

Investigation of the sensitising and cross-sensitising potential of textile dyes and β -lactam antibiotics using a biphasic murine local lymph node assay

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Abstract We used a modified protocol of the murine local lymph node assay (LLNA) to study the cross-sensitising potential of (a) textile dye disperse yellow 3 and its β -lactam antibiotic metabolite 2-amino-*p*-cresol, (b) two antibiotics, penicillin G and cefotiam. The test substances were applied in a biphasic manner, i.e. first on the shaved skin of the back followed by application on the dorsal side of the ears after 2 weeks. The end-points analysed included thickness and weight of an ear-biopsy, weight and cell number of the draining lymph node, and lymphocyte cell surface markers analysed by flow-cytometry. Disperse yellow 3 and its metabolite significantly altered the various end-points at both the tested concentrations (0.5 and 1%), thus demonstrating the sensitising potential of the two substances. The cross-sensitisation study showed significant modulation of the tested variables in the treated group as compared to the control, signifying cross-sensitisation potential of the two substances. Penicillin G and cefotiam showed significant changes in various end-points, pointing towards their sensitising potential. However, even at 50% concentration of the β -lactams no significant change in any end-point indicating absence of cross-reactivity of the antibiotics was noticed. We conclude that a biphasic, modified protocol of the LLNA is a suitable approach to test for a cross-reactivity potential of two related compounds.

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Many chemicals are capable of stimulating immune responses, leading to adverse reactions such as allergy or autoimmune-like diseases (DÖcker 2000). Auto-immuno-logical and allergic reactions are a major problem in clinical use of drugs and the development of new substances. The sensitising potential of antibiotics such as penicillins, cephalosporins, carbapenems and monobactams is the major limiting factor for their therapeutic use, because they may lead to life threatening allergic reactions (Uetrecht 1999). Occupational allergic contact dermatitis from penicillins and cephalosporins has been reported in healthcare workers (Gielen and Goossens 2001). Hypersensitivity to cephalosporins has been reported in patients with penicillin allergy with a prevalence ranging from 3 to 18% (Kelkar and Li 2001). Pumphrey and Davis (1999) reported fatality resulting from anaphylaxis attributed to cephalosporin treatment of a patient allergic to penicillin. However, not all reports support the notion that patients allergic to penicillin are at increased risk for an allergic reaction to cephalosporin (Anne and Reisman 1995).

Reports of dye-related contact sensitization include exposure to dyes in clothing (Brandao et al. 1985), cosmetics (Calnan 1976), spectacle frames (Dooms-Goossens et al. 1981), felt-tip marker pens (Miller et al. 1978), shoes (Correia and Brandao 1986) and a variety of other consumer products. Contact sensitization due to occupational exposure has been reported during the manufacturing of hand-printed fabrics and in clothing factories where

workers were in direct contact with newly dyed garments treated with DMSO on the back from day 1 to 3, followed (Cronin 1980). Disperse yellow 3 (DY) has been classified by application of DY or APC on the ear from day 15 to 17, as an allergen causing allergic contact dermatitis (Hatch and Maibach 1995). We have shown that the human skin back from day 1 to 3 followed by APC or DY treatment bacteria possess the ability to cleave azo dyes in vitro from day 15 to 17, respectively, on the ear. A group of (Platzek et al. 1997). DY forms two metabolites, 4-aminoacetanilide and 2-aminocresol (APC), as a result of azo cleavage.

In an earlier study (Stahlmann et al. 2006), we showed the sensitisation potential of DY and APC at 10 and 30% concentrations. The present study was designed with the aim to examine the cross-sensitisation potential of DY and its metabolite APC and two antibiotics penicillin G and cefotiam, using a modified protocol of mice local lymph node assay (LLNA) with biphasic application.

Materials and methods

Chemicals

DY and APC were purchased from Sigma Aldrich Chemie GmbH, Steinheim, Germany. Both the chemicals were dissolved in dimethyl sulfoxide (DMSO; Merck KGaA, Darmstadt, Germany) at 0.5 and 1% concentrations (w/v). Penicillin G (Penicillin 5 Mega®) and Cefotiam (Spizer®) were purchased from C. Menz GmbH, Aachen, Germany. The antibiotics were dissolved in distilled water at 50% concentration.

Animals

Female BALB/c mice (age: 7 weeks at the start of the experiment; weight: 17–22 g) were kept in Macrofolon cages at room temperature (23°C, 12 h each of light and dark cycle, relative humidity: 45–55%). Pellet feed and tap water were given ad libitum.

Treatment protocol

Mice were shaved over a surface of approximately 2 cm² on their backs, and treated using a biphasic or sensitisation-challenge protocol. The shaved surface was treated once daily on days 1–3 with 50 µl of test solution. Animals remained untreated on days 4–14. On days 15–17, mice were treated with 25 µl of the test solution on the dorsum of both ears. Mice were sacrificed on day 19 with deep anaesthesia, the lymph nodes prepared and various end-points analysed. The results were compared to control group treated with the vehicle alone.

In an attempt to study the cross-reactivity between the dye DY and its metabolite APC, one group of animals was

control animals treated with DMSO alone was also included. The concentration of the dye and its metabolite was 1%. For studying the cross-sensitisation potential of the antibiotics, the animals were divided into three groups and treated with distilled water alone, distilled water followed by cefotiam, and penicillin G followed by cefotiam.

The end-points analysed included ear thickness, ear-biopsy weight, lymph node weight, lymph node cellularity and phenotypic determination of lymphocyte subsets. The ear thickness (mm) was measured with a spring-loaded micrometer (Oditest, Kroeplin, Seeltern, Germany). A section was taken from both ears with a punch of 6 mm diameter and weighed (mg). The draining auricular lymph nodes were excised and weighed (mg). The single cell suspension from each lymph node was prepared by gentle mechanical disaggregation through stainless-steel mesh filter, suspended in phosphate buffered saline (PBS, Biochrom, Berlin, Germany) and counted (million) using the automated cell counter Sysmex F 820 (Sysmex Europe GmbH, Norderstedt, Germany).

Cells were stained using fluorochrome-conjugated antibodies. Antibodies used were phycoerythrin (PE) conjugated rat anti-CD8a, fluorescein isothiocyanate (FITC) conjugated rat anti-CD4, FITC conjugated rat anti-CD45R/B220, PE conjugated rat anti-CD19, FITC conjugated hamster anti-CD69, FITC conjugated rat anti-CD1A, and FITC conjugated rat anti-CD25 (Pharmingen, Heidelberg, Germany). CD4 and CD8 are T-cell markers characterising T-helper and T-cytotoxic cells, respectively. CD45R, also known as B220, and CD19 are B-cell markers. The murine MHC-II corresponds to I-A. CD69, also known as "very early antigen", is an indicator of lymphocyte activation. Flow-cytometry was also done to characterize the activation of antigen-presenting cells (CD69+/I-A+) and B-cells (CD45+/I-A+). Cell suspension from the two lymph nodes of each animal were pooled (10⁶ cells), incubated with antibody in the dark at 4°C for 30 min, then washed twice in PBS and sodium azide 0.02% (Sigma, Heidelberg, Germany). Flow-cytometry was performed with a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). 1 × 10⁴ lymphocytes were counted per sample. Data were

analysed using Winlist 5.0 software (Verity Software House, Topsham, USA).

Statistical evaluation

Mean values were calculated for the lymph node cellular weight, lymph node weight and lymphocyte sub-population of each animal. Results from the treated group were compared to those obtained from vehicle treated control animals using independent *t* test. Statistical analysis was done with the software SPSS 10.0 (SPSS Inc., Chicago, USA).

Results

Treatment with a 0.5% solution of DY led to significant increase in the cell count (74%, changes of the mean in the treated group as compared to the control group are given) and lymph node weight (40%) (Fig. 1, 2). The flow-cytometry results (Table 1) also showed significant modulation in 1A⁺ MHC-II cells (+32%) and CD4⁺ T-helper cells (–11%). There was a significant increase in CD69⁺ 1A⁺ (85%) and CD45⁺/1A⁺ (34%) lymph node cell subset. A non-significant augmentation was also found in CD19⁺ B-cells and CD69⁺ activated lymphocytes by 18 and 28%, respectively.

Application of 1% solution of DY resulted in significant increase in cell count (47%, Fig. 1), lymph node weight (50%, Fig. 2) and ear thickness (4%, Fig. 3). The analysis of surface markers of lymphocytes (Table 1) also showed significant enhancement in CD45⁺ cells (37%), CD19⁺

cells (46%), CD69⁺ cells (49%) and 1A⁺ cells (39%), while CD4⁺ cells were found to be significantly reduced by 12% (Table 1). In the study involving application of the metabolite APC at 0.5% concentration, we detected a consistent significant increase in cell count (88%, Fig. 1), lymph node weight (65%, Fig. 2) and ear punch weight (4%, Fig. 3). The CD69⁺ cells and 1A⁺ cells were found to be significantly enhanced by 37 and 34%, respectively, while the CD4⁺ cells were found to be significantly reduced by 12% (Table 1). The CD19⁺ cells were found to be increased (14%) non-significantly, while CD69⁺/1A⁺ and CD45⁺/1A⁺ cells increased significantly by 118 and 40%, respectively. The application of APC at 1% concentration resulted in detection of significant raise in cell count (60%, Fig. 1), lymph node weight (64%, Fig. 2), ear thickness (9%, Fig. 3) and ear punch weight (10%, Fig. 3). The phenotypic expression of CD45⁺ cells (44%), CD19⁺ cells (53%), CD69⁺ cells (55%) and 1A⁺ cells (50%) were found to be significantly amplified (Table 1). The CD4⁺ cells were found to decrease significantly by 19%. Expansion of CD69⁺/1A⁺ and CD45⁺/1A⁺ cell subtypes occurred by 200 and 68%, respectively.

In the cross-allergy study of the dye DY and its metabolite, we detected significant modulation in CD4⁺ cells (–16%), CD19⁺ cells (+42%) and 1A⁺ cells (+30%) between the DMSO/DY and APC/DY treated

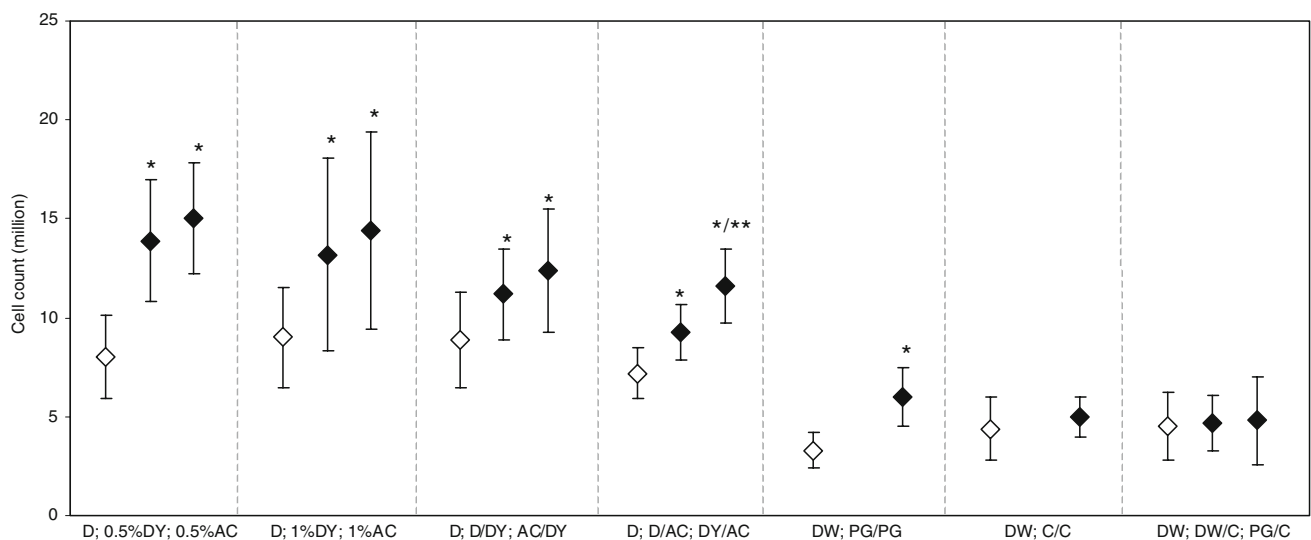


Fig. 1 Cell count (mean $\pm$ SD) values obtained from mice treated with various substances according to the sensitisation-challenge protocol. *D* DMSO, *DY* disperse yellow 3, *AC* 2-aminop-cresol, *DW* distilled water, *PG* penicillin G, *C* cefotiam. Disperse yellow 3 and 2-aminop-cresol were used at 0.5 and 1% concentration to study sensitisation, while at 1% concentration to study cross-sensitisation and sensitisation. Sample size was 13 for the penicillin G study, $n = 5$ for the cefotiam study, $n = 12$ for the penicillin G and cefotiam cross-sensitisation study. White polygons indicate values from animals treated with vehicle which was DMSO in the textile dye study and distilled water in the antibiotic study. The black polygons indicate values obtained from treated animals. *Significant difference at $P < 0.05$ (*t* test) between vehicle control and treated animals, **significant difference at $P < 0.05$ (*t* test) between treated groups

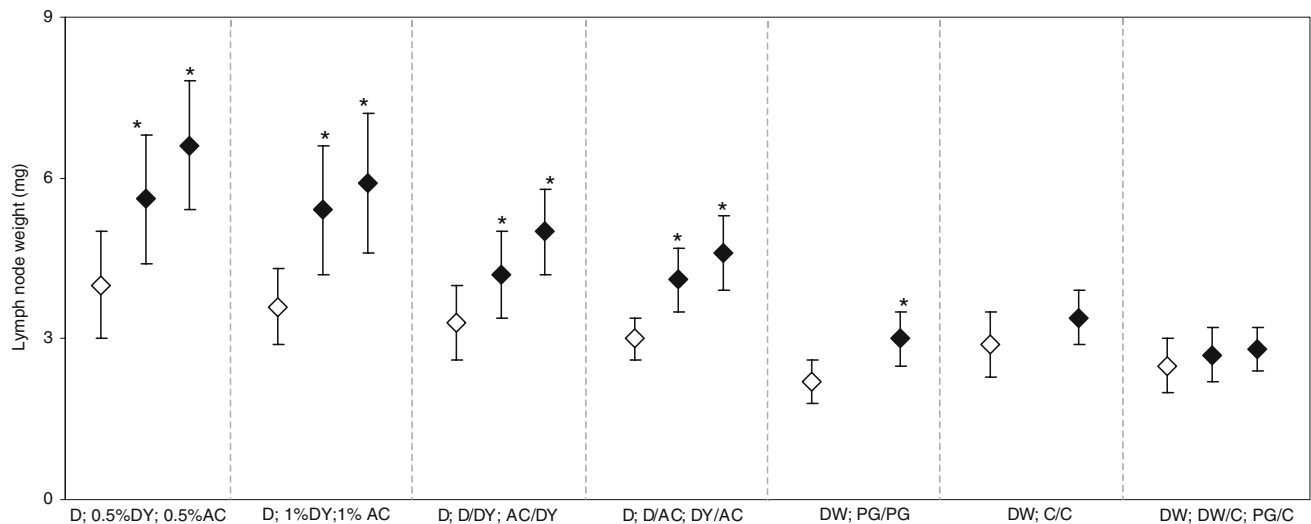


Fig. 2 Lymph node weight (mean $\pm$ SD) values obtained from mice $n = 8$ for the textile dye studies, $n = 13$ for the penicillin G study, treated with various substances according to the sensitisation protocol, $n = 5$ for the cefotiam study, $n = 12$ for the penicillin G and challenge protocol. D DMSO, DY disperse yellow 3, AC 2-amino-*p*-cresol, DW distilled water, PG penicillin G, C cefotiam. Disperse yellow 3 and 2-amino-*p*-cresol were used at 0.5 and 1% concentration to study sensitisation, while at 1% concentration to study cross-sensitisation. Penicillin G and cefotiam were used at 50% concentration to study sensitisation and cross-sensitisation. Sample size was

groups (Table 1). There was a non-significant increase in lymph node weight (+19%, Fig. 2). The CD45⁺ cells and CD69⁺ cells were found to increase non-significantly by 23 and 26%, respectively. The CD69⁺1A⁺ and CD45⁺1A⁺ cells increased significantly by 94 and 33%, respectively. The DMSO/DY treated group showed significant up-regulation in CD19 (42%), CD69⁺ (56%), modulation in cell count (-26%, Fig. 1) and lymph node weight (+27%, Fig. 2) as compared to the DMSO treated group. Non-significant modulation in CD69 cells (+21%) and CD69⁺1A⁺ (+26%) was also observed between the two groups (Table 1).

In the study involving treatment of the animals with DMSO/APC and DY/APC, we observed a significant increase in cell number (25%, Fig. 1), CD69⁺ cells (24%) and CD69⁺1A⁺ (71%), while the CD45⁺1A⁺ cells increased (12%) non-significantly (Table 1) between the two groups. A non-significant modulation in lymph node weight (+12%, Fig. 2) was also seen. There was also noticed significant amplification in cell count (29%, Fig. 1), lymph node weight (37%, Fig. 2) and ear punch weight (9%, Fig. 4) in the DMSO/APC treated group as compared to the DMSO treated group. The CD45⁺ cells were found to be significantly reduced by 6%, in the treated group as compared to the control group (Table 1). The CD45⁺, CD19⁺, CD69⁺, CD69⁺1A⁺ and CD45⁺1A⁺ cells also showed non-significant up-regulation by 11, 10, 20, 30 and 9% respectively, between the two groups.

In the investigation to inspect the allergic potential of penicillin G, there was found significant increase in cell count (82%, Fig. 1), lymph node weight (36%, Fig. 2) and ear thickness (5%, Fig. 3) in the treated group as compared to the control group. Flow-cytometry results showed significant up-regulation in CD19 (42%), CD69⁺ (56%), CD1A⁺ (44%), CD69⁺1A⁺ (183%) and CD45⁺1A⁺ (39%) cells, as compared to the control (Table 1). The CD4⁺ and CD8⁺ cells were found to reduce significantly by 12 and 11%, respectively. The CD45⁺ cells were found to non-significantly augment by 16%.

We found an increase in CD45⁺ cells (63%), CD19⁺ cells (72%) and 1A⁺ cells (56%), between the control and treated groups (Table 1), in the study carried out to examine the allergic potential of cefotiam. The cell count and lymph node weight were found to enhance non-significantly by 14 and 17%, respectively (Figs. 2). The epitope CD4 showed significant decrease (19%) while epitope CD69 showed non-significant increase (29%), in the treated group as compared to the control. The CD69⁺1A⁺ and CD45⁺1A⁺ cell populations increased significantly by 121 and 47% in the treated group as compared to the control.

No relevant differences in the end-points were observed between the treated group and the control group in the study carried out to investigate the cross-allergic potential of penicillin and cefotiam (Figs. 1, 2, 3, 4, Table 1).

Table 1 Immunophenotypic results of the different leucocyte epitope markers obtained from mice treated with various substances according to the sensitisation-challenge protocol

		% Positive cell population (mean $\pm$ SD)						
		CD4+	CD8+	CD45+	CD19+	CD69+	1A+	CD69+/1A+ CD45+/1A+
1.	0.5% solution of disperse yellow and 2-aminop-cresol to study sensitisation							
	D	53.4 $\pm$ 1.9	22.6 $\pm$ 1.9	29.5 $\pm$ 4.0	22.9 $\pm$ 3.4	10.2 $\pm$ 2.5	22.2 $\pm$ 3.2	3.9 $\pm$ 1.3
	DY	47.3 $\pm$ 3.0*	21.4 $\pm$ 1.8	30.3 $\pm$ 5.6	27.0 $\pm$ 5.3	13.0 $\pm$ 2.6	29.4 $\pm$ 5.1*	7.2 $\pm$ 2.4*
	AC	47.2 $\pm$ 3.9*	21.1 $\pm$ 2.3	28.7 $\pm$ 5.6	26.2 $\pm$ 4.6	14.0 $\pm$ 3.0*	29.7 $\pm$ 7.4*	8.5 $\pm$ 2.6*
2.	1% solution of disperse yellow and 2-aminop-cresol to study sensitisation							
	D	55.2 $\pm$ 4.1	21.4 $\pm$ 3.1	21.1 $\pm$ 4.9	17.8 $\pm$ 3.7	10.1 $\pm$ 1.8	20.7 $\pm$ 3.6	3.4 $\pm$ 1.2
	DY	45.1 $\pm$ 4.2*	19.9 $\pm$ 2.7	28.9 $\pm$ 6.0*	25.9 $\pm$ 6.0*	15.0 $\pm$ 2.6*	28.7 $\pm$ 5.9*	9.6 $\pm$ 2.4*
	AC	44.7 $\pm$ 3.6*	19.3 $\pm$ 1.4	30.3 $\pm$ 4.8*	27.2 $\pm$ 4.7*	15.7 $\pm$ 3.5*	31.0 $\pm$ 5.0*	10.2 $\pm$ 3.3*
3.	1% solution of disperse yellow and 2-aminop-cresol to study cross-sensitisation							
(a)	D	54.3 $\pm$ 3.3	19.5 $\pm$ 2.7	27.5 $\pm$ 6.7	19.8 $\pm$ 2.9	12.6 $\pm$ 1.3	24.4 $\pm$ 2.0	7.7 $\pm$ 1.7
	D/DY	55.0 $\pm$ 2.8	21.7 $\pm$ 1.4	23.3 $\pm$ 1.7	17.1 $\pm$ 2.9	15.2 $\pm$ 4.1	24.1 $\pm$ 2.8	9.7 $\pm$ 4.0
	AC/DY	45.5 $\pm$ 2.4*/**	20.2 $\pm$ 1.7	28.7 $\pm$ 6.4	24.2 $\pm$ 5.7**	19.2 $\pm$ 2.8*	31.4 $\pm$ 5.1*/**	14.9 $\pm$ 3.0*/**
(b)	D	53.3 $\pm$ 1.9	20.8 $\pm$ 1.3	25.3 $\pm$ 3.9	18.6 $\pm$ 2.0	11.6 $\pm$ 3.3	25.9 $\pm$ 2.4	6.6 $\pm$ 2.4
	D/AC	50.2 $\pm$ 1.2*	21.8 $\pm$ 3.1	28.0 $\pm$ 4.9	20.4 $\pm$ 3.2	13.9 $\pm$ 1.5	26.9 $\pm$ 3.4	8.6 $\pm$ 1.1
	DY/AC	48.0 $\pm$ 4.2*	21.1 $\pm$ 1.3	26.4 $\pm$ 6.0	20.2 $\pm$ 4.2	17.2 $\pm$ 2.1*/**	28.2 $\pm$ 4.4	11.3 $\pm$ 2.2*/**
4.	50% solution of penicillin G to study sensitisation							
	DW	55.3 $\pm$ 3.6	23.2 $\pm$ 1.9	13.4 $\pm$ 4.3	16.6 $\pm$ 5.4	6.8 $\pm$ 1.6	20.0 $\pm$ 3.2	1.8 $\pm$ 0.8
	PG/PG	48.9 $\pm$ 5.7*	20.7 $\pm$ 2.7*	15.5 $\pm$ 5.5	23.6 $\pm$ 7.2*	10.6 $\pm$ 2.9*	28.8 $\pm$ 7.2*	5.1 $\pm$ 2.4*
5.	50% solution of cefotiam to study sensitisation							
	DW	55.7 $\pm$ 3.3	24.6 $\pm$ 1.2	13.6 $\pm$ 2.8	17.4 $\pm$ 3.2	7.0 $\pm$ 1.4	22.1 $\pm$ 3.2	2.4 $\pm$ 0.8
	C/C	44.9 $\pm$ 4.4*	22.1 $\pm$ 2.4	22.1 $\pm$ 4.7*	30.0 $\pm$ 6.4*	9.0 $\pm$ 2.8	34.5 $\pm$ 7.1*	5.3 $\pm$ 2.0*
6.	50% solution of penicillin G and cefotiam to study cross-sensitisation							
	DW	56.8 $\pm$ 5.6	23.4 $\pm$ 3.4	19.8 $\pm$ 4.6	17.6 $\pm$ 4.8	11.0 $\pm$ 2.2	21.4 $\pm$ 5.6	3.7 $\pm$ 1.4
	DW/C	56.4 $\pm$ 4.2	25.6 $\pm$ 2.8	17.4 $\pm$ 4.1	16.6 $\pm$ 4.5	11.8 $\pm$ 2.2	22.3 $\pm$ 5.2	4.0 $\pm$ 1.7
	PG/C	53.8 $\pm$ 2.6	24.4 $\pm$ 2.1	17.2 $\pm$ 3.4	17.0 $\pm$ 4.1	11.9 $\pm$ 1.9	24.0 $\pm$ 2.4	4.2 $\pm$ 1.0

Disperse yellow 3 and 2-aminop-cresol were used at 0.5 and 1% concentration to study sensitisation, while at 1% concentration to study cross-sensitisation. Penicillin G and cefotiam were used at 50% concentration to study sensitisation and cross-sensitisation. Sample size $n = 13$ for the penicillin G study, $n = 5$ for the cefotiam study, $n = 12$ for the penicillin G and cefotiam cross-sensitisation study

D DMSO, DY disperse yellow 3, AC 2-aminop-cresol, DW distilled water, PG penicillin G, C cefotiam

* Significant difference at $P < 0.05$ (t test) between vehicle control and treated animals, **significant difference at $P < 0.05$ (t test) between treated groups

Discussion

swelling, have been used in a modified form of the LLNA (Ulrich et al. 2001; Ehling et al. 2005). All modifications included once daily sensitisation for three successive days and did not incorporate a challenge treatment. In an earlier study (Stahlmann et al. 2006), we studied the sensitisation potential of four textile dyes and two metabolites using the guinea-pig assays (e.g. guinea-pig maximization test, commonly used sensitisation protocol and the modified Buehler assay) in measuring objective endpoint i.e. lymphocyte proliferation (Sailstad 2002). The need for special facilities to handle radioisotopes hinders the widespread use of conventional LLNA for routine screening of skin sensitisers. Several modifications of the LLNA have been suggested over the past years. Non-radioactive endpoints, such as lymph node weight, cell proliferation and ear and

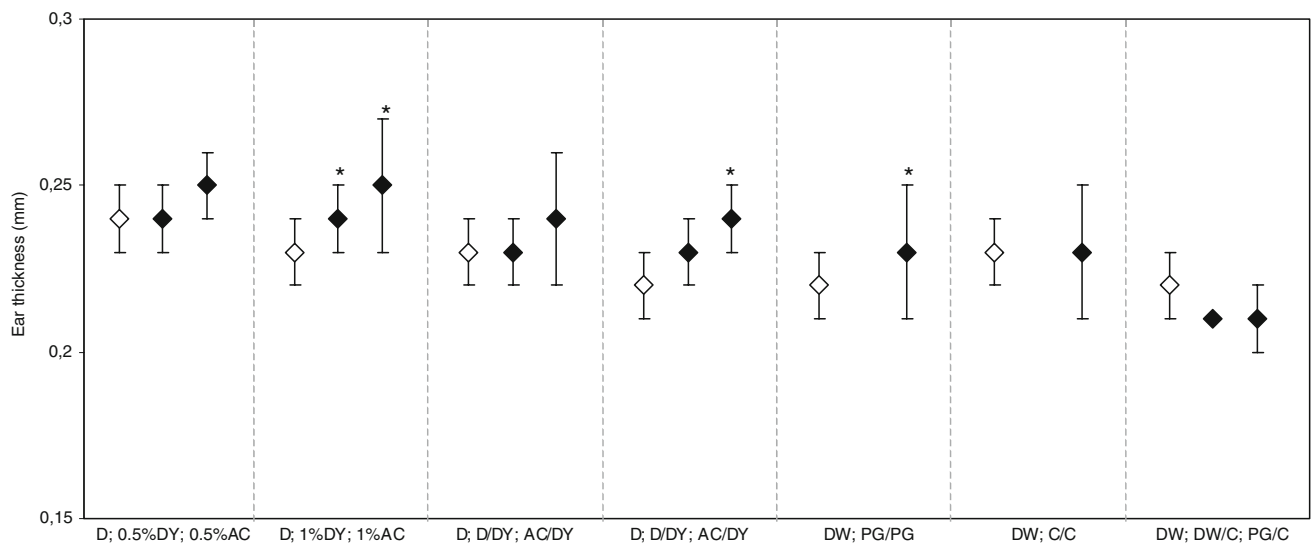


Fig. 3 Ear thickness (mean $\pm$ SD) values obtained from mice treated with various substances according to the sensitisation-challenge protocol. *D* DMSO, *DY* disperse yellow 3, *AC* 2-amino-*p*-cresol, *DW* distilled water, *PG* penicillin G, *C* cefotiam. Disperse yellow 3 and 2-amino-*p*-cresol were used at 0.5 and 1% concentration to study sensitisation, while at 1% concentration to study cross-sensitisation. *White polygons* indicate values from animals treated with vehicle which was DMSO in the textile dye study and distilled water in the antibiotic study. *Black polygons* indicate sensitisation and cross-sensitisation values obtained from treated animals. *Significant difference at $P < 0.05$ (t test) between vehicle control and treated animals. Sample size was 8 for the textile dye studies, $n = 13$ for the penicillin G study, $n = 5$ for the cefotiam study, $n = 12$ for the penicillin G and cefotiam cross-sensitisation study.

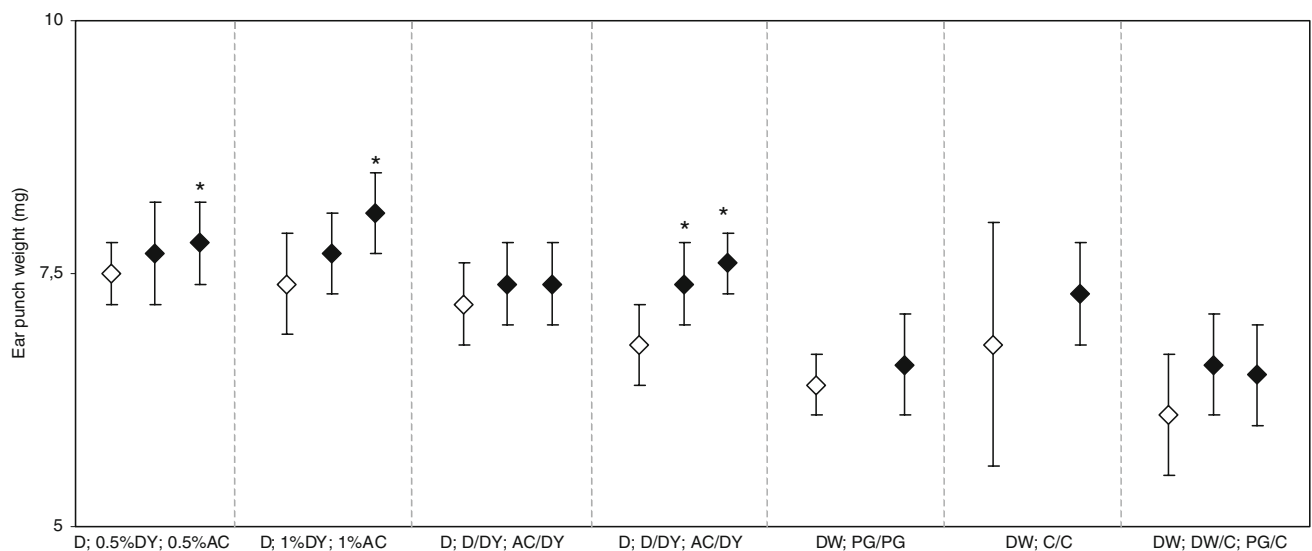


Fig. 4 Ear punch weight (mean $\pm$ SD) values obtained from mice treated with various substances according to the sensitisation-challenge protocol. *D* DMSO, *DY* disperse yellow 3, *AC* 2-amino-*p*-cresol, *DW* distilled water, *PG* penicillin G, *C* cefotiam. Disperse yellow 3 and 2-amino-*p*-cresol were used at 0.5 and 1% concentration to study sensitisation, while at 1% concentration to study cross-sensitisation. *White polygons* indicate values from animals treated with vehicle which was DMSO in the textile dye study and distilled water in the antibiotic study. *Black polygons* indicate sensitisation and cross-sensitisation values obtained from treated animals. *Significant difference at $P < 0.05$ (t test) between vehicle control and treated animals. Sample size was 8 for the textile dye studies, $n = 13$ for the penicillin G study, $n = 5$ for the cefotiam study, $n = 12$ for the penicillin G and cefotiam cross-sensitisation study.

protocol. It helped us to identify weak sensitisers more precisely, and also clarified the ambiguous results obtained on occasion from the sensitisation protocol. Allergies to textiles are likely under-diagnosed (Guidaud-Pons 1999). This may be due to under reporting by the patient, the difficulty in associating a dermatitis with

textiles and the different clinical presentations of a textile into two or more phenyleneamines. Seidenari et al. (1997) reported cross-sensitization between azo dyes and para- and friction are the activating or aggravating factors (Ri- amino compounds in azo-dye sensitive subjects). Dyes are the first, and resin finishes are the second major causes of textile dermatitis. Their presence is dependent on the textile fabric composition. Chemicals such as metals, lubricants, fragrances, biocides and UV absorbers may be other potential textile allergens. Disperse dyes, of which 32 have been documented (Hatch et al. 2003), are the main culprits in causing lymph node cellularity, lymph node weight and the sub-textile dermatitis. They are used on synthetic fibres such as polyesters, acetates, triacetates and nylon or on a combination of fabrics (Lepoittevin 1999). In our previous study (Stahlmann et al. 2006), we showed the sensitising potential of DY and APC at 10 and 30% concentration, in the first time to study the cross-sensitisation, and was found to be suitable enough for the purpose. More extensive studies are required to further validate the suitability of the technique for cross-sensitisation studies. Besides textile dyes, several other classes of chemicals have been reported to produce adverse immune reactions and among these pharmaceuticals are of particular concern. During pre-clinical development, pharmaceutical companies like to know as soon as possible whether a new medical entity has the potential to elicit an adverse immune response resulting in allergic or autoimmune diseases (Adkinson et al. 2002). Our study showed significant sensitisation potential of penicillin G,

The changes in proportion of T- and B-cells observed in our experiments have been previously described (Stahlmann et al. 2006; Sikorski et al. 1996; Hariya et al. 1999). Cefotiam also showed significant modulation in various cell surface markers, but absence of effects on cell count and lymph node parameters. Stewart (1962) reported that cephalosporin C was not cross-allergic with the penicillins in skin tests in man, and several reports have indicated that cephalosporins can safely be taken by patients sensitive to penicillin (Stewart 1967). Schneirson et al. (1964) reported that cephalothin can stimulate immunological reaction in rabbits and guinea-pigs, but they found no evidence of cross-reaction with penicillin. In contrast, clinical reactions on first exposure to cephalosporins have been reported in patients sensitive to penicillin (Kabins et al. 1965). In man, cross-allergenicity

As early as 1949, a correlation between patients with and cross-antigenicity have been demonstrated using skin sensitivity to *p*-phenylenediamine and increased allergic tests (Grieco 1967) and haemagglutination studies (Mol reactions to azo food dyes was reported (Baer and Leidman 1968). The penicilloyl group seems to be largely responsible for the cross-reaction (Ky et al. 1970), and it has been suggested that the lactam ring of cephalosporins may split, giving a cephalosporoyl group with areas of close structural similarity (Feinberg 1968). In our experiment, a brown stocking dye made from a mixture of nitro- and amino anthraquinone dyes. Out of cross-reactivity study between penicillin G and cefotiam, the nine patients that reacted to the azo dyes, three-fourths also reacted to *p*-phenylenediamine. This cross-reactivity has been explained by a cleavage of the azo-dye in the skin concentrations and using other test methods.

In conclusion, the azo-dye DY and its metabolite APC showed sensitising potential at low concentrations of 0.5 and 1%, using a modified mice LLNA. Both the substances also showed cross-allergic potential. Penicillin G showed significant sensitising potential when used as a 50% solution, while cefotiam showed slight allergic potential at the same concentration. We found no cross-allergenicity when both the antibiotics were used subsequently. The modified form of the LLNA along with the flow-cytometric analysis of the selected phenotypic markers was found useful enough for the purpose.

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