

Effect of dark test-substance pigmentation on skin perfusion assessments and effect of test technique on balsam of Peru patch-test results

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13 balsam of Peru (Myroxylon Pereirae) patch-test-positive subjects are re-tested with 25% balsam of Peru in petrolatum and with serial doses printed on polyester squares. All substances are applied with tape strips for 3, 6, 24 (1 day [D]), 48 (2D), 72 (3D) and 96 h (4D) on each subject and for 96 h (4D) with plastic foils. Tests are followed visually and with perfusion assessments from 3 h to 9 days. Results show that pigment remnants following detachment of patches affect perfusion assessments. Such effect due to pigment is supported by readings of patch tests through the petrolatum test substance while applied with transparent foils. For most reactions, good agreement is observed between the assessment techniques when peak assessment values of reactions are compared. There is inter-individual variation in perfusion with identical tests. With the petrolatum test substance, increased visible reactivity was observed when the application time was extended up to 24 h (1D), while extension of application time increased perfusion in most cases except for an extension from 24 (2D) to 48 h (4D) where decreased perfusion resulted in most cases. Dose and application time did not affect the timing of highest reactivity of reactions in most cases.

Key words: pigmented patch-test materials; test procedure; bioengineering methods; laser Doppler perfusion scanning; laser Doppler perfusion imaging; dose; application time; reading time; balsam of Peru; Myroxylon Pereirae. © Munksgaard, 2000.

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Balsam of Peru (Myroxylon Pereirae) is a fragrance material (1) and a common sensitizer (2). It has been shown to test positive in about 50% of cases of perfume allergy (3). Some disagreement exists on its frequency of sensitization in children (4–6).

There have been some reports on inconstant elicitation of balsam of Peru patch-test reactions with the visual assessment approach (7–8). A search for an alternative approach seems appropriate. In this study, we focus on whether superficial perfusion assessments may be used to detect positive balsam of Peru patch tests. For that purpose we use a perfusion scanner, the laser Doppler perfusion imager[®] PIM 1.0 (LDPI) (9). The LDPI makes it possible to assess superficial perfusion without disturbances in perfusion that may be associated with skin touch as with laser Doppler flowmetry. Results of scans are presented as images on a monitor. A software application permits direct assessment of a scanned area or a part thereof. The scanning technique has been used for various investigative purposes (10–13), but

in a very limited way for patch tests. We have primarily used the technique for allergic patch-test assessments and to compare perfusion and visual assessments of patch tests (14–17). We have also used the technique to investigate reactivity caused by diesel oils (18), and to test the effect application devices may have on patch-test results (19). Other LDPI investigations on patch tests concern dithranol (20) and nicotine (21).

The balsam of Peru extract is a pigmented substance. This is the 1st time we have assessed test responses caused by a darkly pigmented test substance with the LDPI. In a recent study, we showed that perfusion assessments may be affected by skin pigmentation (22). The pigmentation of balsam of Peru test substances would probably be of limited interest were it not that some vehicles containing it may leave pigment remnants in place following detachment of patches. Whether such pigment remnants affect LDPI assessments is currently unknown. In this study, we specifically chose to test

with a 25% balsam of Peru test substance in petrolatum applied by Finn Chambers®. The reason for this choice is our experience that this substance is prone to leave visible pigment remnants in place on the skin upon detachment of patches. Additionally, we test the effect of pigmentation on test results by applying that test substance directly under foils and make perfusion readings of tests through the foils and the test substance. We further test with polyester squares printed with balsam of Peru in serial doses and apply these both with tapes and foils. These are much less pigmented with pigmentation fading as dose levels decrease. At the lowest level, pigmentation can hardly be seen. We use the same set-up of the LDPI instrument as we have recently suggested for non-pigmented substances (22) to see if that set-up may also be used with pigmented test substances.

Other aims are to: (A) investigate whether there may be inter-individual variation in perfusion with identical tests; (B) Compare results of patch-test assessments made with the LDPI and visually; (C) Study the effect of some dose levels and application times on test results.

Subjects, Materials and Methods

Study populations

From the outpatient files of patch-tested balsam of Peru positive subjects, randomly selected subjects of skin types I and II were contacted. 13 subjects, 10 women and 3 men (men nos. 4, 5 and 7), aged 30–62 years (median age 49 years) participated. All had been patch tested at the Department of Dermatology or the Department of Occupational Dermatology at the Karolinska Hospital in Stockholm. Apart from positive reactions to a 25% balsam of Peru in petrolatum test substance (Chemotechnique Diagnostics AB, Tygelsjö, Sweden), no subject had tested positive to any more than 1 other patch within 2 years in the Swedish patch-test series. These patches were (with nos. on subjects in parenthesis): nickel sulfate (nos. 3, 9 and 11), quaternium 15 (nos. 10 and 11), colophonium (nos. 8 and 13), fragrance mix (no. 3), potassium dichromate (no. 11), Kathon® CG (no. 11) and thiuram mix (no. 12). Criteria for a positive reaction were at least erythema and infiltration. Readings were performed 24 h (1 day [D]) and 120 h (5D) after 48 h (2D) applications with 9 mm Finn Chambers® on Scanpor® tape (Epitest Ltd., Helsinki, Finland) (23). 8 subjects (nos. 1–4, 6 and 8–10) had clinical relevance between their allergy and some balsam of Peru products, while the others reported no relevance to these. Intensities of prior patch-test readings were unknown to the investigators. Subjects were asked to inform

the investigators of any late reactions appearing after the study. No such reports were received months after the study.

Exclusion criteria

Subjects were excluded if any of the following criteria pertained: medication or disorder known to affect perfusion or allergic reactions; recent or current treatment with ultraviolet radiation; large risk of a patch-test location over a pathological skin condition; atopic disease; history of urticaria, phototoxicity or photoallergy; and patch testing or a history of eczema within the last 2 months.

Test materials and applications

Tape strips were prepared, each containing serial doses of 0.88, 0.28, 0.091, 0.03 and 0.01 mg/cm² balsam of Peru resin in polyvidone, 1 patch of each

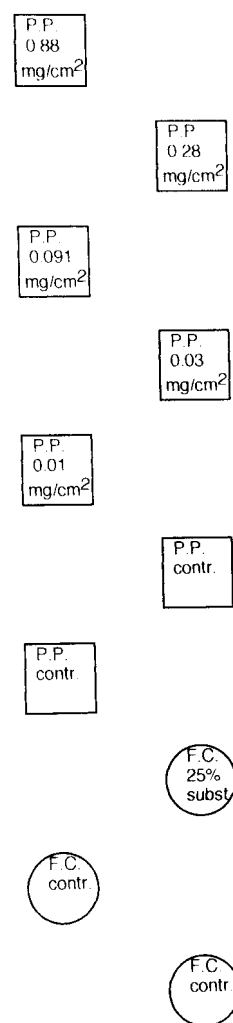


Fig. 1. An example of location of tests on strips. P.P.: polyester patches; F. C.: Finn Chambers®.

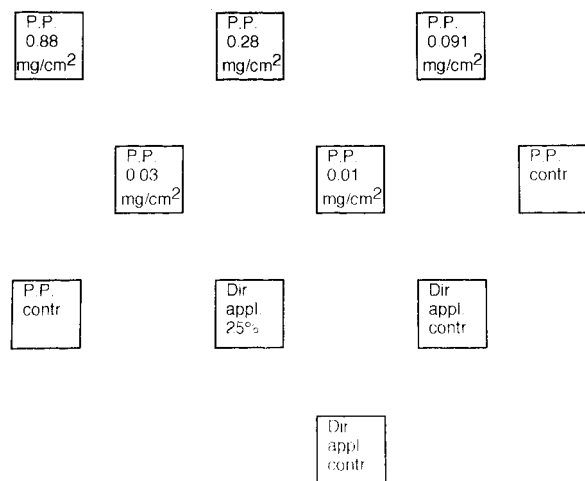


Fig. 2. An example of location of tests on test foils. P.P.: polyester patches; Dir. appl.: direct application.

dose, and 2 control patches with polyvidone only. These test substances were printed on 9×9 mm polyester squares (TRUE Test™ system). The 3 highest doses were chemically analyzed for exact dose (all kindly supplied from Pharmacia A/S, Hillerød, Denmark). Tape strips were 16.5×5 cm long made of non-woven cellulose acetate tape coated with acrylic adhesive (Cederroth BV, Tilburg, The Netherlands). In addition, each strip contained 1 9 mm Finn Chamber® (Epitest Ltd., Helsinki, Finland) (23) filled with approximately 20 µl 25% balsam of Peru in petrolatum (Chemotechnique AB, Tygelsjö, Sweden). For control purposes, 2 identical chambers were filled with approximately the same volume of petrolatum (Apoteksbolaget, Gothenburg, Sweden) and placed on each strip. Patch tests were applied randomly on strips in an order unknown to the investigators in 2 staggered parallel columns 1 cm apart with a 2 cm distance between patches within columns as exemplified in Fig. 1.

An identical set of polyester serial-dose patches including controls were attached to each of Tegaderm™, thin transparent plastic foils (3M Svenska AB, Sollentuna, Sweden). In addition, approximately 20 µl of the 25% test substance was applied directly to each foil as well as 2 control tests with the petrolatum. Patches were randomized in an order unknown to the investigators and distributed 2 cm apart in rows separated by 1 cm. An example of location of patches on foils is shown in Fig. 2.

All application materials except the substances containing petrolatum were kept for a maximum of 4 days following the preparation in a refrigerator at 4–8°C in hermetically sealed and light-proof envelopes until the application. The petrolatum-containing substances were also preserved in

the refrigerator, but applied to Finn Chambers® or foils only a few min before they were applied on the subjects.

Applications

Tape strips were applied vertically and at random on the backs of all subjects during the 1st session. Each subject had tape strips applied for 3, 6, 24 (1D), 48 (2D), 72 (3D) and 96 h (4D) and 1 foil for 96 h (4D). The foil on subject no. 8 detached between the 24 (1D) and 48 h (2D) sessions.

Readings

Patch tests of all detached tape strips were read with both reading techniques at each of the following sessions calculated from the time strips were applied: 3 h, 6 h, 24 h (1D), 48 h (2D), 72 h (3D), 96 h (4D), 168 h (7D) and 216 h (9D). Patch tests applied with foils were read at the same reading times as those applied with tapes, regardless of detachment. Tests applied with foils were read 2× at the session when foils were detached, both prior to and following the detachment. Approximately 5% of planned readings were not performed, mostly explained by subjects who were allowed to skip 3 and/or 6 h readings. Each of 117 reading sessions were completed within 3 h. More than 10,000 readings were performed.

Assessment of readings

Readings and assessments of perfusion were performed with a Laser Doppler Perfusion Imager® PIM 1.0 (LDPI), and PC compatible LDI 2.5 software (both supplied by Lisca Development AB, Linköping, Sweden). An exact reading distance between the laser head and the skin was secured with a 3-mW 670-nm applied laser pen® LP 30 (Applied Laser Systems, Grants Pass, OR, USA) as used previously (22). The same instrument set-up was used as suggested earlier for assessment of non-pigmented test substances tested on white skin (22). The instrument was specially focused at a 17 cm distance from the skin. Perfusion scale values ranged from 0.00–10.00 V, where increased value represents increased perfusion.

Mean value of control patches is subtracted from perfusion values of allergen-containing patches applied equally long and read at the same reading session. The resulting value is termed “adjusted perfusion value” (APV), indicated in V. A standard approved by the North American Contact Dermatitis Group is used for visual assessments (24). A very few reactions disappearing before 72 h (3D) were considered irritant and are ex-

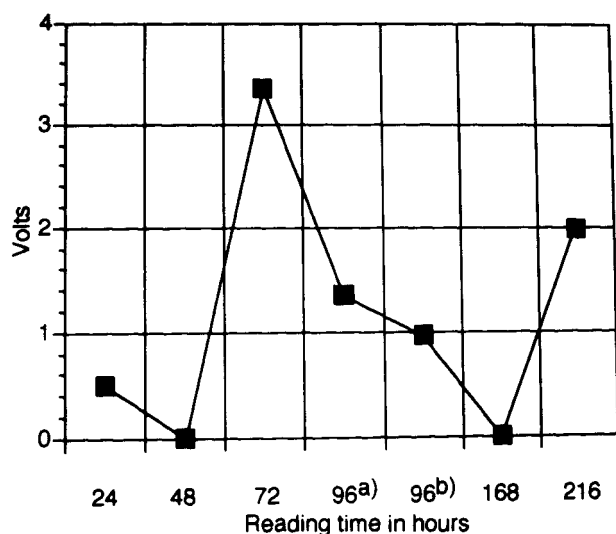


Fig. 3. APV assessments of a test reaction due to application of 25% balsam of Peru in petrolatum directly under a plastic foil for 96 h (4D) (subject no. 10). (a) before detachment of foil, (b) following detachment of foil.

cluded from the analysis of the study's data. No contact urticarial reactions were observed.

Subject related and environmental factors

Prohibited during the study were (1) medications known to affect allergic reactions, perfusion or pigmentation and (2) ultraviolet radiation treatment or sunbathing. Subjects were allowed to pour water over the back only once between the 96 (4D) and the 168 h (7D) reading sessions. No tests were performed during a sunny season. An acclimatization time between detachment of strips and assessment was minimally 30 min. Similarly, at least 30 minutes elapsed between the time a subject arrived for a reading session and the time the readings were taken. Body back temperature ranged between 29–31°C (median 31°C). Ethical approval was obtained from the Ethical Committee of the Karolinska Institute, Stockholm (No. 95/130).

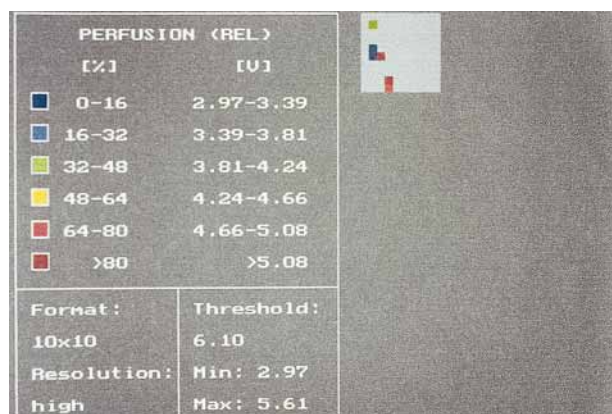


Fig. 4. Reading of perfusion at 72 h (day 3) of the reaction plotted in Fig. 3.



Fig. 5. Pigment remnants left in place following detachment of a Finn Chamber® containing 25% balsam of Peru in petrolatum applied for 3 h with tape (subject no. 1).

Results

Assessment of perfusion

The LDPI makes possible objective non-invasive assessments of balsam of Peru patch tests with the set-up used here. Most reactions have relatively homogeneous spatial perfusion within test sites. Assessment of perfusion of patch tests applied with the 25% test substance was sometimes masked by the pigment. Fig. 3 shows a perfusion profile with

Table 1. Serial doses and application times eliciting visually positive reactions are shown: dose 1: 0.88 mg/cm², dose 2: 0.28 mg/cm², dose 3: 0.091 mg/cm², dose 4: 0.03 mg/cm²

Subject no.	Application time (h)					
	3	6	24	48	72	96
4					1, 2	1, 2, 3
6			1, 2	1	1	1
7			1, 2	1, 2, 3, 4	1, 2	1, 2, 3, 4
8	1, 2, 3	1, 2, 3	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4
10			1	1	1	1
12	1, 2, 3, 4	1, 2, 3, 4	1, 2, 3, 4	1, 2, 4	1, 2	1

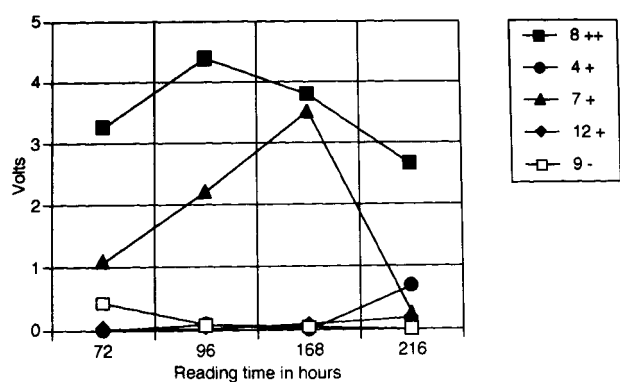


Fig. 6. Variation in APVs and peak visual assessments with 72-h (3D) tape applications of 0.28 mg/cm² balsam of Peru polyester patches.

that substance applied under plastic foil on a subject and Fig. 4 shows the 72 h (3D) reading of the reaction plotted in Fig. 3. Light gray measurement sites in Fig. 4 are sites interpreted as background by the LDPI computer system. Background sites were only observed with the 25% test substance, either while tests were applied with foils or following detachment of foils or Finn Chambers[®]. An example of pigment remnants following detachment of a Finn Chamber[®] is shown in Fig. 5. Lower or higher APVs than expected were infrequently encountered and were not solely associated with readings interrupted by a background interpretation. Such values were detected at different time points in each subject. There was no obvious change in visible erythema associated with such values.

Reactivity of tests

10 of 13 subjects became visually positive with the balsam of Peru in the petrolatum applied in Finn

Chambers[®] (approximately 6.6 mg/cm² balsam of Peru). With the foils and same test substance applied for 96 h (4D), 4 of these 10 subjects became visually positive. Each serial dose level except the lowest one elicited a visually positive reaction in at least some of the subjects. Serial doses and application times eliciting visually positive reactions with the tape applications are indicated in Table 1. With foils, 5 subjects became visually positive (4 highest serial doses).

Inter-individual variation in perfusion

A follow-up of reactivity to identical tests applied equally long on a group of subjects allows inter-individual comparison of adjusted perfusion profiles. An example is shown in Fig. 6.

Comparison between the reading methods

For most reactions, there is good agreement between APV assessments of peak reactivity and assessment values made with the visual assessment approach. Most reactions peaked either at the same reading session with both assessment techniques or earlier with the visual assessment approach.

Effect of test dose on strength of reactions

At each application time used, generally good dose-response relationship was observed regardless of reading methods or whether tapes or foils were used. A comparison between equally long tape applications of the highest serial dose level of 0.88 mg/cm² balsam of Peru and the 25% balsam of Peru test substance in Finn Chambers[®], which contains approximately 6.6 mg/cm² balsam of Peru, generally showed stronger reactivity with the higher dose.

Table 2. Effect on reactivity strength caused by a change of application times with the balsam of Peru in petrolatum test substance applied in Finn Chambers[®]

Subject no.	Adjusted perfusion					Visible reactivity				
	change in application time (from→to)									
	3→6 h	6→24 h	24→48 h	48→72 h	72→96 h	3→6 h	6→24 h	24→48 h	48→72 h	72→96 h
1		↑	↑	↑			↑	—	—	↓
4			↓	↑	↑	↓	↑			
5			↓	↑	↑		↑			
6	↑	↑	↓	↑	↑	↑		↓	↑	—
7	↑		↓	↓						
8	↑	↑	↑	↑		↑	—	—	—	↓
10		↑					↑			
11	↑		↓	↓	↑	—			↓	↑
12	↓	↑								
13		↑	↓	↑		↑				

↑: increase; ↓: decrease; —: no change; empty cells: no reactivity.

Effect of application time on strength of reactions

At each of the 4 highest serial dose levels that elicited positive tests, increased reactivity was generally only observed when the application time was prolonged from 3 or 6 h to 24 h (1D) or longer regardless of reading method. The relationship was unpredictable at other application times. Results with balsam of Peru in the petrolatum vehicle tested in Finn Chambers® are shown in Table 2. As with the serial doses, reactivity with the visual assessment approach generally increased when the application time was extended from 3 or 6 to 24 h (1D) and additionally when it was extended from 3 to 6 h. The finding was different where perfusion is concerned as a good relationship was found between perfusion and application time, except when applications were extended from 24 h (1D) to 48 h (2D) where decreased perfusion was detected in most cases.

Effect on the timing of the highest reactivity of reactions

In most cases, where the timing of highest reactivity of reactions was concerned, little or no change was observed related to a change in dose or application time regardless of reading methods. 3 subjects complained about respiratory and/or oral symptoms which they attributed to the test room.

Discussion

Effect of pigmentation on perfusion

This study supports the idea that the laser Doppler perfusion imaging technique may be used to assess superficial perfusion of patch tests even though pigmented test substances as used in current study are used for testing. However, such assessments may be affected by pigmentation left in place by test substances (Figs. 3, 4). One way to overcome this problem is to attempt removal of the pigment following detachment of tests and before readings. The disadvantage with such attempts is that they may jeopardize accuracy of patch-test perfusion assessments.

Technically, perfusion of patch tests is made by measuring perfusion of each of 100 measurement sites within each patch-test area and where the value of each measurement site contributes equally to a mean perfusion value of each patch test. Pigmentation does not affect such mean perfusion value assessments of test sites, and thereby assessments of patch tests, if both of the following criteria are met: (a) that perfusion of the test sites concerned is homogeneous and all measurement

sites of the test area are not masked by the pigment; (b) that all measurement sites affected by the pigment are interpreted as background by the computer system. The reason why such measurement sites do not affect mean perfusion values of test sites is that their background interpretation by the computer system (Fig. 4) does not generate perfusion values. Lack of compliance to either criteria may be due to:

(A) Heterogeneous spatial perfusion of test sites and partial masking of perfusion by pigmentation. Deviant mean perfusion value of a test site, as the 72 h (3D) reading in Figs. 3 and 4, may be explained by heterogeneity of spatial perfusion and a large number of measurement sites interpreted as background (Fig. 4). Under such circumstances, only one or a few measurement sites determine the mean perfusion value of the test site. If such measurement site(s) are not representative for the perfusion of the whole test area, as may be expected if perfusion is heterogeneous, a false mean perfusion value results and, consequently, false perfusion assessments of the patch tests concerned.

(B) Affected measurement sites that are not interpreted as background by the computer system but are instead given false assessment values by the system. Obviously such values may affect the mean perfusion values of test sites. This effect may explain deviant perfusion values of reactions due to the serial doses where no background interpretations of measurement sites were noted. However, this effect cannot be the sole explanation for such perfusion values with the serial doses. The reason is that reactions with such values may show expected values at the assessment intervals prior to and after the reading with the deviant value. Thus, if pigmentation were the only explanation, the degree of pigmentation would have to change in opposite directions between each of 3 sequential reading intervals of the same reaction.

That factors other than pigmentation may at least partly explain deviant perfusion values is supported by our experience with non-pigmented test substances (15,16) and the finding of the expected values prior to and after the reading with the deviant value with the serial doses above. We found no agreement between such values and which of the balsam of Peru serial doses was used, application time or application device. As such perfusion values were detected at different time points in the same subject, it appears that the values may result from local rather than systemic interaction between the tests and the target organs involved.

That no obvious visible erythema change was associated with deviant perfusion values may suggest an instrument error. However, this cannot be taken for granted as erythema is not the issue with

the LDPI but moving particles in the blood stream (25). This has been shown clinically with port wine stains, where there was a loss of a relationship between LDPI perfusion levels and erythematous intensity, as no difference in perfusion was found between the stains and surrounding skin in 9 of 13 patients investigated (26).

Complete assessment of the effect of pigmentation on positive patch tests with the LDPI is complicated by the fact that no 2 patches contain different degrees of pigment and identical concentration and dose of balsam of Peru. Thus, it is impossible to tell to what degree patch-test perfusion results from an allergy response and to what degree it may be masked by pigmentation.

It is possible that more accurate perfusion is obtained with a closer reading distance and/or a lower background threshold selection with the LDPI when perfusion of patch tests with pigmented substances is assessed. In the current study, this may especially apply for the 25% petrolatum test substance because of its dark pigment and the fact that, following detachment of patches, it is prone to leave in place visible pigment remnants.

Inter-individual comparison of test results in response to identical test materials (Fig. 6) should not be expected to be affected by pigmentation, as pigment problems should be expected to be accounted for once a test substance is used which does not leave visible pigment remnants in place following detachment of patches. However, even though the experience with the serial doses above does not suggest that non-visible pigment solely explains deviant perfusion values, such an effect should not be ignored. This is because pigment may be left on the skin after detachment of patches or be absorbed into the skin during the application.

Agreement between the assessment techniques

The good agreement between APVs and visual reactivity grades is interesting. It suggests that deviant APVs are infrequent, which agrees well with the analysis of more than 5,000 perfusion assessments made in this study. Highest reactivity of reactions is detected either at the same reading session with both assessment methods or at an earlier session with visual assessments. This is different from our experience with other allergens. With both nickel sulfate hexahydrate (14) and Kathon® CG patch tests (15), adjusted perfusion of reactions generally reached its highest value before the visual assessment did, while with budesonide, there was a tendency for highest reactivity of reactions to be detected at the same reading session with both the assessment methods on the same subject (16).

Effect of dose on test results

Good dose-response relationship was generally detected with the balsam of Peru serial doses in the current study regardless of reading technique. The dose relationship between the highest serial dose level and the 25% test substance in petrolatum tested with Finn Chambers® must be interpreted with care as different test techniques are used, including vehicles. Good dose-response relationship was also our experience with nickel sulfate hexahydrate (14) and Kathon® CG (15), but interestingly, not with budesonide where the relationship was unpredictable (16).

Effect of application time on test results

Different results with the serial doses and the Finn Chambers® where application time is concerned may possibly be explained by different doses, vehicles and/or test devices. Better bio-availability of allergens with the Finn Chambers® tests may be a reason why a relationship is established between perfusion and application time with, however, decreased perfusion in most cases with extension of the application time from 24 h (1D) to 48 h (2D) (Table 2). The decrease in perfusion is a mystery to us. It is not due to specific premature detachment of only Finn Chambers® applied for 48 h (2D) supported by a circular peripheral depression at test borders upon detachment of chambers (23). Our experience with other allergens varies very much when it concerns the relationship between reactivity and application times. We found a poor relationship between peak perfusion and length of applications with Kathon® CG (15), while with nickel sulfate hexahydrate we generally experienced a good relationship (14). With budesonide we experienced an unpredictable response when the application time was changed (16).

Timing of highest reactivity of reactions

Little or no change was observed in the timing of highest reactivity of reactions regardless of dose or application time. This finding indicates that once an immunological threshold of stimulation is reached for elicitation of a test response, neither dose nor application time affect the time when reactions reach their peak. This hypothesis is supported by both morphology and perfusion of tests. Our experience with nickel sulfate hexahydrate (14) and with budesonide (16) concerning the timing is similar to that with balsam of Peru, but we found an unpredictable time response with Kathon® CG for both changes in application times

and serial doses regardless of reading techniques indicating a different mechanism (15).

Masking of detection of reactivity

At some dose levels in Table 1, visually positive reactions are elicited at 24-h (1D) applications but not when the application time is extended in some subjects. The reason may be that long applications may mask detection of reactions of short duration while tapes are applied. Another explanation may be that long applications compared to short ones cause earlier reactivity of shorter duration; again, masked by the long application time of tape strips. Assessment of these factors would require assessment of patch tests while tape strips are applied. Similar effects may be associated with assessment of the timing of highest reactivity of reactions.

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