Advanced Skin Test (AST-2000) as a potent *in vitro* tool for the characterization of skin reactions by protein fingerprinting

Health Care
Toxicology

E. Heisler[1], J.J. Hoffmann[2], P. Peters[2], H.-J. Ahr[1], H.-W. Vohr[1]

[1]Institute of Toxicology, Bayer Health Care, Wuppertal, Germany
[2]CellSystems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany

Introduction

Different validation studies (COLIPA, ECVAM, OECD) revealed encouraging data concerning *in vitro* reconstructed human skin models in toxicological research. In cytotoxicity testings based on these skin models, comprehensive results were obtained, which highly correlate to those from well established *in vivo* methods. Therefore, artificial skin models provide fundamental advantages in comparison to single cell culture testings. In our present studies we focussed our research on the full thickness skin model AST-2000 (Advanced Skin Test), obtained from CellSystems® Biotechnologie Vertrieb GmbH, St. Katharinen, Germany. In contrast to more simple reconstructed epidermal models, AST-2000 provides a proliferating, cornified and stratified epidermal layer based on a functional dermal layer and, therefore, provides an architecture which is comparable to normal human *in vivo* skin. In an in-house validation study topical applied compounds were tested according to their irritating or corrosive properties. Furthermore, supernatants were analyzed with respect to the induced production of immunomodulating molecules including proinflammatory cytokines as well as chemokines and matrix metalloproteases which are related to inflammation, tissue damage and wound healing *in vivo*. In this context also a substance with an especially sensitizing potential was tested to find out if AST-2000 may help to discriminate between irritation, corrosion and sensitisation *in vitro*.

Histological overview of AST-2000

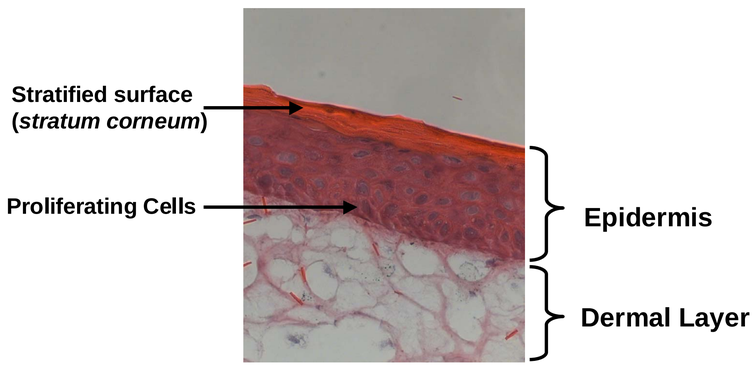

Fig.1: HE- stained section of AST-2000

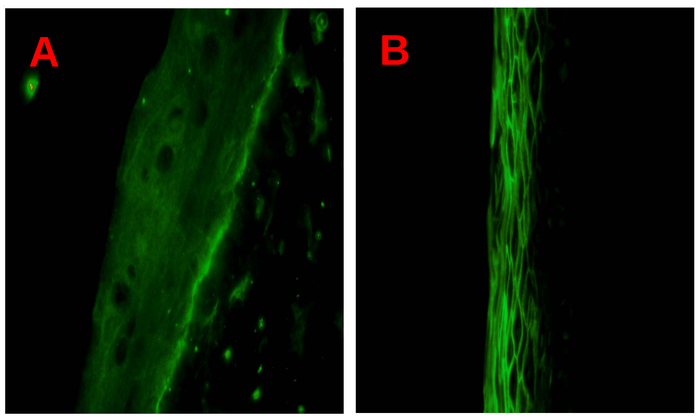

Fig.2: Immunohistochemical staining of **A:** Collagen IV and **B:** Tranglutaminase in AST-2000

Material and Methods

AST-2000 (0,63 cm^2) was obtained from CellSystems® Biotechnologie Vertrieb GmbH, St. Katharinen, Germany.

Culture conditions, pretreatment and treatment: After shipment, tissue cultures were placed in 1 ml medium (supplemented MCDB 153 based medium, supplied by CellSystems®) and later on adapted to cell culture conditions (37°C, 5% CO_2, max. humidity, o.n.). Test substances were dissolved in vehicle (PBS, PEG 400) and applied topically onto the dry *stratum corneum* in a volume of 15µl/cm^2. The medium was replaced previously by fresh and prewarmed medium. AST-2000 was exposed to the compounds for 30 minutes (min.) up to 24 hours (max.). After exposure the reconstructed skin models were washed 3 times in PBS and incubated for a max. of 24 hours (37°C, 5% CO_2, max. humidity).

Validation study: was performed with substances characterized by OECD / EU : SDS, Citronellol, Cyclooctene, Dithiopropionic acid, Heptanal, Potassium hydroxid and 4,4 Methyl bis-(2,6 ditert buthyl) phenol were obtained from Sigma (Deisenhofen, Germany).

Sensitiazation experiments: were performed with Oxazolone (Sigma, Deisenhofen, Germany).

Analysis:
1. Viability of cells after treatment was determined by MMT Test and/or by luminometric detection of Adenylat Kinase release (Toxilight , Cambrax).

2. Release of immunomodulating proteins was determined by Cytometric Bead Array (Human Chemokine CBA, Human Inflammation CBA both obtained from BD Biosciences, Heidelberg, Germany) on a FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany) or by ELISA (R&D, Wiesbaden, Germany).

Author: Eckhard Heisler
Tel: +49-202-36-4355
Fax +49-202-36-4137
em@il eckhard.heisler.eh@bayer-ag.de

Results

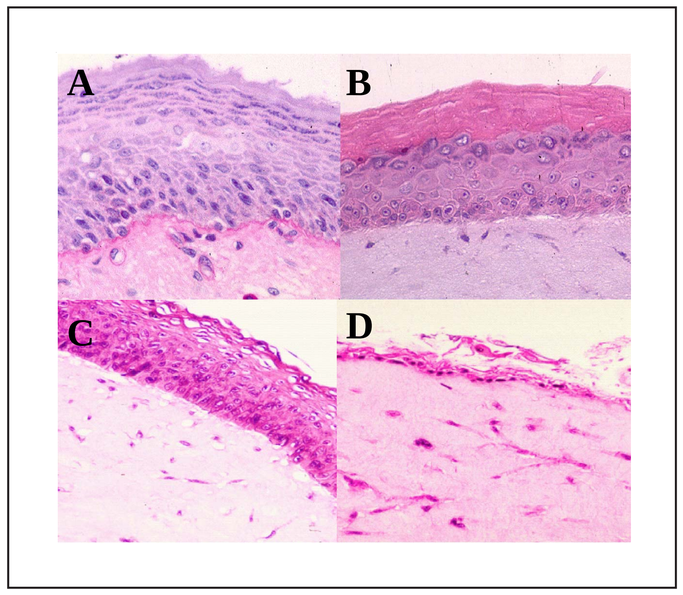

Fig.3: HE stained sections of **A.** Normal human in vivo skin, **B.-C.** AST-2000 reconstructed human full thickness skin. **B:** AST-2000 without treatment, **C:** AST-2000 after exposure to vehicle (PBS), **D:** AST-2000 after exposure to 1% Sodium Lauryl Sulfate (SLS)

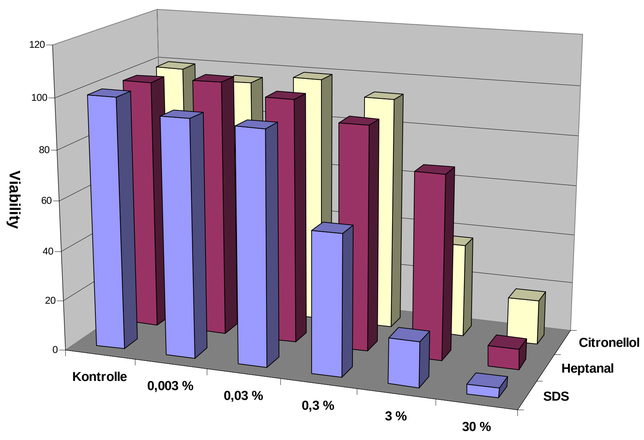

Fig. 4: Decrease of cell viability after exposure of AST 2000 to substances (Heptanal, SDS, Citronellol) with skin irritating properties after 5 hours of incubation. The compounds were dissolved in vehicle (PBS, PEG 400) and applied topically onto the dry *stratum corneum* in a volume of 15 µl. The cell viability was determined by MTT-test.

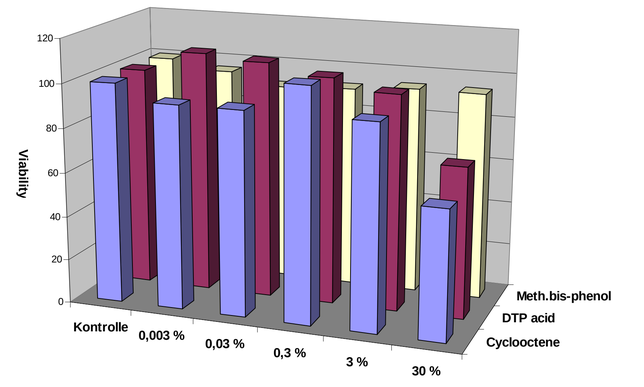

Fig. 5: Decrease of cell viability after exposure of AST 2000 to substances (Cyclooctene, Dithiopropionic acid, 4,4 Methylen bis-(2,6 ditert-buthyl) phenol) with no- or slight irritating properties after 5 hours of incubation. The compounds were dissolved in vehicle (PBS, PEG 400) and applied topically onto the dry *stratum corneum* in a volume of 15 µl. The cell viability was determined by MTT-test.

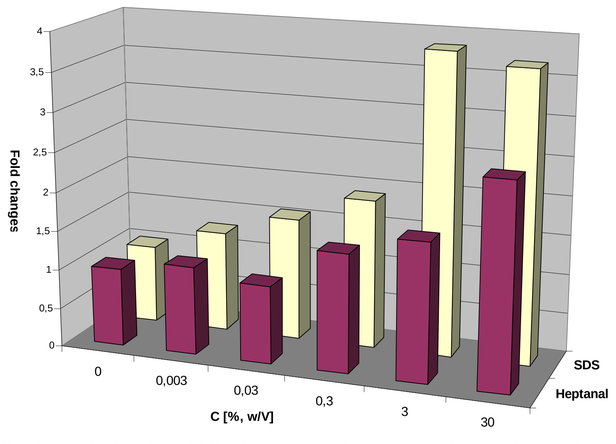

Fig. 6: Additional determination of cell viability after exposure of AST 2000 to substances (Heptanal, SDS) with irritating properties after 5 hours of incubation. The compounds were dissolved in vehicle (PBS, PEG 400) and applied topically onto the dry *stratum corneum* in a volume of 15 µl. The cell viability was determined by luminometric measurement of Adenylat Kinase release.

Substance	AST2000 LD50 / LD30	AST2000	OECD / EU
Citronellol	0,3% / 0,3%	I	I / SLI
cis-Cyclooctene	non / 3%	NI / SLI	NI / SLI
1,1-Dithiopropionic acid	non /30%	NI / (SLI)	NI / NI
Heptanal	3% / 3%	I	I / I
Methylene-bisphenol	non / non	NI	NI / NI
Potassium hydroxid	5% / 5%	I	I / I
SDS	0,3% / 0,3%	I	I / I

Tab. 1: Results of an in-house validation study with compounds characterized by OECD / EU as irritating, non- or slight irritating. Substances were dissolved in vehicle (PBS, PEG 400) and applied topically to AST-2000 in a volume of 15 µl. The decrease of cell viability, measured by MTT, was determined as the degree of irritancy. LD50 was determined as the loss of 50%, LD30 as the loss of 30 % of cell viability. Threshold concentrations are given in % (w/V). Time of exposure was limited to 5 hours. NI = non irritating, SLI = slight irritating, I = irritating.

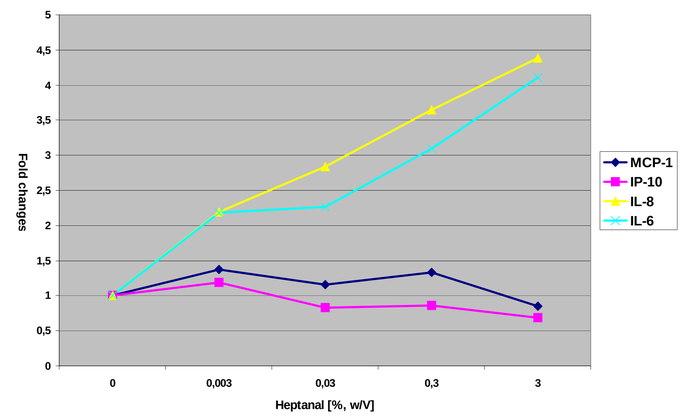

Fig. 7: Release of immunorelevant Proteins (MCP-1, IP-10, IL8, IL-6) after exposure of AST-2000 to Heptanal, as a compound with irritating properties. The threshold concentration was fixed below the LD50 concentration of the substance, determined by MTT. Time of incubation was fixed to a total of 24 hours.

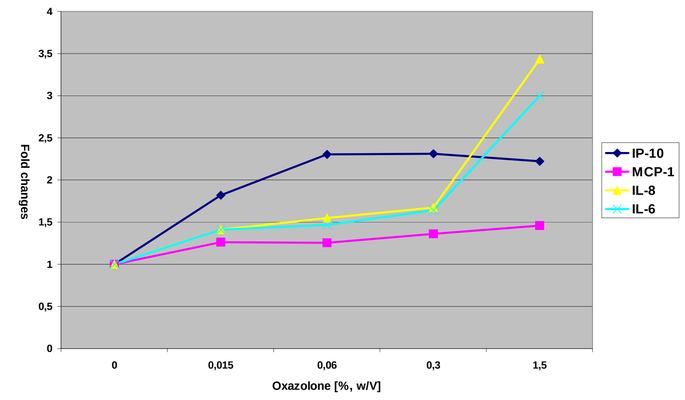

Fig. 8: Release of immunorelevant Proteins (MCP-1, IP-10, IL8, IL-6) after exposure of AST-2000 to Oxazolone, as a compound with sensitizing properties. The threshold concentration was fixed below the LD50 concentration of the substance, determined by MTT. Time of incubation was fixed to a total of 24 hours.

Cytokine / Chemokine	Release AST2000	Irritant	Sensitizer
IL-6	+++	>>	>>
IL-8	+++	>>	>>
MCP-1	+++	<	>
RANTES	+/-	<>	<>
IP-10	++	<	>
MIG (CXCL 9)	-	-	-
active MMP-1	+++	<	**n.d.**
MMP-3 (total)	+++	<>	**n.d.**

Tab. 2: Release of immunomodulating proteins from AST-2000 after topically treatment with an irritating or a sensitizing compound. (+++ = strong release, ++ = slight release, +/- = detectable, - = undetectable, >> = increase, > = slight increase, < = slight decrease, <> = not affected, n.d. = not determined.

Conclusions

1. In histological examinations and immunohistochemical analysis of Transglutaminase and Collagen IV distribution in AST-2000 it was shown that the human skin model AST-2000 provides a microarchitecture which is similar to the structure of human *in vivo* skin.

2. The aim of this study was to demonstrate the use of AST-2000 in toxicological and immunotoxicological research. In an in-house validation study with irritating, slight- and non-irritating compounds, we were able to provide results in good correlation to those from OECD / EU validation studies.

3. Furthermore, analysis of released immunomodulating proteins from AST-2000 after topically treatment with a sensitizer revealed new opportunities for the discrimination of substances with irritating properties from those with a sensitizing potential. Proinflammatory mediators like IL-8 and IL-6 were found to be released after treatment of AST-2000 with both, an irritant (Heptanal) and a sensitizer (Oxazolone). However, we determined a slight increase of MCP-1 and IP-10 after exposure to Oxazolone, while the release of both chemokines decreased after treatment of AST-2000 with Heptanal. IL-6 and IL-8 seemed to be produced by this threedimensional skin model as a consequence of cytotoxicity. Due to the slightly increased release of IP-10 and MCP-1 after exposure to Oxazolone we propose both chemokines to be involved in mechanisms that lead to a specific local immune reaction.

4. The release of matrix-metalloproteases MMP-3 and active MMP-1 from AST-2000 was not analyzed in details so far. However both proteases play a central role in wound healing and may be important parameters for recovery experiments. A release of MMPs from the more simple reconstructed epidermal models is not described so far.