

Patch testing with the textile dyes Disperse Orange 1 and Disperse Yellow 3 and some of their potential metabolites, and simultaneous reactions to para-amino compounds

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Summary

Background. It is known that, *in vitro*, human skin bacteria are able to split disperse azo dyes into the corresponding aromatic amines, some of which are sensitizers in the local lymph node assay. We hypothesize that the molecules of disperse dyes migrate onto the skin while garments are worn, and are metabolized and degraded by commensal skin bacteria. These molecules penetrate the skin and induce sensitization.

Objectives. To evaluate the elicitation capacities of the possible azo-degradation products of the selected azo disperse dyes in patients allergic to them and to compare it with the elicitation capacities of other para-compounds.

Methods. Ten patients allergic to Disperse Yellow 3 (DY3) and/or Disperse Orange 1 (DO1) were patch tested with a dilution series of the purified dyes 4-nitroaniline and *p*-aminodiphenylamine in concentrations equimolar to those of purified DO1 in the dilution series, as well as 4-aminoacetanilide and 2-amino-*p*-cresol in concentrations equimolar to those of purified DY3 in the dilution series.

Results. Three patterns of patch test reactions could be seen. The 6 patients who were positive to DO1 and DY3 also reacted to *p*-aminodiphenylamine and 2-amino-*p*-cresol. Two patients were positive to DO1 only, and both reacted to *p*-aminodiphenylamine, but to neither 4-aminoacetanilide or 2-amino-*p*-cresol. Two patients did not react to DO1 or DY3 on this occasion.

Conclusion. We show that it is possible that the major sensitizers in contact allergy to DO1 and DY3 are their metabolites, *p*-aminodiphenylamine and 2-amino-*p*-cresol, respectively, which might be formed by the azoreductase pathway of skin bacteria.

Key words: 2-amino-*p*-cresol; 4-aminoacetanilide; 4-nitroaniline; allergic contact dermatitis; disperse dyes; *p*-aminodiphenylamine.

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Disperse dyes (DDs) constitute a large dyestuff class, accounting for 22% of all dyes produced in the world (1). They are used to colour textiles, plastics, cosmetics, and food. The chemical structure of disperse azo dyes is characterized by the presence of one or more azo groups ($-N=N-$).

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One potential hazard to human health with azo dyes is allergic contact dermatitis, which appears in the sites where textile coloured with azo dyes comes into close contact with the skin (2).

Azo dyes can be split into aromatic amines by the liver microsomal system and the intestinal microflora in mammals (3–5).

Commensal microflora are present on all body surfaces that are covered by epithelial cells and exposed to the external environment (gastrointestinal and respiratory tract, skin, etc.). The number of bacteria colonizing mucosal and skin surfaces exceeds the number of cells forming the human body. The number of skin bacteria approaches 10^{12} , and this population includes mainly gram-positive bacteria, obligate aerobes (*Micrococcus*), and facultative anaerobes (e.g. *Staphylococcus* and *Corynebacterium*) (6, 7). In recent years, it has been demonstrated *in vitro* that human skin bacteria are able to split azo dyes into the corresponding aromatic amines, some of which are sensitizers in the local lymph node assay (8). The gene encoding azoreductase from *Staphylococcus aureus* was identified and cloned, and it was shown that this azoreductase was able to cleave azo dyes to the corresponding amines (5, 9). It was also shown experimentally that skin bacteria from the genera *Staphylococcus*, *Corynebacterium*, *Micrococcus*, *Demacoccus* and *Kocuria* were able to efficiently reduce and cleave the azo dyes Methyl Red and Orange II *in vitro* (by 74–100% in 24 hr) (10).

In vitro percutaneous absorption studies comparing human, mouse and guinea pig skin showed that human skin was the least permeable to selected azo dyes: ~30% of the applied dose was absorbed in 24 hr, and ~30% of the absorbed dose underwent azoreduction in the skin (11).

Coloured textile clothing is assumed to be the most widespread source of skin exposure to DDs. The external exposure to DDs by this route is between 1 ng/cm²/event and 1 µg/cm²/event. However, in the case of garments that have not been dyed according to the state-of-the-art technology, one has to assume higher release rates and exposures (12).

Thus, we can hypothesize that the DDs migrate onto the skin garments while garments are being worn, and that they are metabolized by commensal skin bacteria. The metabolites then penetrate the skin, and may induce sensitization and/or elicitation.

In a recent study, we found Disperse Orange 1 (DO1) to be the most common DD allergen among the eight DDs investigated in the mix, with a contact allergy rate of 0.5% among consecutively tested dermatitis patients, followed by Disperse Yellow 3 (DY3) (13). Combined sensitization to disperse azo dyes and other

para-amino compounds [e.g. black rubber mix and *p*-phenylenediamine (PPD)], probably based on true cross-sensitization or on simultaneous positive reactions, has frequently been described (14–16). Para-amino compounds are a group of substances in which hydrogen in a benzene molecule is substituted with amino and other groups in opposite positions (17). There are experimental studies showing that a group in the para position induces a stronger immune response than the same group in other positions (e.g. ortho or meta) (18). Common or similar metabolites may also contribute to the frequently reported simultaneous reactions.

The chemical structures of DO1 and DY3, as well as their possible azo-degradation products, are shown in Fig. 1.

The aim of the present study was to evaluate the elicitation capacities of the possible azo-degradation products of DO1 and DY3, and of black rubber mix ingredients and PPD (Fig. 2), and to compare these with the elicitation capacities of purified DO1 and DY3 in patients allergic to DO1 and/or DY3.

Materials and Methods

Study population

The study population consisted of 10 patients allergic to DO1 and/or DY3. There were 3 women and 7 men (mean age 46.1 years, range 19–70 years). All 10 patients had had dermatitis. Their previous patch test reactivities to DO1, DY3, PPD and black rubber mix, and the clinically relevant contact allergies, are shown in Table 1.

Chemicals

Acetone of analytical grade was obtained from Scharlau Chemie S.A. (La Jota, Barcelona, Spain). The chemicals used for DO1 and DY3 solutions had been previously purified and identified at the Malmö department from commercial DO1 and DY3, purchased from Chemotechnique Diagnostics (Vellinge, Sweden) (19). DO1 and DY3 were purified, and their purity was >99%. These dyes were used to prepare the solutions for patch testing.

PPD and black rubber mix ingredients [*N*-cyclohexyl-*N'*-phenyl-4-phenylenediamine (CPPD), *N*, *N'*-diphenyl-4-phenylenediamine (DPPD), and *N*-isopropyl-*N'*-phenyl-4-phenylenediamine (IPPD)] were bought from Chemotechnique Diagnostics. 4-Nitroaniline, *p*-aminodiphenylamine, 4-aminoacetanilide and 2-amino-*p*-cresol were bought from Sigma Aldrich (Steinheim, Germany). The general purity of 4-aminoacetanilide

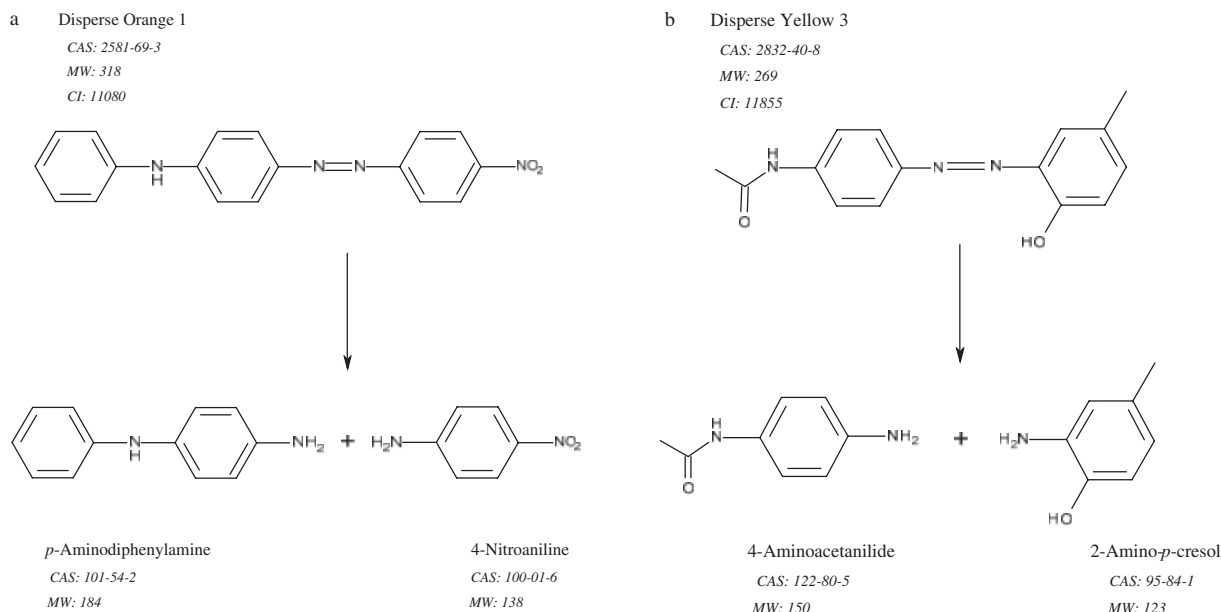


Fig. 1. Possible azo degradation pathways of Disperse Orange 1 (a) and Disperse Yellow 3 (b) and chemical structures. CAS numbers, Colour Index (CI) numbers and molecular weight (MW) of the investigated dyes and their metabolites are also shown.

<i>N</i> -Cyclohexyl- <i>N'</i> -phenyl-4-phenylenediamine	CAS: 101-87-1 MW: 266
<i>N,N'</i> -Diphenyl-4-phenylenediamine	CAS: 74-31-7 MW: 260
<i>N</i> -Isopropyl- <i>N'</i> -phenyl-4-phenylenediamine	CAS: 101-72-4 MW: 226
<i>p</i> -Phenylenediamine	CAS: 106-50-3 MW: 108

Fig. 2. Chemical structure, CAS numbers and molecular weight (MW) for the investigated black rubber mix ingredients and *p*-phenylenediamine.

was 99%, that of 2-amino-*p*-cresol was 97%, that of *p*-aminodiphenylamine was 98%, and that of 4-nitroaniline was >99%. Purified DO1 and DY3 were analysed with high-performance liquid chromatography for the presence of 4-aminoacetanilide, 2-amino-*p*-cresol, *p*-aminodiphenylamine, and 4-nitroaniline. These substances were not detected.

Patch test preparations

All patch test solutions were prepared in our department from the same batches. Approximately 20 mg each of purified DO1, purified DY3, PPD, CPPD, DPPD and IPPD was accurately weighed and dissolved in acetone, yielding a 1.0% wt/vol preparation. From this stock solution, further dilutions, from 1.0×10^{-1} to 1.0×10^{-6} % wt/vol, were prepared. All 10 patients were patch tested with one or more dilutions of the purified DY3 and DO1, depending on their previous patch test reactivity; those who previously had a +++ reaction to 1.0% were patch tested starting with a 100-fold lower concentration (0.01%), and those who previously did not react were patch-tested with the highest concentration (1.0%) only. If patients had a positive reaction on D3 or D4, they were also tested with the corresponding dilution series.

Dilution series of 4-nitroaniline and *p*-aminodiphenylamine were prepared in concentrations equimolar to the dilution series of DO1, starting at 1.0% wt/vol

Table 1. Patch test reactivity to Disperse Orange 1, Disperse Yellow 3, *p*-phenylenediamine and black rubber mix, when patch testing was performed earlier, in conjunction with the patients' clinical work-up

Substance		Patient number, year									
		1, 1999	2, 1999	3, 1999	4, 2005	5, 2004	6, 2007	7, 1999	8, 2005	9, 2004	10, 1999
DO1	Tested concentration	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.
	Reaction*	+++	+++	+	+++	++	++	+++	–	++	++
DY3	Tested concentration	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.	0.5% pet.	1.0% pet.	0.5% pet.
	Reaction*	+	++	+	–	–	+	–	+	++	++
PPD	Tested concentration	1.0% pet.	0.94% pet.	0.94% pet.	0.94% pet.	0.94% pet.	0.94% pet.	1.0% pet.	0.94% pet.	0.94% pet.	1.0% pet.
	Reaction*	+++	+++	+	–	–	–	–	++	+++	++
BRM†		–	++	–	+	–	++	+++	–	–	–
Clinically relevant contact allergy		Disperse dyes	PPD	Not found	Disperse dyes	Disperse dyes	Not found	BRM	PPD	PPD	Not found

BRM, black rubber mix; DO1, Disperse Orange 1; DY3, Disperse Yellow 3; PPD, *p*-phenylenediamine.

*The results of patch testing are based on the strongest reaction either on D3/4 or D7.

†Black rubber mix was tested at 0.6% pet. concentration in all patients.

(31 mM). 4-Aminoacetanilide and 2-amino-*p*-cresol dilution series were prepared in concentrations equimolar to the dilution series of DY3, starting at 1.0% wt/vol (37 mM). All dilution series were prepared in acetone.

Patch test technique

Finn Chambers® (Ø 8 mm; Epitest Ltd, Tuusula, Finland) on Scanpor® tape (Norgesplaster A/S, Vennesla, Norway) were used for patch testing with the dilution series of the dyes. Fifteen microlitres of the test solution was applied with a micropipette to the filter paper disc in each test chamber. The chambers were left on the back for 48 hr, and readings were performed on D3/D4 and D7 by a dermatologist. The reactions were scored according to the guidelines of the International Contact Dermatitis Research Group (20).

Patch testing

The patients were all patch tested with the same series, which included purified DO1 and DY3 (19), PPD, and the black rubber mix ingredients DPPD, CPPD, and IPPD, at 1.0% wt/vol (all in serial dilutions in acetone, starting at 1.0%). Patients were also tested with the presumed DO1 and DY3 metabolites in serial dilutions in concentrations equimolar to the parental compound (Fig. 3). If the patients were positive on the first reading to the lowest concentration of the respective substance tested, they were additionally tested with the lower concentrations.

Controls

Consecutively patch tested dermatitis patients served as controls: 16 for 4-nitroaniline, 5 for *p*-aminodiphenylamine, 118 for 4-aminoacetanilide, and 27 for 2-amino-*p*-cresol.

In order to show a relationship of the metabolites to the parental dyes, we also selected dermatitis patients positive to a textile dye mix – a mixture of eight DDs, consisting of Disperse Blue 35, DY3, DO1, Disperse Orange 3, Disperse Red 1, and Disperse Red 17 (at 0.5% wt/wt pet.), and Disperse Blue 106 and Disperse Blue 124 (at 0.1% wt/wt pet.), giving a total concentration of 3.2% – but negative to DO1 and/or DY3. We tested 4 control patients positive to textile dye mix but negative to DO1 with *p*-aminodiphenylamine 0.58%, and 7 with 4-nitroaniline 0.43%.

We tested 7 patients positive to textile dye mix but negative to DY3 with 4-aminoacetanilide 0.56% and 4 with 2-amino-*p*-cresol 0.46%. These control patients were tested with higher concentrations than the 118 controls chosen from consecutive dermatitis patients, in order to allow comparison of the results with the positive reactions to equimolar concentrations of DY3 and DO1.

Statistical analysis

Fisher's two-sided exact test was used, and we regarded $p < 0.05$ as being statistically significant when patch tests results of control patients were compared with the

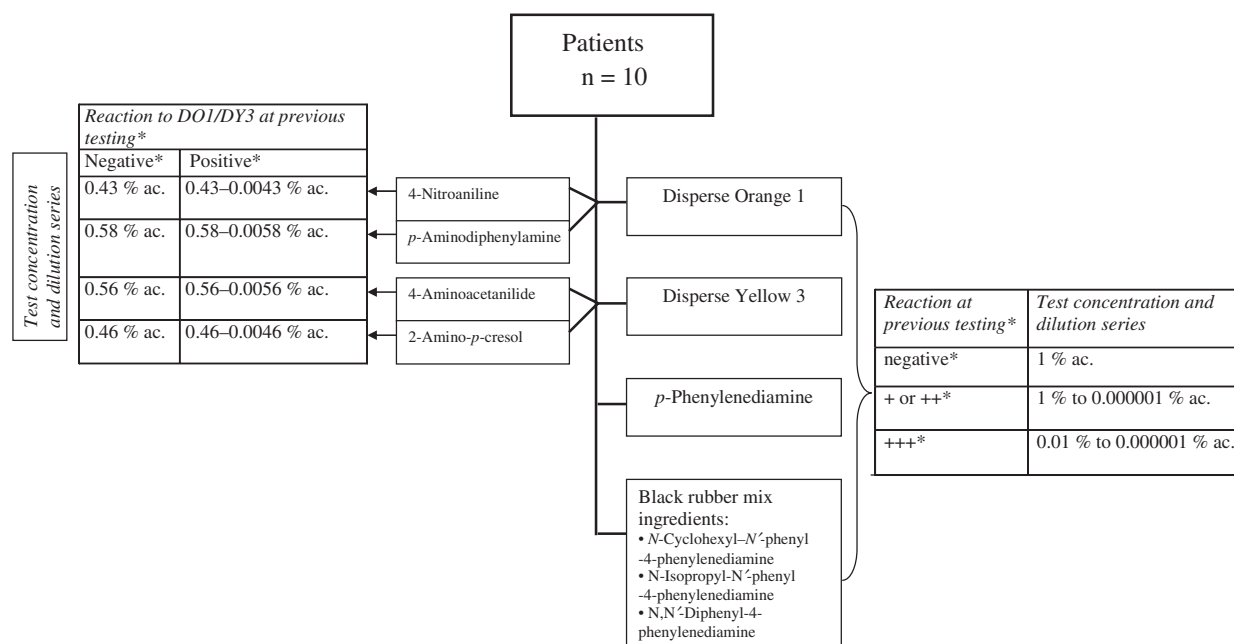


Fig. 3. The protocol for testing with Disperse Orange 1, Disperse Yellow 3, their metabolites, black rubber mixture ingredients, and *p*-phenylenediamine. + or ++ or ++++, previous positive patch test reactions. *The results of patch testing (negative, positive) are based on earlier patch test results prior to this study (see Table 1). Ac: Acetone.

results of the investigated patients. We used the test to calculate the number of controls needed to show that the concentration used was not irritant for each individual allergen ($p < 0.05$).

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

All results are presented in Table 2.

The results indicated three different reaction patterns among patients:

- (1) Six positive to DO1 and DY3 – all reacted to *p*-aminodiphenylamine, PPD, and 2-amino-*p*-cresol, and 5 reacted to IPPD (Table 2). In the latter group, 1 patient had previously reacted only to DO1, but on this occasion also reacted to DY3.
- (2) Two positive to DO1 and not to DY3 – both reacted to *p*-aminodiphenylamine, and none reacted to CPPD, PPD, 4-aminoacetanilide, or 2-amino-*p*-cresol.

- (3) Two did not react to DO1 or DY3 – 1 of them had reacted previously to DY3, but on this occasion he was positive to PPD only. The other patient, previously found to be allergic to DO1, now showed a late (D14) positive reaction to *p*-aminodiphenylamine (21).

Ten patients were tested with the dilution series of DO1, and 8 of them were positive: the lowest concentrations giving a positive reaction were 0.1% in 3 patients, 0.001% in 4 patients, and 0.000001% in 1 patient.

Six of 10 patients tested with a dilution series of DY3 were positive. Four patients reacted down to 0.01%, 1–0.1%, and 1–1.0%.

The pattern of concomitant reactivity to DO1, DY3, their potential metabolites, PPD and black rubber mix ingredients is shown in Table 3.

Patch testing with the potential metabolites of DO1: *p*-aminodiphenylamine and 4-nitroaniline

Of the 8 patients positive to DO1, all reacted to *p*-aminodiphenylamine. One of these patients, previously shown to be allergic to DO1, reacted only to *p*-aminodiphenylamine. All reactions to *p*-aminodiphenylamine were already strong (++/+++) on the D3 reading.

Table 2. Results of patch testing with the dilution series of Disperse Orange 1 (DO1), Disperse Yellow 3 (DY3), their potential metabolites, black rubber mix ingredients, and *p*-phenylenediamine

Tested substance*	Patient number Days of testing									
	1 D4 D7	2 D4 D7	3 D4 D7	4 D4 D7	5 D4 D7	6 D4 D7	7 D4 D7	8 D4 D7	9 D4 D7	10 D4 D7
DO1										
1.0%	NT	NT	—	(+)	+++	NT	NT	—	+++	(+)
			—	+	NR			—	NR	++
0.1%	+++	NT	—	(+)	+++	NT	NT	NT	+++	(+)
	tr		—	+	NR				NR	++
0.01%	(+)	++	—	—	+++	+++	+	NT	(+)	—
	(+)	+++	—	—	NR	+++	+++		++	(+)
0.001%	—	++	—	—	+++	+	(+)	NT	(+)	—
	—	+++	—	—	NR	+++	++		+	—
0.0001%	—	(+)	—	—	++	(+)	—	NT	—	—
	—	(+)	—	—	NR	(+)	—		—	—
0.00001%	—	—	—	—	+	—	—	NT	—	—
	—	—	—	—	NR	—	—		—	—
0.000001%	—	—	—	—	+	—	—	NT	—	—
	—	—	—	—	NR	—	—		—	—
4-Nitroaniline										
0.43%	—	(+)	—	—	++	—	—	—	++	+
	—	+	—	++	NR	—	—	—	+++	++
0.043%	—	—	—	—	(+)	—	—	NT	(+)	—
	—	—	—	(+)	NR	—	—		+	—
0.0043%	—	—	—	—	—	—	—	NT	—	—
	—	—	—	—	NR	—	—		—	—
<i>p</i> -Aminodiphenylamine										
0.58%	+++	+++	†	++	+++	+++	+++	—	+++	++
	tr	+++		+	NR	tr	+++	—	+++	+++
0.058%	+++	+++	—	(+)	+++	+++	++	NT	+++	—
	tr	+++	—	(+)	NR	tr	+++		+++	++
0.0058%	++	+++	—	—	+++	+++	+	NT	+++	—
	+	+++	—	—	NR	tr	++		+++	—
0.00058%	—	—	NT	NT	NT	—	—	NT	—	—
	—	—				—	—		—	—
DY3										
1.0%	+++	(+)	—	—	++	+++	—	—	+++	(+)
	tr		—	—	NR	NR	—	—	+++	+
0.1%	+++	(+)	NT	NT	NT	+	NT	—	+++	—
	tr	(+)				NR		—	+++	+
0.01%	++	++	NT	NT	NT	(+)	NT	—	++	—
	+	+				+++		—	++	—
0.001%	(+)	—	NT	NT	NT	—	NT	—	—	—
	—	—				(+)		—	—	—
0.0001%	—	—	NT	NT	NT	—	NT	—	—	—
	—	—				—		—	—	—
4-Aminoacetanilide										
0.56%	++	—	—	—	—	—	—	—	++	(+)
	+	—	—	—	NR	—	—	—	+++	++
0.056%	—	—	NT	NT	NT	—	NT	—	+	—
	—	—				—		—	(+)	—
0.0056%	—	—	NT	NT	NT	—	NT	—	—	—
	—	—				—		—	—	—
2-Amino- <i>p</i> -cresol										
0.46%	+++	+++	—	—	++	+	—	—	+++	+
	tr	+++	—	—	NR	+++	—	—	+++	++
0.046%	—	—	NT	NT	NT	+	NT	—	(+)	—
	—	—				++		—	(+)	—
0.0046%	—	—	NT	NT	NT	—	NT	—	—	—
	—	—				—		—	—	—
CPPD										
1.0%	—	+++	—	—	++	NT	—	—	—	—

Table 2. Continued

Tested substance*	Patient number Days of testing									
	1	2	3	4	5	6	7	8	9	10
	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7	D4 D7
0.1%	— NT	+++ +++ +++	— NT	— —	NR NT	— +++ tr	(+) —	— NT	— NT	— NT
0.01%	NT	++ ++	NT	— —	NT	+++ tr	— —	NT	NT	NT
0.001%	NT	— —	NT	NT	NT	++	NT	NT	NT	NT
0.0001–0.0000001%	NT	— —	NT	NT	NT	— —	NT	NT	NT	NT
DPPD										
1.0%	— —	++ +++	— —	— —	+ NR	NT	— +	— —	— —	— —
0.1%	NT	++ ++	NT	— —	NT	+ ++	— —	NT	NT	NT
0.01%	NT	+++ (+)	NT	— —	NT	+ +	— —	NT	NT	NT
0.001%	NT	— —	NT	NT	NT	— —	NT	NT	NT	NT
IPPD										
1.0%	+ (+)	+++ +++	— —	(+) —	+++ NR	NT	++ +++	— —	+++ +++	— —
0.1%	NT	+++ ++	NT	— —	NT	+++ tr	+ +++	NT	++ NR	NT
0.01%	NT	+ (+)	NT	— —	NT	+++ tr	(+) ++	NT	— NR	NT
0.001%	NT	++ NR	NT	NT	NT	NT	— —	NT	— NR	NT
0.0001%	NT	— NR	NT	NT	NT	NT	— —	NT	— NR	NT
PPD										
1.0%	NT	NT	— —	— —	+++ NR	+++ tr	— —	++ NR	NT	NT
0.1%	NT	NT	— —	NT	NT	NT	NT	+ NR	NT	NT
0.01%	+++ tr	(+) ++	NT	NT	NT	NT	NT	— —	+++ +++	+++ +++
0.001–0.000001%	— —	— —	NT	NT	NT	NT	NT	— —	— —	— —

CPPD, *N*-cyclohexyl-*N'*-phenyl-4-phenylenediamine; DPPD, *N,N'*-diphenyl-4-phenylenediamine; IPPD, *N*-isopropyl-*N'*-phenyl-4-phenylenediamine; NR, not read; NT, not tested; PPD, *p*-phenylenediamine; tr, reaction treated with potent topical corticosteroid.

*Concentration (wt/vol) in acetone.

†Case report published elsewhere (21).

Regarding the dilution series of *p*-aminodiphenylamine, there were 8 positive patients among 9 tested (Table 2). The majority of the reactions were strong, and disappeared abruptly from one tested concentration to the nearest lower concentration.

Five of the 8 patients positive to DO1 reacted to 4-nitroaniline. The patch test reactions to 4-nitroaniline were weaker than those to *p*-aminodiphenylamine.

Patch testing with the potential metabolites of DY3: 4-aminoacetanilide and 2-amino-*p*-cresol

Of the 10 patients patch-tested with 4-aminoacetanilide, 3 were positive; all reacted to DY3 and 2-amino-*p*-cresol.

Of the 10 patients tested with 2-amino-*p*-cresol, 6 were positive, with strong reactions on D3. All of these patients were also allergic to DY3.

Table 3. Pattern of concomitant reactivity to Disperse Orange 1 (DO1), Disperse Yellow 3 (DY3), their potential metabolites, *p*-phenylenediamine (PPD) and black rubber mix (BRM) ingredients in 10 patients allergic to DO1 and/or DY3

	DO1	NA	PADPA	DY3	AAA	AC	CPPD	DPPD	IPPD	PPD
DO1	8	5	8	6	3	6	3	4	6	6
NA	5	5	5	4	2	4	2	2	3	4
PADPA	8	5	8	6	3	6	3	3	5	6
DY3	6	4	6	6	3	6	3	3	5	6
AAA	3	2	3	3	3	3	0	0	2	3
AC	6	4	6	6	3	6	3	3	5	6
CPPD	3	2	3	3	0	3	3	3	3	3
DPPD	4	2	3	3	0	3	3	4	4	3
IPPD	6	3	5	5	2	5	3	4	6	5
PPD	6	4	6	6	3	6	3	3	5	7

AAA, aminoacetanilide; AC, 2-amino-*p*-cresol; CPPD, *N*-cyclohexyl-*N'*-phenyl-4-phenylenediamine; DPPD, *N,N'*-diphenyl-4-phenylenediamine; IPPD, *N*-isopropyl-*N'*-phenyl-4-phenylenediamine; NA, 4-nitroaniline; PADPA, *p*-aminodiphenylamine.

Of the 6 patients tested with the <0.56% concentration of 4-aminoacetanilide, only 1 was positive, and reacted to 0.056%.

Of the 6 patients tested with the <0.46% concentration of 2-amino-*p*-cresol, 1 was positive, and reacted to a 0.046% (diluted 10-fold).

Test reactivity

In the 8 patients who reacted positively to DO1, the majority of positive D7 reactions were stronger than D3 reactions (Table 2). In contrast, the majority of strong positive reactions to *p*-aminodiphenylamine appeared on the first reading, that is, D3/D4.

Regarding patterns of reactions to DY3, this tendency was not so strong. The majority of reactions to DY3 and to 2-amino-*p*-cresol were seen on D3/D4.

For both DO1 and DY3, the elicitation thresholds for the proposed metabolites were, in most cases, higher than those for the parent compound. In 3 of 8 patients, the thresholds for DO1 and *p*-aminodiphenylamine were similar.

Patch testing of the control patients

None of 118 consecutively patch tested dermatitis patients reacted to the test solution with 4-nitroaniline 0.043%, *p*-aminodiphenylamine 0.0058%, 4-aminoacetanilide 0.056% or 2-amino-*p*-cresol 0.046% in acetone. The concentration chosen for control testing of each substance was the lowest concentration giving a positive patch test reaction in a reasonable proportion of the patch-tested patients with respect to controls needed for statistical

significance. Positive reactions to *p*-aminodiphenylamine were linked to positive reactions to DO1, but not to positive reactions to other ingredients of the textile dye mix ($p < 0.05$).

Of 5 control patients positive to textile dye mix but negative to DO1 who were tested with *p*-aminodiphenylamine 0.58%, 1 was positive ($p = 0.018$). Of 7 patients positive to textile dye mix but negative to DO1 who were tested with 4-nitroaniline 0.43%, 2 were positive ($p > 0.05$). All 7 patients positive to textile dye mix but negative to DY3 who were tested with 4-aminoacetanilide 0.56% were negative ($p < 0.05$), and all 4 who were tested with 2-amino-*p*-cresol 0.46% were negative ($p = 0.0079$).

Patch testing with the ingredients of black rubber mix

Of the 4 patients previously shown to be allergic to black rubber mix, two reacted to all ingredients (CPPD, DPPD, and IPPD), 1 to DPPD and IPPD, and 1 to black rubber mix only and not to the separate ingredients. Three patients previously not shown to be positive to black rubber mix, and now not tested with black rubber mix, but with ingredients, were positive: 3 to IPPD, 1 to DPPD, and 1 to CPPD. Of 8 patients positive to DO1 and *p*-aminodiphenylamine, 6 reacted to IPPD.

Five patients were tested with the dilution series of black rubber mix ingredients, and 4 were positive (Table 2). Two patients were positive to the dilution series of all three ingredients, and 2 were positive only to the dilution series of IPPD.

Of 8 patients positive to DO1, 6 (75%) reacted to at least one black rubber mix ingredient, and of 6 positive to DY3, 5 (83%) reacted to at least one black rubber mix ingredient.

Patch testing with PPD

Seven patients of 10 tested were positive to PPD. One patient previously shown to be positive to PPD did not react in the present testing. Of the 7 PPD-positive patients, 5 also reacted to IPPD.

All 6 patients who were allergic to both DO1 and DY3 were positive to PPD, but 2 patients who reacted only to DO1 did not react to PPD. One patient who was previously shown to be allergic to DY3 now reacted only to PPD. Also, all 6 patients who reacted positively to DY3 and PPD reacted to 2-amino-*p*-cresol, one of the metabolites of DY3. Reactions to PPD were strongly related to positive reactions to DY3 and to one of its metabolites, 2-amino-*p*-cresol ($p < 0.05$).

Five patients were tested with the dilution series of PPD; 4 of them reacted to PPD at the lowest concentration of 0.01%, and one to 0.1%.

Discussion

Reductive cleavage of the azo bond in DDs on the surface of the skin or in the skin could potentially lead to the formation of aromatic amines, which are absorbed by the skin (22).

In the present study, we wanted to compare the elicitation capacities of patch test preparations containing purified DO1 and DY3 and their potential metabolites. If the primary sensitizer is a metabolite of the DD, the strength of the reaction to these metabolites will probably be stronger, and imply a lower elicitation threshold.

There was a good agreement in patch test results between DO1 and *p*-aminodiphenylamine, as all patients positive to DO1 reacted to *p*-aminodiphenylamine, a potential metabolite of the former. A difference was observed between D4 reactions to *p*-aminodiphenylamine and those of DO1. Positive reactions to *p*-aminodiphenylamine were stronger than those to DO1 at D4 in 6 of 9 patients positive to DO1 and/or *p*-aminodiphenylamine. Reactions to the other metabolite of DO1, 4-nitroaniline, were weaker and less prevalent than the reactions to *p*-aminodiphenylamine. However, testing with the equimolar dilution series of DO1 showed a lower elicitation threshold than that for *p*-aminodiphenylamine, except in 3 of 8 patients, for whom the thresholds were similar to those of the substances. Testing of control patients positive to textile dye mix but negative to DO1 showed that positive reactions to *p*-aminodiphenylamine are linked to positive reactions to DO1 but not to positive reactions to other ingredients of the textile dye mix, suggesting that the concentration of *p*-aminodiphenylamine chosen, which was equimolar to that of the maternal compound, was not toxic or irritative.

This raises the question of whether *p*-aminodiphenylamine could be the main sensitizer in DO1 contact allergy. Actually, *p*-aminodiphenylamine has already been identified as a strong sensitizer in the local lymph node assay and the guinea pig maximization test (8, 23). Also, *p*-aminodiphenylamine is a known contact allergen for hairdressers and consumers using hair dyes (24).

One possible explanation based on experimental studies of some DDs and 4-nitroaniline could be the following. *In vitro* experiments have shown that, after 24 hr, 70% of DDs remain on human skin, whereas almost 50% of 4-nitroaniline is absorbed through human skin during this time period (11, 25). This means that the kinetics of DDs and those of at least one of the possible metabolites, 4-nitroaniline, are different. DD molecules stay on the skin for long enough to interact with azoreductases from skin bacteria. The metabolites formed penetrate the skin

and initiate the immune response. When DDs are applied on the skin, they induce an immune reaction slowly, because the molecules penetrate slowly, and metabolism by skin bacteria to form immunoreactive substances also takes time. The influx of the molecules into the skin takes longer to achieve the concentration needed to induce an immune response, and the influx is constant, so positive reactions can be seen with lower concentration of the DDs. On the other hand, when already formed metabolites are applied on the skin, as in our study, they penetrate and induce an immune response more quickly, because many of the same molecules can penetrate the skin at the same time, inducing an immune response and positive patch test reactions. Because of the instant penetration of the molecules, degradation also starts instantly, and the concentration of the molecules in the skin is quickly reduced, so a lower concentration of the applied substance (fewer molecules) does not induce an immune response.

Perhaps this may also apply to *p*-aminodiphenylamine, which is a stronger sensitizer than 4-nitroaniline: testing with *p*-aminodiphenylamine provokes strong and early reactions (on D3), whereas the appearance of positive patch test reactions to DO1 requires more time.

Another explanation is that application of DO1 creates two possible allergens – *p*-aminodiphenylamine and 4-nitroaniline – that might act synergistically, inducing an allergic reaction.

In this study, we observed strong positive patch test reactions to DO1 and *p*-aminodiphenylamine that ended abruptly in the dilution series. Taking into account the similarity of the molecules of PPD and *p*-aminodiphenylamine, perhaps their metabolism or degradation in the skin is similar. It has been shown that various aromatic amines undergo N-acetylation in keratinocytes, and that N-acetylated derivatives are not capable of inducing dendritic cell activation or a positive local lymph node assay response (23, 26). Thus, the main substances responsible for the sensitization to PPD are oxidation products of PPD (27). However, it has also been shown that the skin has a very high acetylation capacity (28). Aromatic amines may be acetylated, and when the amount of substrate molecules exceeds the acetylation capacity, then oxidative metabolism could become more important in the skin (27). Therefore, when *p*-aminodiphenylamine is applied at a lower concentration, N-acetylation may detoxify it to low or non-immunogenic compounds.

Testing of control patients positive to textile dye mix but negative to DO1 did not show a statistically significant link between positive reactions to DO1 and to 4-nitroaniline at equimolar concentrations. We speculate that this could

be caused by similar metabolite formation from Disperse Orange 3, as some of the control patients were positive to this DD. Also, there are data showing that, during hair dyeing when PPD is mixed with the oxidizing agent, 4-nitroaniline is formed (29). As most of our control patients were also positive to PPD, they might have been exposed and sensitized to 4-nitroaniline when dyeing their hair in the past.

Regarding DY3, 2-amino-*p*-cresol was positive in all 6 patients who were positive to DY3. As with *p*-aminodiphenylamine, positive reactions were strong and occurred early, but there was no tendency for them to appear earlier than reactions to DY3; when the dilution series was tested, the allergic reactions also ceased at a 10-fold or 100-fold higher concentration than that of DY3. As it is also an aromatic amine, perhaps the same hypothetical mechanisms as in the *p*-aminodiphenylamine case can be assumed. It was shown previously *in vitro* and in a local lymph node assay that 4-aminoacetanilide is a weaker sensitizer than 2-amino-*p*-cresol; however, they are said to cross-react (8, 30). When control patients positive to textile dye mix but negative to DY3 were tested, it appeared that positive reactions to 4-aminoacetanilide and 2-amino-*p*-cresol were statistically significantly linked to positive reactions to DY3.

The observed overrepresentation of the simultaneous positive reactions to DO1 and *p*-aminodiphenylamine as well as to DY3 and 2-amino-*p*-cresol may also have another cause. These substances might be closely related to the raw materials used for production of the dyes, and remain as impurities in the DDs used for dyeing textiles. There could also be cross-reactivity between the DDs and these substances, as they represent exact copies of the ends of the parental molecules of the dyes. Chemical investigation for purity is therefore necessary. In our study, high purity of DO1 and DY3 was established by chemical analysis. The possibility that *p*-aminodiphenylamine or 2-amino-*p*-cresol could be contaminated with DO1 or DY3, respectively, is therefore not supported by the patch test reaction pattern. It should be expected that reactions to these substances would be weaker than to the respective dyes, as the concentration of the DDs would be lower than in the parental dye, but this was not the case.

It is possible that the levels of metabolites required for the elicitation of the positive patch reactions in our study are formed during the wearing of garments dyed with the DDs. A standard textile garment contains approximately 100 µg/cm² of the dye, for example DO1 (12). In our study, the lowest concentration of *p*-aminodiphenylamine to which patients reacted was

0.0058%. This corresponds to 1.7 µg/cm². This amount of *p*-aminodiphenylamine could be formed from 3 µg/cm² DO1, which corresponds to a patch test with 0.01% DO1. In our study, some patients were positive to even lower concentrations of DO1. If just a low percentage of the dye in a fabric is broken down, this could produce a relevant amount of the metabolite. Several other circumstances can favour the development of an allergic reaction. New textiles can contain higher concentrations of dyes. The exposure to dyes when wearing a garment can mimic a repeated application test rather than a regular patch test. In certain skin areas, there can be close contact that, in combination with increased sweating and friction, leads to enhanced extraction from the fabric and better penetration of the allergens.

Reactions to PPD were strongly related to positive reactions to DY3 and to one of its metabolites, 2-amino-*p*-cresol. Patients who reacted only to DO1 did not react to PPD. This is in contrast to the results of other authors, who found that PPD sensitivity is not related or is only weakly related to DY3 sensitivity (16, 31). We were unable to find a study indicating cross-reactivity between PPD and 2-amino-*p*-cresol.

p-Aminodiphenylamine, the possible metabolite of DO1, is considered to be an allergen in hair dyes, with strong cross-reactivity to PPD (27). In our study, we found that, of 9 patients positive to *p*-aminodiphenylamine, 6 (66.7%) were positive to PPD, and an additional patient was positive to PPD but not to *p*-aminodiphenylamine.

All but 1 of the patients positive to PPD were positive to IPPD, a constituent of the black rubber mix. It has previously been shown that PPD cross-reacts with IPPD, probably because of the often seen cross-reactivity between PPD and para-amino compounds (15).

Recently, cross-reactivity between IPPD and *p*-aminodiphenylamine was reported (27). In our study, 66.7% (6/9) of patients positive to DO1 and *p*-aminodiphenylamine also reacted to IPPD. On the other hand, 1 patient who was shown previously to be positive to black rubber mix did not react to the individual ingredients, but only to the black rubber mix 0.6% pet. This might be caused by a compound effect.

In this study, we consecutively tested dermatitis patients with 4-nitroaniline, 4-aminoacetanilide, and 2-amino-*p*-cresol. Patch test reactions to dilutions of these substances together with negative reactions in control patients and the macroscopic appearance of the positive patch test reactions consistent with an allergic reaction strongly support the sensitizing capacity of the chemicals.

In conclusion, the simultaneous reactions observed to DO1 and *p*-aminodiphenylamine, as well as to DY3 and

2-amino-*p*-cresol, could be attributable to cross-reactivity or to these chemicals being potential metabolites, formed by the azoreductase pathway of skin bacteria. This means that *p*-aminodiphenylamine and 2-amino-*p*-cresol would be the primary sensitizers in cases of contact allergy to DO1 and DY3, respectively. To fully establish the nature of the positive reactions to these

substances, sensitization studies in the guinea pig are required.

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