

Patch testing of patients allergic to Disperse Blue 106 and Disperse Blue 124 with thin-layer chromatograms and purified dyes

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Background: Former investigations have demonstrated that patch test preparations containing Disperse Blue (DB) 106 and DB 124 are impure.

Objective: To investigate the significance of impurities in preparations of disperse dyes with regard to contact allergy.

Materials/methods: 21 patients allergic to DB 106 and/or DB 124 were tested with dilution series of commercial and purified DB 106 and DB 124. All patients were additionally tested with thin-layer chromatography (TLC) strips made from the commercial preparations containing DB 106 and 18 of them also with TLC strips containing DB 124.

Results: 13 of 21 and 12/18 patients tested positively to the strips of DB 106 and DB 124, respectively. Among these patients, 4/13 and 5/12, respectively, did not react to the main spot but reacted to other spots. Dilution series of purified DB 106 and DB 124 were positive in 10 patients each, while 16 and 15 patients, respectively, tested positively to the dilution series of the corresponding commercial disperse dye.

Conclusion: Approximately 25% of the patients, diagnosed as contact allergic to DB 106 and DB 124, only reacted to impurities in the patch test preparations. This has implications for diagnosis and prevention.

Key words: contact allergy; Disperse Blue 106; Disperse Blue 124; impurities; patch test preparations; purified reference substance; textile dye; thin-layer chromatography. © 2009 John Wiley & Sons A/S.

Conflicts of interest: The authors have declared no conflicts.

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Among textile dyes, Disperse Blue (DB) 106 and DB 124 are well-known contact sensitizers, which have been identified as the most frequent disperse dye allergens in several studies (1–4). According to three studies from Italy, 1.7–2.1% of consecutively patch-tested dermatitis patients were allergic to DB 124 (5–7), and a study from 2001/2002, which included 13 European centres, reported contact allergy to a mix of DB 106/DB 124 at

1% in petrolatum in 1.3% of 3041 patch-tested patients, with varying results from no reactions in two centres to 6.2% in Basel, Switzerland (8). Many authors recommend DB 106 and DB 124 as screening allergens for textile dye dermatitis (4, 8, 9). A high frequency of simultaneous reactivity to DB 106 and DB 124 has been described and been attributed to a close structural similarity (10, 11). The chemical structures of DB 106 and DB 124,

which are defined substances with individual Colour Index numbers, are shown in (Fig. 1). A study at the Department of Occupational and Environmental Dermatology in Malmö (12) confirmed the results from other studies (1, 13), showing that the raw materials used for preparing DB 106 and DB 124 patch test preparations were impure. This has possible consequences for the diagnosis and prevention of contact allergy and allergic contact dermatitis from DB 106 and/or DB 124 and when comparing test results from various studies as the number and the concentrations of the potentially sensitizing impurities in the test preparations may vary and also the concentrations of the chemically declared disperse dyes.

The aim of the present study was to investigate the significance of the impurities found in patch test preparations of DB 106 and DB 124 with regard to contact allergy by patch testing the patients with known contact allergy to these dyes with thin-layer chromatography (TLC) strips made from the aforementioned patch test preparations (Fig. 2) (14). Furthermore, we wanted to compare the elicitation potential of patch test preparations containing commercial and purified DB 106 and DB 124 by patch testing the patients with dilution series of these dyes.

Materials and Methods

Study population

The study population consisted of 21 patients; 11 patients (nos. 1–11; Table 1) were patch tested at the Department of Occupational and Environmental Dermatology, Malmö University Hospital, Malmö, Sweden, in May or in December 2006 and 10 patients (nos. 12–21) at the Department of Dermatology, Contact Allergy Unit, University Hospital, K.U. Leuven, Belgium, in October 2006. There were 18 women (mean age 47.3 years, range 26–75 years) and 3 men (mean age 51.0 years, range 30–72 years). All 21 patients

were allergic to DB 106, and all but 5 patients (numbers 9, 12, 13, 20, and 21) were previously patch tested positively to DB 124.

Chemicals

Chloroform and acetone of analytical grade were obtained from Scharlau Chemie S. A. (La Jota, Barcelona, Spain) and acetonitrile for the TLC system from Lab-Scan (Dublin, Ireland). The raw materials for preparing the solutions for the patch test preparations and the thin-layer chromatograms were bought from Chemotechnique Diagnostics (Vellinge, Sweden).

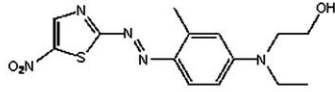
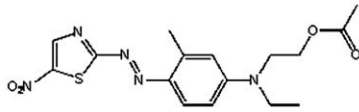
Patch test preparations

The purification and identification of DB 106 and DB 124 was carried out in the Malmö department, and the purity of the substances was >99% (12). All patch test solutions and thin-layer chromatograms were prepared at the department in Malmö from the same batches. About 20 mg of each disperse dye, commercial and purified, was accurately weighed and dissolved in acetone, yielding a 1.0% w/v preparation. From this stock solution, further dilutions from $10^{-1}\%$ to $10^{-6}\%$ w/v were prepared. All 21 patients were patch tested with dilution series of commercial and purified DB 106, whereas all but 1 patient were tested with dilution series of commercial and purified DB 124. Some patients, who had strong allergic reactions when previously patch tested to DB 106 and DB 124, were not tested with the highest concentration of the patch test preparations in the dilution series (Table 2).

Preparation of thin-layer chromatograms

The chromatograms were made according to a procedure earlier described by Bruze et al. (14). From a TLC plastic roll, 500×20 cm silica gel 60F254 (Merck KGaA, Darmstadt, Germany)

Fig. 1. Chemical structures, Chemical Abstracts Service (CAS), and Colour Index (CI) numbers as well as molecular weight (MW) for disperse dyes Disperse Blue (DB) 106 and DB 124.

DB 106 	CAS: 68516-81-4 CI: 111935 MW: 335
DB 124 	CAS: 15141-18-1 CI: 111938 MW: 377

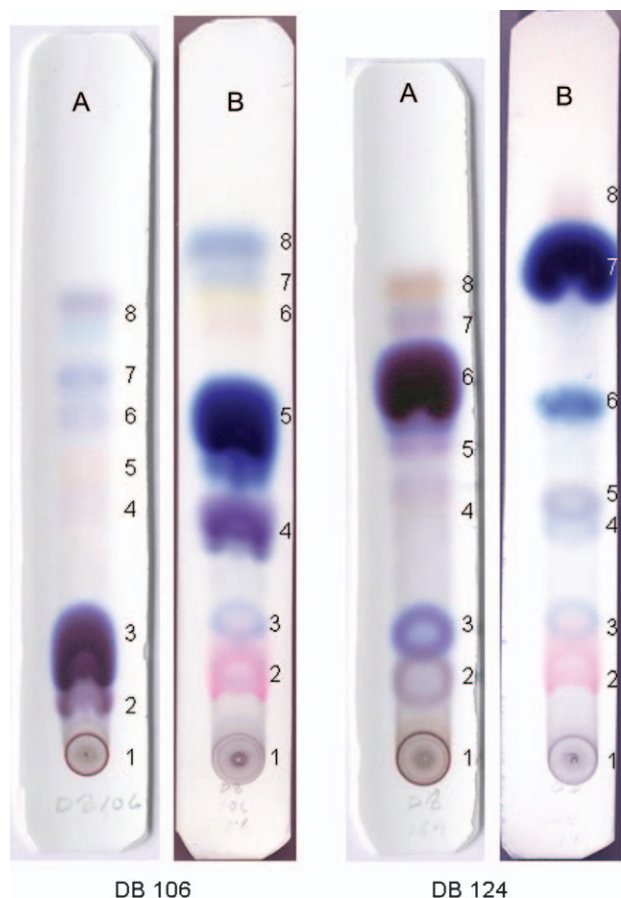


Fig. 2. The thin-layer chromatograms A and B of disperse dyes Disperse Blue (DB) 106 and DB 124 patch test preparations, respectively. The spots on each chromatogram are numbered from 1 to 8, with spot number 1 as the application spot.

18 × 18 cm strips were cut out. Three microlitres of the samples to be tested was repeatedly applied with a micropipette, until 75 µl had been deposited to one spot each for every 2.5 cm along a line on the lower part of the silica gel plate. Double spots were applied for each sample, one to be used as a patch test and the other to be used as a reference when reading the test. Initially, a mixture of chloroform and acetonitrile, 86/14 (v/v), was used as the mobile phase (chromatogram A), but to obtain a better separation of the spots, the eluent was modified to a mixture of chloroform and acetonitrile, 70/30 (v/v) (chromatogram B). After separation, visually less substance remained on the application spot and a band of well-defined and separated spots could be seen. The thin-layer chromatograms were all inspected in visible light and in ultraviolet radiation (254 and 366 nm), with no additional spots visualized. The DB 106 and DB 124 chromatograms A and B can be seen in (Fig. 2). A total of 3 patients were patch tested with DB 106 chromatogram A and 2 patients with

DB 124 chromatogram A, whereas 18 and 16 patients were tested with DB 106 and DB 124 chromatogram B, respectively. Some patients were tested with chromatograms made from the 0.1% solutions of the commercial DB 106 and DB 124 preparations because of strong allergic reactions when previously patch tested with the two dyes (Table 1).

Patch test technique

Finn Chambers[®] (Ø8 mm; Epitest Ltd, Tuusula, Finland) on Scanpor[®] tape (Norgesplaster A/S, Vennessla, Norway) were used for the patch testing with the dilution series of commercial and purified dyes. Fifteen microlitres of the test solution was applied with a micropipette to the filter paper disc in each test chamber. The thin-layer chromatograms with the separated spots were cut out in pieces of about 2.5 × 16 cm and were then applied on the upper back, to the left, and to the right of the spine, of each tested patient, using Scanpor tape. The chambers and the thin-layer chromatograms were left on the back, on the left, and on the right of the spine for 2 days, and the readings were performed on D3 or D4 and on D6 or D7. The reactions were scored according to guidelines from the International Contact Dermatitis Research Group (15), with two classifications added between the three usual positive gradings: strong + and ++ reactions were graded as ++(+) and ++(+), respectively (16). The strongest patch test reactions were used for registration in the present study irrespective of the reading occasion.

Statistical analysis

The Fisher's exact test was used, and we regarded two-sided $P < 0.05$ as statistically significant.

Ethics

The study was approved by the regional ethics review board in Lund, Sweden, and conducted in accordance with ethical standards specified in the Declaration of Helsinki. All patients gave informed written consent to participate in the study.

Results

Patch testing with chromatogram A

All 3 patients (nos. 1–3) patch tested with DB 106 thin-layer chromatogram A reacted to the main spot and to areas near the application area (Table 1; Fig. 3). Both the patients (numbers 1 and 3) patch tested with the DB 124 chromatogram A showed a corresponding pattern of

Table 1. The results of the patch testing with disperse dyes Disperse Blue (DB) 106 and DB 124 thin-layer chromatograms A and B^a

Patient number	TLC conc. ^b (%)	DB 106 chromatogram A								DB 124 chromatogram A							
		1	2	3 ^c	4	5	6	7	8	1	2	3	4	5	6 ^c	7	8
1	1	++	++	++					?	+++	+++	+++			+++		
2	0.1	+++	+++	+++	+++			++	+	NT							
3	1		+++	+++								+++		+++	+++		
Patient number	TLC conc. ^b (%)	DB 106 chromatogram B								DB 124 chromatogram B							
		1	2 ^d	3	4	5 ^c	6	7	8	1	2 ^d	3	4	5	6	7 ^c	8
4	1		+++			(+)					+++						
5	0.1	+	++							+	+++						
6	0.1					+++										+++	
7	0.1		(+)	(+)		+++					(+)	(+)				+++	
8	0.1		(+)	(+)							(+)	(+)					
9	0.1		+(+)	+(+)							+(+)	+(+)				(+)	
10	0.1					+++										+++	
11	1										+					(+)	
12	1																
13	1																
14	1		(+)								++						
15	1					+											
16 ^e	1	++	++	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
17	1																
18	1																
19	1					+										+	
20	1									NT							
21	1		+++	++						NT							

Conc., concentration; NT, not tested; TLC, thin-layer chromatography.

^aThe spots on each chromatogram are numbered in accordance with the registration in Fig. 2, with number 1 as the application spot. Empty cells indicate negative test results.

^bThe w/v concentration in acetone of the test preparations used for application on the TLC strips.

^cThe main spot on each chromatogram is marked in dark colour.

^dThe pink spots, number 2 on the chromatograms B.

^ePatient number 16 had allergic reactions covering the whole area corresponding to the DB 106 and DB 124 chromatograms, respectively.

reactivity, reacting to the main spot and to spots closer to the application area (Table 1; Fig. 3).

Patch testing with chromatogram B

1 patient, number 16, strongly reacting to the dilution series of both commercial and purified DB 106 and DB 124, presented with severe allergic reactions corresponding to the area from the application spot to spot number 8 on the DB 106 and DB 124 chromatograms, respectively (Table 1). Because of the widespread reactions, discerning allergic reactions to specific spots was impossible. This patient was therefore excluded in (Fig. 3). Of the remaining 17 patients patch tested with the DB 106 chromatogram B, 9 reacted positively to at least one spot, 5 of them (nos. 6, 7, 10, 15, and 19) to the main spot, whereas 4 patients (nos. 4, 5, 9, and 21) solely reacted to spots near the application area (Table 1; Fig. 3). Correspondingly, 9 of the remaining 15 patients patch tested with the DB 124 chromatogram B reacted positively to at least one spot: 4 of 9 (nos. 6, 7, 10, and 19) to the main spot and 5 (nos. 4, 5, 9, 11, and 14) only to other spots (Table 1; Fig. 3).

Patch testing with the dilution series

The results of patch testing with the dilution series of the commercial and purified DB 106 and DB 124 are shown in Table 2. A total of 16 of 21 and 15 of 20 patients tested positively to the dilution series of the commercial DB 106 and DB 124, respectively, while dilution series of the purified disperse dyes were positive in 10 patients each. The strongest allergic reactions to the dilution series were seen in two patients (nos. 2 and 16), both reacting to the lowest concentrations tested, i.e. 0.010 p.p.m. (0.010 µg/ml) of the purified DB 106. Only one of these patients (no. 16) was tested with the dilution series of DB 124, but this patient tested positively down to 0.010 p.p.m. of the purified DB 124 as well. The patients testing positively to the main spot on the DB 106 chromatograms more often had a higher or equal reactivity to the purified than the commercial DB 106 (nos. 1, 2, 3, 6, 7, 10, 15, and 19 versus 0) compared with those only reacting to other spots on the DB 106 chromatograms (no. 9 versus nos. 4, 5, and 21; $P = 0.018$). The corresponding calculation for the patients reacting to the main spot on the DB 124

Table 2. The results of the patch testing with the dilution series of commercial and purified disperse dye Disperse Blue (DB) 106 and DB 124

Patient number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16 ^a	17	18	19	20	21
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C
DB 106																					
Conc.	+++	NT	+++	+++	+++	NT	NT	++	+	NT	++			++	++	NT	++	++	++	++	++
1.0%	+++	+++	+++	+++	+++	NT	+++	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻¹ %	+++	NT	+++	+++	+++	NT	+++	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻² %	+++	+++	+++	+++	++	+++	+++	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻³ %	+++	+++	+++	+++	+++	+++	+++	+++	+	+++	++	++	++	+	++	++	++	++	++	++	++
10 ⁻⁴ %	+++	+++	+++	+++	+++	+++	+++	+++		+	++	++	++		++	++	++	++	++	++	++
10 ⁻⁵ %	+++	+++	+++	+++	+++	+++	++	++			++	++	++		++	++	++	++	++	++	++
10 ⁻⁶ %	++	++				++	++									++	++	++	++	++	++
Main spot ^b	x	x	x	x	x	x	x		x					x		x		x			x
Other spots ^b	x	x	x	x	x			x								x					
DB 124																					
Conc.	+++	NT	+++	+++	+++	NT	NT	++	+	NT	+++	++	++	++	++	NT	++	++	++	++	++
1.0%	+++	NT	+++	+++	+++	NT	+++	++	++	NT	++	++	++	++	++	++	++	++	++	++	++
10 ⁻¹ %	+++	NT	+++	+++	+++	NT	+++	++	++	NT	++	++	++	++	++	++	++	++	++	++	++
10 ⁻² %	+++	NT	+++	+++	++	+++	+++	++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻³ %	+++	NT	+++	+++	++	+++	+++	+++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻⁴ %	+++	NT	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻⁵ %	+++	NT	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	++	++	++	++	++	++	++	++
10 ⁻⁶ %	+++	NT	+++	+++	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Main spot ^b	x	x	x	x	x	x	x		x							x		x	++	NT	NT
Other spots ^b	x	x	x	x	x			x								x				NT	NT

C, commercial; conc., concentration; NT, not tested; P, purified.

^aPatient number 16 had allergic reactions covering the whole area corresponding to the DB 106 and DB 124 chromatograms, respectively. Empty cells indicate negative test results.^bPositive reactions to the main spot and/or to other spots on the thin-layer chromatograms for each patient are indicated with 'x'.

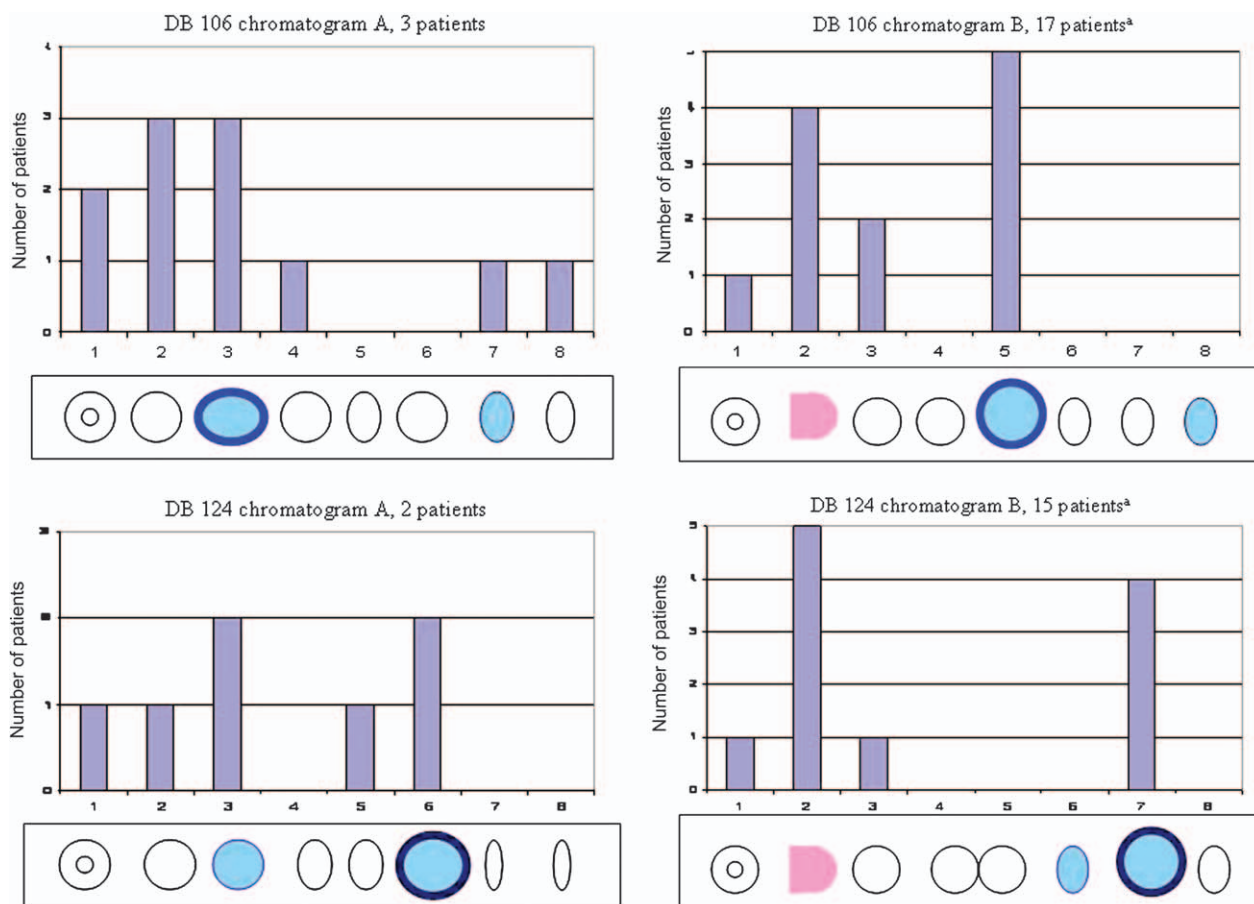


Fig. 3. The number of patients reacting to the different spots on the disperse dye Disperse Blue (DB) 106 and DB 124 thin-layer chromatograms A and B. The strong blue rings represent the main spot and the faint-coloured rings represent the low content of DB 124 found in the DB 106 test preparations and vice versa. The spots on each chromatogram are numbered in accordance with the registration in Fig. 2. The pink spots are number 2 on the chromatograms B. ^aPatient number 16 is excluded.

chromatograms (nos. 1, 3, 6, 7, 10, and 19 versus 0) compared with those testing positively exclusively to other spots (no. 9 versus nos. 4, 5, 11, and 14; $P = 0.015$) gave a similar pattern. Negative reactions to the dilution series of DB 106 and DB 124 were noted in 5 patients (nos. 12, 13, 17, 18, and 20).

The reading on D7 detected 1 more patient (no. 15) with positive reactions to both the commercial and the purified DB 106 and DB 124 patch test preparations and to the thin-layer chromatogram of DB 106. Another 2 patients (nos. 14 and 19) reacted to the dilution series of the dyes on D3 but to the chromatograms only on the second reading on D7.

Discussion

Patch testing with thin-layer chromatograms has been developed from a technique using paper chromatograms, described in a Dutch study (17). This method has been further developed and

refined at the Malmö department (14) and has demonstrated to be a valuable tool in the process of detecting individual sensitizers in compound materials, as reported in previous studies (18–20). The results in the present study verified that patch testing with thin-layer chromatograms of DB 106 and DB 124 patch test preparations was applicable to demonstrate positive patch test reactions to the main spot as well as to the impurities of the preparations. However, after the patch testing of the first patients (nos. 1–3), the method for preparing the thin-layer chromatograms was improved as the adding of more acetonitrile, which has more polar properties than chloroform, resulted in a better separation of the spots. A pink spot was now visualized near the application area (Fig. 2, spot numbers 2 on the DB 106 and DB 124 chromatograms B). Of the patients patch tested with the DB 106 and DB 124 chromatograms B, 22% (4/18) and 31% (5/16), respectively, reacted to the pink spots but not to the main spot of the respective chromatograms.

Consequently, the results demonstrated that approximately 25% of the patients, earlier diagnosed as contact allergic to DB 106 and/or DB 124, instead reacted to impurities in the patch test preparations.

Furthermore, we wanted to compare the elicitation potential of patch test preparations containing commercial and purified DB 106 and DB 124. A tendency was found for a higher reactivity to the purified than to the commercial dyes (Table 2) in the patients reacting to the dilution series of both the commercial and the purified dyes. These results are in accordance with the results found, both in a study from Germany (13) and confirmed in a study from our department in Malmö (12), showing that the mean concentration of purified DB 106 and DB 124 in commercial patch test preparations was only 25–30% of the labelled concentration.

As mentioned before, 2 patients reacted to the lowest concentration tested, i.e. 0.010 µg/ml of the purified DB 106. One of these patients, who was also tested with the dilution series of DB 124, tested positively down to 0.010 µg/ml of the purified DB 124 as well. It had been of a great value to identify the lowest concentrations giving positive allergic reactions in the two disperse dyes, but our patients denied further testing. Nevertheless, both patients tested positively to concentrations corresponding to 0.00030 µg/cm² of the purified DB 106, and one of them also to the corresponding dose per square centimetre of the purified DB 124, which is comparable to the lowest doses reported to give positive reactions in allergens such as some phenol formaldehyde resins (21, 22) and the perfume contact allergen chloroatranol (23), all regarded as very potent sensitizers. Both DB 106 and DB 124 have also previously been identified as strong allergens by several authors (1, 24, 25), and the sensitization potential of DB 106 is estimated as being similar to 2,4-dinitrochlorobenzene when investigated with the local lymph node assay (24).

For the individual patients, there was a good agreement for the two dyes as the patients patch tested with the chromatograms and with the dilution series of both DB 106 and DB 124 and reacting to the DB 106 preparations also reacted to the corresponding DB 124 preparations and vice versa. This raises the question if the pattern of reactivity was a consequence of the fact that the test preparations of DB 106 contained a low content of DB 124 and the reverse, as found in a previous study at the Malmö department (12). In that study, the highest possible concentration of DB 124 in the preparations of DB 106 labelled 1.0% was 0.002%, and the corresponding concentration

of DB 106 in the DB 124 preparations was 0.06%. As the reactivity to the dilution series of DB 106 and DB 124 was equal for the individual patients, this could not be the reason for the corresponding test results. Another explanation to the agreement in the allergic reactions to the two dyes in the individual patients may also be the fact that the acetate of DB 124 could be hydrolysed in the skin and give DB 106. However, the possibility that the test results were a consequence of cross-reactivity between the two dyes was not investigated in this study because this would need animal experiments. In any case, the results raise the question if separate DB 106 and DB 124 patch test preparations are needed to trace the patients with contact allergy to the individual disperse dyes, or if a mix of the two disperse dyes is acceptable, as discussed in an article by Uter et al. (11).

The pattern on the chromatograms also demonstrated that the test preparations of DB 124 contained a low content of DB 106 and vice versa (Fig. 2). However, in the present study, only 1 patient (no. 2) reacted to the spot corresponding to DB 124 on the DB 106 chromatogram A and 2 patients (nos. 1 and 3) reacted to the spot corresponding to DB 106 on the DB 124 chromatogram A (Fig. 3). All the 3 patients had strong allergic reactions to the dilution series of both the commercial and the purified dyes (Table 2).

Moreover, 2 patients (nos. 11 and 15; Table 2) with known contact allergy to DB 106 and DB 124 tested positively to the dilution series of both the disperse dyes but only to one of the thin-layer chromatograms each. One explanation to the results, with positive tests to the dilution series but not to the corresponding chromatogram, could be that patch testing with the preparations on a limited area of 50 mm², using Finn Chambers, could give additive and/or synergistic effects between the allergens compared with the patch testing with chromatograms, where the allergens were separated into different spots. Other reasons could be that the individual dose/cm² of the allergens on the thin-layer chromatograms was too low, when spreading on a larger area, or that the availability of the allergens was decreased because of the adhesion to the silica on the chromatograms compared with the availability of the allergens when patch tested in a solution on the filter papers in the Finn Chambers. This could give false-negative reactions, unless it was not compensated for by a larger volume of the allergens applied on the thin-layer chromatograms.

Negative patch test reactions to any of the patch test preparations and to the thin-layer chromatograms were found in 5 patients with earlier diagnosed contact allergy to DB 106 (nos. 12, 13, 17,

18, and 20) and in 2 patients (nos. 17 and 18) earlier diagnosed as allergic to DB 124 (Table 2). However, 2 patients (nos. 9 and 21), one from Malmö and one from Leuven, previously testing negatively to DB 124 patch test preparations reacted to the dilution series of the corresponding dye. These results raise the question whether there was a variation regarding the actual patch test concentration or qualitative differences with respect to impurities in the patch test preparations used for the previous and present patch testing, as demonstrated in former studies (12, 13).

Isolation and identification of the different components in the patch test preparations can be made based on the TLC patch testing. From a reference chromatogram, the silica on the area corresponding to the positive reactions can be scraped off, extracted, and chemically analysed (14). However, the nature and the similarities between the pink spots on the respective chromatograms are not yet elucidated as the sensitizers in the chromatograms are not yet identified.

The results in the present study raise the question if the patch test preparations should be changed to contain only the chemically defined disperse dye without the impurities. However, the disperse dyes used in the textile industry are impure (26), and our results rather emphasize the importance of identifying the various sensitizers in the disperse dyes responsible for allergic reactions from clothes and other textiles, as stressed in other articles (13, 26), in which the use of TLC is also mentioned as an important tool to identify the sensitizers.

Conclusions

As some patients did not react to the main spot on the DB 106 and DB 124 thin-layer chromatograms but only to other spots near the application area, the results in the study demonstrate that these patients, diagnosed as contact allergic to DB 106 and/or DB 124, only reacted to impurities in the patch test preparations. Although both DB 106 and DB 124 are chemically defined in the Colour Index, the present study confirmed that DB 106 and DB 124 patch test preparations contained impurities. Furthermore, patch testing with thin-layer chromatograms proved to be a useful tool to demonstrate positive patch test reactions to the impurities of the preparations. The isolation and identification of the respective sensitizers in DB 106 and DB 124 patch test preparations can be performed based on the TLC patch testing as the individual spots can be chemically analysed with regard to different contact allergens. The results from these investigations may have conse-

quences not only for individual diagnosis and prevention but also for the comparison of test results from various studies.

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