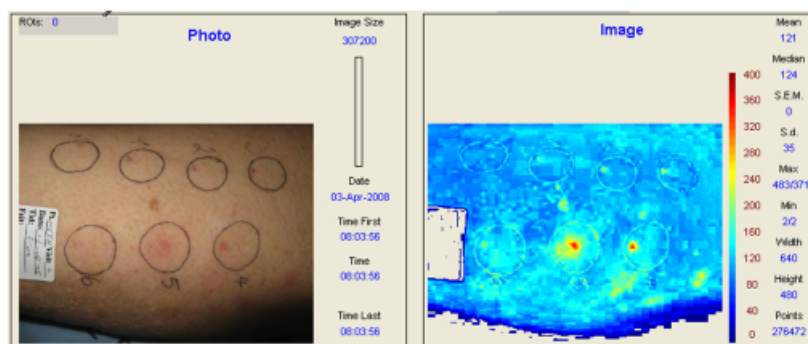


Fig. 5. Polarized photo and imaging of redness and hyperaemia of test fields of the person illustrated in Fig. 3 taken with the TiVi skin perfusion imager (Whealsbridge AB, Linköping, Sweden). The skin surrounding the needle injection in field 5, where intradermal insulin detemir was given, is hyperaemic. Tiny hyperaemic spots in fields 4 and 5 may represent needle injection reflux with fiery hyperaemia of the needle tract.

after 3 months were compared with baseline before insulin detemir injections of the study. Thus, there was no indication that insulin detemir allergy was present at the start or was acquired during the experiment. Antibody levels were not higher in patients with reactions compared with those without.

Insulin prick test vs. histamine controls and saline showed no significant wheal-and-flare response from insulin detemir, despite positive reactions to histamine positive control.

Observations in an illustrative case are shown in Figs 4–6.

Discussion

Clinical evaluation, laser Doppler flowmetry and colorimetry perfectly agreed that insulin detemir (Levemir®) injections into skin under experimental

conditions designed to sense even weak reactions frequently cause delayed inflammatory reactions, especially after intradermal injection. Reactions peaked after about 48 h. In contrast, the same methods demonstrated no significant immediate reaction of insulin detemir injection vs. saline injection. Furthermore, skin prick with insulin detemir produced no wheal-and-flare, and accordingly Levemir® is not a pharmacological histamine releaser.

The delayed reactions to insulin detemir were *not* allergic in nature as patients were insulin detemir naïve and as measurement of specific insulin antibodies was normal at baseline and on follow-up. Moreover, the histopathologic pattern was not consistent with the traditional typing of allergic responses.

We found, thus, no biphasic reaction mode and only observed delayed reactions. Histopathology

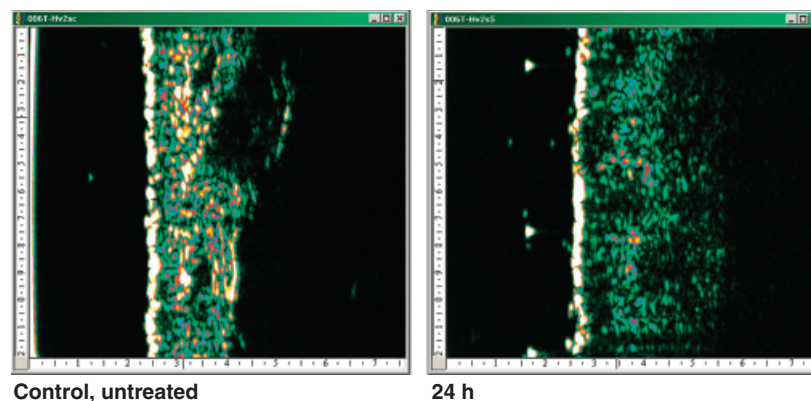


Fig. 6. Twenty MH2 ultrasound scan (Dermascan[®], Cortex Technology, Hadsund, Denmark) from the test person illustrated in Fig. 3, regional control of untreated skin and cross-sectional scan of the dermis at site 5 injected in the dermis with insulin detemir. The skin is thickened with increased distance between the entrance echo (left side) and the interface to subcutaneous fat identified with no visible echoes. The echostructure of the dermis is weakened with echo loose fields in the outer dermis and in the inner dermis towards the fat as a manifestation of inflammatory oedema widely distributed in the dermis. Scan field is 12.4 mm vertical.

was dominated by inflammation with eosinophilia. This mode of reaction is a biological response often seen in skin reactions from different medicines and the pattern is also observed in others situations, such as after insect bites. Thus, the histopathology is neither special nor specific for insulin detemir.

Our findings are in accordance with the clinical cases of mainly delayed reactions reported in the literature. Our observations may argue that suggestions or direct claims of an allergic mechanism behind the published cases are unlikely and remain unsupported.

Immediate local reactions to insulin detemir as reported in the literature might occur even after the very first injection. This occurs if the insulin detemir injection is given in an ongoing delayed reaction in the subcutis resulting from some recent injection of another insulin, and the predisposing reaction not observed by the patient. The needle and injection trauma might burst such subclinical reaction under healing with release of cytokines, leading to an immediate clinical reaction that is not an expression of specific allergy to the insulin detemir injection given immediately before the reaction burst and became clinically visible. Patients may without knowing it carry a number of such 'landmines' hidden in their preferred injection field, which may be a surprisingly limited area.

Insulins may exert local tissue effect due to their hormonal effect directly on the tissue, and patients with diabetes for many years often have inflammatory and sclerotic skin manifestations locally or more generalized (8, 9). Local effects of

insulins after short-term treatment and after chronic treatment deserve in depth study.

In the present study, insulin was injected strictly adherent to manufacturer's instructions, by an experienced study nurse, and the needle was kept *in situ* for 10 s to avoid insulin reflux to the dermis on needle retraction. Results were not simply the outcome of poor technique, and proper technique may anyhow not eliminate reflux via the needle tract. It appears not realistic to maintain the needle *in situ* beyond 10 s. We found significantly more severe and more delayed reactions after intradermal injections in comparison with subdermal and subcutaneous injections, and the dermis seems to be more sensitive to local reactions to insulin than deeper layers of the skin. However, dermis is more superficial and more accessible for visual inspection and measurement in comparison with deeper structures, and this might also explain stronger reactions observed in the dermis vs. deeper injection level. With an 8 mm injection needle and injection under an angle of about 45°, the vertical distance between subdermal and subcutaneous injection is anyhow small, a few millimetres, and we thus found no difference between subdermal and subcutaneous injections. Both are in a very outer subcutaneous fatty layer typically of some centimetres of thickness on the thigh. Thus, we cannot conclude whether longer injection needle would overcome the problem in the clinic as 6, 8 and 12 mm needles are all short. It shall be kept in mind that the high rate of delayed reactions in our study is a result of the experimental design aimed to sense even weak

reactions, and our results do not directly image adverse reactions as experienced in clinical routine, where we know that the frequency of spontaneously reported clinically relevant reactions is much lower, at the level of about 1–2% of patients treated over a longer period.

Diabetes patients challenge a special problem with repeated daily injections in the same field of limited size. Many inject more or less the same site, and many may inject on the top of some 'landmine' with silent inflammatory reaction due to previous injection and then unexpectedly elicit a burst of mediators of inflammation as mentioned above. Clinically, such a combined event may have various presentations and includes both immediate and delayed reactions mimicking allergic and biphasic responses. In our study, delayed reactions are frequent and stronger after intradermal injections compared with subcutaneous injections and also seen after injections in the subcutaneous layer. Knowing from histopathology that the reactions often last for at least 1 week, our best advice to insulin users would be

to be especially careful with rotation of injections within the preferred injection field and *not* inject the same site or spot until after some weeks. This is combined with the advice to use proper technique with sufficient injection depth well away from dermis and to avoid injection reflux to the dermis, which is best prevented when injections are only given slowly into a subcutis that is felt perfectly soft and pliable and thus suited for injection. As distance in depth away from dermis plays a role, the need for longer needles beyond the present 6–12 mm comfortable range for insulin injection needles might be logical to overcome the problem with injection site reactions.

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