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An in vitro model for detecting skin irritants: methyl green-pyronine staining of human skin explant cultures

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Abstract

We evaluated the potential of human organotypic skin explant cultures (hOSECs) for screening skin irritants. Test chemicals were applied to the epidermis of the skin explants which were incubated for 4, 24 or 48 h in tissue culture medium. A decrease in epidermal RNA staining, visualised in frozen sections using a modified methyl-green pyronine (MGP) staining procedure, was used as a marker of irritancy. A decrease in epidermal RNA after a 4-, 24- or 48-h exposure to a certain concentration of a test chemical equated to a MGP score of 3, 2 or 1, respectively. The MGP score was 0 if there was no keratinocyte cytotoxicity after a 48-h exposure. A minimum of three donors were used per chemical and the average MGP score was used to classify the chemical as irritant or not. Chemicals with an average MGP score $\geqslant 1.5$ were classified as irritants (R38), at that concentration. Chemicals with a MGP score < 1.5 were not classified (NC), at that concentration. The results obtained using human skin in vitro were compared with published data obtained using cultured porcine skin, the cutaneous Draize test (from this point referred to as the "rabbit skin irritation test") and volunteer studies. There was an excellent correlation between the classification of a chemical, as R38 or NC, based on hOSEC and results of volunteer studies. The hOSEC model predicted perfectly the irritation hazard of the 22 chemicals for which volunteer data were available. The porcine OSEC correctly predicted the classification of 21 of 22 (95%) chemicals and the rabbit skin irritation test correctly predicted the classification of 14 of 15 chemicals (93%) for which data were available. In conclusion, MGP staining of human skin explant cultures can be used to predicted human skin irritancy in vivo. In addition, the data validate the use of porcine skin as an alternative to human skin for screening for dermal irritants in vitro.

Keywords: Human skin; Pig skin; Methyl-green pyronine; Irritant testing; Alternative method

1. Introduction

Chemicals may be hazardous to the skin, for example they could induce skin irritation, and regulatory guidelines, such as those from the European Union (EC, 1988), require that information on the irritancy of new chemicals be provided. In addition, the irritancy potential of existing substances may need to be re-evaluated if current data are judged to be insufficient. A large number of animals, especially rabbits, are still used to test the dermal irritancy of chemical compounds (Draize et al., 1944). For ethical reasons the use of experimental animals for skin irritation studies is not desired. A number of alternative methods for skin irritation testing have been proposed and several have been evaluated in an ECVAM prevalidation trial. However, no method has been successfully prevalidated (Fentem et al., 2001). Proposed methods include (reconstituted) skin explant culture and human keratinocyte cultures (van de Sandt et al., 1993a,b; Botham et al., 1998). In general, the putative irritant is added to cell cultures or applied to the epidermis of the skin or a skin equivalent. Cellular or cutaneous toxicity is then quantified as a measure of irritancy. Keratinocyte toxicity can be measured by quantifying a reduction in the metabolism of dimethylthiazol diphenyltetrazolium bromide (MTT) or by or leakage of lactate dehydrogenase (LDH) from cells or neutral red from preloaded cells (Hoh et al., 1987, 1988;

Abbreviations: DDAB, dimethyl dodecyl aminobetaine; DNCB, 1-chloro-2,4-dinitrobenzene; DPBS, Dulbecco's phosphate buffered saline; hOSECs, human organotypic skin explant cultures; LDH, lactate dehydrogenase; MGP, methyl-green pyronine; pOSEC, porcine organotypic skin explant cultures; SDS, sodium dodecyl sulphate.

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Triglia et al., 1991; Osborne and Perkins, 1994; Osborne et al., 1995; Brosin et al., 1997; Botham et al., 1998; Demetrulias et al., 1998). However, these markers of cell toxicity have disadvantages, as some irritants enhance MTT metabolism and other chemicals, can kill cells without disrupting cell membrane integrity (Augustin and Damour, 1995). In view of the disadvantages associated with current markers of cellular toxicity, it has been proposed that additional endpoints should be developed to improve the sensitivity and specificity of such tests (Fentem et al., 2001).

Using porcine organotypic skin explant cultures (pOSEC), we recently reported that keratinocyte cytotoxicity (measured as the disappearance of keratinocyte RNA) could be used as a marker of irritancy. Intracellular RNA was visualised using a modified methyl green-pyronine (MGP) stain (Jacobs et al., 2000). In the present paper we report that human organotypic skin explant culture (hOSEC) correctly predicted the irritancy of all 22 test chemicals for which there were in vivo human data at normal test concentrations. When solutions of test chemicals were diluted to borderline irritant concentrations, the correlation between human in vivo and hOSEC classifications was still greater than 90%. In addition, there was a 95% agreement between the classification obtained using pOSEC and hOSEC, validating the use of porcine skin as a valuable alternative to human skin when screening for dermal irritants.

We conclude that the MGP method, using either human or porcine skin, is a simple, accurate, robust and reproducible alternative technique for screening for irritant chemicals which can be used for both hazard and risk assessment.

2. Materials and methods

2.1. Chemicals

The brand of olive oil was Bertolli classic. Decanoic acid, decanol, isopropanol, isopropyl palmitate, lauric (dodecanoic) acid, methyl caproate, methyl laurate, methyl palmitate and octanoic acid were all obtained from Aldrich; Dulbecco's phosphate buffered saline (DPBS) was obtained from BioWhittaker; acetone, hibitane and ethanol,were obtained from Merck. Cobalt (II) chloride, croton oil, 1-chloro-2,4-dinitrobenzene (DNCB), eugenol, isopropanol, mineral oil, neomycin sulphate, nickel sulphate, nonanoic acid, potassium dichromate and sodium dodecyl sulphate (SDS) were obtained from Sigma. Dr Lesley Earl (Unilever, UK) supplied 20% dimethyl dodecyl aminobetaine (DDAB, code name Empigen). Croton oil, DNCB, eugenol and nonanoic acid were dissolved in mineral oil; all other chemicals in distilled water. Aminosilane (3-aminopropyltriethoxysilane) was obtained from Aldrich; methyl green was obtained from Fluka and pyronine was obtained from Merck.

2.2. Human organotypic skin explant culture (human OSEC; hOSEC)

The method used was based on that described previously for hOSEC (Pistor et al., 1996) and was the same as previously described for porcine (p) OSEC (Jacobs et al., 2000). The human breast skin used was a waste product of cosmetic surgery and was obtained with the informed consent of the patient. Excess fat and connective tissue was removed from the skin which was then cut into squares of about 0.25 cm^2. The skin explants were then placed, dermal-side down, in 200 μl culture medium in 24-well plates. The epidermis remained above the medium/air interface. The culture medium consisted of Dulbecco's modified Eagle's medium:Ham's F12 (3:1) and glutamax (all obtained from Life Science Technologies), supplemented with 10% foetal calf serum (Sigma). The test chemicals were preheated to 37 °C and painted onto the epidermis of the explant. The skin explants were cultured for 4, 24 or 48 h at 37 °C in a humid incubator in an atmosphere containing 5% CO_2. After the incubation the skin biopsies were embedded in Tissue-Tek® (OCT compound, Sakura Finetek Europe BV), frozen in liquid nitrogen, and stored at −70 °C. Each chemical was tested using skin from at least three donors and in triplicate per donor.

2.3. Methyl-green pyronine (MGP) staining of frozen sections

The MGP staining of cryostat sections was a modification of the method of Moffitt (1994) as described by Jacobs et al. (2000). In brief, 5-μm thick cryostat sections were cut and dried. They were then stained using a freshly prepared MGP solution (0.5% methyl green, 0.1% pyronine in a 0.2 M sodium acetate buffer, pH 4.0) for 20 min at room temperature. The MGP was then poured off the sections which were then washed in tap water three times for 1 s. After drying in a blow-dryer, the sections were embedded in Pertex and evaluated using light microscopy, for the presence of RNA.

2.4. The examination and scoring of MGP-stained cryostat sections

MGP stains DNA (nuclei) bluish green and RNA (cytoplasm) pink. Areas without RNA were considered to have suffered a toxic insult. When more than 25% of the nucleated epidermis of a biopsy was MGP negative the skin was considered to be dead. If the cell nuclei did not stain blue (no DNA present) the cells were also considered to be non-viable. In all other cases the epidermis was classified as viable. The edges of biopsies

were not included when scoring sections as these areas would have been damaged when preparing the explants.

MGP staining was performed after 4, 24 and 48 h of exposure of OSECs to the test chemicals. The time needed for the development of epidermal cytotoxicity was used to generate the MGP score (Jacobs et al., 2000). Chemicals inducing epidermal cytotoxicity after a 4-h exposure were scored as 3. Cytotoxicity after a 24-h exposure was scored as 2 and cytotoxicity after a 48-h exposure was scored as 1. If there was no cytotoxicity after a 48-h exposure the MGP score was 0. The EU guidelines define 20% SDS as a minimal irritant in the rabbit skin irritation test. Using this test, chemicals are classified as irritant (R38) or not (NC) if they are more, or less, irritant than 20% SDS (EEC, 1988, 1991; OECD, 1992). Based on our results for 20% SDS using pOSEC, we set the MGP-score cut-off value for an irritant chemical at 1.5 (Jacobs et al., 2000). This value was retained for tests using hOSEC.

3. Results

Published data have been used in the Results section in order to facilitate the comparison of hOSEC with pOSEC, rabbit skin irritation test and volunteer data. The classification of a compound as R38 (irritant) or NC (non-irritant) is usually restricted to chemicals tested using the rabbit skin irritation test. We have used these classifications throughout this paper, irrespective of the test system, for reasons of simplicity.

3.1. Use of the hOSEC model for hazard assessment

The pOSEC and hOSEC MGP scores for each test preparation (stock preparations and dilutions of each chemical) are given in Table 1. The classifications predicted using pOSEC, hOSEC and the rabbit skin irritation test are also compared with classifications based on volunteer studies. The classifications (R38 or NC) of 27 chemicals tested using hOSEC agreed with those generated in volunteer studies or inferred from the concentrations of chemicals used in the human allergic patch test (see footnote to Table 1) at normal test concentrations. If inferred data are not included in the comparison, hOSEC correctly predicted the human classification of the 22 chemicals for which volunteer irritancy data were available. The pOSEC model correctly predicted the classification of 21 of these 22 test chemicals while the rabbit skin irritation test predicted the classification of 14 of the 15 for which data were available. Both the rabbit skin irritation test and pOSEC models classified methyl laurate as an irritant while it is non-irritant when tested on volunteers.

The absolute MGP scores per chemical obtained with human and porcine skin correlated very well with each other at the highest concentration of each chemical tested (regression analysis; slope = 0.93; $R^2 = 0.90$) (Fig. 1).

3.2. Use of the hOSEC model for risk assessment

In order to obtain information on the relative sensitivity of the OSEC model for risk assessment purposes, croton oil, DNCB, nonanoic acid and SDS were diluted up to eight-fold. These four chemicals were used as there was volunteer data. The MGP score at each dilution of a chemical was compared with the human classification at that concentration (Table 1, Fig. 2). Inferred classifications are given in Table 1 but not used when comparing volunteer and OSEC results. There were only minor differences in the classifications of the different chemical solutions between hOSEC and pOSEC. In general, hOSEC was more sensitive to the cytotoxic effects of low concentrations of the test chemicals than porcine skin.

The hOSEC model correctly predicted the human classification of 88% of the dilutions of croton oil, DNCB, nonanoic acid and SDS tested while the pOSEC model was 84% correct. In total, the hOSEC model correctly predicted the human classification of 95% of all chemicals tested (stock and dilutions). The pOSEC model correctly predicted the classification of 91% of all chemicals tested (Table 3b). These data indicated that the OSEC models can predict human skin irritancy even at borderline irritant concentrations.

3.3. The influence of intra- and inter-donor variation on the classification of test chemicals

Replicate skin samples from any one donor always gave the same classification for a given test preparation (data not shown). This lack of intra-donor variation is in agreement with data generated using porcine skin (Jacobs et al., 2000). The predicted classification based on skin from any single human donor was correct in 93% of all tests performed (Table 2b). Classifications based on individual porcine skin samples were 88% accurate (Table 2b). In about 80% of all tests pOSEC and hOSEC replicates gave the same, correct, classification for a test solution (Table 3b).

4. Discussion

Despite efforts from a number of different laboratories, there are no validated in vitro methods available for testing for skin irritancy (Fentem et al., 2001). In a key ECVAM report on the development of keratinocytes and human skin models for predicting skin irritation, the authors make several recommendations as to the composition of the test matrix, the variability in the response of the matrix to test chemicals and the range of chemicals to be tested (van de Sandt et al., 1999).

Table 1
A comparison of irritancy classifications based on human (h) and porcine (p) OSEC MGP scores with rabbit and human classifications in vivo

Concentration	Chemical	Solvent	pOSEC		hOSEC		MGP prediction	HPT	RSIT	Ref.
			MGP score	S.E.M.	MGP score	S.E.M.				
Pure	Aqua (milli Q)	=	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	a,b
1× concn	DPBS	=	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	a
Pure	Mineral oil	=	**0.0**	0.0	**0.0**	0.0	NC	NC		a
Pure	Acetone	=	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	a
4:1	Acetone:olive oil	=	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	a
10%	Cobalt chloride	Aqua	**2.0**	0.0	**2.0**	0.0	R38	>PT		c
5%	Cobalt chloride	Aqua	**1.0**	0.6	**2.0**	0.0	h R38	>PT		c
2%	Cobalt chloride	Aqua	**0.2**	0.2	**2.0**	0.0	h R38	>PT		c
1%	Cobalt chloride	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		c
3%	Croton oil	Min.oil	**2.0**	0.0	**2.0**	0.0	R38	R38		d
1%	Croton oil	Min.oil	**1.4**	0.2	**2.0**	0.0	h R38	R38		d
0.5%	Croton oil	Min.oil	**1.0**	0.0	**2.0**	0.0	h R38	NC		d
0.2%	Croton oil	Min.oil	**1.0**	0.0	**1.3**	0.3	NC	NC		d
0.1%	Croton oil	Min.oil	**0.9**	0.1	**1.0**	0.0	NC	NC		d
0.05%	Croton oil	Min.oil	**0.5**	0.3	**0.3**	0.3	NC	NC		d
0.02%	Croton oil	Min.oil	**0.0**	0.0	**0.0**	0.0	NC	NC		d
20%	DDAB	Aqua	**2.0**	0.0	**1.7**	0.3	R38	R38	R38	e
Pure	Decanoic acid	=	**3.0**	0.0	**2.7**	0.3	R38	R38	R38	f
Pure	Decanol	=	**3.0**	0.0	**2.3**	0.3	R38	R38	R38	f
1%	DNCB, 1-chloro 2,4-dinitrobenzene	Min.oil	**2.0**	0.4	**2.0**	0.0	R38	R38		g
0.5%	DNCB	Min.oil	**2.0**	0.4	**2.0**	0.0	R38	?		g
0.2%	DNCB	Min.oil	**0.5**	0.5	**0.7**	0.7	NC	?		g
0.1%	DNCB	Min.oil	**0.5**	0.5	**0.3**	0.3	NC	NC		g
0.05%	DNCB	Min.oil	**0.3**	0.3	**0.0**	0.0	NC	NC		g
70%	Ethanol	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		h
20%	Eugenol	Min.oil	**3.0**	0.0	**3.0**	0.0	R38	>PT		i
10%	Eugenol	Min.oil	**2.8**	0.3	**3.0**	0.0	R38	>PT		i
5%	Eugenol	Min.oil	**2.7**	0.3	**2.7**	0.3	R38	>PT		i
2%	Eugenol	Min.oil	**1.0**	0.4	**1.3**	0.7	NC	?		i
1%	Eugenol	Min.oil	**0.5**	0.3	**0.0**	0.0	NC	NC		i
0.5%	Eugenol	Min.oil	**0.0**	0.0	**0.0**	0.0	NC	NC		i
1%	Hibitane	Aqua	**0.3**	0.3	**0.0**	0.0	NC	NC		h
Pure	Isopropanol	=	**0.0**	0.0	**0.3**	0.3	NC	NC		b,e
Pure	Isopropyl palmitate	=	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	b,e
1%	Potassium dichromate	Aqua	**1.5**	0.5	**1.7**	0.3	R38	>PT		n
0.5%	Potassium dichromate	Aqua	**0.5**	0.5	**1.3**	0.3	NC	NC		n
0.2%	Potassium dichromate	Aqua	**0.0**	0.0	**0.7**	0.3	NC	NC		n
0.1%	Potassium dichromate	Aqua	**0.0**	0.0	**0.3**	0.3	NC	NC		n
0.05%	Potassium dichromate	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		n
10%	Potassium chloride	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC	NC	m
Pure	Lauric (dodecanoic) acid	=	**1.0**	0.0	**0.7**	0.7	NC	NC	NC	e
Pure	Methyl caproate	=	**0.8**	0.3	**0.3**	0.3	NC	NC	NC	b,e
Pure	Methyl laurate	=	**1.7**	0.2	**0.3**	0.3	pR38	NC	R38	e
Pure	Methyl palmitate	=	**0.0**	0.0	**0.0**	0.0	R38	R38	NC	l
40%	Neomycine sulphate	Aqua	**0.8**	0.5	**0.3**	0.3	NC	>PT		j
20%	Neomycine sulphate	Aqua	**0.8**	0.5	**0.0**	0.0	NC	NC		j
10%	Neomycine sulphate	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		j
20% (sat.)	Nickel sulphate	Aqua	**1.7**	0.3	**2.0**	0.0	R38	>PT		k
10%	Nickel sulphate	Aqua	**1.5**	0.3	**2.0**	0.0	R38	>PT		k
5%	Nickel sulphate	Aqua	**0.3**	0.2	**0.7**	0.3	NC	NC		k
2%	Nickel sulphate	Aqua	**0.3**	0.2	**0.0**	0.0	NC	NC		k
1%	Nickel sulphate	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		k
40%	Nonanoic acid	Min.oil	**3.0**	0.0	**2.7**	0.3	R38	R38		l
10%	Nonanoic acid	Min.oil	**3.0**	0.0	**2.3**	0.3	R38	R38		l
5%	Nonanoic acid	Min.oil	**2.8**	0.3	**1.7**	0.7	R38	NC		l
2%	Nonanoic acid	Min.oil	**1.8**	0.6	**1.7**	0.7	R38	NC		l
1%	Nonanoic acid	Min.oil	**1.3**	0.5	**0.7**	0.3	NC	NC		l
0.5%	Nonanoic acid	Min.oil	**0.0**	0.0	**0.3**	0.3	NC	NC		l
0.2%	Nonanoic acid	Min.oil	**0.0**	0.0	**0.0**	0.0	NC	NC		l

(*continued on next page*)

Table 1 (*continued*)

Concentration	Chemical	Solvent	pOSEC		hOSEC		MGP prediction	HPT	RSIT	Ref.
			MGP score	S.E.M.	MGP score	S.E.M.				
Pure	Octanoic acid	=	**3.0**	0.0	**3.0**	0.0	R38	R38	R34	b
20%	SDS	Aqua	**1.7**	0.2	**2.0**	0.0	R38	R38	R38	o,b,p
10%	SDS	Aqua	**2.0**	0.0	**2.0**	0.0	R38	R38		b,p
5%	SDS	Aqua	**1.0**	0.3	**1.7**	0.3	h R38	R38		p
2%	SDS	Aqua	**0.5**	0.2	**1.3**	0.3	NC	NC		p
1%	SDS	Aqua	**0.3**	0.2	**1.0**	0.6	NC	NC		p
0.5%	SDS	Aqua	**0.0**	0.0	**0.3**	0.3	NC	NC		p
0.2%	SDS	Aqua	**0.0**	0.0	**0.3**	0.3	NC	NC		p
0.1%	SDS	Aqua	**0.0**	0.0	**0.0**	0.0	NC	NC		p

HPT, human patch test; RSIT, rabbit skin irritation test; hR38, only irritant using hOSEC; pR38, only irritant using pOSEC; NC, not classified (EU classification); R38, EU risk phrase skin irritant; ? doubtful classification; PT, patch test; >PT, concentration is higher than used in allergic patch test and thus may be irritant; ICD, irritant contact dermatitis; ref, references: a, solvent in PT; b, (Basketter et al., 1997); c, 1% cobalt chloride in PT (Nordlind and Liden, 1992); d, 0.8% croton oil required for ICD (Willis et al., 1988); e, (Fentem et al., 1998); f, R38 (Basketter et al., 1997) R38/NC (Fentem et al., 1998); g, 0.1–0.5% DNCB in PT; 1% DNCB causes ICD (Krawiec and Gaafar, 1975); h, used for skin decontamination; i, 1–2% eugenol in PT (Groot and Frosch, 1997); j, 20% neomycin in PT (Schnuch et al., 1997); k, 5% nickel sulphate in PT (Schnuch et al., 1997); l, 10% nonanoic acid causes ICD (Wahlberg and Maibach, 1980); m, salt solution; n, 0.5% potassium dichromate in PT (Nordlind and Liden, 1992; Schnuch et al., 1997); o, EC definition, minimal irritant; p, 5% SDS required for ICD (Willis, 1988, p. 93).

Recommendations relevant to this article are: (a) it would be preferable that any alternative test for screening for dermal irritants should use a preparation with an intact stratum corneum; (b) variability in the response to test chemicals must be taken into account when evaluating the test; and (c) a range of positive and negative controls should be included in the panel of test chemicals used. In this paper we demonstrate that OSEC, which has an intact stratum corneum, can be used to predict accurately the irritancy of a wide range of different classes of chemicals in humans with a high degree of reliability (Tables 2 and 3). We have compared our classifications, based on in vitro data, with those based on volunteer and rabbit skin irritation test. As the rabbit skin irritation test is the standard test for predicting irritancy, it is important that classifications

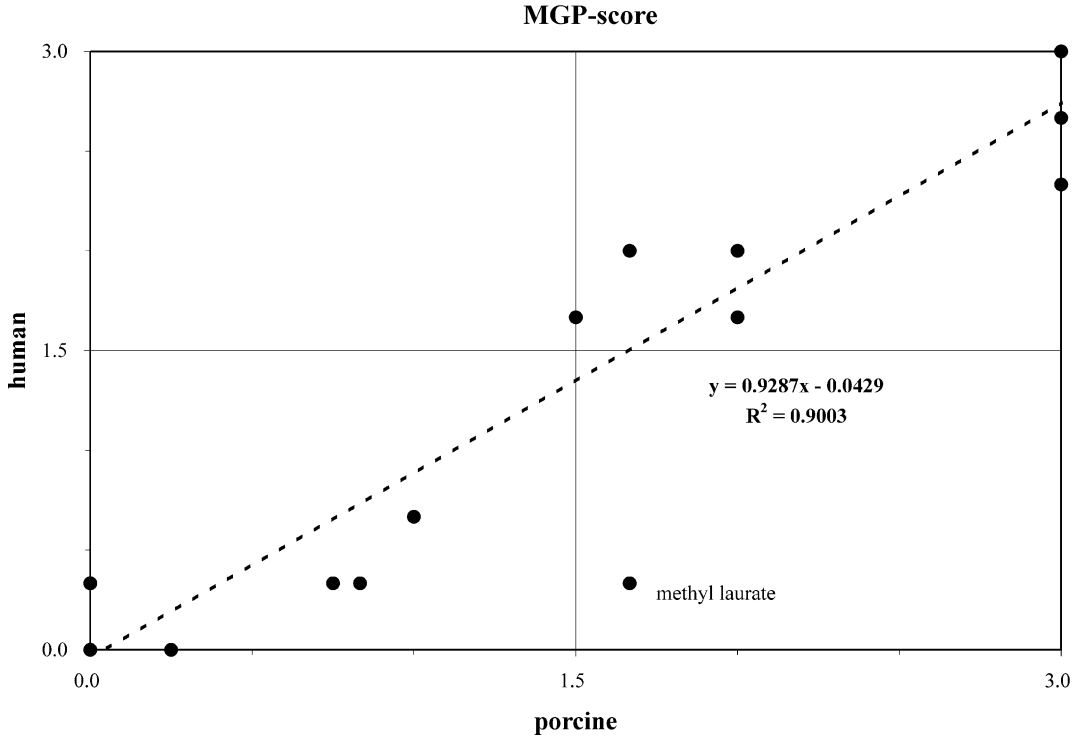

Fig. 1. A comparison of human and porcine MGP scores for 22 chemicals. The lines at MGP score of 1.5 indicate the threshold for R38 vs NC classification. The only compound—methyl laurate—classifying differently according to human and porcine OSEC, is labelled.

Table 2
Test performance of human (h) and porcine (p) OSEC based on data from individual donors

	Number of explants			
	hOSEC	%	pOSEC	%
(a) Twenty-two chemicals at normal test concentrations				
Correct prediction	64	97	86	91
Incorrect prediction	2	3	8	9
Total	66		94	
(b) Twenty-two chemicals at all (55) concentrations tested				
Correct prediction	153	93	214	88
Incorrect prediction	12	7	28	12
Total	165		242	

The table gives the number of correct and incorrect predictions per chemical based on results from one donor. For hOSEC n = three donors per test preparation; for pOSEC $n \geqslant$ three donors per test preparation. The volunteer data used to calculate the percentages were obtained from the references provided in the legend to Table 1. (a) Gives the data for the 22 chemicals at normal test concentrations. (b) Shows the reliability of predictions obtained using those dilutions of nine chemicals for which volunteer studies had provided a clear classification (NC or R38). Thus, inferred classifications such as "> PT concentration" were not used to generate the percentages.

Table 3
Hazard and risk assessment using replicate groups of human (h) and porcine (p) OSEC

	Number of preparations tested in triplicate			
	hOSEC	%	pOSEC	%
(a) Twenty-two chemicals tested at normal test concentrations				
All right	20	91	18	82
Inconsistent right	2	9	3	14
Inconsistent wrong	0	0	1	5
All wrong	0	0	0	0
Total	22		22	
(b) Twenty-two chemicals at all (55) concentrations tested				
All right	45	82	43	78
Inconsistent right	7	13	7	13
Inconsistent wrong	2	4	4	7
All wrong	1	2	1	2
Total	55		55	

Table shows the number times replicate groups (hOSEC, $n = 3$, pOSEC $n = \geqslant 3$) gave the same correct, or incorrect, prediction. The volunteer data used to calculate the percentages were obtained from the references provided in the legend to Table 1. (a) Shows the data for 22 chemicals at normal test concentrations. (b) Shows the reliability of predictions obtained using those dilutions of nine chemicals for which volunteer studies had provided a clear classification (NC or R38). Thus, inferred classifications, such as "> PT concentration", were not used to generate the percentages. "all right"—all replicates of that group gave the same correct prediction in agreement with volunteer data; "all wrong"—all replicates of that group gave the same incorrect prediction in comparison with volunteer data; "inconsistent right"—majority of the replicates gave a correct classification; "inconsistent wrong"—majority of the replicates gave an incorrect classification.

based on OSEC are at least as good as those based on the rabbit test. However, we consider human data to be more relevant when validating alternative methods for predicting irritancy in humans. Thus, where the predicted classification using volunteers differs from that using the rabbit test, we assume the volunteer classification to be correct. Human irritancy data is not readily available for a large series of chemicals and we have relied mainly on the data set published by Basketter et al. (1997) and the data provided by ECVAM for its prevalidation study (Fentem et al., 2001) for most of the human classifications. In some cases we used data from human skin sensitisation tests to obtain an inferred classification. It is recommended that chemicals be tested for their sensitising potential at the highest minimally irritant concentration (ECETOC, 2000) and we have assumed that concentrations of chemicals higher than those used in a human skin sensitisation test will be irritant. For ease of comparison we have used R38 (irritant) and NC (non-irritant) for classifying chemicals tested using all the methods mentioned in this article.

The correlation between the classification of irritancy predicted using the in vitro tests and the classification based on the human patch test was 100% for human skin and 95% for pig skin at the highest concentrations of the chemicals tested. When all the data generated during the course of this study were analysed (including dilutions of chemicals), hOSEC correctly classified 95% of samples and pOSEC 91% (Table 3b). The OSEC models were also sensitive, correctly predicting human irritancy over a range of dilutions of the maximum concentrations used (Table 1). When there were differences between classifications based on human and porcine skin, the human skin appeared to be more sensitive. This was more apparent at low dilutions of chemicals (Fig. 2).

The intra-donor response to test chemicals was very reproducible. In contrast, some variation was noticed in the response of hOSEC to test chemicals when skin was obtained from different donors. A similar inter-donor variation has also been reported for pig ear skin (Jacobs et al., 2000) and volunteers (Judge et al., 1996; McFadden et al., 1998). In order to reduce the chance of false positive or negative results, each experiment was performed with skin from three donors. The influence of

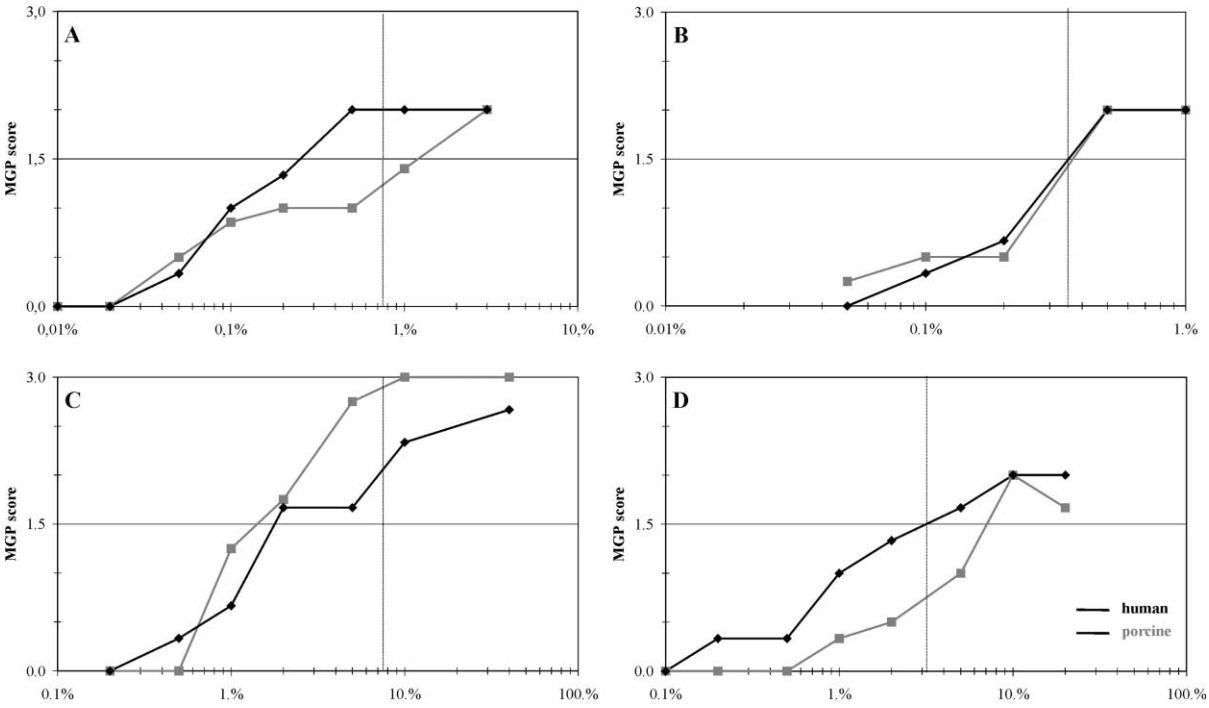

Fig. 2. Dose responses, using human (◆) and porcine (■) OSEC scores for (A) croton oil, (B) DNCB, (C) nonanoic acid and (D) SDS. The lines at MGP score of 1.5 indicate the threshold for R38 vs NC classification. The vertical line indicates the approximate border between irritant (R38) and non-irritant (NC) concentrations based on volunteer studies.

inter-donor variation was investigated by determining how often the MGP score for replicates straddled the MGP-score cut-off value of 1.5. This occurred in 17% of all solutions tested using hOSEC and 20% using pOSEC (Table 3b). When replicate MGP scores lie just either side of cut-off, it may be necessary to increase the number of donors used for hazard and risk assessment. False or inconsistent (individual data straddled cut-off value) predictions were almost only found when borderline irritant dilutions of chemicals were used. In total, triplicates gave the same correct classification in 82% (hOSEC) and 78% (pOSEC) of all experiments performed (Table 3b).

Taken together, the results indicate that the OSEC models are robust and can be used for accurate, sensitive and reproducible hazard and risk assessment. The good correlation between classifications obtained using human and porcine skin further reinforces the use of porcine skin as an alternative to human skin when testing for dermal irritants.

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