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Genotoxicity of Textile Dyestuffs in Normal Human Epidermal Keratinocytes and a Three Dimensional Reconstituted Human Epidermis Model

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Introduction

– Potential health risks resulting from exposure to textile dyestuffs have not been adequately considered in the past, and at the same time the knowledge on their toxicological properties is insufficient. Textile dyes can exhibit allergic and skin-irritant properties, as has been found in toxicological testing, but can also possess a genotoxic potential.

– In previous studies, it could been demonstrated that textile dyestuffs customary in trade from the chemical classes of azo dyes, azo metal complex dyes as well as anthraquinone dyes or their mixtures, respectively, are direct-acting genotoxic substances in mutagenicity screening using the Salmonella microsome assay, as well as in immortalized human keratinocytes (HaCaT cell line) when using the in vitro micronucleus assay and the Single Cell Gel Electrophoresis/ Comet assay [1].

– Because dermal uptake is an important pathway referring to actual exposition of human beings and starting from the outcomes of mutagenicity testing in the bacterial culture and in an immortalized cell line, the aim of the present studies was to investigate the mutagenic potential of selected textile dyestuffs in normal human epidermal keratinocytes. Furthermore, a three-dimensional (3D) human epidermis model should be used in genotoxicity testing. The reconstituted epidermis model is assumed to closely reproduce human skin behaviour in vivo and effects of textile dyestuffs applied topical can be modulated by the magnitude of percutaneous absorption.

Materials and Methods

– Textile dyestuffs were kindly provided by Dr. J. Rieker, Research Institute Hohenstein, Bönnigheim. Details according to table M-1 were taken from the manufacturer's safety data sheets.

– The individual textile dyestuffs listed in table M-1 were considered.

– The dyestuffs have been characterized by their UV-VIS absorption spectra. In aqueous solution, the UV-VIS spectra of the dyes appeared unchanged during 0-1.5 hours (data not given).

– Normal human epidermal keratinocytes (NHEK) were purchased from CellSystems (St. Katharinen) and cultivated in keratinocyte growth medium (KGM) supplemented with 0.1 ng/ml human recombinant epidermal growth factor (hEGF), 5.0 g/ml insulin, 0.5 g/ml hydrocortisone, 50 g/ml gentamicin, 50 ng/ml amphotericin-B, 0.15 mM calcium, 7.5 mg/ml bovine pituitary extract (BPE).

– The reconstituted epidermis model AST-2000 (Advanced Skin Test) was supplied by CellSystems (St. Katharinen). The in vitro skin model was cultured with maintenance medium and incubated at 37 OC in a 5 % CO_2, humidified atmosphere for at least 6-8 hours or over night before testing.

Genotoxicity assays with NHEK cells and the 3D skin model were performed according to standard protocols.

– The in vitro micronucleus (MN) assay (MP procedure) [2] with NHEK cells was performed without metabolicactivation. NHEK cells were seeded onto microscopic slides in QuadriPERM dishes. Treatment of cells was continued for 24 h between 1.5 up to 150 µg dye/ml. Subsequently, the medium was replaced by 1,5 % trisodium citrate-2-hydrate. The hypotonic solution was removed immediately and replaced twice by an ethanol-acetic acid solution (3:1), containing 0,74 % formaldehyde for cell fixing. Cells were air dried, stained with Giemsa and analysed for MN. Micronuclei were scored per 1,000 cells. Medium and positive controls (0,02 µg/mL colcemid) were performed within each test series.

– The standard procedure described by Tice et al. [3] with modifications was used for the Single Cell Gel Electrophoresis/Cometassay (SCGE) (cell lysis for at least 1 h or maximal overnight, alkaline hydrolysis for 40 min at pH > 13, electrophoresis for 20 min at 24 V and 290 to 300 mA). NHEK cells were treated with dye concentrations of 5 to 150 µg/mL for 1 h and without metabolic activation. 22 µl/ml H_2O_2 (diluted 1:100 from a 30 % solution) or EMS (1µL/mL) served as positive control.

– In the case of AST-2000, following topical treatment with 100 µL of the dissolved dye in the dose range of 50-500 µg/mL for 3 h, keratinocytes as well as fibroblasts were separated from the 3D tissues and analysed by SCGE.

– Interactive examination of the slides was carried out by image analysis (Comet Assay II, Perceptive Instruments, Haverhill, UK). Each time, 100 nuclei were analysed.

Table M-1: Textile dyestuffs

Textile dye, commercial name (Abbreviation)	Chemical classes of dyes	Colorant/Dye (CAS RN)	Colour Index Name	Metal content of organo metal complex
Acidol Black M (ABM)	Azo metal complex		C.I. Acid Black 194	
Irgalan Navy B (INB)	Azo metal complex		C.I. Acid Blue 229	3.4% Cr (III)
Isolan Grey S-GL (IGS)	Azo metal complex		C.I. Acid Black 220	3.2% Cr (III)
Lanaset Black B (LBB)	Azo and azo metal complex, mixture	Chromate(3-), bis[3-(hydroxy-κO)-4-[[2-hydroxy-κO)-1-naphthalenyl]azo--κN1]-7-nitro-1-naphthalenesulfonato(3-)]-, trisodium **(57693-14-8)**	C.I. Acid Black 172	3.2% Cr (III)
Lanaset Bordeaux BA (LBBA)	Azo metal complex, mixture			2.3% Cr (III), 0.2% Co
Lanaset Green B (LGB)	Anthraquinone	Benzenesulfonic acid, [(9,10-dihydro-9,10-dioxo-1,4-anthracenediyl)bis(imino-4,1-phenyleneoxy)]bis-, disodium salt **(70161-19-2)**		
Lanaset Navy R (LNR)	Azo and azo metal complex, mixture	2-Anthracenesulfonic acid, 1-amino-4-[[3-[[chloracetyl) amino]methyl]-2,4,6-trimethylphenyl]amino]9,10-dihydro-9,10-dioxo-, monosodium salt **(70209-96-0)**	C.I. Acid Blue 225	1.3% Cr (III)
Lanaset Orange RN (LOR)	Azo and azo metal complex, mixture	Benzenesulfonic acid, 4-[4-[[5-[(2-bromo-1-oxo-2-propenyl) amino]-2-sulfophenyl]azo]-4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-1-yl]-2,5-dichloro-, disodium salt **(70247-70-0)**	C.I. Reactive Yellow 39	1.1% Cr (III)
Lanaset Red G (LRG)	Azo metal complex, mixture			3.0% Cr (III)
Lanaset Yellow 2R (LYR)	Azo metal complex, mixture	Cobaltate(4-), 2-[[[3-[[1-[[(2-chlorophenyl) amino]carbonyl]2-oxo-κO)propyl]azo-kN1]-4-(hydroxy-κO)phenyl]sulfonyl] amino]benzoato(3-)]-, tetrasodium **(70851-34-2)**	C.I. Acid Yellow 220	1.4% Cr (III), 0.7% Co

Fig. 1: MN (MP) NHEK

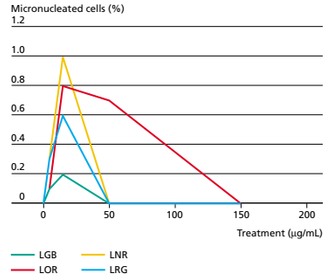


Fig. 2: NHEK SCG/Comet assay

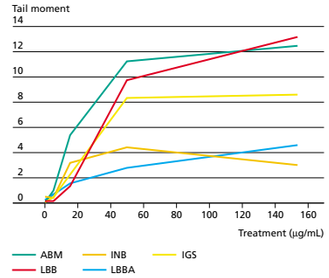


Fig. 3: NHEK SCG/Comet assay

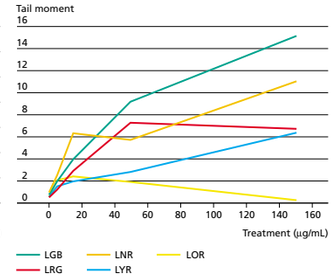


Fig. 4: AST-2000 SCG/Comet assay

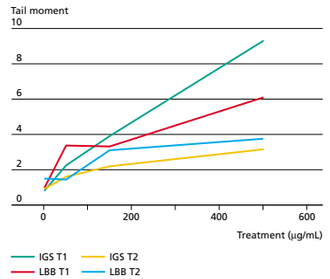


Fig. 5: AST-2000 SCG/Comet assay

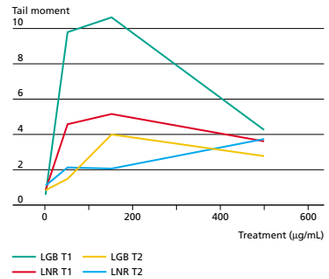
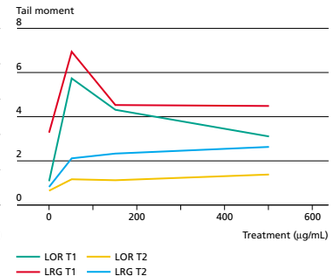


Fig. 6: AST-2000 SCG/Comet assay



Results

– MN assay
Clastogenic effects in the in vitro micronucleus (MN) assay on NHEK have been observed in the case of four dyestuffs investigated in the dose range of 1.5 - 150 µg/mL and with maximum MN frequencies between 0.2 - 1.0 % compared to negative controls with 0 - 0.1 % micronucleated cells (cf. Fig. 1). Cytotoxicity has been observed in part starting at 50 µg/mL.

– SCG/Comet assay
In the Single Cell Gel/Comet assay, all ten dyestuffs investigated caused DNA damage in NHEK cells in the dose range of 5 - 150 µg/mL (cf. Fig. 2 and 3). Maximum tail moments of the individual dyes have been found between 2.41 (LOR) and 15.17 (LGB) compared to a mean tail moment for medium controls of 0.45 (n = 10).

Following treatment of the reconstituted human epidermis model with dyes in the concentration range of 50 - 500 µg/mL, keratinocytes as well as fibroblasts exhibited DNA damage (cf. Fig. 4 to 6). The magnitude of effects is obviously more pronounced in the upper keratinocyte stratums. From six dyes investigated, maximum tail moments of keratinocytes were found in the range of 5.13 (LNR) - 10.63 (LGB) and those of fibroblasts between 1.37 (LOR) - 3.98 (LGB), respectively; compared to controls with mean tail moments of 1.24 (n = 6) or 0.84 (n = 5 without LRG T1) and 0.97 (n = 6).

Discussion

– Generally, the data base on mammalian cell mutagenicity in vitro of textile dyestuffs is limited. If at all published, mutagenicity testing of textile dyes usally was done using the Salmonella microsome assay [4].

– We could demonstrate that normal human epidermal keratinocytes (NHEK) are suitable for mutagenicity testing of dyestuffs. All dyes were found on NHEK to be direct-acting mutagens in the MN assay as well as the SCG/Comet assay.

– The reconstituted epidermis model is assumed to closely reproduce human skin behaviour in vivo; effects of the textile dyestuffs applied topically can be modulated by the magnitude of percutaneous absorption.

– The specific composition of each of the commercial products is unknown. Hence, metabolic pathways for the dyes according to Table M-1 can be discussed only on a general level of structural alerts to DNA reactivity such as those aromatic amino/nitro-type compounds (e. g., -N=N-, -NH_2, -NO_2,) or electrophiles including reactive halogens. Mutagenic metabolites resulting from reductive-cleavage of the azo bond have been reported for azo dyes [5]. The dyes C.I. Reactive Yellow 39 and C.I. Acid Blue 225, which are components in LOR and LNR, contain the a-bromoacrylamido- and chloroacetyl- structural alerts capable of forming covalent bonds.

Conclusions

– Dermal contact is a major route of exposure concerning occupational safety and exposure of the general public towards dyes and dyed materials. Because most textile dyes in use are "existing chemicals" which have not been adequately tested, the identification and substitution of genotoxic dyes is meaningful.

– Cultured normal human epidermal keratinocytes and the three-dimensional reconstituted human epidermis model were qualified models to study the genotoxic potential of some selected textile dyestuffs by the in vitro MN assay and the SCG/Comet assay.

– Under the conditions of these screening tests, the textile dyes investigated are direct-acting genotoxic substances. Genotoxicity of the textile dyes formerly observed towards HaCaT cells now has been confirmed in NHEK cells and the epidermal skin model.

– Furthermore, the studies with the reconstituted human skin model are a contribution to the application of alternative methods in toxicology. The test strategy could be used to study alterations of the human skin barrier function and as screening method for other topically applied substances.

References

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