Organotypic skin explant cultures to identify skin irritants and contact allergens

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Organotypic skin explant cultures to identify skin irritants and contact allergens / Johannes Jacobus Louis Jacobs.
Proefschrift Universiteit van Amsterdam. – Met lit. opg. –
Met samenvatting in het Nederlands
ISBN 90-9017486-9
Trefw, contact dermatitis, allergy, irritancy, alternative methods

Printed by grafisch bedrijf Ponsen & Looijen BV

Cover. Front Lag (blue) CD1a (red) stained epidermis, with LC migration from top to bottom. Back MGP stained epidermis; top viable (pink); bottom dead.

The research described in this thesis was financially supported by the ZonMw Dutch Programme on Alternatives to Animal Testing (proj.nr 3170.0003).

ORGANOTYPIC SKIN EXPLANT CULTURES TO IDENTIFY SKIN IRRITANTS AND CONTACT ALLERGENS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof.mr. P. F. van der Heijden

ten overstaan van een door het College voor Promoties ingestelde commissie in het openbaar te verdedigen in de Aula der Universiteit

op maandag 24 november 2003, te 13.00 uur

door Johannes Jacobus Louis Jacobs

geboren te Arcen en Velden

Promotiecommissie

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Abbreviations

ACD	allergen contact dermatitis
AEC	3,3-amino-9-ethyl carbazole
Aminosilane	3-aminopropyltriethoxysilane
AP	alkaline phosphatase
APC	antigen presenting cell
aq.	aquous
BA	basal accumulation
bLC	basal LC; LC in basal epidermis
BSD	basal-suprabasal distribution
CD1	cluster of determination (for antibodies) number 1
CHAPS	3-{(cholamidopropyl)-dimethylammonio}-propanesulfonate
CHS	contact hypersensitivity
CIMA	cutaneous immune modulating activity
DC	dendritic cell
DDAB	dimethyldodecyl aminobetaine
DMEM	Dulbecco's modified Eagle's medium
DNCB	dinitrochlorobenzene (1-chloro 2,4-dinitrobenzene)
DPBS	Dulbecco's phosphate buffered saline
EC	european community
EC_3	effective concentration inducing cell proliferation 3 times the basal proliferation (in LLNA)
eLC	epidermal LC
FBB	fast blue base
GPMT	guinea pig maximisation test
HLA	human leukocyte antigen
HMT	human maximisation test
hOSEC	human OSEC, human organotypic skin explant culture
HPTA	human patch test allergen
HRP	horseradish peroxidase
I	irritant
ICD	irritant contact dermatitis
iDC	immature DC
IL-1	Interleukin 1
LC	Langerhans cell
LDH	lactate dehydrogenase
LLNA	local lymph node assay
LWIC	lowest weak irritant concentration
mDC	mature DC
MGP	methyl-green pyronine
MHC	major histocompatibility class
MI	migration index
min.	solved in mineral oil
MTT	dimethylthiazol diphenyltetrazolium bromide
NC	not classified (as skin irritant)
NI	non-irritant
OSEC	organotypic skin explant culture
PBS	phosphate buffered saline
pOSEC	porcine OSEC, porcine organotypic skin explant culture
QSAR	quantitative structure-activity relationship
R34	risk sentence indicating EU classification of skin corrosives
R38	risk sentence indicating EU classification of skin irritants
sat.	saturated
sbLC	suprabasal LC; LC in suprabasal epidermis
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
TCA	trichloroacetic acid
TER	transcutaneous electrical resistance
TEWL	trans-epithelial water loss
TNCB	2,4,6-trinitrochlorobenzene
TNF-α	tumour necrosis factor α

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Chapter 1
Introduction

1.1. Aim

Hazard identification is an important issue for chemicals released on the market. Important hazards are the possibility that compounds may cause irritant or allergen contact dermatitis. Skin irritants are currently assessed with the Draize test on rabbits, requiring 3 to 6 rabbits per compound [1]. Contact allergens are currently assessed with the guinea pig maximisation test (GPMT) or the local lymph node assay (LLNA). The GPMT requires 24 to 32 guinea pigs and the LLNA requires 16 to 30 mice per chemical [2]. There are considerable pressures, ethical and legislative, to develop alternative models for risk assessment which do not need animals (see next section). The subject of this thesis is the development of such alternatives for the screening of skin irritants and contact allergens.

1.2. Ethical and legal background

In 1859, Charles Darwin published *'The Origin of Species'* and suggested an evolutionary relationship between man and animals. His book provided a biological rationale on the use of animal experiments for human biomedics and toxicology. Six years later, Claude Bernard wrote *'Introduction á l'étude de la medicine experimentale'*, which proclaimed the use of animals for experimental science. The use of animal experiments has been a great help for the progress of medical and toxicological research. This has lead to an increase of the number of animals used for experiments to a maximum of 1.5 million in 1978 in The Netherlands. In 1993 the number had decreased to 780,000 due to laws, education, ethics, dialogue with animal protection organisations, and the development of alternatives. More than three quarters of the test animals are mice and rats. Almost 25% of all experimental animals are used for pharmaceutical research, about 21% for biologicals (three quarters of these for quality control), 10% for cancer research, and about 8% for toxicology, and the rest for different kinds of research, diagnostics, experimental surgery, education, etc. [3,4].

Product safety is an important ethical issue. For this purpose guideline 67/548/EEC has introduced registration codes, e.g. R34, R35, R38 and R43 to label dangerous compounds. The codes R34 and R35 label corrosive chemicals and severe corrosives, respectively. Code R38 classifies chemicals as causing skin irritation. The code R43 classifies chemicals as a putative cause of skin allergy. The code NC is used for compounds ‘not classified’ by a registration code. The EC also provides guidelines for assessment of registration codes to chemicals. Preferably chemical classification is assessed by validated test methods, but many of the current test methods are only selected on historical grounds.

In 1959, Russell and Burch wrote *'The Principles of Humane Experimental Technique*' [5], which introduced ethics into laboratory animal research for a broad audience [3,4]. These principles can be summarised by the three Rs, Replacement, Reduction and Refinement. The aim of these three Rs is not to abolish animal use

for experiments, but to minimise animal suffering, while maintaining the scientific value of the experiments [6]. The definition of an alternative test is then: "all procedures which can completely replace the need for animal experiments, reduce the number of animals required, or diminish the amount of pain or distress by animals in meeting the essential needs of man and other animals" [7]. Replacement alternatives mean that an in vivo animal test is completely replaced by an alternative test that permits the achievement of a given purpose, without conducting experiments or other scientific procedures on animals [8]. Thus any test or procedure which meets the essential needs for a study but does not include animal suffering, can be regarded as a replacement alternative. Reduction alternatives mean that less animals are used to achieve the same scientific result. Refinement alternatives are methods which alleviate or minimise potential pain suffering and distress, and which enhance animal well being.
The Declaration of Helsinki (1964) was designed to provide recommendations to guide physicians in biomedical research involving human subjects. At the 3rd world congress on Alternatives and Animal Use in the Life Sciences (1999) it was proposed to amend the Declaration of Helsinki. The amendment proposes tot limit limit animal experimentation to those that are necessary, relevant and reliable for their stated purposes [9]. Most countries have laws to regulate the use of animal experimentation. The Netherlands have two laws regulating the use of living vertebrate animals for all kinds of biomedical and toxicological research, namely *Wet op dierproeven* (1977; modified in 1995) and *Dierproeven besluit* (1985). The principles of the three Rs are implemented in these laws [4]. Moreover, The Netherlands has prohibited animal experimentation for cosmetics in 1997, and this ban is due to become an EU wide ban in 2009. Thus besides laws requiring safety testing by using animal experimentation, there are laws limiting the use of animal experiments by requiring alternative methods [10].

1.3. Biomedical background

Skin biology. The skin covers the outside of mammalian bodies to protect against water loss, physical, chemical and biological stress. It also plays an important role in heat regulation, excretion of various substances, synthesis of vitamin D and transmission of several stimuli (pain, pressure, temperature and touch). The skin is comprised of a stratified epidermis at the outside and an underlying dermis (Figure 1.1a). These are separated from each other by the basal membrane. The dermis is often divided into two layers, in the epidermal ridges is the papillary dermis, and underneath the reticular dermis. Food supply and immune cells enter the epidermis through arteria and capillaries in the papillary dermis, and waste products and immune cells leave again in the papillary dermis using capillaries and venae or lymph vessels. The dermis consists of fibroblasts, which make connective tissue with collagen and, in the reticular dermis, also elastic fibres to maintain skin strength and elasticity. The dermis contains many blood vessels. Besides feeding (epi)dermal cells, these blood vessels are important in skin immunology and warmth regulation. Below the reticular dermis is a fatty layer, the subcutis, underneath the subcutis is the internal body [11].

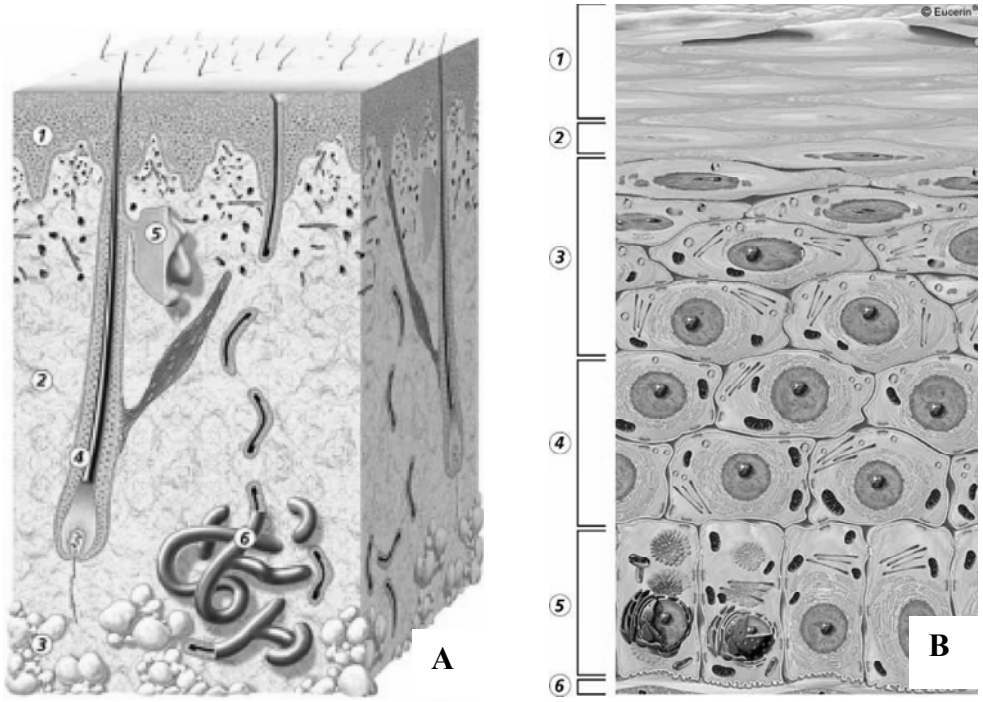

Figure 1.1. (A) Overview of the skin. 1 epidermis; 2 dermis; 3 subcutis; 4 hair follicle; 5 Sebaceous gland; 6 Sweat gland. (B) Overview of the epidermis. 1-2 horn layer, it grows from layer 2; 3-5 different layers of keratinocytes: the keratinocytes in layer 5 (*stratum basale*) divide, and push the other keratinocytes upwards towards layer 4 (*stratum spinosum*) and finally layer 3 (*stratum granulosum*) where the keratinocytes are flattened and contain the granules that will be form the horn layer; 6 basale membrane.
Pictures taken from http://www.ccunix.ccu.edu.tw/~chenmsl/tea/SKIN_910721.htm.

Skin epidermis consists of keratinized squamous epithelial tissue (Figure 1.1b). Just above the basal membrane in the stratum basale are basal keratinocytes, which are capable of dividing, and take care of epidermal growth. Due to the increase in number, cells will be moved upward to the stratum corneum. The layer above the stratum basale is the stratum spinosum, keratinocytes in this layer have a cubic shape and are attached to each other with desmosomes. Above the stratum spinosum lies the stratum granulosum. These cells are flattened, polygonal and have flattened, pycnotic nuclei. Gradually the stratum granulosum becomes the stratum lucidum, if visible, and finally the top layer, stratum corneum or horn layer. Cells in this layer do not have nuclei, are flat and completely filled with keratin. Keratin provides a very good protection against damage, drying out and bacterial infections [11].
The skin protects the body against water loss and all kinds of external stresses. For this purpose, the skin consists of a number of barriers. The outer barrier is the horn layer, consisting of dead keratinocytes filled with keratin. A second barrier lies in between the viable keratinocyte layers. Keratinocytes are connected to each other with desmosomes, which consist of protein fibers. A desmosome is built by two neighbouring cells to attach these together and providing a barrier. The third line of

defence is the basal membrane, which forms a barrier between the epidermis and the dermis. This is partly built by basal keratinocytes which attach their part of a desmosome (hemidesmosome) to the basal membrane. In the dermis special forms of collagens (e.g. collagen type IV) are synthesized by dermal fibroblasts; these collagens function as the counterpart of the basal membrane. Interactions between dermal collagens and epidermal hemidesmosomes, integrins and other proteins are the bases for a solid basal membrane barrier.

Beside keratinocytes, the epidermis also contains melanocytes, Merkel cells and Langerhans cells. Melanocytes are located between the keratinocytes of the stratum basale and the stratum spinosum. Melanocytes are dendritic shaped cells with oval nuclei. The melanocytes produce melanin, a pigment that determines skin colour and protects against ultraviolet radiation. This pigment is injected by way of dendritic processes into epidermal and hair cells. Merkel cells are tactile cells associated with nerve plates. They are embedded between the basal membrane and the basal keratinocytes, and have desmosomes together with these cells. [11]

Epidermal Langerhans cells are named after Paul Langerhans, and are structurally, ontogenetically and functionally completely unrelated to the Langerhans islets containing insulin-producing beta-cells in the pancreas. Langerhans cells (LCs) are dendritic cells, which originate from the bone marrow and have migrated into the epidermis. In the epidermis, LCs cell will capture and process antigens, after which they migrate to the draining lymph node to induce immune responses.

Contact eczema. Eczema is a distinctive pattern of inflammatory response of the skin, and is characterized histologically by spongiosis (intercellular oedema) and clinically by vesicle formation. In time the different stratae will thicken in sequence of their generation, i.e. *stratum spinosum* (acanthosis) in the subacute phase, followed by the *stratum granulosum* (granulosis), and the *stratum corneum* (hyperkeratosis) in the chronic situation. Eczema and dermatitis are often regarded as synonymous. Eczema can be caused by endogeneous or exogenous factors acting singly or in combination. Endogenous eczemas include atopic dermatitis (IgE mediated), seborrhoeic dermatitis, nummular eczema, pompholyx, pityriasis alba. Exogeneous eczema includes infective dermatitis and both irritant and allergen contact dermatitis [11]. Exposure of the skin to chemicals causes occupational (contact) dermatitis, which is the most frequent occupational health problem [11,12]. Development of new substances and new formulations, e.g. for cosmetic use, increases the number of chemicals that may cause contact dermatitis. This number may be as high as 2,000 new chemicals per year. Proper assessment of chemicals as putative causes of skin problems may lead to regulation that minimizes exposure and thus discomfort.

Skin irritants and contact allergens can induce contact dermatitis or eczema. Skin irritants cause inflammation by a toxicological mechanism. Allergens invoke an immune response only after sensitisation. In clinical practice this means that any exposure to a strong irritant causes eczema, but the reaction will differ from person to person after exposure to a weak irritant. Contact allergens only cause eczema when an individual has been sensitized by sufficient previous exposure [13,14]. Regulatory guidelines, like those from the European Union [15], require that

information on the irritancy or allergenicity of chemicals is provided for new substances and, increasingly, for existing substances.

Skin irritation. Irritant contact dermatitis (ICD) accounts for about 70% of the contact dermatitis cases, and is thus a more common problem than allergic eczema [16]. In clinical praxis, both causes of dermatitis look very much alike. Five different types of irritant dermatitis have been classified:

I - acute irritant contact dermatitis, due to contact with a powerful irritant;

II - irritant reaction and cumulative irritant dermatitis. This is the most common form and often occurs in people who do repetitive wet work (nurses, hairdressers, bar personnel);

III - delayed acute irritant contact dermatitis, due to irritants that cause an acute reaction after a delay of 8 to 24 hours (e.g. dithranol, podophyllin, epichlorhydrin);

IV - mechanically induced irritant contact dermatitis, due to (repetitive) skin damage by mechanical forces;

V - pustular and acneiform dermatitis. Pustules, papules and coedone can be formed by contact with metals, cutting oils (used as coolants in metal work), greases and tar [12].

Most often ICD is caused by chemicals, but it may also be caused by sunburn, mechanical or physical damage. Known groups of skin irritants are water (after repeated exposure), skin cleansers, detergents, alkalies, acids, many oils, many organic solvents, oxidizing agents, reducing agents, topical medicaments, as well as plant and animal products. As many different types of chemical may induce skin irritation, they initiate inflammation by diverse mechanisms, which depends on the physicochemical properties of the irritant and the circumstance of exposure. Skin irritation is a toxic insult of the skin. Two main mechanisms of skin irritation exist. Some irritants destroy the horn layer, so that the epidermal cells come in direct contact with the outer environment. Loss of the horn layer leads to increased water loss and / or increased penetration of irritant substances. Next in direct contact with the keratinocytes, these compounds are toxic. Disruption of the stratum corneum integrity and barrier function is measured in tests for corrosives. [12,13]. The same or other chemicals penetrate through the horn layer and are toxic for the keratinocytes. The skin damage induces the secretion of chemokines and other cytokines which attract inflammatory cells leading to a cutaneous inflammation. This can be done by (a) disruption of cellular membranes in the (epi)dermis leading to synthesis of proinflammatory prostaglandins etc. (b) Perturbation of keratinocytes leading to the release of proinflammatory cytokines. and (c) cytotoxicity leading to release of mediators/tissue destructive enzymes etc [12,13] A third, but rare, mechanism in ICD is the direct effect on dermal blood vessels and cell surface adhesion molecules leading to an inflammatory infiltrate [12]. The threshold for skin irritancy may vary significantly depending on the individual tested. Using human volunteers, the lowest SDS concentration causing skin irritancy can vary from 0.1% to more than 20% [17]. This variation may be explained by differences in composition of the human stratum corneum [18].

Skin immunology. Dendritic cells and memory and effector leukocytes are the only immune cells that enter the skin. Immunity to skin antigens is acquired in lymphoid

organs outside of the skin, i.e. the draining lymph node. From a skin immunological point of view, these lymphoid structures are part of the skin immune system. Antigen presenting cells (APCs) will migrate from the skin to the draining lymph node to induce an immune response. Two types of antigen presenting cells (APCs) exist, professional and non-professional, and both types APCs may restimulate lymphocytes. Only professional APCs can stimulate naive lymphocytes [19]. Dendritic cells (DCs) are the only professional APCs. Their precursors originate from bone marrow and migrate to peripheral organs, e.g. skin, where they differentiate to immature dendritic cells (immature DCs; iDCs) [20], with tissues-specific markers. Upon activation, iDCs become activated, maturate and migrate through lymph vessels to the draining lymph node. In the lymph node, mature DCs (mDCs) encounter large numbers of naive lymphocytes to activate the rare antigen-specific lymphocytes [21-24].

All somatic cells have major histocompatibility class I (MHC I) to present antigen. Besides MHC I, APCs also have MHC II and often CD1 antigen presenting molecules. All these molecules present antigens to the T cell receptor. MHC I present peptide antigens to $CD8^+$ T cells, MHC II peptide antigens to $CD4^+$ T cells. CD1 molecules present lipid antigens to either $CD4^+$, $CD8^+$ or double negative T cells. The type I CD1 family consist of CD1a, CD1b, CD1c and CD1e, the type two family of CD1d. Type I CD1 molecules can be found on different skin dendritic cells, and present antigens from different cellular compartments. CD1a samples antigens from the recycling endosome, CD1c possible from the early endosome, and CD1b from the acidified late endosome, where also MHC II resides [25]. Skin DCs with different CD1 molecules will thus sample antigens from different cellular compartments. LCs are unique DCs, that express much CD1a, little CD1c, and usually no CD1b [26-28]. LCs are also unique in expressing Birbeck granules, which can be detected by the socalled lag and langerin antibodies [28,29]. Birbeck granules can be used to detect of LCs using electron microscopy. LCs can also be recognized by antigen presenting molecules such as MHC-II and CD1a [28]. Langerhans cells (LCs), named after Paul Langerhans, are DCs situated between the keratinocytes of the epidermis. Human epidermis contains 450 to 730 LCs mm^{-2}, this is 2.9 to 4.7 % of all epidermal cells [30]. Direct and indirect studies showed that migration of epidermal LCs *in vivo* causes increased proliferation of local lymph node cells. [31-39]. Remarkably, this relation was also found for skin irritants (e.g. SDS), which suggests that proliferation of local lymph node cells does not necessarily imply that a state of immunity is generated. Dispite its used in many sensitization experiments, a contact hypersensitivity reaction against SDS has never been shown. Under steady state conditions, spontaneous migration of LCs occurs. Spontaneous migrating LCs might be immature, and immature DCs cause tolerization. Thus it has been suggested that homeostatic migration LCs are involved in maintenance of tolerance. [40-43]

Contact allergy. Contact allergy or allergen contact dermatitis (ACD) is a type IV or delayed type hypersensitivity response, also known as contact hypersensitivity (CHS). This disease was first studied in 1936 by Karl Landsteiner and John Jacobs [44]. Antibodies do not play a major role, but antibodies do play a major role in atopic dermatitis, which is an IgE mediated or type I allergy. ACD requires the

induction of memory T cells by dendritic cells. ACD consists of two phases, the induction and the elicitation phase. In the induction phase, sensitization occurs, most often without clinical dermatitis. In general, an allergen is a reactive hapten. The hapten penetrates, and will bind to skin proteins forming a hapten-carrier complex. By some means, possibly its reactive nature, the hapten also activates skin DCs, most notably the LCs in the skin. The migration of LCs to the draining lymph node is required to sensitize an individual, and thus crucial to the induction of ACD.

Agents causing contact allergy are antimicrobial agents (e.g. phenoxyetanol, organic mercury compounds, DNCB), anti-oxidants, balsams, perfumes, flavouring agents and spices, clothes, cosmetics, many metals (e.g. nickel, chromium cobalt, gold) explosives, formaldehyde, hydrazines, hyydroxyquinolines, oils, organic dyes, pesticides. Photographic chemicals, many plastics, quarternary ammonium compounds, rubber chemicals, tars, thiuram sulphides, tars, turpentine,

Differences and interrelation between skin irritants and contact allergens. It may be very difficult to discriminate between ACD and irritant contact dermatitis (ICD) due to the limited number of clinical differences (Table 1.1). Moreover, within both causes of contact dermatitis differences in clinical manifestation can be present due to different molecular mechanism of different agents, or agents in different concentrations. This makes it more difficult to discriminate between ACD and ICD using molecular parameters, such as the measurement of cytokines. The major differences between these occupational diseases reside in the different mechanisms causing the diseases. Irritation is caused by toxicity, whereas allergy is caused by activation of the specific immune system.

Toxicological mechanisms generally show a strict dose-response reaction. In contrast, most immunological reactions do not show a strict dose-response relation. One of the few exceptions is ACD, which is an immunological reaction, but shows a strict dose-response curve in contrast to e.g. related DTH reactions in the skin. Although mechanical differences between ACD and ICD exist, in practice contact eczema is often a combination of both. Irritation potentates and increases the ACD reaction. Moreover, at least for one model allergen, irritation appears to be necessary to induce ACD [45].

Table 1.1. Comparing dermatitis due to immunogens, allergens, weak and strong irritants

Cause	DTH immunogen	contact allergen	weak irritant	strong irritant
Persons affected	few [a]	few [a]	few [b]	everyone
Elicitation after exp.	0.5 – 3 weeks	1 –3 weeks	after rep. exposure	0-2 days
Strictly dose related	No	Yes	yes	yes
Reappearance	immunogen contact	allergen contact	variable	variable
Passive transfer (Ly)	Yes	Yes	No	No
Healing	slow	slow (1-2 weeks)	not prompt	prompt [c]
Early cells	Mononuclear cells	Mononuclear cells	PMNs or MNCs	PMNs

Data after [19,103]; [a] only sensitized persons; [b] depending on individual sensitivity for a certain agent; [c] after avoid; ly = lymohocytes; MNCs = Mononuclear cells; PMNs = polymorphonuclear cells.

1.4. Risk assessment of skin irritants

The aim of risk assessment of skin irritants is to assess the risks for humans. Thus the relevant reference data are the human data. For this purpose a human volunteer 4-hour patch test has been developed. This test is gradually built up from shorter exposure to avoid strong irritant reactions [46, 47]. The protocol is designed to avoid the production of more than mild irritant reaction and meets the highest ethical standards. Chemicals that are allowed to be tested, should be known to be non-corrosive and without other toxicological or carcinogenic effects [12,48]. In practice this means that many new chemicals cannot be tested on humans *in vivo*. Nevertheless, sufficient data of the human 4-hour patch test exists to serve as reference data for validating (alternative) tests assessing skin irritation.

Current animal models. A large number of animals, especially rabbits, are still used to test the dermal irritancy of chemical compounds [1]. The most used animal test to assess skin irritants is the Draize rabbit test. In the Draize rabbit test, the test chemical is applied to sites on the dorsal skin of three to six albino rabbits. This method gives a scaling for irritation in the primary irritation index (PII) score by grading erythema and oedema. A PII of < 2 is considered to be mild, 2-5 moderate, and > 5 severe [1]. The Draize rabbit model has been shown to be a poor predictor of human skin irritation hazard [12,49]. For ethical reasons, the use of animal experiments for skin irritation studies is not desirable. In the next sections, I will discuss briefly some alternative methods to assess skin irritants.

Theoretical models to assess irritants. Quantitative structure activity relationship analysis has been carried out on organic acids and bases, phenols, and neutral and electrophilic chemicals to predict their skin irritation/corrosion potential [12]. This computer model is capable of predicting hazard of chemicals having similar physiochemical properties of other compounds, which are known with regard to their skin irritation/corrosion potential.

Assessment of skin irritants in vitro. Cultures of keratinocytes and / or fibroblasts, and air exposed keratinocyte cultures (epidermal or skin equivalents) are used to study skin irritants. In some models the keratinocyte culture is differentiated *in vitro* to generate a reconstituted human skin [50]. Also intact skin may be used [51-55]. It should be noted keratinocyte toxicity is observed very rapidly in *in vitro* reconstituted skin cultures, and that the results have a very limited predictivity [56-58]. Limited differentiation of the *stratum corneum*, but also of epidermal barriers may be the cause of this low toxicity threshold and low predictivity.

The cells or reconstituted skin structures are exposed to the putative irritant and cutaneous toxicity is measured to determine irritancy. *In vitro* assays for skin irritation are based on markers of cell toxicity such as on reduction in cellular metabolism, leakage from cells or the secretion of inflammatory cytokines [50,59-64]. However, these markers of cell toxicity have disadvantages. Some irritants, like sodium dodecyl sulphate (SDS), can enhance cellular and mitochondrial metabolic function, resulting in enhanced MTT metabolism [65]. Other chemicals, such as cadmium chloride, can kill cells without disrupting cell membrane integrity [65]. Moreover, different skin irritants induce different patterns of cytokine synthesis [66].

Corrosive chemicals ans skin irritants can be screened by a functional measurement of the skin barrier function, and especially that of the horny layer. A reduction

electrical resistance of skin is tested by applying test material directly to a skin disc *in vitro* for 24 hours. Loss of stratum corneum integrity and barrier function is measured as a fall in the trans cutaneous electrical resistance (TER). When this resistance drops below a predetermined threshold the substance is regarded as corrosive [12]. A similar method using Trans Epidermal Water Loss can also be used to test corrosives or skin irritants [67,68]. The methods in these tests detect the mechanism of corrosive chemicals, and these methods may have very good predictive value for corrosives [12,13]. Corrosive chemicals are the most aggressive skin irritants and can be assessed by testing skin barrier integrity. Since many irritants have a different mechanism of toxicity,[13] these methods may be less reliable in assessing skin irritants.

Comparing skin irritation models. Alternative methods for assessing skin irritants are compared in Table 1.2. Reliable assessment of skin irritants requires a human

Table 1.2. Alternative methods for assessing skin irritants

skin model	Reconstituted skin				OSEC	intact skin	
Normal barrier present in model	-	-	+	+	+	+	+
Irritation is measured by:	MTT [a]	NR/LDH [b]	MTT	NR	cytokines [c]	RNA [d]	barrier [e]
Theoretical possible to detect:							
strong corrosive, rapid toxicity	+	+	+	+	-	+	+
weak corrosive	+	+	+/-	+/-	+/-	+/-	+
non-corrosive penetrating irritant	+	+	+	+	+	+	-
Non-cytolytic irritant	+	-	+	-	+	+	+/-
Non-inflammatory irritant	+	+	+	+	-	+	+
Toxic metabolic enhancer	-	+	-	+	+	+	+

+, either property present (normal barrier) or normal detection of skin irritant group; -, either property absent (normal barrier) or no detection of skin irritant group; +/- intermediate property, or detection possibility. [a] reduction in the metabolism of dimethylthiazol diphenyltetrazolium bromide (MTT); [b] uptake of neutral red (NR); leakage of lactate dehydrogenase (LDH); [c] cytokine or prostaglandin production; [d] presence of RNA, assessed by pyronine staining; [e] measurement of skin barrier function by trans epithelial electric resistance (TER) or trans epithelial water loss (TEWL).

or similar hairless skin and a reliable parameter of skin irritation, such as cytotoxicity. A cytotoxicity test should be robust and detect cell death irrespective of the pathway of toxicity. The methyl-green pyronine (MGP) staining is a reliable predictor of toxicity. Organotypic skin explant cultures contain intact skin, of either human or similar (in the case of the pig) composition. This may be an appropriate model to assess skin irritants.

1.5 Risk assessment of contact allergens

Allergic contact dermatitis is a common skin disease that affects many people. ACD is a hypersensitivity reaction that can be induced by many different chemicals and substances. Contact dermatitis is caused by chemicals, which, for instance, are present in perfumes or soaps. ACD is best prevented by avoiding contact with the allergen. So it is important to know if a chemical has a sensitizing effect. In order to screen chemicals for a sensitizing effect, several research models are being developed or have already been developed. Since the aim is risk assessment for human, human allergen data is the gold standard. Human allergen data come from

two different laboratory tests. The human maximisation test (HMT) is a deliberate attempt to sensitize human against a chemical. After attempted sensitization the volunteers are challenged by a non-irritating concentration. Skin reaction is monitored after 24 and / or 72 hours after challenge. If there is a skin reaction then the compound is per definition an allergen. This test is only ethical if subsequent occupational exposure can be easily avoided for the volunteers, and if the safety of the chemical is determined. The non-natural synthetic chemical dinitrochloro-benzene (DNCB) is an example of a sensitizer detected by the HMT. The other source of human data are the human patch test allergens (HPTAs). HPTAs are chemicals to which some people have been sensitized 'naturally' due to occupational exposure. In order to be positive in the HPTA a sensitizer must be used by humans in such a way that accidental sensitization may occur. The metal nickel present in many metal alloys, and the perfume eugenol are examples of human sensitizers detected as HPTAs. All chemicals positive in either the HMT or the HPTA are considered to be human allergens.

Current animal models to assess allergens. Currently, two animal tests exist to assess contact allergens. Because different concentrations need to be tested, these tests use a lot of animals. The first is the guinea pig maximisation test of Magnusson and Kligman (GPMT). The GPMT is a deliberate attempt to sensitize guinea pigs. After attempted sensitization, a non-irritating concentration of the chemical is applied to the shaven skin of the animal. The animal is monitored for clinical symptoms, like redness of the skin after approximately 48 and / or 72 hours. The GPMT requires 24 to 32 guinea pigs to assess the sensitization potential of a single chemical. The GPMT predicts the answer to this 'yes or no' question with an accuracy of 73% [2].

The other validated animal test is the murine local lymph node assay (LLNA). The LLNA measures the proliferation of cells in the draining lymph nodes. A strong response of the immune system after contact with a sensitizer, will result in a substantial increase in the size of the draining lymph nodes and the number of cells in it. The LLNA requires 16 to 30 mice to assess the sensitization potential of a single chemical. The LLNA has a prediction accuracy of 72%, however false positive results obtained with various skin irritants are of great concern [2]. This may be related to the fact that the LLNA has no internal control to exclude skin irritants, and many contact allergens are only positive in the LLNA, when tested at irritating concentrations.

Computer model predictions for allergenicity. Computer simulation and prediction models: expert systems and quantitative structure-activity relationship (QSAR) models. Computer models are based on what is known about the chemical and biological basis of skin sensitization. With this knowledge, it becomes possible to predict the effect of a substance on a biosystem like the human skin. The chemical properties of a test chemical are compared to those of other chemicals known to be a sensitizer or not. Developed QSAR models can reduce the number of chemicals that need to be tested to some compounds per class, in stead of all compounds. It would thus classify as an important reduction alternative, but not as a replacement alternative. The need to test less chemicals will be a cost and effort reduction for both animal and alternative tests.

Testing allergens by cell culture. In vitro culture systems for dendritic cells. DCs can be generated in vitro from human peripheral blood monocytes. These DCs resemble Langerhans' cells, but are not identical to LCs [29]. After in vitro exposure to an allergen, DCs may be directly activated. The absence of functional skin in vitro requires the use of markers to assess DC activation. One of these markers is the upregulation of IL-1β mRNA, which can be detected by quantitative RT-PCR. [69-71]. Production of IL-1β mRNA by DCs has been suggested as the basis for an in vitro assay [72], however it was not found to be reproducible with DC derived from different donors [73]. This may be related to the fact that both IL-1β and TNF-α can activate LCs [74]. Haptens can directly activate DCs, causing the relocation of MHC II from cytoplasmic vesicles to the cell membrane, or the expression of co-stimulatory molecules [24,75]. However different haptens may activate DCs in different ways [76].

Activated DCs function to stimulate naive antigen-specific T-cells. This is the principle of the use of DC and T-cell co-culture systems. The read-out would be T lymphocyte blastogenesis or activation [77]. From a theoretical immunological point of view, activation of antigen-specific T lymphocytes in the naive repertoire would be an extremely rare event [19].

A third cell culture system is the culture of keratinocytes. The idea behind this test is that allergens and non-allergens, including skin irritants have different effects on keratinocytes. Different activation of keratinocytes could cause different cytokine production. But only for a few of related benzene-compounds an allergen-specific effect has been found [78,79].

Organotypic skin explant culture to screen for allergens. Both the sensitization and the elicitation of contact allergy are initiated in the skin *in vivo*. Thus organotypic skin explant cultures (OSECs) probably contain all cells and factors important early in the induction phase of ACD. LC migration is important in the induction and elicitation phase of ACD. Migration of epidermal LCs can be studies in human OSEC (hOSEC) [80,81], where they migrate out through lymphatic vessels [82]. Applying contact sensitizers topically on the skin in vivo accelerates LC migration out of the epidermis [21,31-34,83,84]. This also occurs in hOSEC, where compound-induced LC migration has been used as a predictive assay for contact allergens [83,84]. This LC migration is not dependent on previous sensitisation of the donor skin [84]. However, also skin irritants and accelerate LC migration in vivo [21,31-34,83,84], and possibly also in hOSEC. This means that it might be needed to exclude skin irritation in order to use LC migration as a predictive test for contact allergens.

Comparing model systems to assess allergens. Different tests to assess contact allergens have different advantages and disadvantages. In order to generate an overview of these, the data are summarized in Table 1.3. Computer models like QSAR are only a reduction alternative, as they cannot replace all animal testing. Animal models like the GPMT, MEST and LLNA cannot detect allergens, which only sensitize human, and have false positives with human non-sensitizers that sensitize guinea pigs. Animal ethics are a strong argument against animal testing techniques. Cell culture techniques miss certain cell types, and also important skin structures and barriers. Many allergens are small molecular compounds (haptens), and small molecular compounds are likely to penetrate the skin easily. Thus skin

penetration may indeed play an important role in contact sensitization. This may seriously limit their applicability to accurately assess contact allergens in cell culture. Therefore the OSEC models may have the potential to be best alternatives of all models compared.

Table 1.3. Alternative methods for assessing skin contact allergens

	Dendritic cells [a]	Keratinocytes [b]	OSEC [c]
Resource [d]	+	+	+/-
Lipophils [e]	-	-	+
New chemicals [f]	+	+	+
Skin barrier [g]	-	-	+
Metabolism [h]	-	+	+
Langerhans cells [i]	+/-	-	+
Keratinocytes [j]	-	+	+

[a] culture of Dendritic cells, derived from peripheral blood monocytes; [b] Skin reconstructed from keratinocytes cultured at the liquid-air interface; [c] Organotypic Skin Explant Culture; [d] Availability of resources; [e] Possibility to testing of water insoluble chemicals; [f] Suitability of model to test new chemicals; [g] Taking skin penetration in account; [h] Metabolism is possible in skin systems, also important for the detection of prohaptens; [i] Role of LCs in test system; [j] Role of keratinocytes in test system.

1.6 Animal skin as a model for human skin

Human skin is limited available as a waste product, but animal skin may be available in higher quantities. In order to be a functional replacement of human skin, animal skin should be very similar to human skin. But not every animal skin is equally suited be used as a model for human skin, since most animal skin differs from human skin in many ways. A model for human skin should be as close as possible to human skin with respect to skin morphology, histology, and permeability.

Human skin differs significantly from the skin of most animals, with respect to histological properties, which may be related to the absence of a fur (Figure 1.2). Rat epidermis for instance has a much more developed stratum granulosum than human and pig skin. The stratum corneum in guinea pig and rat skin is much thicker, and that of the mouse skin is much thinner than the human horny layer. On first sight the pink pig skin is a rare exception among mammals in that it resembles human skin. In contrast to the furry rodents, hair growth on pig skin is usually as sparse as that on human skin. A typical furry rodent skin has only two layers of epidermal cells, whereas the pink human and porcine skins have four to five keratinocyte layers. Also the presence of epidermal ridges is unique to human and porcine skin. It is suggested that these ridges may play a role in skin penetration. In histological sections, only pig skin closely resembles human skin [85]. It could be hypothesized that a dense hair coat might affect the accessibility of skin to chemical and other influences. Dense hair growth offers extra protection to the skin and affects differentiation of the skin. Density of the pelage could therefore be a disturbing factor in animal models for human skin. In line with this reasoning hairless rodents are used as models for skin research, i.e. hairless (not nude!) guinea pigs [86]. Skin penetration is similar in human and pig skin [87,88].

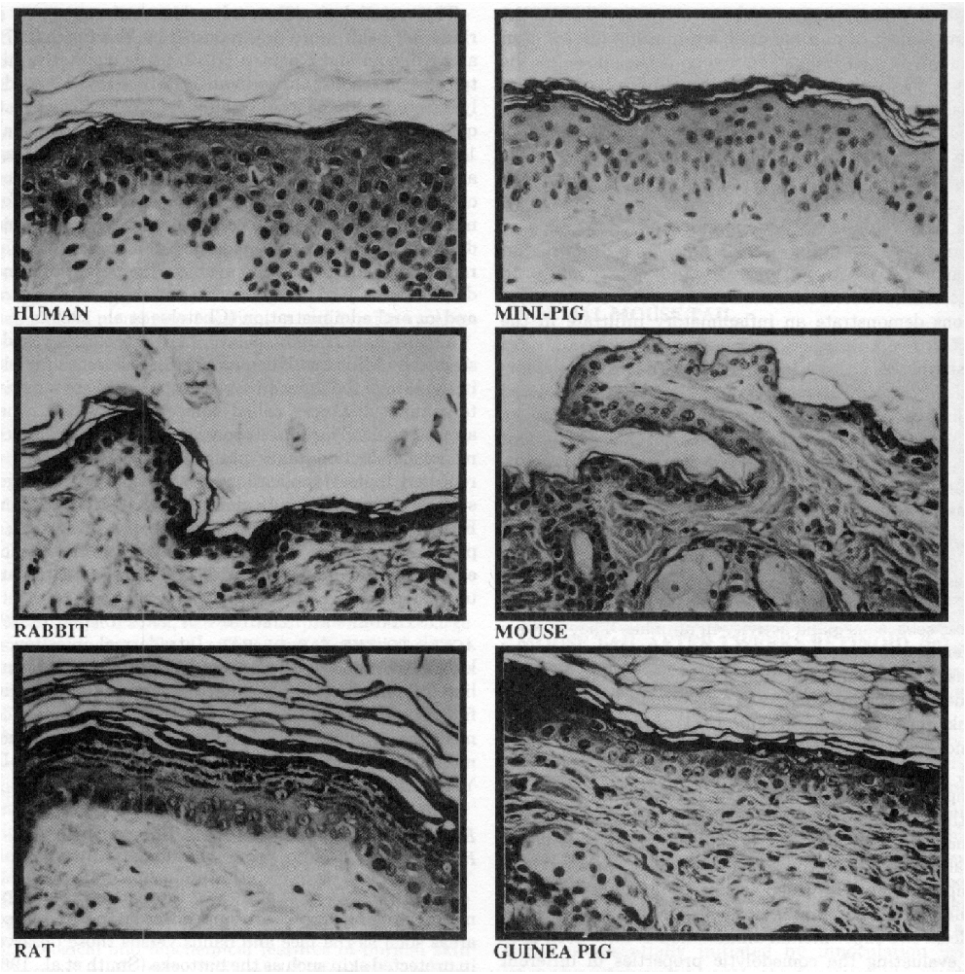

Figure 1.2.
Histological aspect of the skin from various species. Picture taken from ref. [86].

A minor difference between human and pig skin, is the absence of melanocytes in the latter. This might affect results of skin exposed to UV, e.g. for the assessment of photoirritants or photosensitizers. Based on histology, however, no real differences between human and pig skin in relation to assessment of skin irritants or contact sensitizers is expected. The skin has to protect the body from stresses from outside. For this purpose the skin contains a number of barriers, which look similar in pig and in man. Comparison of skin histology suggests that human and porcine skins are very similar. Also the presence of different types of fatty compounds in this barrier shows more similarity between human and porcine skin, than between human and rodent skin. Skin penetration experiments have shown that porcine skin has a similar permeability compared to human skin, at least for a number of compounds.
Genetically humans and pigs are fairly close, and this is reflected in the genes encoding for enzymes involved in skin metabolism. Most rodents differ considerably with humans in skin enzymes, their activity and / or substrate

specificity of these enzymes. However these porcine skin enzymes are mostly similar to those in human skin [89].
Another important feature is skin immunology. Small, preferably lipophilic compounds can penetrate the skin. Lipid antigens are presented by CD1 molecules [25] which are present on Langerhans cells of humans [90-94], cats [95], dogs [96], sheep [97], bovine, equine [98], guinea pigs [99,100], pigs [101] (unpublished data), but not on Langerhans cells of rats and mice, as these species lack type I CD1 molecules.

1.7 Contents of this thesis

The aim of this thesis is to develop alternative methods to assess skin irritants and contact allergens. These models will be developed using human OSEC. Also the possibility to use porcine OSEC to replace human OSEC for these assessments will be investigated. OSECs have the ethical advantage that the skins are waste products. So animals (and humans) do not suffer. Nevertheless they remain close to the *in vivo* situation, as skin structures are intact, resulting in a normal skin barrier function. Also the LCs are present in their normal habitat, suggesting that they will behave normally.
Proper assessment of skin irritants requires skin structures similar to human skin *in vivo* or in the OSEC. It is also important to assess toxicity by caused by different mechanisms. RNA is essential for all living cells, but quickly degraded after cell death. The methyl-green pyronine (MGP) staining allows the detection of RNA by pyronine in OSEC [102]. The application of this method is described in Chapter 2 and 3. Extensive studies show that the combination of OSEC-MGP is a robust method for screening skin irritants.
Assessment of contact allergens is *in vivo* only possible at non-irritating concentrations. The original *in vitro* protocol lacked a reliable method for assessing skin irritants, but this method is described in the previous chapters. After excluding skin irritants, LC migration may be used to assesss contact allergens. LC migration is calculated by counting LCs before and after culture, thus the counting of LCs should be accurate and reliable. An autmated method for counting LCs is described in Chapter 4. After excluding skin irritation and effects thereof, automated counting of LCs allows appropriate assessment of contact allergens (Chapter 5). These improvements result in reliable model for assessing contact allergens. The similarity of porcine skin to human skin for the assessment of skin irritants is shown in Chapter 2 and 3.
Alternative test methods need to be validated prior replacing animal tests. Validation means that a set of reference chemicals with known properties is tested in order to assay the validity of the new test method, with regard to reproducibility and predictive value. The predictive value of the alternative test will be compared with the predictive value of current animal tests. It is important that the known properties of the reference chemicals are established in a relevant system. Since risk assessment is most often required for humans, human data is thus the only relevant data. The animal test predictions should thus not be used as reference data when validating an alternative test. It is important that human data are the reference for both the alternative test and for the to be replace animal test.

Chapter 2

The use of porcine skin cultures to assess skin irritants.

Published with slight modifications as:
J.J.L. Jacobs, C. Lehé, K.D.A. Cammans, P.K. Das, G.R. Elliott
Screening of skin irritant by RNA detection as a viability marker in porcine organotypic skin explants.
Progress in the Reduction, Refinement and Replacement of Animal Experimentation eds. M Balls, Zeller, A.-M.v. & Halder, M.E. (2000). Elsevier Science B.V., Amsterdam, The Netherlands.601-607 pp.
and
J.J.L.Jacobs, C. Lehé, K.D.A. Cammans, P.K. Das, G.R. Elliott (2000).
Methyl green-pyronine staining of porcine organotypic skin explant cultures: an alternative model for screening skin irritants.
ATLA (Alternatives to Laboratory Animals) 28: 279-292.

Abstract

We describe a new alternative method for screening for irritants, using fresh intact porcine skin biopsies. Test chemicals were applied to the epidermis of skin biopsies that were then incubated for different times in tissue culture medium at 37ºC and 5% carbon dioxide. A decrease in epidermal keratinocyte RNA, visualized in frozen sections using a modified methyl-green pyronine staining procedure, was used as a marker of irritancy. If a decrease in keratinocyte RNA was observed after a 4-hours incubation (strong irritant) the chemical had a MGP score of 3, after a 24-hours incubation (moderate irritant) the MGP score was 2 and after 48-hours (weak irritant) the MGP score was 1. If no keratinocyte cytotoxicity was observed after a 48-hours incubation the chemical was classified as a non-irritant (MGP score 0). A minimum of 3 ears was used per chemical. The average MGP score was used to classify the chemical. Based on the MGP score for 20% sodium dodecyl sulphate (SDS), chemicals classified as strong or moderate irritants using the MGP test were grouped together as category R38 chemicals. Weak or non-irritants were none classified (NC). The MGP staining was able to correctly identified 23 of 25 skin irritants for which reference data were available.

2.1 Introduction

Chemical substances may be hazardous, e.g. by their potential to cause skin irritations. Regulatory guidelines, like those from the European Union [104], require that information on the irritancy of chemicals be provided for new substances and, increasingly, for existing substances. A large number of animals, especially rabbits, are still used to test the dermal irritancy of chemical compounds [1]. For ethical reasons the use of experimental animals experiments for skin irritation studies is not desired. Currently, several alternative methods for skin irritation testing are being developed. These methods include skin explant and human keratinocyte cultures [53,54]. In some cases the keratinocyte culture is differentiated *in vitro* to generate a reconstituted human skin [50]. The cells or skin are exposed to the putative irritant and cutaneous toxicity is measured to determine irritancy. Keratinocyte toxicity is usually measured by quantifying a reduction in the metabolism of dimethylthiazol diphenyltetrazolium bromide (MTT) or by leakage of neutral red from pre-loaded cells, or leakage of lactate dehydrogenase (LDH) [50,59-64]. However, these markers of cell toxicity have disadvantages. Some irritants, like sodium dodecyl sulphate (SDS), can enhance cellular and mitochondrial metabolic function, resulting in enhanced MTT metabolism [65] while other chemicals, such as cadmium chloride, can kill cells without disrupting cell membrane integrity [65]. The synthesis and release of cytokines has also been used as a biochemical marker of skin irritation. However, different skin irritants induce different patterns of cytokine synthesis [66] (and own unpublished observations)

The desired material for screening for irritant compounds *in vitro* is human skin. However, intact human skin is not routinely available in large quantities and we used intact porcine skin for these experiments. In contrast to rodent skin, porcine skin has been shown to be very similar to human skin morphologically, biochemically and immunologically [85]. In addition, the porcine ear skin used in these experiments is a waste product obtained from an abattoir. For these experiments we assumed that chemicals that were cytotoxic for epidermal keratinocytes were also irritants. The marker of keratinocyte vitality chosen was the presence of cytoplasmic RNA, which was visualized by methyl green-pyronine (MGP) staining [105-107].

In order to validate the MGP method we screened 41 chemicals (the 10 chemicals used in the ECVAM challenge of May 1998 were also included) and the results of our experiments were compared with available animal and human data. We conclude that the MGP method is a useful and simple alternative technique for screening for irritant chemicals.

2.2 Materials and methods

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; Pertex was obtained from Klinparth, The Netherlands; Acetone, benzalkonium chloride, ethyl acetate, hibitane, hydrochloric acid, methyl acetate, potassium chloride, silver nitrate, ethanol, sodium hydroxide and sodium oxalate were obtained from Merck; xylene was obtained from Solvesso Xylene, Exxon Chemicals Holland, Schiedam, The Netherlands (technical solution: 11% o-xylene, 49% m-xylene, 20% p-xylene, 19% ethyl benzene). CHAPS, cobalt (II) chloride, croton oil, 1-chloro 2,4-dinitrobenzene (DNCB), eugenol, isopropanol, mercury chloride, mineral oil, neomycin sulphate, nickel sulphate, nonanoic acid, nonidet P-40, potassium dichromate, salicylic acid, sodium dodecyl sulphate (SDS), trichloroacetic acid (TCA) and triton X-100 were obtained from Sigma. Dr. Lesley Earl supplied 20% dimethyldodecyl aminobetaine (DDAB, code name Empigen) Aminosilane (3-aminopropyltriethoxysilane) was obtained from Aldrich; culture media DMEM and F12, foetal calf serum and glutamax were all obtained

from Life science technologies; foetal calf serum was obtained from Sigma; methyl green was obtained from Fluka and pyronine was obtained from Merck.

Porcine organotypic skin explants cultures (pOSEC). Pig ears were obtained as a waste product from an abattoir. In the laboratory the pig ears were washed with tap water and 70% ethanol. Subsequently ears were decontaminated with 1% hibitane, which was removed using 70% alcohol. After disinfecting, sterile biopsies were cut of approximately 0.25 cm^2. These were placed dermal-side down in 200 □l culture medium in wells in 24-well plates. The epidermis remained above the medium/air interface. The culture medium consisted of DMEM : F12 (3:1), 10% foetal calf serum, and glutamax. The test chemicals were painted on the epidermis. Porcine organotypic skin explants were cultured for 4, 24 or 48 hours at 37^oC in a humid incubator in an atmosphere containing 5% CO_2. After the incubation the skin biopsies were embedded in Tissue-Tek® (O.C.T. compound, Sakura Finetek Europe B.V.), frozen in liquid nitrogen, and stored at -70^oC. Each single experiment for a chemical was done with 3 skin biopsies per ear per exposure time.

Methyl-green pyronine staining of frozen sections. The MGP staining of cryostat sections was a modification of the method of Moffitt [107]. Five □m thick cryostat sections were cut and dried onto aminosilano-coated slides [108] for 2 hours to overnight. Care was taken to avoid the introduction of RNAses e.g. from fingers. A fresh MGP-staining solution (0.5% methyl green, 0.1% pyronine in a 0.2 sodium-acetate buffer, $_pH$ 4.0) was made for each experiment. Stock solutions of 2% methyl green and 2% pyronine were stored for a maximum of 6 months after chloroform extraction. The MGP-staining solution was applied to the cryostat sections using a Pasteur pipette and incubated for 20 minutes at room temperature. The MGP was then poured off the sections that were then washed in tap water for 3 times 1 second. The sides of the slides were hit on paper to remove any remaining drops of water and the slides were subsequently dried using a blow dryer. After drying, the sections were embedded in pertex and evaluated using light microscopy, for the presence of RNA. Washing with water for more than 5 or 10 seconds dramatically decreased the staining intensity. We also found that the MGP-staining intensity and reproducibility was reduced if the sections were dehydrated using alcohol.. These modifications resulted in a very good reproducible MGP-staining protocol.

Examination of methyl-green pyronine stained cryostat sections. MGP stains DNA (nuclei) bluish green and RNA (cytoplasm) pink (Figure 2.1). Areas without RNA were considered to have suffered a toxic insult. The edges of biopsies were not included when sections were evaluated. When more than 75% of the epidermis of a biopsy was viable, the skin was considered to be viable. In other cases the epidermis was classified as non-viable.

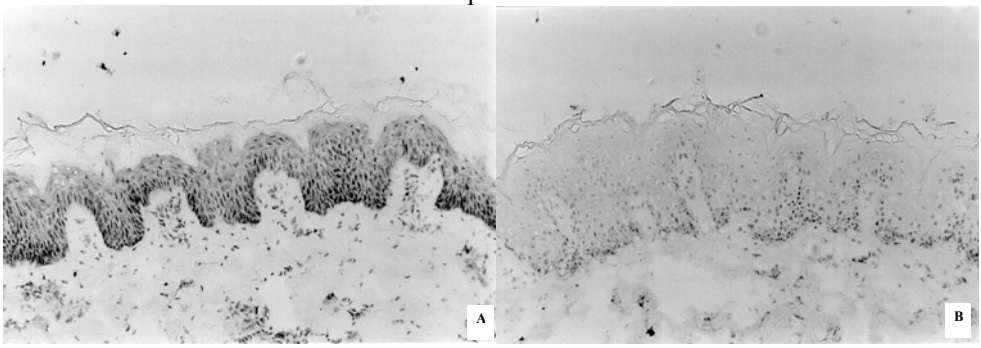

Figure 2.1. Photographs of methyl green-pyronine staining on cryosections. **A**: shows a cryostat section of skin with RNA present in the epidermis after 4-hours incubation (skin treated with iso-propanol). **B**: shows a section without RNA in the epidermis after 4-hours incubation (skin treated with decanol). Viable epidermal sections are pink; dead epidermal sections are white; both have blue nuclei.

2.3 Results

Visualization of the cytotoxic effects of irritants and its kinetics using MGP staining. MGP staining was performed after 4, 24 and 48-hours exposure to the test chemical. Pilot experiments showed that an 8-hour exposure revealed no additional information (data not shown). Cytotoxicity after 4, 24 and 48-hours exposure was interpreted as being due, respectively, to either a strong, moderate or weak irritant. When no cytotoxicity was observed the compound was considered a non-irritant.

MGP-scoring system. The time needed for the development of epidermal cytotoxicity was used to generate the MGP score. Chemicals inducing epidermal cytotoxicity after a 4-hours incubation were scored as 3. Cytotoxicity after a 24-hours incubation was scored as 2 and after a 48-hours incubation was scored as 1. We found that skin biopsies from different ears can have different kinetics of tissue damage when exposed to the same chemical. For this reason we used at leased 3 ears for each test compound. For each time point in we analyzed the results of triplicates for each ear. An average irritation potential was used to assess irritancy. In practice we generated an average based on the number of ears used as the replicates from the same ear gave uniform results. The method used is described in Tables 2.1 and 2.2.

Table 2.1. Calculation of the MGP scores

	Culture time			
	4 hours	24 hours.	48 hours	MGP score
Pig ear 1	V	V	D	1
Pig ear 2	V	V	V	0
Pig ear 3	V	V	V	0
Pig ear 4	V	V	D	1
Pig ear 5	V	D	D	2
Pig ear 6	V	D	D	2
Pig ear 7	V	V	D	1
Average (n = 7)				1.0

If no RNA could be detected in the epidermis after 4-hours of cultures the chemical was given a score of 3. If RNA was disappeared after 24-hours exposure the score was 2 and if a 48-hours incubation was needed before RNA disappearance the score was 1. The data presented were obtained with 5% SDS. Abbreviations used: V, viable; D, dead; n = number of different ears.

Table 2.2. MGP scores for ears from different pigs.

Chemical	n	MGP score of pig number						MGP score	
		#1	#2	#3	#4	#5	#6	average	SEM
Decanoic acid	3	3	3	3				3.0	0.0
Decanol	3	3	3	3				3.0	0.0
Octanoic acid	3	3	3	3				3.0	0.0
20% DDAB (aq.)	3	2	2	2				2.0	0.0
Methyl laurate	6	1	2	2	2	2	1	1.7	0.2
20% SDS (aq.)	6	1	2	2	2	2	1	1.7	0.2
Lauric acid	3	1	1	1				1.0	0.0
Methyl caproate	6	2	1	0	1	1	0	0.8	0.3
Isopropanol	3	0	0	0				0.0	0.0
Isopropyl palmitate	3	0	0	0				0.0	0.0
Methyl palmitate	3	0	0	0				0.0	0.0

Number of ears with indicated MGP scores. MGP scores 3, 2 and 1 are assigned to cytotoxic effects after 4, 24, or 48 hours, respectively. If there was no toxicity after a 48-hours incubation the ears were given a score of 0. SEM = standard error of mean.

Dose-response for SDS. The relationship between cytotoxicity and exposure time was investigated using 20% SDS, a concentration used by the EU as a standard for validating methods for screening for irritants [104,109,110]. As mentioned above, we found that the kinetics of epidermal cytotoxicity was not the same for each ear used. For example, in 4 out of 6 ears, 20% SDS was toxic after a 24-hours exposure period, while in the other two ears toxicity was seen after 48-hours (Table 2.3). Using the MGP-scoring system, a dose-response for SDS was calculated (Figure 2.2).

Validation of the pOSEC-MGP model for screening of irritant chemicals. The average MGP scored varied from 0 to 3. The classification of chemicals as R38 or NC was performed according to the EU guidelines [104,109,110]. These guidelines define 20% SDS as a minimal irritant. Chemicals that are significantly less irritating than 20% SDS are non-

Table 2.3. Dose-response for SDS.

SDS concentration	Total number of skins	Number of skins with MGP score: score 3	score 2	score 1	score 0	Average MGP score
20% SDS (aq.)	6	0	4	2	0	1.7
10% SDS (aq.)	3	0	3	0	0	2.0
5% SDS (aq.)	7	0	2	3	2	1.0
2% SDS (aq.)	6	0	0	3	3	0.5
1% SDS (aq.)	9	0	0	3	6	0.3
0.5% SDS (aq.)	4	0	0	0	4	0.0
0.2% SDS (aq.)	4	0	0	0	4	0.0
0.1% SDS (aq.)	4	0	0	0	4	0.0

Number of ears with indicated MGP scores. MGP score 3, 2 and 1 are assigned to cytotoxic effects after 4, 24, or 48 hours, respectively. If there was no toxicity after a 48-hours incubation the ears were given a score of 0.

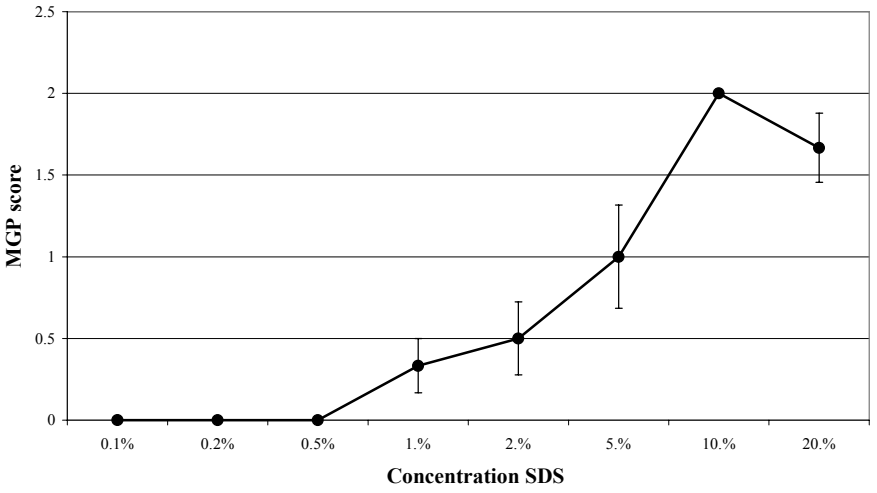

Figure 2.2. Dose-response sodium dodecyl sulphate with pOSEC-MGP score. Average MGP scores are shown for various concentrations of SDS in distilled water (+/- SEM). MGP = methyl green-pyronine; pOSEC = porcine Organotypic Skin Explant culture.

classified. For this reason we calculated the cut-off value for an irritant chemical as the average value for 20% SDS minus its standard error of mean , i.e. 1.7 – 0.2 = 1.5. We further divided the chemicals in 4 groups according to their MGP scores. Strong irritants were defined as having an MGP value of above 2.5. Moderate irritants had a score of between 1.5 and 2.4 whilst weak irritants had a MGP score of 0.5 to 1.4. If the score was less than 0.5 the compound was considered to be none irritant. For the purpose of classifying compounds for legislative reasons, we grouped strong and moderate irritants together as category R38 while weak and non irritants were NC. The results are listed in Tables 2.4a–d. The classification as R38 or NC, according to the MGP score, was compared with current classification based on various test systems. The MGP classification agreed with the consensus of the current classification for 23 of the 25 chemicals for which there was reliable experimental data (Table 2.5).

Dose-response relationship for skin irritants. With exception of mercury chloride, all irritants tested at more than 2 concentrations showed a dose-response relationship between irritancy and the concentrations of the chemicals used (Figure 2.3).

Table 2.4a. Overview MGP results for strong irritants, MGP score 2.5 - 3.0 (classified as R38).

Concentrations	Chemical	n	MGP scores	Estimated classification	Results other tests (references)
	Decanoic acid	3	3.0	R38	Table 2.5
	Decanol	3	3.0	R38	Table 2.5
20% (min.)	Eugenol	4	3.0	R38 ?	1–2% P.T.* [116]
10 % (min.)	Nonanoic acid	6	3.0	R38	10% ICD human [117]
	Octanoic acid	3	3.0	R38	Table 2.5
10% (min.)	Eugenol	4	2.8	R38 ?	1–2% P.T. [116]
5% (min.)	Nonanoic acid	4	2.8	NC ?	10% ICD human [117]
10% (aq.)	Benzalkonium chloride	3	2.7	R38	Table 2.5
5% (min.)	Eugenol	3	2.7	R38 ?	1–2% P.T. [116]
10% (aq.)	Sodium hydroxide	3	2.7	R38	Table 2.5
30% (aq.)	Trichloroacetic acid	3	2.7	R38	Table 2.5
1% (min.)	DNCB	2	2.5	R38	0.1 - 0.5% P.T.; 1% ICD [118]

Overview of all chemicals tested in the pOSEC-MGP model. Controls, ear skin cultured under medium (n=4) and the negative control (nothing applied) (n=9), were viable at each time point. n = number of experiments performed with different ears. min., solvent is mineral oil; aq., solvent is distilled water. sat. = saturated. ICD = causing irritant contact dermatitis. P.T. = concentration used in allergic patch test. *The concentration used in allergic patch tests is in general a high non-irritating concentration. Abbreviations of test chemicals, see materials and methods.

Table 2.4b. Overview MGP results for moderate irritants, MGP score 1.5 - 2.4 (classified as R38).

Concentrations	Chemical	n	MGP scores	Estimated classification	Results other tests (references)
10% (aq.)	Salicylic acid	3	2.3	NC ?	10% P.T.* [119]
5% (aq.)	Benzalkonium chloride	3	2.0	R38	Table 2.5
10% (aq.)	Cobalt chloride	3	2.0	R38 ?	1% P.T. [120]
3% (min.)	Croton oil	3	2.0	R38	0.8% optimal ICD [121]
20% (aq.)	DDAB (Empigen)	3	2.0	R38	Table 2.5
0.5% (min.)	DNCB	4	2.0	?	0.1 - 0.5% P.T.; 1% ICD [118]
1% (aq.)	Mercury chloride	3	2.0	R38 ?	0.01-0.1% P.T. [122,123]
0.3% (aq.)	Mercury chloride	3	2.0	R38 ?	0.01-0.1% P.T. [122,123]
0.1% (aq.)	Mercury chloride	3	2.0	NC ?	0.01-0.1% P.T. [120,122,123]
10% (aq.)	SDS	3	2.0	R38	Table 2.5

10% (aq.)	Trichloroacetic acid	3	2.0	?	30% (R38) 3% (NC)
5% (aq.)	Triton X-100	3	2.0	NC	Table 2.5
	Xylene	3	2.0	R38	
2% (min.)	Nonanoic acid	4	1.8	NC ?	10% ICD human [117]
	Methyl laurate	6	1.7	?	Table 2.5
20% (aq.)	Nickel sulphate (sat.)	3	1.7	R38 ?	5% P.T. [124]
20% (aq.)	SDS	6	1.7	R38	Table 2.5
1% (aq.)	Triton X-100	3	1.7	NC	Table 2.5
50% (aq.)	Neomycin sulphate	2	1.5	R38 ?	20% P.T. [124]
10% (aq.)	Nickel sulphate	4	1.5	R38 ?	5% P.T. [124]
1% (aq.)	Potassium dichromate	2	1.5	R38 ?	0.5% P.T. [120,124]

Legend, see Table 2.4a.

Table 2.4c. Overview MGP results for weak irritants, MGP score 0.5 - 1.4 (classified as NC).

Concentrations	Chemical	n	MGP scores	Estimated classification	Results other tests (references)
1% (min.)	Croton oil	5	1.4	R38	0.8% optimal ICD [121]
2% (aq.)	Benzalkonium chloride	3	1.3	?	5% (R38), 1% (NC)
1% (min.)	Nonanoic acid	4	1.3	NC ?	10% ICD human [117]
5% (aq.)	Cobalt chloride	4	1.0	R38 ?	1% P.T. [124]
0.5% (min.)	Croton oil	3	1.0	NC ?	0.8% optimal ICD [121]
0.2% (min.)	Croton oil	3	1.0	NC ?	0.8% optimal ICD [121]
2% (min.)	Eugenol	4	1.0	NC ?	1–2% P.T. [116]
	Lauric acid	3	1.0	NC	Table 2.5
5% (aq.)	SDS	7	1.0	NC	EC definition
0.1% (min.)	Croton oil	7	0.9	NC	0.8% ICD
	Methyl caproate	6	0.8	NC	Table 2.5
40% (aq.)	Neomycin sulphate	4	0.8	R38 ?	20% P.T. [124]
20% (aq.)	Neomycin sulphate	4	0.8	NC	20% P.T. [124]
10% (aq.)	Hydrochloric acid	3	0.7	?	Table 2.5
2% (aq.)	Salicylic acid	3	0.7	NC	10% P.T. [119]
5% (aq.)	Sodium oxalate	3	0.7	NC ?	low conc.
0.05% (min.)	Croton oil	4	0.5	NC	0.8% ICD [121]
0.2% (min.)	DNCB	4	0.5	NC	?0.1 - 0.5% P.T.; 1% ICD [118]
0.1% (min.)	DNCB	4	0.5	NC	0.1 - 0.5% P.T.; 1% ICD [118]
1% (min.)	Eugenol	4	0.5	NC	1–2% P.T. [116]
0.5% (aq.)	Potassium dichromate	4	0.5	R38 ?	0.5% P.T. [120,124]
2% (aq.)	SDS	6	0.5	NC	EC definition

Legend, see Table 2.4a.

Table 2.4d. Overview MGP results for non irritants, MGP score 0.0 - 0.4 (classified as NC).

Concentrations	Chemical	n	MGP scores	Estimated classification	Results other tests (references)
1% (aq.)	Benzalkonium chloride	3	0.3	NC	Table 2.5
	Methyl acetate	3	0.3	NC	Table 2.5
5% (aq.)	Nickel sulphate	6	0.3	NC	5% P.T. [124]
2% (aq.)	Nickel sulphate	6	0.3	NC	5% P.T. [124]
1% (aq.)	SDS	9	0.3	NC	EC definition
0.2% (aq.)	Triton X-100	3	0.3	NC	Table 2.5
0.05% (min.)	DNCB	4	0.3	NC	0.1 - 0.5% P.T.; 1% ICD [118]
1% (aq.)	Hibitane	4	0.3	NC	skin decontamination
2% (aq.)	Cobalt chloride	6	0.2	R38 ?	1% P.T. [124]
	Acetone	3	0.0	NC ?	solvent P.T. (Table 2.5)

4:1	Acetone : Olive oil	3	0.0	NC	solvent P.T.
3% (aq.)	Silver nitrate	3	0.0	NC	1-2% P.T.[120]
0.5% (aq.)	Benzalkonium chloride	3	0.0	NC	1%, Table 2.5
0.6% (aq.)	CHAPS	3	0.0	NC	unaggressive soap
1% (aq.)	Cobalt chloride	6	0.0	NC	1% P.T. [124]
0.01% (min.)	Croton oil	5	0.0	NC	0.8% ICD [121]
70% (aq.)	Ethanol	4	0.0	NC	Table 2.5
	Ethyl acetate	3	0.0	NC	Table 2.5
0.5% (min.)	Eugenol	3	0.0	NC	1–2% P.T. [116]
1% (aq.)	Hydrochloric acid	3	0.0	NC ?	moderate irritant [125]
0.1 % (aq.)	Hydrochloric acid	3	0.0	NC ?	weak irritant [125]
	Isopropanol	3	0.0	NC	Table 2.5
	Isopropyl palmitate	3	0.0	NC ?	Table 2.5
0.2% (aq.)	Potassium dichromate	4	0.0	NC	0.5% P.T. [120,124]
	Methyl palmitate	3	0.0	NC ?	Table 2.5
10% (aq.)	Neomycin sulphate	2	0.0	NC	20% P.T. [124]
1% (aq.)	Nickel sulphate	6	0.0	NC	5% P.T. [124]
0.5% (min.)	Nonanoic acid	3	0.0	NC ?	10% ICD human [117]
200 ppm (aq.)	Nonidet P-40	3	0.0	NC ?	low conc. soap
10% (aq.)	Potassium chloride	3	0.0	NC	salt solution
0.5% (aq.)	SDS	4	0.0	NC	EC definition
1% (aq.)	Sodium hydroxide	3	0.0	R38/NC	Table 2.5 [121,125]
1% (aq.)	Sodium oxalate	3	0.0	NC ?	low conc. soap
3% (aq.)	Trichloroacetic acid	3	0.0	NC	Table 2.5
	Aqua	10	0.0	NC	solvent, Table 2.5
10x	DPBS	3	0.0	NC	solvent
2x	DPBS	6	0.0	NC	solvent
1x	DPBS	6	0.0	NC	solvent
0.5x	DPBS	6	0.0	NC	solvent
	Mineral oil	8	0.0	NC	solvent

Legend, see Table 2.4a.

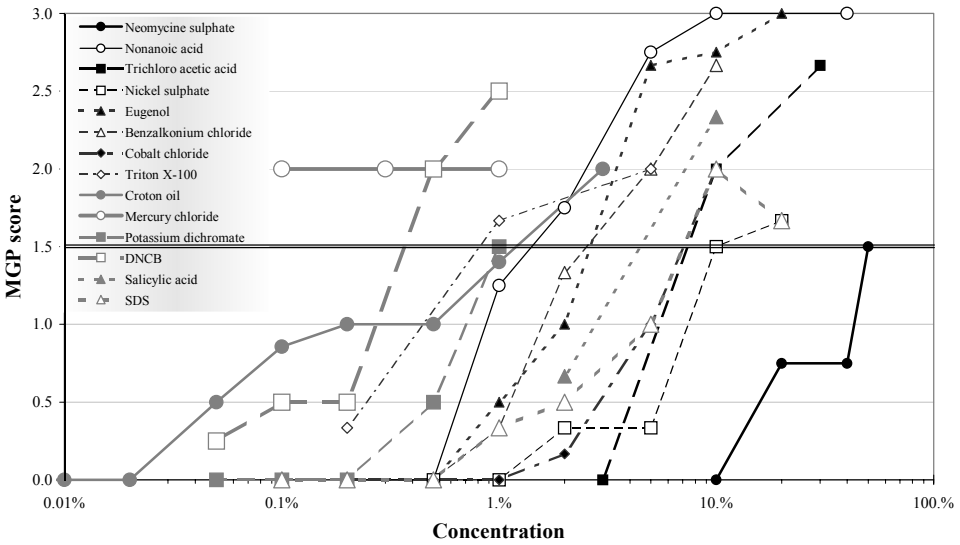

Figure 2.3. Dose-response relationship for 14 skin irritants. Skin irritants were selected which showed cytotoxicity in at least 2 different concentrations out of at least 3 concentrations, tested. The double line indicates the threshold of R38 classification.

Table 2.5. Comparison MGP with current classification methods.

Conc.	Chemical	MGP-score	EC	Human	HPT	Rabbit	PII	MMAS	consensus	Agree
Strong and moderate irritants		**1.5 - 3.0**							(R38)	**10/11**
STRONG IRRITANTS*		**2.5 - 3.0**							**R38**	**6/6**
	Decanoic acid*	**3.0**	R38	R38	$^{19\text{-}17}/_{19}$	R38/NC			**R38**	**Yes**
	Decanol*	**3.0**	R38	R38/NC	$^{11\text{-}17}/_{31}$	R38	3.33		**R38**	**Yes**
	Octanoic acid*	**3.0**		R38		R34			**R38**	**Yes**
10% (aq.)	Benzalkonium chloride	**2.7**	R34		$^{4\text{-}8}/_{27}$				**R38**	**Yes**
10% (aq.)	Sodium hydroxide	**2.7**						108	**R38**	**Yes**
30% (aq.)	TCA	**2.7**						106	**R38**	**Yes**
MODERATE IRRITANTS*		**1.5 - 2.4**							**R38**	**4/5**
5% (aq.)	Benzalkonium chloride	**2.0**						83.8	**R38**	**Yes**
20% (aq.)	DDAB (Empigen)*	**2.0**	R38	R38	$^{30\text{-}27}/_{32}$	R38			**R38**	**Yes**
10% (aq.)	SDS	**2.0**	R38						**R38**	**Yes**
5% (aq.)	Triton X-100	**2.0**						33.05	**NC**	**No**
	Methyl laurate*	**1.7**		NC		R38	3.89		**?**	**-**
20% (aq.)	SDS	**1.7**	R38				6.78		**R38**	**Yes**
Weak and non irritants		**0.0 - 1.4**							(NC)	**13/14**
WEAK IRRITANTS*		**0.5 - 1.4**							**NC**	**2/2**
	Lauric acid*	**1.0**		NC		NC	0.44		**NC**	**Yes**
	Methyl caproate*	**0.8**	NC	NC	$^{0\text{-}17}/_{29}$	NC	2.78		**NC ?**	**Yes**
10% (aq.)	Hydrochloric acid	**0.7**	R38		$^{6\text{-}23}/_{32}$				**?**	**-**
NON IRRITANTS[12]		**0.0 - 0.4**							**NC**	**11/12**
1% (aq.)	Benzalkonium chloride	**0.3**						45.3	**NC**	**Yes**
	Methyl acetate	**0.3**						39.5	**NC**	**Yes**
	Acetone	**0.0**	NC[$$]					65.8	**NC**	**Yes**
70% (aq.)	Ethanol	**0.0**						24	**NC**	**Yes**
	Ethyl acetate	**0.0**						15	**NC**	**Yes**
	Isopropanol*	**0.0**	NC	NC	$^{0\text{-}17}/_{31}$	NC	0.78	30.5	**NC**	**Yes**
	Isopropyl palmitate*	**0.0**	R38	NC	$^{0\text{-}17}/_{29}$	NC	1.44		**NC ?**	**Yes**
	Methyl palmitate*	**0.0**	NC	NC	$^{1\text{-}17}/_{29}$	R38	4.56		**NC ?**	**Yes**
1% (aq.)	Sodium hydroxide	**0.0**	R38	NC[##]	$^{20\text{-}4}/_{33}$[#,$]			25,8[#]	**R38 ?**	**No**
3%	TCA	**0.0**						6.7	**NC**	**Yes**
	Aqua	**0.0**	NC						**NC**	**Yes**

Chemicals are classified according their MGP scores (see Table 2.4a). Abbreviations: conc. = concentration. EC, Current EC classifications: R38 = irritant, R34 = corrosive, NC = not classified (not irritating), data from ref. [104,109] [57]. Human = human skin irritation data. Rabbit = rabbit skin irritation. Both human and rabbit skin irritation data are from [57]. HPT = 4-hours human *in vivo* patch test data [47]; (number of positive test chemical - number of positive SDS) / total number of test persons. PII = primary irritation index data [112]. MMASs = mean Modified Maximum Average Scores in Draize test for rabbit eye irritation [126]. Consensus = consensus of current classification methods is obtained by comparing EC, Human, HPT, Rabbit, PII and MMAS data; ?, indicates discrepancy in consensus. Agree, Agreement MGP scoring with consensus of current classification methods. * Chemical used in the ECVAM challenge May 1998. # 0.5% sodium hydroxide tested. ## Application of 2% sodium hydroxide did not result in ICD [121]. $ 1-hour human patch test. $$ Acetone is used as a solvent in patch tests, indicating that it is non irritant; Skin irritation by acetone is usually induced by rubbing acetone into the skin.

2.4 Discussion

We noted a wide variation in the response of skin explants from different ears to a chemical (Table 2.1). Such a wide variation also exists in human volunteers [17,111]. We propose therefore, that skin cultures from at least 3 different pigs should be evaluated before classifying a putative (non-) irritant. In contrast to the variation between ears, the response

of replicates to a particular chemical was reproducible. Analysis of duplicate biopsies is sufficient to give a reliable MGP score for any one ear.

In order to calculate an MGP score, an arbitrary scale was introduced based on the exposure time before loss of epidermal MGP staining was observed (Table 2.1). The EC directive considers a 20% SDS solution to be a borderline irritant [104,109,110,112]. The MGP score for 20% SDS was 1.7 ± 0.2 (SEM) (Table 2.1). For this reason a MGP score of 1.5 (1.7 – 0.2 = 1.5) was considered to be the threshold value for classifying chemicals as irritant (R38) or not (none classified) using the pOSEC–MGP test.

We found a good correlation between irritancy based on the MGP test and the generally accepted classification of the same chemicals as reported in the literature. The pOSEC–MGP model showed a good correlation (9/10 chemicals, Table 2.5) compared with human and rabbit data for the chemicals used in the ECVAM challenge [57]. Differences were only for chemicals having different effects in human and rabbit test systems. When the MGP scores were compared with human data only methyl laurate (NC in volunteers, R38 in the rabbit) with an MGP score of 1.7, equal to 20% SDS, was overclassified as a skin irritant. When the MGP scores were compared to the rabbit data, methyl palmitate was under classified as a non-irritant (NC in volunteers).

Different test systems to assess skin irritation potential may yield different results. In order to compare the pOSEC-MGP results with those obtained with other test, we compare our results with the consensus of these tests. The pOSEC-MGP results agreed with the consensus skin irritation classification, based on multiple tests, for 23/25 chemicals (Table 2.5). An exception was acetone, which was not a skin irritant in our pOSEC-MGP, but has been shown to be an irritant in various other test. However, acetone is only an irritant if rubbed into the skin. The pOSEC-MGP may also underestimate the irritation potential of dilutions of acids and bases. While 10% sodium hydroxide was classified as an irritant, 1% sodium hydroxide was not (Tables 2.4, 2.5). In the human patch test 0.5% sodium hydroxide induces a very strong irritant response after only a one hour exposure [47]. Strong acids ($_pH < 2.0$) or bases ($_pH > 11.5$) are considered to be irritants without testing [50]. However these prove difficult to detect *in vitro* and the irritation potential of acids and bases was also underestimated in the MTT assay performed at *in vitro* using differentiated human keratinocytes [113]. While most chemicals exert their irritation potential through killing keratinocytes, some (*i.e.* acids and bases) may be irritating by dissolving the *stratum corneum*, leading to barrier disruption [13]. Only at high concentrations, possibly when the *stratum corneum* is sufficiently dissolved, can the acid or base penetrate to the epidermis. Barrier perturbation undermines the most important homeostatic function of the skin, and may lead to irritant contact dermatitis through cytokine release [114]. This suggests that certain irritants, such as dilutions of corrosive compounds, could be tested for their irritating potential by assessing barrier disruption, *e.g.* by measuring trans-epithelial water loss (TEWL) [115]. Preliminary data obtained using pig ear skin indicate that the irritant effects of dilute solutions of acids and basis may be detected using an increase in trans-epidermal water loss as a parameter of disruption of the dermal barrier function (unpublished data).

Strong irritants are assessed in the pOSEC-MGP by causing cell death within 4 hours. Skin corrosion is assessed by direct loss of vital metabolism in cultured keratinocyte death within 3 minutes [56]. Thus, some compounds classified as strong irritants in the pOSEC-MGP may be corrosive chemicals (R34), causing *e.g.* tissue destruction (necrosis, burns). Certain strong irritants, *e.g.* 30% trichloroacetic acid or 10% benzalkonium chloride, lead to disappearance of keratinocyte nuclei. Further research will be required to fully examine the relation between *in vitro* strong irritants in the pOSEC-MGP model and skin corrosives *in vivo*. In summary, the pOSEC–MGP model is a promising new "animal-saving" system for screening skin irritants.

Chapter 3

The use of human skin cultures to assess skin irritants.

Published with slight modifications as:

J.J.L.Jacobs, C. Lehé, K.D.A. Cammans, P.K. Das, G.R. Elliott (2002).
An in vitro model for detecting skin irritants: methyl green-pyronine staining of human skin explant cultures.
Toxicology in Vitro 16: 581-588.

Abstract

We evaluated the potential of human organotypic skin explant cultures (human OSECs) for assessment of skin irritants. Test chemicals were applied to the epidermis of explants prior to incubation. Irritancy was assessed by detecting epidermal keratinocyte RNA using methyl-green pyronine (MGP) of cryostat sections. A decrease in epidermal RNA after a 4 h, 24 h or 48 h exposure equated to a MGP score of 3, 2, 1, respectively. Minimums of 3 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 ≥ 1.5 were classified as irritants (R38), other chemicals were not classified (NC).

The results obtained using human skin were compared with published data on human skin irritancy. The human OSEC predicted perfectly the irritation hazard of the 22 chemicals for which human patch test was available. The porcine OSEC and the cutaneous Draize test correctly predicted the classification of 21 of the 22 (95 %) and 15 out of 16 (94%) of the tested chemicals.

In conclusion, both the human and the porcine OSEC–MGP models are promising "animal-saving" models for screening skin irritants.

3.1 Introduction

Chemical substances may be hazardous, e.g. they could induce skin irritation. Regulatory guidelines, such as those from the European Union [104], require that information on the irritancy of chemicals be provided for new substances and, increasingly, for existing substances. A large number of animals, especially rabbits, are still used to test the dermal irritancy of chemical compounds [1]. For ethical reasons the use of experimental animals experiments 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 [58]. Proposed methods include (reconstituted) skin explant and human keratinocyte cultures [50,53,54]. The cells or skin are exposed to the putative irritant and cutaneous toxicity is measured to determine irritancy. Keratinocyte toxicity can be measured by quantifying a reduction in the metabolism of dimethylthiazol diphenyltetrazolium bromide (MTT), the leakage of lactate dehydrogenase (LDH) from cells or neutral red from pre-loaded cells [50,59-64]. 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 [65]. Although most alternative methods use MTT metabolism as an end point, additional endpoints may be needed to improve the sensitivity and specificity of these tests [58].

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 visualized using a modified methyl green-pyronine (MGP) stain [102,127]. In this article we demonstrate that human organotypic skin explant culture is a very good model for screening irritant chemicals correctly predicting the classification (based on published human data) of all 22 test chemicals used. In addition, there was a 95% agreement between the classification obtained using porcine and human skin. The Draize test correctly predicted 15/16 of the chemicals for which there is rabbit data. Both the Draize and pOSEC models incorrectly classified methyl laurate as an irritant.

We conclude that the MGP method is a simple, accurate, robust and reproducible alternative technique for screening for irritant chemicals and that both human and porcine skin can be used for this purpose.

3.2 Materials and Methods

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 supplied 20% dimethyldodecyl aminobetaine (DDAB, code name Empigen). Croton oil, DNCB, eugenol and nonanoic acid were dissolved in mineral oil; all other chemicals in aqua dest.. Aminosilane (3-aminopropyltriethoxysilane) was obtained from Aldrich; methyl green was obtained from Fluka and pyronine was obtained from Merck.

Organotypic skin explant cultures. The method used was based on that already described for porcine OSECs [102,127]. Human breast skin was obtained as a waste product of cosmetic surgery with the informed consent of the patient. Excess fat and connective tissue was removed from the skin that was then cut into squares of about 0.25 cm^2. The biopsies 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 Modification of Eagles Medium : Ham's F12 (3:1) and glutamax (all obtained from Life science technologies), supplemented with 10% foetal calf serum (obtained from Sigma). The

preheated (37°C) test chemicals were painted on the epidermis. Organotypic skin explants were cultured for 4, 24 or 48 hours 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 B.V.), frozen in liquid nitrogen, and stored at -70°C. Each chemical was tested using skin from at least 3 donors and in triplicate per donor.

Methyl-green pyronine (MGP) staining of frozen sections. The MGP staining of cryostat sections was a modification of the method of Moffitt [107] as described [102,127]. In brief, five 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 sodium-acetate buffer, pH 4.0) for 20 minutes at room temperature. The MGP was then poured off the sections that were then washed in tap water for 3 times 1 second. After drying in a blow dryer, the sections were embedded in pertex and evaluated using light microscopy, for the presence of RNA.

Examination 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 keratinocytes 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 sections were evaluated.

MGP-scoring system. MGP staining was performed after 4, 24 and 48-hours exposure of OSECs to the test chemical. The time needed for the development of epidermal cytotoxicity was used to generate the MGP score. Chemicals inducing epidermal cytotoxicity after a 4-hours exposure were scored as 3. Cytotoxicity after a 24-hours exposure was scored as 2 and cytotoxicity after a 48 h exposure was scored as 1. If there was no cytotoxicity after a 48-hours exposure the MGP score was 0. The EU guidelines define 20% SDS as a minimal irritant. Chemicals are classified as irritant (R38) or not (NC) if they are more, or less, irritating than 20% SDS [104,109,110]. Based on our results for 20% SDS using pOSECs, we set the MGP-score cut-off value for an irritant chemical at 1.5 [102,127].

3.3 Results

In order to facilitate the comparison of different methods, we refer in the results section to published pOSEC, Draize test and volunteer data.

Specificity of the hOSEC model. Using hOSEC, it proved possible to correctly predict the classification (based on volunteer data) of all 22 test chemicals used for this study (Table 3.1). The pOSEC model correctly predicted the classification of 21 of the 22 test chemicals whilst the Draize test predicted the classification of 15 of the 16 for which data was available. Both the Draize and pOSEC models classify 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 (regression analysis; slope = 0.93; $R^2 = 0.90$) (Figure 3.1).

Sensitivity of the hOSEC model. In order to obtain information on the sensitivity of the hOSEC model we performed dose-response experiments for four chemicals (croton oil, DNCB, nonanoic acid and SDS) using human and porcine skin (Figure 3.2). For these chemicals there was reference data for between 5 and 8 dilutions per chemical (25 dilutions in total). The hOSEC model correctly predicted the classification (compared to volunteer data) of 72% of all the chemical solutions and the pOSEC model 68%. These data indicated that even at borderline irritant concentrations, the OSEC models are suitable for predicting skin irritants. There were only minor differences in the classifications of the different chemical solutions generated using human and porcine OSEC. However, pOSEC was less sensitive than hOSEC to 1% and 0.5% croton oil and 5% SDS.

Table 3.1. Prediction skin irritants

Conc.	Chemical	MGP Human	MGP Porcine	in vivo Human	in vivo Rabbit	ref.
	Acetone	NC	NC	NC	NC	a
4:1	Acetone : Olive oil	NC	NC	NC	NC	a
	Aqua	NC	NC	NC	NC	a,b
10%	Cobalt chloride	R38	R38	R38 ?	-	c
3%	Croton oil	R38	R38	R38	-	d
20%	DDAB (Empigen)	R38	R38	R38	R38	e
	Decanoic acid	R38	R38	R38	R38	f
	Decanol	R38	R38	R38	R38	f
1%	DNCB	R38	R38	R38	-	g
	DPBS	NC	NC	NC	NC	a
70%	Ethanol	NC	NC	NC	-	h
20%	Eugenol	R38	R38	R38 ?	-	i
1%	Hibitane	NC	NC	NC	-	h
	Isopropanol	NC	NC	NC	-	b,e
	Isopropyl palmitate	NC	NC	NC	NC	b,e
	Lauric acid	NC	NC	NC	NC	e
	Methyl caproate	NC	NC	NC	NC	b,e
	Methyl laurate	NC	R38	NC	R38	e
	Methyl palmitate	NC	NC	NC	NC	b,e
	Mineral oil	NC	NC	NC	-	a
40%	Neomycin sulphate	NC	NC	R38 ?	-	j
20%	Nickel sulphate (sat.)	R38	R38	R38 ?	-	k
40%	Nonanoic acid	R38	R38	R38	-	l
	Octanoic acid	R38	R38	R38	R34	b
10%	Potassium chloride	NC	NC	NC	NC	m
1%	Potassium dichromate	R38	R38	R38 ?	-	n
20%	SDS	ref.	ref.	ref.	ref.	o
10%	SDS	R38	R38	R38	-	b,o
	correct predictions	22	21	22	15	
	total predictions	22	22	22	16	
	% right	100%	95%	100%	94%	

I, irritant; NI, non-irritant; h I, human I - porcine NI; p I, human NI - porcine I; NC, not classified (EU classification); R38, EU risk phrase skin irritant; ? doubtful classification; PT = patch test; generally a high non-irritant concentration is used. >PT, concentration is higher than used in allergic PT, and thus may be irritant. ICD, irritant contact dermatitis. SEM, standard error of mean; ref, references: a, solvent in PT; b, [49]; c, 1% cobalt chloride in PT [120]; d, 0.8% croton oil required for ICD [121]; e, [57]; f, R38 [49] R38/NC [57]; g, 0.1-0.5% DNCB in PT; 1% DNCB causes ICD [118]; h, used for skin decontamination; i, 1–2% eugenol in PT [116]; j, 20% neomycin in PT [124]; k, 5% nickel sulphate in PT [124]; l, 10% nonanoic acid causes ICD [117]; m, salt solution; n, 0.5% potassium dichromate in PT [120,124]; o, EC definition, minimal irritant; p, 5% SDS required for ICD [121].

The total predictive value of the OSEC models, using data from all chemicals and all dilutions of the test chemicals, was 95% for human skin and 91% for porcine skin (Table 3.2). The correlation between the results of both OSEC models was 91% (individual data not shown).
Reproducibility. There was no intra-donor variability with respect to the classification of a chemical as R38 or NC (data not shown), which is in agreement with data generated using porcine OSECs [102]. In contrast, there was inter-donor variation (Table 3.3). However, most test predictions based on the results of one biopsy per donor, in either OSEC, were correct.

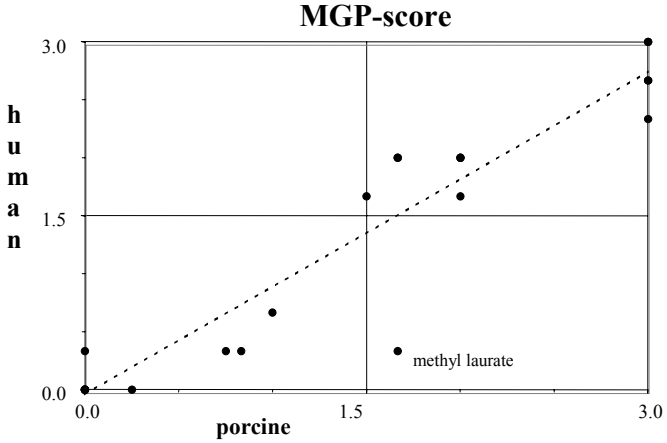

Figure 3.1. Comparing MGP score of pig and human. y = 0.9287x -0.0429. R^2 = 0.9003.

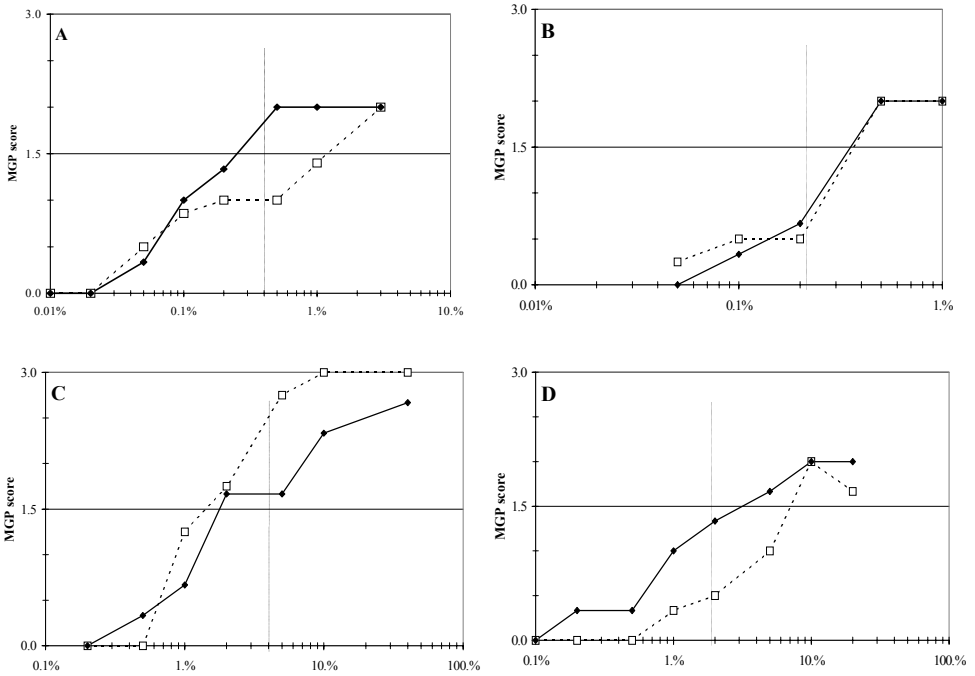

Figure 3.2. Comparison of dose-response for MGP score in human (solid lines and squares) and porcine (broken lines and open squares) OSECs. Dilutions are shown of (A) croton oil, (B) DNCB, (C) nonanoic acid and (D) SDS. The vertical dotted line indicates the approximate border between irritant (R38) and non-irritant (NC) concentrations.

The correlation with hOSEC and volunteer data, for all dilutions of test chemicals, was 93% and for pOSEC 88% (Table 3.4). The influence of inter-donor variation on the predictive value of the OSEC models was further investigated by determining how often the MGP score for replicates straddled the MGP-score cut off value of 1.5. This occurred for 16% of all chemical solutions tested using hOSEC and 20% using pOSEC (individual data not shown). However, this spread of results only resulted in two and four false classifications versus seven correct classifications in human and porcine OSEC (Table 3.5).

Table 3.2. Comparison human-porcine OSEC

Conc.	Chemical	human	porcine	T-test	MGP	class.	ref.
	Acetone	-	-	-	NI	NC	a
4:1	Acetone : Olive oil	-	-	-	NI	NC	a
	Aqua	-	-	-	NI	NC	a,b
10. %	Cobalt chloride	2.0	2.0	-	I	>PT	c
5. %	Cobalt chloride	2.0	1.0	-	h I	>PT	c
2. %	Cobalt chloride	2.0	0.2	p<0.01	h I	>PT	c
1. %	Cobalt chloride	-	-	-	NI	NC	c
3. %	Croton oil	2.0	2.0	-	I	R38	d
1. %	Croton oil	2.0	1.4	-	h I	R38	d
0.5 %	Croton oil	2.0	1.0	p<0.01	h I	NC	d
0.2 %	Croton oil	1.3	1.0	-	NI	NC	d
0.1 %	Croton oil	1.0	0.9	-	NI	NC	d
0.05 %	Croton oil	0.3	0.5	-	NI	NC	d
0.02 %	Croton oil	-	-	-	NI	NC	d
20. %	DDAB (Empigen)	1.7	2.0	-	I	R38	e
	Decanoic acid	2.7	3.0	-	I	R38	f
	Decanol	2.3	3.0	-	I	R38	f
1. %	DNCB	2.0	2.5	-	I	R38	g
0.5 %	DNCB	2.0	2.0	-	I	?	g
0.2 %	DNCB	0.7	0.5	-	NI	?	g
0.1 %	DNCB	0.3	0.5	-	NI	NC	g
0.05 %	DNCB	-	0.3	-	NI	NC	g
1x	DPBS	-	-	-	NI	NC	a
70. %	Ethanol	-	-	-	NI	NC	h
20. %	Eugenol	3.0	3.0	-	I	>PT	i
10. %	Eugenol	3.0	2.8	-	I	>PT	i
5. %	Eugenol	2.7	2.7	-	I	>PT	i
2. %	Eugenol	1.3	1.0	-	NI	?	i
1. %	Eugenol	-	0.5	-	NI	NC	i
0.5 %	Eugenol	-	-	-	NI	NC	i
1. %	Hibitane	-	0.3	-	NI	NC	h
	Isopropanol	0.3	-	-	NI	NC	b,e
	Isopropyl palmitate	-	-	-	NI	NC	b,e
	Lauric acid	0.7	1.0	-	NI	NC	e
	Methyl caproate	0.3	0.8	-	NI	NC	b,e
	Methyl laurate	0.3	1.7	p<0.05	p I	NC	e
	Methyl palmitate	-	-	-	NI	NC	b,e
	Mineral oil	-	-	-	NI	NC	a
40. %	Neomycin sulphate	0.3	0.8	-	NI	>PT	j
20. %	Neomycin sulphate	-	0.8	-	NI	NC	j
10. %	Neomycin sulphate	-	-	-	NI	NC	j
20. %	Nickel sulphate (sat.)	2.0	1.7	-	I	>PT	k
10. %	Nickel sulphate	2.0	1.5	-	I	>PT	k
5. %	Nickel sulphate	0.7	0.3	-	NI	NC	k
2. %	Nickel sulphate	-	0.3	-	NI	NC	k
1. %	Nickel sulphate	-	-	-	NI	NC	k
40. %	Nonanoic acid	2.7	3.0	-	I	R38	l
10. %	Nonanoic acid	2.3	3.0	-	I	R38	l
5. %	Nonanoic acid	1.7	2.8	-	I	NC	l
2. %	Nonanoic acid	1.7	1.8	-	I	NC	l
1. %	Nonanoic acid	0.7	1.3	-	NI	NC	l
0.5 %	Nonanoic acid	0.3	-	-	NI	NC	l
0.2 %	Nonanoic acid	-	-	-	NI	NC	l
	Octanoic acid	3.0	3.0	-	I	R38	b

10. %	Potassium chloride	-	-	-	NI	NC	m
1. %	Potassium dichromate	1.7	1.5	-	I	>PT	n
0.5 %	Potassium dichromate	1.3	0.5	-	NI	NC	n
0.2 %	Potassium dichromate	0.7	-	-	NI	NC	n
0.1 %	Potassium dichromate	0.3	-	-	NI	NC	n
0.05 %	Potassium dichromate	-	-	-	NI	NC	n
20. %	SDS	2.0	1.7	-	I	R38	o
10. %	SDS	2.0	2.0	-	I	R38	b,o
5. %	SDS	1.7	1.0	-	h I	R38	o,p
2. %	SDS	1.3	0.5	-	NI	NC	o,p
1. %	SDS	1.0	0.3	-	NI	NC	o,p
0.5 %	SDS	0.3	-	-	NI	NC	o,p
0.2 %	SDS	0.3	-	-	NI	NC	o,p
0.1 %	SDS	-	-	-	NI	NC	o,p
	right	52	50				
	wrong	3	5				
	score	95%	91%				

Legends see Table 3.1

Table 3.3. Interdonor variations in dose response SDS

		Donor number				
Concentration		#1	#2	#3	average	SEM
20%	SDS	2	2	2	2.0	0.0
10%	SDS	2	2	2	2.0	0.0
5%	SDS	2	2	1	1.7	0.3
2%	SDS	1	1	2	1.3	0.3
1%	SDS	2	1	0	1.0	0.6
0.5%	SDS	1	0	0	0.3	0.3
0.2%	SDS	1	0	0	0.3	0.3
0.1%	SDS	0	0	0	0.0	0.0

Table 3.4. Test performance by human and porcine OSEC at individual donors

Specificity (22 chemicals)	hOSEC	%	pOSEC	%
right	64	97%	86	91%
wrong	2	3%	8	9%
all	66		94	
Sensitivity (dose-response)	**hOSEC**	**%**	**pOSEC**	**%**
right	66	88%	89	81%
wrong	9	12%	21	19%
all	75		110	
27 chemicals at 55 concentrations	**hOSEC**	**%**	**pOSEC**	**%**
right	153	93%	214	88%
wrong	12	7%	28	12%
all	165		242	

3.4 Discussion

The data presented here clearly show that MGP staining of either human or porcine OSEC can be used to accurately predict the irritancy of a wide range of different classes of chemicals in humans (Table 3.1). The agreement between the irritancy classification based on *in vitro* tests and that obtained using volunteers was 100% for human skin and 95% for pig skin. The only false positive, methyl laurate, is also a false positive irritant in the rabbit Draize test. When all the data generated during the course of this study were analyzed (concentrated chemicals and dilutions), the hOSEC correctly classified 95% of solutions and the pOSEC 91%. The OSEC

models were also sensitive, correctly identifying irritant test solutions at dilutions only 2 fold more concentrated than those inducing an irritation in volunteers. In general, human skin *in vitro* appeared to be more sensitive to the cytotoxic effects of chemicals than porcine skin at lower dilutions (Figure 3.2a,d). However, the predictive power of porcine OSEC model is similar to that of human OSEC and at least equal to that of the Draize test.

Table 3.5. Risk assessment by triplicates of human and porcine OSEC

Specificity (22 chemicals)	hOSEC	%	pOSEC	%
right	20	91%	19	82%
inconsistent right	2	9%	3	14%
inconsistent wrong	0	0%	1	5%
wrong	0	0%	0	0%
all	22		22	
Sensitivity (dose-response)	hOSEC	%	pOSEC	%
right	18	72%	17	68%
inconsistent right	4	16%	4	16%
inconsistent wrong	2	8%	3	12%
wrong	1	4%	1	4%
all	25		25	
27 chemicals at 55 concentrations	hOSEC	%	pOSEC	%
right	45	82%	43	78%
inconsistent right	7	13%	7	13%
inconsistent wrong	2	4%	4	7%
wrong	1	2%	1	2%
all	55		55	

Test - right, the prediction of a test agrees with human patch test data; e.g. toxicity within 24 hours present or not present for chemicals labeled R38 or NC respectively; test -wrong, the prediction of a test does not agree with human patch test data; Chemical - right, all tests for a chemical have a 'right' prediction; Chemical - wrong, all tests for a chemical have a 'wrong' prediction; Chemical - discussion, discrepancy between the test results for a chemical, i.e. some are 'right' and others are 'wrong'.

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 the skin was obtained from different donors. A similar inter-donor variation has also been reported for pig ear skin [102] and a large variation in the dermal response to chemicals has also been reported for volunteers [17,111]. In order to minimize the chance of false-positive or negative results, each experiment was performed with skin from three donors. The influence of 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 16% of all chemical solutions tested using hOSEC and 20% using pOSEC. When replicates of the individual MGP scores lay just either side of cut off value it may be necessary to increase the number of donors used to obtain a more reliable MGP score. It should be emphasized that, with the exception of methyl laurate, false or inconsistent (triplicate results not all R38 or NC) classifications were only found using dilutions of chemicals that were close to being non-irritant in volunteer studies. In total, triplicate biopsies gave the same, correct, classification in 82% (hOSEC) and 78% (pOSEC) of all experiments performed (Table 3.5). These results indicate that the OSEC models can be used for the specific, sensitive and reproducible screening of irritant chemicals. It confirms that a classification as R38 or NC based on the MGP staining of hOSEC or pOSEC is robust sensitive and specific.
In conclusion, both the human and the porcine OSEC–MGP models are promising "animal-saving" models for screening skin irritants.

Chapter 4

Software-aided quantification of epidermal Langerhans cells.

Published with slight modifications as:
J.J.L. Jacobs, C. Lehé, K.D.A. Cammans, P.K. Das, G.R. Elliott (2001).
An automated method for the quantification of immunostained human Langerhans cells.
J. Immunol. Methods 247: 73-82.

Abstract

Allergic contact dermatitis is a frequent and increasing health problem. For ethical reasons, the current animal tests used to screen for contact sensitizers should be replaced by *in vitro* alternatives. Contact sensitizers have been shown to accelerate Langerhans cell (LCs) migration from human organotypic skin explant cultures (hOSECs) more rapidly than non-sensitizers and it has been proposed that the hOSEC model could be used to screen for sensitizers. However, chemical induced decreases in epidermal LC numbers need to be accurately quantified if the alterations in epidermal LC number are to form the basis of an alternative system for screening contact sensitizers in vitro. As manual counting of LCs is labour intensive and subjected to intra- and interpersonal variation we developed an image analysis routine, using the Leica QWin image analysis software, to quantify LCs *in situ* using immunohistochemically stained skin sections. LCs can be identified using antibodies against the membrane molecule CD1a or the Lag antibody, which recognizes cytoplasmic Birbeck granules. Quantification of epidermal LC number using the image analysis software had a much lower inter-person variation than when the same specimens were counted manually, using both the anti-Lag and CD1a antibodies. The software-aided quantification of epidermal LCs provides an accurate method for measuring chemical-induced changes in LC numbers.

4.1 Introduction

Allergic contact dermatitis is a frequent health problem. Contact allergens are currently screened using animal models, such as the guinea pig maximisation test (GPMT) and the murine local lymph node assay (LLNA). The assessment of the sensitisation potential of a single chemical requires 24 to 32 guinea pigs or 16 to 30 mice. The accuracy of both the GPMT and the LLNA for predicting human contact sensitisers is about 70%, [2]. Differences in the response of the immune system and skin morphology could account for part of the low efficiencies [85].

Immature Dendritic Cells (DCs), such as Langerhans cells (LCs) in the epidermis, take up antigen in the peripheral tissue [20]. After activation, e.g. induced by contact allergens, LC migrate to the draining lymph node and maturate [21-24]. Mature LCs or DCs stimulate the development of hapten-specific naive T cells leading to antigen-specific sensitisation [128-130]. The subsequent application of a contact allergen on the skin elicitates an allergic contact dermatitis [19].

Migration of epidermal LCs can be studied in human organotypic skin explant cultures (hOSECs), where LCs spontaneously migrate out through lymphatic vessels [80-82]. The topical exposure of hOSEC to contact sensitisers accelerates LC migration out of the epidermis, relative to spontaneous migration and the migration induced by control chemicals. This contact sensitizer induced acceleration of epidermal LC migration may be used as a screening system for contact allergens [83,84]. Manual counting of epidermal LCs is labour intensive and subjected to intra- and interpersonal variation. For these reasons we developed an image analysis routine using Leica QWin image analysis software that can be used to quantify LCs in immunohistochemically stained skin sections *in situ*.

4.2 Materials and methods

Human organotypic skin explant cultures (human OSECs, hOSECs). Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker, Verviers, Belgium), mineral oil, nickel sulphate, potassium dichromate and sodium dodecyl sulphate (SDS) (all Sigma-Aldrich Fine Chemicals BV, Zwijndrecht, The Netherlands) were preheated to 37°C, prior to application onto the skin. Human breast skin was obtained as a waste product of cosmetic surgery. Sterile biopsies were cut of approx. 0.25 cm^2, and these were dermal-side down incubated in DMEM : F12 (3:1) with glutamax (all from Life Technologies BV, Breda, The Netherlands) and supplemented with 10% foetal calf serum (Sigma-Aldrich Fine Chemicals BV, Zwijndrecht) [102]. Test chemicals were applied topically on the epidermis, using a cotton tip. Human organotypic skin explants were cultured for 24 or 48 hours at 37°C in a humid incubator under 5% CO_2. Each experiment was performed in triple using skin from at least three different donors. After the incubation, the culture medium was removed, and the cultured skin biopsies were embedded in Tissue-Tek® (OCT compound, Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), frozen in liquid nitrogen, and stored at -70°C.

Methyl-green pyronine (MGP) staining of frozen sections. Viability of hOSECs was confirmed using an MGP staining of cryostat sections, as we described before [102,127,131]. In brief, air-dried five □m thick cryostat sections were incubated for 20 minutes at room temperature in a fresh MGP-staining solution, 0.5% methyl green (Sigma-Aldrich Fine Chemicals BV, Zwijndrecht, The Netherlands), 0.1% pyronine (Merck KGaA, Darmstadt, Germany) in a 0.2 sodium-acetate buffer, $_p$H 4.0). Subsequently, the MGP was poured off the sections and the sections were washed, dried and embedded in pertex (Klinipath, Duiven, The Netherlands). Pyronine staining of cytoplasmic RNA (pink) in the epidermis was considered a marker of keratinocyte viability.

Immunohistochemistry. Immunohistochemistry was performed using standard methods on cryostat sections of hOSEC using antibodies for MHC II (HLA-DR), CD1a (All CLB,

Amsterdam, The Netherlands). Dr Kozo Yoneda, Dept. of Dermatology, Faculty of Medicine, Kyoto University, kindly provided the Lag antibody against LC's Birbeck granules [28]. Polyclonal secondary antibodies, rabbit-anti-mouse conjugated with horseradish peroxidase (HRP) and goat-anti-mouse conjugated with Alkaline Phosphatase (AP), were obtained from Dako A/S, Glostrup, Denmark. Rat monoclonal antibodies against mouse IgG1 (AP labelled) and IgG2b (biotin labelled), were obtained from Pharmingen, Woerden, The Netherlands. Avidin-HRP was obtained from (Bio-Rad Laboratories, Hercules, CA, USA. Enzymatic staining with either fast blue base (FBB) revealed antibody binding for AP and staining with 3,3-amino-9-ethyl carbazole (AEC) for HRP. After staining, slides were embedded in glycerine-gelatine and examined within 6 months.

Software-aided quantification of epidermal LCs. Leica Leitz microscope with 16x planapo objective (Leitz Wesler, Germany); CCD/RGB colour video camera (Sony, Japan); Matrox Meteor frame grabber (Matrox Electronic Systems ltd., Canada), and Leica QWin image processing and analysis system version 2.2a (Leica Imaging Systems Ltd, Cambridge, England). Images consisted of 764 x 574 pixels (1 pixel = 1.05 µm at magnification of 16x), each had one of 256 possible levels of red, green and blue. Two image analysis routines (macros) were developed in the Leica QWin program, Lag-blue.Q5R to count FBB-stained objects and Lag-red.Q5R to count AEC-stained objects (Figure 4.1). Image acquisition and analysis took about 5 minutes for each sample. The image analysis routines generate data about the number of epidermal LCs (eLCs), the surface of the epidermis measured (mm^2 epidermis), and the length of the epidermis measured (mm). These data are exported to a Microsoft Excel spreadsheet file. The number of epidermal LCs (eLCs) is calculated per mm horn layer length (eLC/mm) and per mm^2 epidermal surface (eLC/mm^2). The percentage stained is calculated by dividing the eLC area by the epidermal area (%eLC). The chemical-induced migration is calculated by comparing the chemical values with the solvent values. The Migration index (MI) is defined as changes in eLC/mm counts of solvent-treated skin versus chemical-treated skin: MI = 100% - (('solvent' - 'chemical') / 'solvent' * 100%).

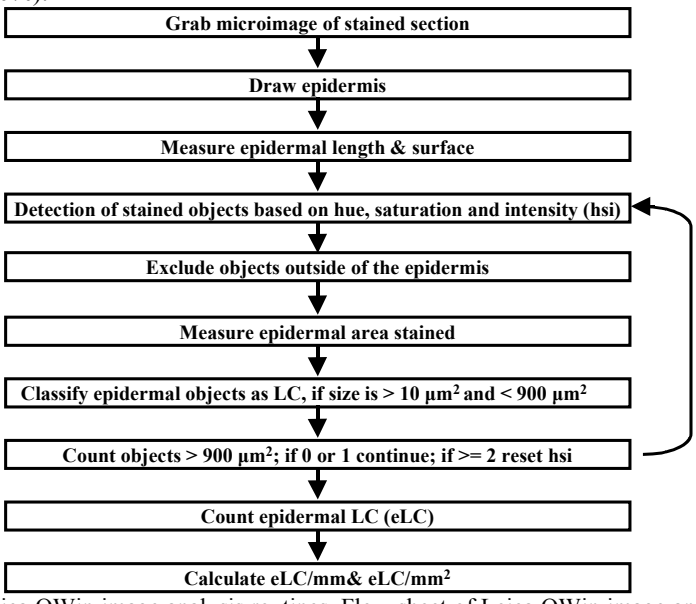

Figure 4.1. Leica QWin image analysis routines. Flow sheet of Leica QWin image analysis routines, lag-blue.Q5R and Lag-red.Q5R for counting epidermal LCs in immunohistochemical stained skin sections.

4.3 Results

Visual examination of eLC stainings. LCs were stained in cryostat sections using MHC-II, CD1a, or Lag antibodies (Figure 4.2a,b,c). MHC-II staining of human LCs was not specific as in some experiments all keratinocytes in the epidermis were MHC II positive (data not shown). For the studies reported in this article, skin LCs were defined as Lag^+ or $CD1a^+$. In practice, all epidermal and dermal $CD1a^+$ cells were Lag^+ and *vice versa* (data not shown). Lag stains the Birbeck granules, which are present in the LC body, and positive cells appear rounded with little branching. In contrast, CD1a stains both the cell body and the dendrites (Figure 4.2a,b). The LCs' dendrites form a network, also referred to as wire-netting [132] in the epidermis with which to capture antigen. This network is also known as the reticuloendothelial trap [20]. While Lag^+ LCs were relatively easy to quantify, due to the limited distribution of the antigen, CD1a positive cells were more difficult. In order to visualise individual $CD1a^+$ LCs, thin sections were cut, preferably < 5 μm and the contribution of LC dendrites was limited by introducing a minimum size for a LC. In addition, a control was introduced to identify erroneous counting of multiple groups of LCs as a single cell, by recognising objects above a certain size. The image analysis routines screened for objects larger than 900 μm^2 (oversized objects), and tolerated no more than one oversized object in every 100 analysed frames. When more than one oversized object was counted, the series of fields were re-analysed at less sensitive parameters for hue, saturation and / or intensity (Figure 4.1). These optimisations allowed a reproducible counting of CD1a stained LCs, albeit less reproducible than counting Lag stained LCs (Table 4.1).

Table 4.1. Difference in average of 6 measurements between different IHC experiments of the same biopsies

method	Lag stained	Lag versus CD1a
# eLC / mm epidermal length	21%	36%
# eLC / mm^2 epidermal surface	24%	35%
% eLC / epidermal surface	70%	79%

Relative difference between LC counts of 50 biopsies run in two independent experiments involving cryostat sectioning, immunohistochemistry and LC quantification on consecutive sections of the same biopsy. Lag, counts of LC stained with Lag; CD1a, counts of LCs stained with CD1a; eLC-

Reproducibility of LC counts. The reproducibility of the LC counts obtained using the Lag and CD1a antibodies were assessed using images recorded from five different histological sections for each antibody. Three analysts counted the numbers of LCs in the 10 different images manually, each image in triplicate. The intra- and inter-person variation for each antibody is given in Table 4.2. The same analysts also quantified the number of LCs in the same sections using the computer program. The intra- and inter-personal variations were considerably lower (Table 4.2).

Table 4.2. Differences between LC counts from the same five microscope images

	Lag stained LC		CD1a stained LC	
method	intrapersonal	interpersonal	intrapersonal	interpersonal
Leica	1.0%	1.8%	0.8%	1.1%
Manual	5.3%	15.9%	13.7%	28.8%

Difference between repeated LC counts from the same five microscope images. Manual counts: three different persons each conducted five independent counts.

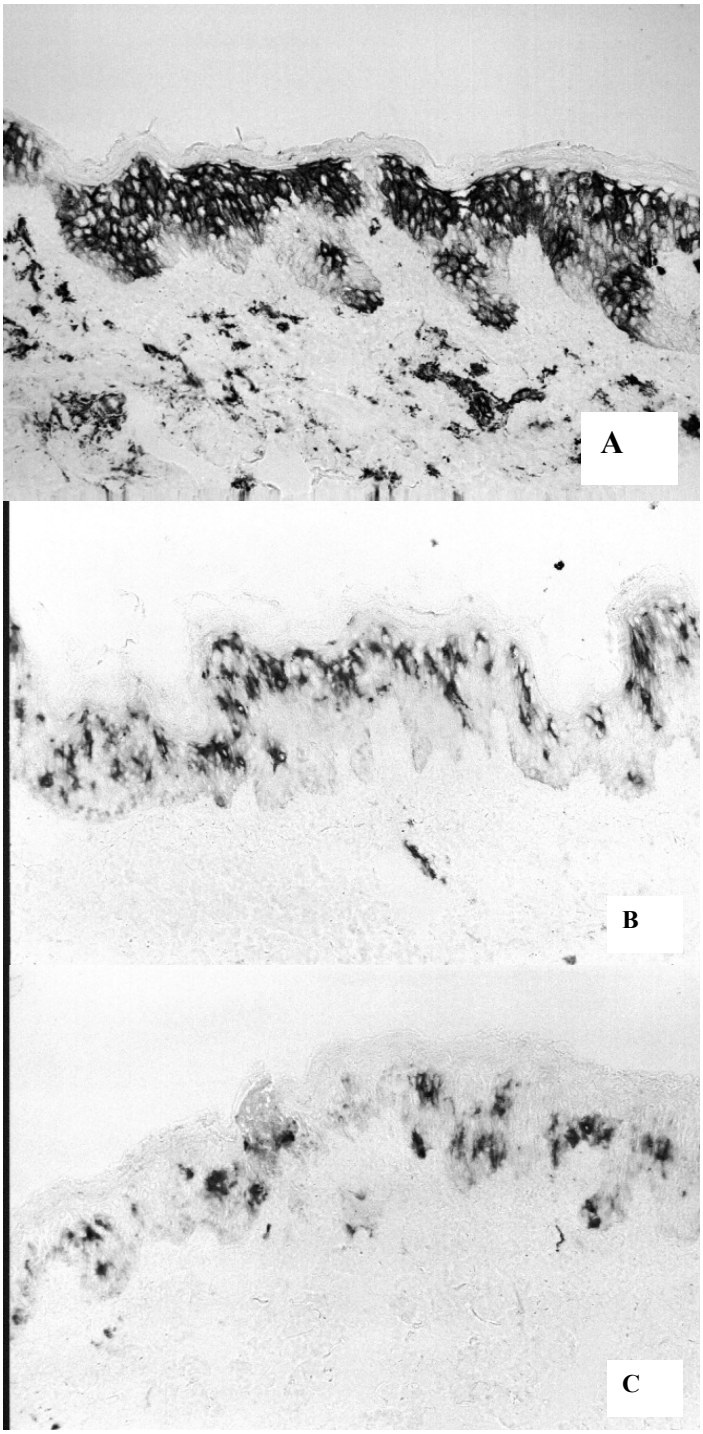

Figure 4.2.
Immunohistochemistry of epidermal Langerhans cells
Immunohistochemistry showing (A) MHC-II (B) CD1a, (C) Lag in the epidermis.

After comparing the variation of software-aided counts with those of the manual counts, we compared the average LC counts per biopsy. A comparison of manual and software-aided counts revealed a good correlation for the number of eLCs/mm *stratum corneum* (Figure 4.3). Similar results were obtained with FBB and AEC-developed sections, when manual counts were compared with software-aided counts. These result indicate that software-aided counts give comparable numbers of LCs but with a much lower variation.

The distribution of LCs within the epidermis varied from field to field within the same biopsy. In order to quantify this variation, the numbers of LCs ± S.D. were quantified in 50 biopsies with differing numbers of LCs (six different fields from the same biopsy). The average standard deviation (S.D.) for the number of LCs/biopsy was 41% and the average standard error of mean (SEM) was 17%.

A variation in LC count between sections from the same biopsy could also be caused by differences in immunohistochemical staining intensity of the LCs. In order to quantify the variation associated with the immunohistochemical staining, a series of biopsies (n = 50) with different numbers of LCs were cut and stained for LCs using the Lag antibody. Later, a new series of sections from the same biopsies were cut and stained. The average difference (SEM) in LC/mm between the 2 series was 21%. This is only slightly higher than the intra-biopsy variation in LC number of 17% (average SEM).

Method of LC quantification. LCs can be quantified per mm epidermal length, per mm^2 surface area or, alternatively, as a percentage of the epidermal surface area stained with a LC marker. The method chosen for quantification of epidermal LC can drastically influence the conclusions drawn from an experiment [133]. For example, epidermal length can be calculated at the level of the stratum corneum or the basal membrane. The latter parameter however, will be greatly influenced by the number and size of ridges [133]. For this reason LCs were counted per mm stratum corneum and the results compared with those obtained using when LC number was standardized with reference to the basal membrane. Both methods were reproducible and their results correlated well (Figure 4.4; Table 4.1). In

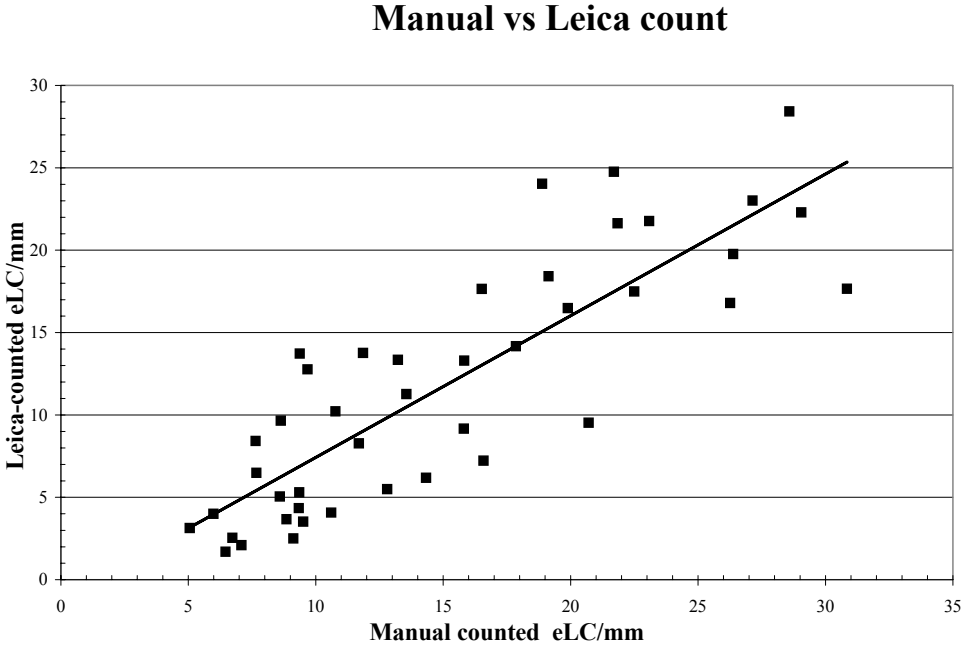

Figure 4.3. Manual versus software-(Leica Qwin) LC counts. y = 0.8601x - 1.1787. R^2 = 0.7127.

addition, the number of LCs/mm^2 epidermis was compared with the percentage of the epidermis surface area stained with LC markers. Although the results of these methods correlated well (R^2=0.8672, data not shown), there was a much larger variation when LC number was expressed as a percentage of the surface area stained with LC markers (Table 4.1).

Assessment of LC migration. Human OSEC were cultured for 24 hours with the test chemicals and the Lag positive epidermal cells quantified. Three experiments, each in triplicate using skin from different donors, were performed. The results presented are from one typical experiment. There was a spontaneous migration of LCs from biopsies during the 24-hours incubation, ranging from 10 to 50% (Table 4.3a). There was a small, but not significant, decrease in LC numbers when biopsies were treated with the vehicles used to apply the test chemicals (Table 4.3b). The number of LCs decreased statistically when incubated with SDS, $NiSO_4$ or $K_2Cr_2O_7$ (Table 4.3c).

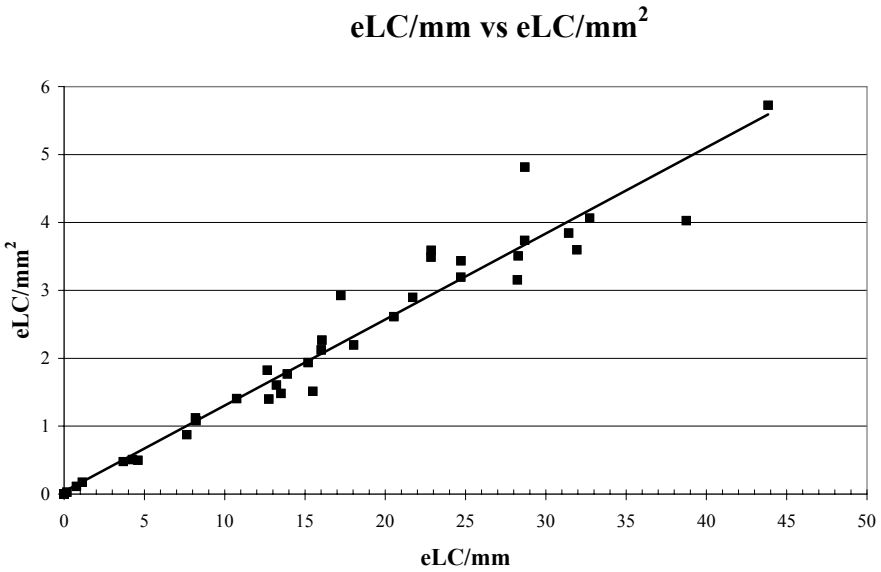

Figure 4.4. Epidermal LCs per epidermal length versus per surface area. y = 0.1267x + 0.0367. R^2 = 0.9544 .

4.4 Discussion

Detection of LCs. Epidermal LCs can be visualized using a number of unique markers. Adequate and accurate counting of LCs requires that these markers are stable and uniquely expressed on LCs. We stained LCs in cryostat sections with three antibodies, anti-MHC-II, CD1a and Lag (Figure 4.2a-c). LCs could be visualized using CD1a or Lag staining, and all, epidermal and dermal, LCs double stained for both CD1a and Lag markers (Data not shown). Our results are in agreement with *in vivo* data showing that LCs emigrating from human epidermis into the dermis remain Lag-CD1a double positive [134]. MHC-II staining of human LC is not a reliable method since immune modulation of the skin can induce MHC-II expression in keratinocytes, as has been shown in psoriasis, after UV-A irradiation and in graft-versus-host disease [135-137]. Moreover, in immunohistochemistry experiments, MHC II is not consistently detected on all CD1a$^+$ LCs [132]. We noted upregulation of epidermal MHC II in some experiments, without noticing an increase in epidermal LCs, as assessed by Lag and / or CD1a staining (data not shown). These data suggest that CD1a and Lag are suitable markers to detect LCs, and that MHC II is not reliable for this purpose.

Table 4.3. LC migration relative to various controls

	eLC/mm	SEM	% migration	M.I.	t-test
(A) LC migration relative versus 0-hours control					
hOSEC time					
0-hours control	43.8	6.4	ref.	ref.	ref.
24-hours incubation	29.1	4.3	34%	3.4	n.s.
(B) Vehicle-induced LC migration relative versus culture control					
Treatment					
Culture control	29.1	4.3	ref.	ref.	ref.
Aqua	20.5	2.4	29 %	2.9	n.s.
DABS	24.7	5.0	15 %	1.5	n.s.
Mineral oil	21.7	3.1	25 %	2.5	n.s.
(C) Chemical-induced LC migration relative versus aqua-solvent control					
Solvent control	*20.5*	*2.4*	*ref.*	*ref.*	*ref.*
SDS	9.3	5.4	55 %	5.5	$p < 0.05$
$NiSO_4$	4.6	1.4	78 %	7.8	$p < 0.001$
$K_2Cr_2O_7$	0.8	0.6	96 %	9.6	$p < 0.001$

Data are from one representive out of three experiments. eLC/mm = epidermal LC/mm epidermis calculated average of 6 counts. Student's t-test versus ref.; ref. = reference. n.s. = no significant effect; SEM= Standard Error of Mean; M.I., Migration index = 10* % LC migration.

Quantification of LCs. In order to quantify LCs in a reproducible manner, a number of factors were taken in account. The first one is the reproducibility of the LC counts within the analysed field. The reproducibility of manual counting greatly depends on the clarity of the staining pattern. The intra-person variation, for repeated counting of a field, was much lower when cells were stained with Lag (4%) than with CD1a (14%), which stained the cell body and the network of dendrites. The interpersonal variation was even larger, 16% for the Lag antibody and 29% for CD1a. By introducing software-aided LC counts, we reduced both in intra- and interpersonal variation to less than 1% and 2%, respectively. Secondly, the distribution of LCs within the epidermis of a biopsy is not uniform, and varies from field to field within the same biopsy. Using 40 to 50 biopsies, LCs were counted in six different fields from each biopsy. The average SEM was 17%. As the inter-experimental variation is below 2%, the variations in LC numbers are due to biological differences in LC distribution. Indeed, the site-to-site variation in LC number is responsible for most of the variation in LC counts between fields [91]. However, an additional factor, which could increase the variation in LC count, is the inter-experimental variation in staining intensity associated with immunohistochemistry. When this is minimised, by staining all sections simultaneously and cutting uniformly thin sections (5 μm), the inter-experiment variation (two experiments, n= 50 biopsies) was 21% (Table 4.1). This variation, due to biological and experimental uncertainties, is the inter-field variation that would be expected when calculating LC numbers in any two fields. In order to compensate for this variation, a difference in LC numbers between vehicle and chemical treated biopsies was only considered to be significant when at least 1/3 of the eLC had migrated from the epidermis. LC migration would be significant increased when comparing vehicle with chemical-treated skin biopsies.

Expression of LC number. The number of epidermal LCs (eLCs) can be expressed using different methods, *e.g.* eLC per mm *stratum corneum* length (eLC/mm), eLC per mm^2 epidermal surface area (eLC/mm^2) and as the percentage of the epidermis stained with an LC marker (% eLC). The relative counts for Lag stained LCs correlated very well between eLC/mm and eLC/mm^2 and both methods produced reproducible results (Figure 4.4; Table

4.1). Reproducibility, however, was very low when counting the percentage of surface area stained with LC marker, making this an unsuitable method for expressing LC numbers. We reasoned that during hOSEC, the length of the epidermis would be constant, but the epidermal cross-section surface might change due to chemicals that induce cell swelling or shrinkage [138]. Thus, expression of eLC/mm might be a better method when comparing LC numbers of chemical-treated and solvent-treated skin to assess LC migration.

Measurement of chemical-induced LC migration. Initial experiments showed that only very few, if any, LCs could be determined in non-viable sections as assessed by MGP staining. For this reason we quantified LC numbers in biopsies exposed to the highest non-toxic concentration, assessed after a 24-hours exposure. The spontaneous LC migration and its donor-to-donor variation were similar to the spontaneous migration described [80]. While application of the solvent vehicle did not enhance LC migration significantly (Table 4.3b), both sensitizers tested, $NiSO_4$ and $K_2Cr_2O_7$, caused a significant decrease in epidermal LC numbers (Table 4.3c). Both contact allergens are correctly classified when tested using the GPMT. However, nickel is classified as a non-sensitizer in the murine LLNA [2]. SDS is a non-sensitising skin irritant. However, it is wrongly classified as a sensitizer in the LLNA [2]. SDS also induced LC migration in vitro (Table 4.3c) and, at similar concentrations, it induces human LC migration to the draining lymph node in volunteers *in vivo* [34]. The reason for these false positive effects are not clear but recent experiments with SDS and sensitizers indicate that the sensitivity and specificity of the hOSEC model can be greatly improved by using concentrations of the test chemicals that non-toxic after longer exposure periods.

Summarising remarks. In conclusion, an automated analysis routine was developed for quantifying epidermal LCs cells in skin sections stained for Lag and CD1a using immunohistochemical techniques. Both antibodies give similar results (Figure 4.5), but as shown before CD1a staining results in higher variation. Besides its use in quantifying LCs for screening sensitizers, the procedure may be helpful in assessing LC numbers and migration in skin diseases such as atopic dermatitis, psoriasis, virus infections, tumours and UV-induced immune suppression.

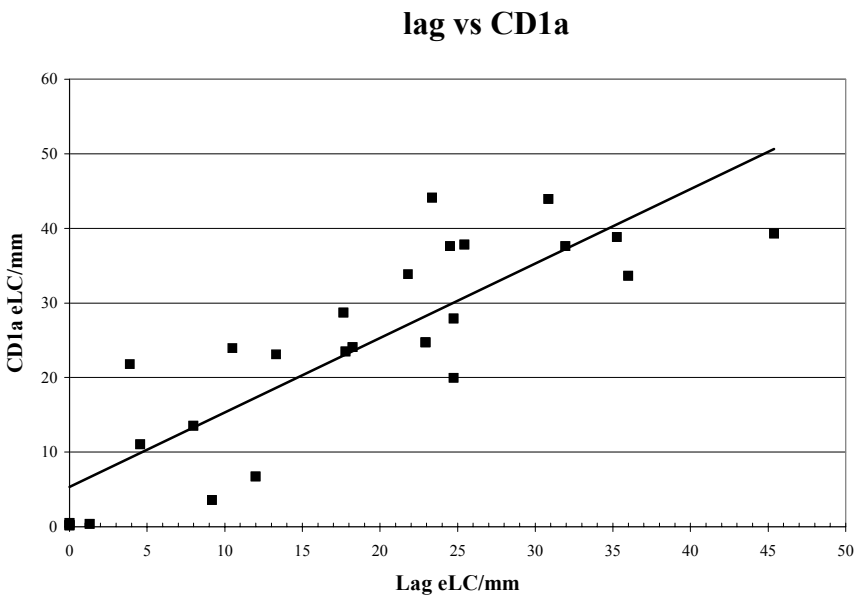

Figure 4.5. Counts of LCs stained with Lag versus CD1a. y=0.9976x+5.3389; R^2=0.7367.

Chapter 5

A two centre evaluation of the human organotypic skin explant culture model for screening contact allergens.

In press with slight modifications as:
C.L. Lehé, J.J.L. Jacobs, G.R. Elliott, P.K. Das.
A two centre evaluation of the human organotypic skin explant culture model for screening contact allergens.
ATLA (Alternatives to Laboratory Animals)

Abstract

Animal models are the gold standard to determine the potential contact allergenicity of small molecular weight chemicals. However, governmental regulations and ethical objections restrict the use of animals for such purposes. There is a need, therefore, for in vitro alternative models. The human organotypic skin explant culture model (hOSEC) has been reported to be promising for the predictive testing of contact allergens. The accelerated migration of Langerhans cells out of the epidermis, upon treatment with contact allergens, is used to identify potential chemicals capable of inducing a delayed type hypersensitivity. In this study, the model is further refined and used in two independent laboratories to screen 23 small molecular weight compounds of known classification for their allergenicity. Both laboratories were able to accurately detect the contact allergens despite small differences in the protocols used. However, the classification of dermal irritants, which were often falsely classified as allergens, varied between the laboratories. Despite the current limitations in the hOSEC model, the accuracy of the predictions made (sensitizer or not) compare favourably to classifications obtained using commonly used animal models. The hOSEC model has the potential to be developed further as an in vitro alternative to animal models for screening for contact allergens.

5.1 Introduction

Chemically reactive compounds of small molecular weight, often present in daily household, cosmetic and industrial products, can cause an irritant contact dermatitis (ICD) and an allergic contact dermatitis (ACD). An ACD is a delayed type hypersensitivity (DTH) reaction initiated by pre-sensitization of the host by small molecular weight chemicals called haptens whereas an ICD is a non-immunologic inflammatory reaction triggered by a toxic insult to epidermal cells [139-142]. Since many chemicals can potentially induce either ICD or ACD, European regulations require that any new chemical must be screened for irritancy and/or allergenicity (sensitizing) properties before they are introduced into marketable products [143].

The development of an ACD after topical exposure to a hapten consists of two distinct physiological process, the sensitization and the elicitation phases. The sensitization phase is primarily dependent on epidermal Langerhans cells (LC), immature dendritic cells which form a cellular network in suprabasal layers of the epidermis [144,145]. LC trap sensitizers penetrating through the skin, process them intracellularly and express them as haptens on the cell surface. Haptenated LCs become activated and migrate from the skin to the regional lymph node where they present the hapten to naïve T cells. Activation of T cells, either in the draining lymph node or in the skin after a re-challenge, forms the basis of all the *in vivo* animal models developed for discriminating between contact sensitizers and irritants [146-150]. The animal models detailed in the OECD test guidelines 406, are the guinea pig maximization test (GPMT) [146,148] and the murine local lymph node assay (LLNA)[149]. These models require large numbers of animals and are expensive. Moreover, ethical objections have lead to governmental restrictions on the use of animals for the predictive testing of potential sensitizers.

Ideally, screening of the contact sensitization potential of chemicals should be performed in the species of interest in order to exclude problems introduced by cross-species extrapolation. However ethical considerations restrict the use of the human patch test allergen (HPTA) and the human maximization test (HMT) [147,151]. Following the principal proposal by the European Centre for the Validation of Alternative Methods (ECVAM) [152] several novel approaches have been developed, for example the analysis of quantitative structure activity relationships (QSAR)[153,154] and skin cell culture systems [155]. Skin cell systems have the disadvantage that they do not reproduce either the full barrier function of the skin, an important parameter in determining the rate at which a chemical comes in contact with LCs, or the natural environment of the LCs. In addition, it can be difficult to screen some types of formulations, such as ointments and creams. For these reasons the human organotypic skin culture model (human OSEC; hOSEC) was developed for determining the allergenic potential of low molecular weight chemicals *in vitro* [81,84]. Human OSEC uses full thickness healthy human skin with an intact stratum corneum and allergen-accelerated migration of LCs from the epidermis is used as a measurement of allergenicity [84,156]. LC characteristically express CD1a and Langerin and these markers can be used to visualize them immunohistochemically [145]. As dermal irritants can also accelerate LC migration it is important to use concentrations of chemicals which are non-irritant under the conditions of the experiment. In this study we report the results obtained when OHSEC was used to independently screen 23 chemicals of known classification (contact allergen or dermal irritant) at two centres. The results demonstrate that the method can reliably detect contact allergens although dermal irritants can give false positive results. Thus, the hOSEC model needs further refinement before it can be used to reliably discriminate between contact sensitizers and dermal irritants. However, even in its present form it was as good as the GMT and the LLNA in predicting allergenicity in humans.

5.2 Materials and Methods

Collaborating centers. The two centres participating in this study were:- centre I; the Department of Pathology, Amsterdam Medical Center/University of Amsterdam and centre II; the Department of Pharmacology, Prins Maurits Laboratorium-TNO.

Human organotypic skin explant culture (hOSEC) model. Discarded fresh human adult skin was obtained with the informed consent of patients undergoing either mammary reduction - or abdominoplasty. The method of Pistoor et al [84] was used with modifications at centres I and II and is explained briefly below. *Centre I*: Briefly, 0.5mm thick split-skin sheets were prepared using a dermatome (Aesculap-wagner GB231R). Explants were prepared using a 6 mm punch biopsy (Stiefel laboratories, Germany) and incubated on nitrocellulose filters (Millipore, pore size 1.2 μm) placed on stainless steel grids in 6 well culture plates (Costar). Each well contained Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% pooled complement inactivated normal human serum (CLB, Amsterdam, the Netherlands) penicillin (100 U/ml) / streptomycin (100 μg/ml) (Gibco/Brl). Explants were cultured for 24 hr at 37^{o}C in 5%CO_2 humidified air at air liquid interface, epidermal side up. *Centre II*: At centre II the method was as described by Jacobs et al [157]. Briefly, fat and connective tissue were removed from the skin using scissors and explants of 0.25 cm^2 were prepared. These were placed dermal-side down into 300 □l culture medium in a 24-well plate with the epidermis above the air-liquid interphase. The culture medium consisted of DMEM: HAM F12 (3:1), 10% foetal calf serum, and 1% glutamax. Skin explants were cultured for 24 hr at 37^{o}C in a humid incubator under 5% CO_2. At both centres each chemical was evaluated in triplicate for each donor, using skin from at least 3 different donors (n = ≥ 3). Cultured biopsies were harvested and either fixed in formalin for paraffin embedding, or embedded in Tissue-Tek® (OCT compound, Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) snap frozen in liquid nitrogen and stored at -80^{o} C.

Chemicals. The chemicals were applied topically using cotton tips. At both centres hydrophilic compounds were dissolved in distilled water. Hydrophobic compounds were dissolved in acetone / olive oil (4:1) at centre I and in mineral oil at centre II. The choice of solvent did not modify the toxicity of the test compounds used in this study (data not shown). *Vehicles.* Dulbecco's phosphate-buffered saline (PBS)(BioWhittaker, Verviers, Belgium); olive oil (Bertolli classic, Rotterdam, The Netherlands); acetone (Merck, Amsterdam, The Netherlands) and mineral oil (Sigma-Aldrich, Zwijndrecht, the Netherlands). *Irritants.* sodium dodecyl sulphate (SDS); croton oil and nonanoic acid are obtained from Sigma-Aldrich. *Contact allergens.* α-hexylcinnamaldehyde; eugenol; methacrylic acid (MMA); 1-bromohexane; 2,4-dinitrofluorbenzene (DNFB); potassium dichromate ($K_2Cr_2O_7$); neomycin; oxazolone; 2-methyl-4,5-trimethylene-4-isothiazolin-3-one (kathon c.g); hydroxy citronellal; 1-bromo-pentadecane; 2-hydroxyethylmethacrylate (HEMA), 2-mercaptobenzothiazol and benzalkonium chloride all obtained from Sigma-Aldrich. Nickel sulphate ($NiSO_4$. 6 H_2O) and cobalt (II) chloride ($CoCl_2$. 6 H_2O) were both obtained from Merck. Classification of the chemicals was based on previous literature; where discrepancies existed between animal- and human classifications the latter was used as the reference classification.

Determination of the highest non-toxic Concentration. The highest non-toxic concentration (HNTC) for each chemical was determined before analyzing allergenicity in order to eliminate irritant (toxic) effects on LC migration. *Centre I*: Three staining techniques were used to screen for toxicity. Histomorphology analysis of the explant was studied from HE stainings, wherein e.g. vacuolization of epidermal cells and epidermolysis were considered parameters for toxicity. Furthermore, paraffin sections stained with methyl green pyronine (MGP)[107] and cryostat sections stained for lactate dehydrogenase (LDH) activity [158] were used to asses toxicity. MGP stains DNA (nuclei) greenish blue and RNA (cytoplasm) red absence of RNA indicated the explants are non-viable due to toxic insult. LDH activity is

demonstrated by a brown formazan precipitate of a Tetra Nitro Blue Tetrazolium salt (TNBT) absence of a formazan precipitate indicated a toxic effect. A given concentration of a chemical was scored as toxic when LDH and RNA was absent from 2 of 3 biopsies for each donor. A maximum test concentration of a chemical at which all three stainings, HE, MgP and LDH, showed no toxicity was considered the HNTC. At least 3 donors were used to determine the HNTC for each chemical. *Centre II*: The toxicity of each concentration of a chemical used was determined at the end of the 24 hr exposure period using a modified MGP staining [102,127]. Briefly, 5µm thick cryostat sections were cut and dried. The sections were incubated for 20 minutes at room temperature in a freshly prepared MGP-staining solution (0.5% methyl green, 0.1% pyronine in a 0.2 sodium-acetate buffer, $_{p}$H 4.0). Subsequently, the MGP was poured off the sections; the sections were washed 3 times for about 1 second in tap water dried, mounted and then evaluated using light microscopy. When approximately more than 25% of the epidermal cells were dead (MGP negative) the chemical, at that concentration, was considered to be toxic. When sections from 2 of 3 donors were MGP positive the chemical, at that concentration was considered to be non-toxic. The HNTCs, determined at centre II using only the MGP stain, were the same as those determined at centre I. The HNTC and half the HNTC (50% HNTC) were used at both centres to study chemical-induced LC migration.

Immunohistochemistry. Cryo-sections from the skin explants were stained for the presence of $CD1a^+$ and $HLA\text{-}DR^+$ LC cells. *Centre I*. A three step method was used. Briefly, sections were first incubated with the primary mouse monoclonal antibody against CD1a (Becton Dickinson UK Ltd., Oxford, UK, dilution 1:50) or HLA-DR (Becton Dickenson UK Ltd., Oxford, UK, dilution 1:100) for 1hr. then a rabbit anti-mouse biotin labelled secondary antibody (Dako Ltd.,High Wycombe Bucks, UK.; dilution 1:200) for 30 minutes. Followed by a peroxidase labelled streptavidin/biotin complex (DAKO). The peroxidase activity was visualized using 3-amino 9-ethyl carbazole (AEC) from Sigma as the substrate. *Centre II*. Immunohistochemical analysis was performed using a two step staining method [107]. Antibodies against HLA-DR and CD1a were obtained from CLB (CLB, Amsterdam, The Netherlands) and polyclonal secondary antibodies, rabbit-anti-mouse conjugated with horseradish peroxidase (HRP) were obtained from Dako (DAKO, A/S, Glostrup, Denmark). The peroxidase activity was visualized as in centre I by AEC.

Migration scores and criteria for classifying chemicals as contact sensitizers. The number and distribution of LCs in each explant was analyzed independently in a blind fashion by three different investigators at each centre. The investigators divided LC distribution pattern within the explant into three regions i.e. the supra basal epidermis, the basal layer (epidermal/dermal junction) and the upper (papillary) dermis. The changes in LC number and distribution were expressed in terms of an Migration score (MS) which was then used to predict allergenicity of the chemicals in humans. The system used to generate the Migration score (MS) is given in Table 5.1.

5.3 Results

The highest non-toxic concentrations (HNTC) of the test chemicals (24hr exposure period). Alterations in the pattern of HE, LDH and MGP staining, induced by exposure of explants to toxic concentrations of a test chemical, were used for evaluating the HNTC. These concentrations were confirmed at centre II.

The Migration scores (MS) for all concentrations of each test chemical. At the HNTC, both irritants and contact sensitizers induced considerable LC migration and there were no differences in the average group MS values (average ± s.d. of all irritants vs. average ± s.d. of all contact sensitizers). The average MS values obtained at centre I were; 0 for the vehicles, 2.5 ± 0.2 for non-sensitizers and 2.5 ± 0.17 for sensitizers (ratio of average MS

Table 5.1. LC migration caused by chemicals

Chemical	sbLC	bLC	pdLC	Migration Score (MS)
non inducer	5+	1+	1+	0
weak inducer	4+	1+	1+	1
moderate inducer	2+ to 3+	1+ to 2+	1+ to 2+	2
strong inducer	1+ to 0	3+ to 0	0 to 3+	3

For vehicles (non-allergens) suprabasal a network of CD1a+ LC are seen which are scored as 5+, in the basal membrane and the papillary dermis few cells are seen this is scored as 1+. If the allergeinicity potential of a chemical increases a decrease is seen of the number of LC in the suprabasal epidermis simultaneously with an increase in the basal membrane and/or papillary dermis. LC distribution pattern is translated into a MS of 0 to 3. sbLC = suprabasal epidermal LC; bLC = basal-epidermal LC; pdLC = papillary dermal LC.

values, non-sensitizers: sensitizers, was 1: 1). At centre II the MS values were; 0 for the vehicles, 1.9 ± 0.4 for non-sensitizers and 2.4 ± 0.16 for sensitizers (ratio of average MS values, non-sensitizers: sensitizers, was 1:1.3). When the concentrations of the test chemicals were reduced by 50%, to the 50% HNTC, there was a difference in the average MS values between groups, i.e. irritants (non-sensitizers) and contact sensitizers (Table 5.2). At centre I the average MS for non-sensitizers decreased to 1.1 ± 0.2 while the MS

Table 5.2. Migration score of chemicals at 50% of the highest non-toxic concentration (HNTC).

Chemical	HNTC	Centre 1. (AMC)					Centre 2.(TNO)				
		#1	#2	#3	Avg	SEM	#1	#2	#3	Avg	SEM
Aceton-oliveoil		nd	nd	nd			nd	nd	nd		
Aqua		nd	nd	nd			nd	nd	nd		
Mineral oil		nd	nd	nd			nd	nd	nd		
PBS		nd	nd	nd			nd	nd	nd		
Croton oil	0.2%	2	1	2	1.7	0.3	1	2	1	1.3	0.3
Nonanoic acid	1%	1	0	1	0.7	0.3	0	0	0	0.0	0.0
Sodium dodecyl sulphate	2%	1	1	1	1.0	0.0	0	1	0	0.3	0.3
Benzalkonium chloride	2%	1	1	1	1.0	0.0	2	1	1	1.3	0.3
Bromo-hexane-1	pure	2	3	2	2.3	0.3	2	2	1	1.7	0.3
Bromo-pentadecane-1	50%	1	1	1	1.0	0.0	0	2	1	1.0	0.6
$CoCl_2$	2%	3	3	3	3.0	0.0	1	2	1	1.3	0.3
Dinitrofluorobenzene	0.2%	2	2	2	2.0	0.0	1	1	1	1.0	0.0
Eugenol	2%	2	3	3	2.7	0.3	1	2	1	1.3	0.3
HEMA-2	50%	3	3	3	3.0	0.0	3	3	2	2.7	0.3
Hexyl cinnamic aldehyde	10%	2	3	3	2.7	0.3	2	1	2	1.7	0.3
Hydroxy citronellal	10%	3	3	3	3.0	0.0	3	3	2	2.7	0.3
$K_2Cr_2O_7$	2%	3	3	3	3.0	0.0	3	3	2	2.7	0.3
Kathon c.g	0.5%	3	3	3	3.0	0.0	nd	nd	nd		
2-Mercaptobenzothiazol	2.5%	3	3	3	3.0	0.0	2	2	3	2.3	0.3
MMA	pure	2	2	2	2.0	0.0	1	1	2	1.3	0.3
Neomycine sulphate	20%	1	1	2	1.3	0.3	1	1	2	1.3	0.3
$NiSO_4$	10%	3	3	3	3.0	0.0	2	2	2	2.0	0.0
Oxazolone	2%	1	3	3	2.3	0.7	1	1	1	1.0	0.0

Migration score (MS) of the 23 chemicals studied at half the highest non-toxic concentration (50% HNTC). Results of 3 individual experiments from both centres are shown.

value for sensitizers remained the same (2.4 ± 0.21). As a result, the ratio of average MS values, non-sensitizers: sensitizers decreased to 1 : 2.2. At centre II the average MS values were; 0.6± 0.2 for non-sensitizers and 1.7 ± 0.16 for the sensitizers. The average MS ratios, non-sensitizers : sensitizers, decreased to 1 : 3.0. The results indicated that the

discrimination between non-sensitizers and sensitizers could be improved by decreasing the concentrations of the test chemicals to below the HNTC measured after a 24 hr exposure.
Based on MS values obtained using the 50% HNTC, a cut off MS of ≥1.0 was used to predict allergenicity (Table 5.2). The classification of chemicals using the MS was compared with the classifications evaluated by human tests (the HMT and the HPTA), the GPMT and the LLNA as obtained from literature (Table 5.3). When there were discrepancies between classifications based on animal and human studies, we used human test data for reference purposes. At 50% HNTC, allergens and vehicles were correctly classified at both centres. However, at centre I two irritants (croton oil and SDS) were falsely classified as allergens. At centre II, only one of the three irritant chemicals, croton oil, was classified falsely as an allergen.

Table 5.3. The predictions of allergenicity based on hOSEC data obtained from half the highest non-toxic concentration (50%HNTC) compared with human tests (HMT[a] and HPTA) and animal data (GPMT[b] and LLNA[c]).

Chemical	Clas.	Ref.	Human[a]	GPMT[b]	LLNA[c]	Centre I	Centre II
						50% HNTC	
Aceton-oliveoil	V	NA	-	-	-	-	-
Aqua	V	NA	-	-	-	-	-
Mineral oil	V	NA	-	-	+	-	-
PBS	V	NA	-	-	-	-	-
Croton oil	N/I	84,162	?	?	+	+	+
Nonanoic acid	N/I	117,162	-	?	?	-	-
Sodium dodecyl sulphate	N/I	151,162,163	-	-	+	+	-
Benzalkonium chloride	A	160,162,164	+	-	-	+	+
Bromo-hexane-1	A	165	?	+	+	+	+
Bromo-pentadecane-1	A	165	?	+	+	+	+
$CoCl_2$	A	151,163,162	+	+	+	+	+
Dinitrofluorobenzene	A	164,166	?	+	+	+	+
Eugenol	A	35,162	+	+	+	+	+
HEMA-2	A	162,167	+	+	?	+	+
Hexyl cinnamic aldehyde	A	35,162,164	+	+	+	+	+
Hydroxy citronellal	A	162,164	+	+	+	+	+
$K_2Cr_2O_7$	A	151,162,164	+	+	+	+	+
Kathon c.g	A	164	+	+	+	+	ND
2-Mercaptobenzothiazol	A	151,164,168	+	+	+	+	+
MMA	A	167	+	+	?	+	+
Neomycine sulphate	A	151,162,163	+	-	-	+	+
$NiSO_4$	A	151,163,162	+	+	-	+	+
Oxazolone	A	35,164	+	+	+	+	+

The symbol '+' indicates classification as a sensitizer and '-' classifies a non-sensitizer. V: vehicle, N/I: non-sensitizer (irritant), A: sensitizer (allergen), NA: not applicable, ND: not done, ?: not known, Clas. = Classification; Ref. = Reference; [a] human patch test allergen and/or human maximization test; [b] Guinea pig maximization test; [c] local lymph node assay.

Reproducibility of the Migration scores based on individual replicate data. The inter-donor variation, with respect to the predicted classification (either sensitizer or non-sensitizer) was small. At centre I the triplicate experiments gave the same classification for 22 of the 23 chemical tested, the exception being nonanoic acid. At centre II the triplicate experiments gave the same classification for 20 of the 22 chemicals tested, the exceptions being SDS and bromo-pentadecane-1. The predicted classifications for these chemicals, based on the average of triplicate incubations, were correct.

5.4 Discussion

In this study we have confirmed that contact allergens accelerate the migration of LCs from hOSEC epidermis and that this model could be developed as an in vitro alternative to animal tests for screening for contact allergens. In addition the basic model can successfully transferred to another center. The hOSEC model needs further refinement before it can be used reliably to differentiate between contact allergens and irritants. However, even in its present form, the accuracy of classifications generated at both centre I and II, using hOSEC, are comparable to those published by researchers using the GPMT and the LLNA.

Despite the (small) differences in the protocols used all contact sensitizers were correctly classified at both centers. At centre I the irritants, croton oil and SDS were falsely classified as allergens. At centre II only croton oil was misclassified. There was a 96% agreement between the two centers in the classifications predicted for the chemicals tested. The inter-donor variation in classification, for any one chemical, were also small. There were larger inter-centre differences in the absolute AS values for each chemical; for almost half the irritant and allergenic chemicals tested the difference in average MS between centre I and centre II was greater than 25% (Table 5.2). As the inter-donor variation was small, at both centers, the inter-centre differences in absolute MS probably reflect methodological differences, like differences in the source of the skin (breast or stomach), the technicians performing the analysis and differences in the protocols used. Studies are now being performed to quantify the influence of these factors on the absolute MS.

We found that irritants can accelerate hOSEC LC migration, even at the 50% HNTC. However, the ratio of average MS values, non-sensitizers : sensitizers decreased at both centres I and II when the 50% HNTC was used in place of the HNTC, indicating that the discrimination between non-sensitizers and sensitizers could be improved by further decreasing the concentrations of the test chemicals. OSEC (porcine and human) can also be used to determine dermal irritancy [102,127,131]. Using OSEC, it was shown that both the HNTC and the 50% HNTC of a chemical, classified as non-irritant after a 24hr culture period, could be toxic when the exposure period was increased to 48hr. A preliminary study has shown that an optimal discrimination between non-sensitizers (irritants) and contact sensitizers, using LC migration at 24hr as the end point, can be obtained by reducing the concentration of chemicals further, to a level at which they are non-toxic after a 48 hr exposure (Chapter 6). It appears that sensitizer specific LC migration may only be observed at concentrations of test chemicals which are not considered to be even weakly irritant.

The classifications obtained using hOSEC were compared with those generated using animal models and found to be equally accurate (Table 5.3). While SDS is classified correctly as a non-allergen using the GPMT (we could find no data for the other irritants) the LLNA falsely classifies SDS and croton oil as allergens. The GPMT and the LLNA correctly predict the allergenicity of almost all of the allergens tested with the exception of neomycin sulphate and benzalkonium chloride (both) and nickel sulphate (the LLNA) [2,39]. These chemicals were correctly classified by groups I and II using hOSEC. Although there is some uncertainty as to the correct classification of benzalkonium chloride (irritant, corrosive or sensitizer), available data indicate that it is a contact allergen in humans [159,160].

The question arises whether results obtained using hOSEC would be invalidated if skin from allergen sensitized individuals were used. This study cannot answer this question, as the donors were anonymous. The fact that we found no exceptional responses when explants were exposed to NiSO4 may indicate that prior-sensitization does not modify LC responses to allergens. About 20% of females are sensitized to NiSO4 [161] and we exposed explants from about 50 donors to NiSO4 during the course of our studies (data not shown). We would expect, therefore, that about 10 volunteers would be sensitive to NiSO4. However, we found no abnormal responses to nickel in our group of volunteers.

The three Rs (Reduction, Refinement and Replacement of animals) remain the aims of research programs aimed at developing non-animal alternatives for screening of novel compounds [155]. The GPMT requires 24 to 32 guinea pigs and the LLNA requires 16 to 30 mice per chemical [2]. Using hOSEC, 15 chemicals at 5 concentrations could be tested routinely, using fresh skin from only 3 donors. The hOSEC model could, therefore, play an important role in reducing the number of animals used for screening for allergens. However, the model needs to be refined still further before it can be used routinely to specifically predict allergenicity in humans. We are currently defining the role of irritation in LC migration and investigating the potential of markers of LC activation to discriminate between irritants and allergens.

5.5 Conclusion

The hOSEC model can be used to predict allergenicity in humans with an accuracy comparable to that of current animal models. Although the model needs to be further refined and validated, the data presented suggest that it has the potential to become an alternative to animal models for the predictive testing of potential allergens.

Chapter 6

Dissection of allergen and irritant properties of chemicals in human skin cultures.

Submitted for publication as:
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Assessement of contact allergens by dissociation of irritant and sensitizing properties.
and
J.J.L. Jacobs, Hitoshi Hasegawa, P.K. Das, G.R. Elliott.
Langerhans cell migration and maturation caused by a skin irritant and a contact sensitizer

Abstract

The human organotypic skin explant culture (hOSEC) model is a promising alternative *in vitro* model for screening contact allergens. In this model, the chemical-induced migration of Langerhans cells (LCs) out of the epidermis is used as a measure of sensitizer potential. However, skin irritants may give false positive results. Recently, we published that skin irritants cause epidermal toxicity in the hOSEC model. Determination of LC migration after 24-hours, while excluding toxicity after a 48-hours' exposure period eliminated false positive results due to irritation, while maintaining true positives. Next, we analysed cells emigrating out of hOSEC for markers of LCs (CD1a), mature dendritic cells (DCs; CD83) and lymph node homing receptor (CCR7). After exposure to an irritant, an increase of $CD1a^+$ $CD83^+$ LCs was found in the culture medium. After exposure to a sensitizer, $CD1a^+$ and $CD83^+$ skin emigrants were increased, but 43% of $CD1a^+$ LCs were $CD83^-$ (immature). CCR7 was found on > 90% of all DC subpopulations of skin emigrants, including immature LCs. Thus LC migration may occur without LC maturation. In conclusion, the hOSEC model predicted allergenicity in humans better than animal tests, i.e. guinea pig maximization test and local lymph node assay in mice.

6.1 Introduction

Exposure to small molecular weight chemicals, often found in industrial, cosmetic and household products, can induce contact dermatitis. The mechanism involved can be either toxic (irritant contact dermatitis) or immunological (type IV delayed type hypersensitivity reaction). In both cases inflamed skin is infiltrated by activated memory T lymphocytes and other leukocytes [169]. Since skin irritants and contact allergens are the major cause of contact dermatitis, European Community (EC) regulations require that any new chemical must be screened for irritancy and / or allergenicity (sensitizing) properties before they are introduced into products [143]. Contact allergens are currently screened using animal models, such as the guinea pig maximization test (GPMT) and the local lymph node assay (LLNA) in mice. The assessment of the sensitization potential of a single chemical requires 24 to 32 guinea pigs or 16 to 30 mice. The accuracy of both the GPMT and the LLNA for predicting human contact sensitizers are 73 and 72%, respectively [2]. Possible causes of the relative low efficiency of the LLNA and GPMT are differences between the skin of these animals and human skin, immunological differences [85], and false positive reactions with (non-sensitizing) irritants [2].

New EC regulations restrict the use of animals to identify irritant and allergenic chemicals for ethical reasons. Consequently, several alternatives to replace animal use for identifying skin sensitizers and irritants are being developed, based on the understanding of the chemistry and immunobiology of skin sensitization [170]. Important features in skin sensitization are the skin barrier function and epidermal Langerhans cells (LCs). The human organotypic skin explant culture (human OSEC; hOSEC) model is the only non-animal, non-volunteer alternative test that includes both. Epidermal LCs are a unique set of Dendritic cells (DCs) [29], important and sufficient in sensitization to a contact allergen [130]. Immature DCs process antigen in the peripheral tissue, maturate and migrate to the draining lymph node after activation by e.g. contact allergens [21,171,172]. The CCR7 marker is important for homing of DCs to the lymph node [173-175]. Mature DCs express CD83, and stimulate hapten-specific naive T cells leading to antigen-specific sensitization [129,130,145]. The elicitation phase (allergic contact dermatitis) is induced when the allergen is applied on to the skin of a sensitized animal [19].

Migration of epidermal LCs can be studied in hOSEC [80,81] where they migrate out of the skin explants through lymphatic vessels [82]. Applying contact sensitizers topically on hOSEC, accelerates LC migration out of the epidermis, and compound-induced LC migration has been used as a predictive assay for contact allergens [83,84]. However, the migration of human epidermal LCs can also be induced by skin irritants in vivo and in vitro [31-34]. Thus chemicals have to be screened for allergenicity at non-irritating concentrations. The threshold for skin irritancy may vary significantly depending on the individual tested; the threshold concentration may differ up to 200-fold [17]. This variation may be explained by differences in composition of the human stratum corneum causing differences in skin penetration between individuals [18]. Recently, we have developed a new method for assessing irritancy using methyl-green pyronine (MGP) staining of OSEC to assess skin irritation potential [102,176]. In this test, moderate and weak irritants are defined by toxicity after 24 and 48 hours of OSEC, respectively. Twofold and fourfold dilutions of the lowest moderate irritant concentration still induce LC migration of some non-sensitizers [157] (and our unpublished data). In this study, the lowest weak irritant concentration (LWIC) is evaluated to serve as the irritancy threshold for discriminating between sensitizers and non-sensitizers. This leads to predictions that correspond better with human contact sensitizer data than results obtained using either the guinea pig maximization test or the local lymph node assay.

We also studied the cells migrating out of the hOSEC after exposure to 10% SDS or 1% nickel sulphate, as a model non-sensitizing skin irritant and a model non-irritating

sensitizer, respectively. For this purpose we needed large pieces of skin (9 cm^2) that were placed in medium, and cultured for 24 hours, similar to the hOSEC of small biopsies. Skin immigrants were collected and analysed for CD1a, CD83 and CCR7 expression to verify and study LC migration and maturation.

6.2 Materials and methods

Chemicals. Chemicals used are Dulbecco's phosphate buffered saline (DABS), acetone, cobalt (II) chloride, croton oil, 1-chloro 2,4-dinitrobenzene (DNCB), eugenol, mineral oil, nickel sulphate, nonanoic acid, potassium dichromate and sodium dodecyl sulphate (SDS). Test chemicals were preheated to 37^{o}C, prior to application onto the skin. The following dilutions of the test chemicals were used, SDS (0.1, 0.2, 0.5, 1, and 2%), Cobalt chloride (0.2, 0.5, 1, and 2%), potassium dichromate (0.05, 0.1, 0.2, 0.5, and 1%), Neomycin sulphate (5, 10, 20, and 40%), Nickel chloride (0.5, 1, 2, 5, and 10%), all as aqueous solutions, and croton oil (0.02, 0.05, 0.1, 0.2, 0.5, and 1%), nonanoic acid (0.5, 1, 2, 5, and 10%), DNCB (0.05, 0.1, 0.2, and 0.5%) and eugenol (0.5, 1, 2, and 5%), all dissolved in mineral oil. Methyl green was obtained from Sigma-Aldrich Fine Chemicals BV, Zwijndrecht, The Netherlands; pyronine was obtained from Merck KGaA, Darmstadt, Germany; collagenase D was obtained from Boehringer Mannheim, Germany.

Human organotypic skin explants cultures (human OSEC, hOSEC). Human breast skin was obtained as a waste product of cosmetic surgery with informed consent of the patient. Sterile biopsies were cut of approx. 0.25 cm^2 each for routine experiments, or 9 cm^2 when skin emigrants were isolated. The biopsies were dermal-side down incubated in DMEM : F12 (3:1) medium supplemented with 10% foetal calf serum.[102] Test chemicals were applied on top of the epidermis, using a cotton tip. Human organotypic skin explants were cultured for 24 or 48 hours at 37^{o}C in a humid incubator under 5% CO_2. Each experiment was performed in triple using skin from at least three different donors. After the incubation, the culture medium was removed, and the cultured skin biopsies were embedded in Tissue-Tek® (OCT compound, Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), frozen in liquid nitrogen, and stored at -70^{o}C.

Methyl-green pyronine (MGP) staining of frozen sections. The MGP staining of cryostat sections was performed as previously described [102,176]. In brief, air-dried five □m thick cryostat sections were incubated for 20 minutes at room temperature in a fresh MGP-staining solution (0.5% methyl green, 0.1% pyronine in a 0.2 mM sodium-acetate buffer, pH 4.0). Subsequently, the MGP was poured off the sections and the sections were washed, dried and embedded in pertex. Absence of pyronine (RNA) staining in the epidermis was regarded to be a toxic effect.

Analysis of skin emigrants. Skin emigrants were isolated as described before [80]. Briefly, after 24 hours of culture, the skin explants were taken out and the culture medium was incubated for another hour at 37^{o}C in the presence of collagenase D. The cells (skin emigrants) were collected, washed and stained with antibodies for flow cytometry. Antibodies used for flow cytometry were FITC- and PE-labelled isotype controls and CD1a, PE-labelled CD14, and FITC-labelled CD83 and anti-mouse IgG1 and the CCR7.6B3 mouse IgG1 monoclonal antibody [177]. Flow cytometry was performed with the FACScan (BD). The cells were gated for leukocytes using forward and sideward scatter characteristics.

Immunohistochemistry. Immunohistochemistry was performed using standard methods on cryostat sections of hOSEC using antibodies for MHC II (HLA-DR), CD1a. The Lag antibody against LC's Birbeck granules [28] was a kind gift from Dr. Kozo Yoneda, Dept. of Dermatology, Faculty of Medicine, Kyoto University. Secondary a antibodies were rabbit-anti-mouse conjugated with horseradish peroxidase (HRP), goat-anti-mouse conjugated with Alkaline Phosphatase (AP), rat monoclonal antibodies against mouse IgG1 (AP

labelled) and IgG2b (biotin labelled), and avidin-HRP. Staining with fast blue base (FBB) revealed antibody binding for AP and staining with 3,3-amino-9-ethyl carbazole (AEC) showed HRP. After staining, slides were embedded in glycerin gelatin and examined within 6 months.

Automated quantification of epidermal LCs. LC numbers were measured using the Leica QWin image processing and analysis system version 2.2a (Leica Imaging Systems Ltd, Cambridge, England) with two macros, Lag-blue.Q5R and Lag-red.Q5R, to count FBB and AEC-stained objects, respectively [157]. The Leica QWin macros generate data on the number of epidermal LCs (eLCs/mm) and LC in the basal epidermal area (bLCs/mm). The chemical-induced effects are calculated by comparing the chemical values with the solvent values. Migration index (MI) is defined as changes in eLCs/mm counts of solvent treated skin compared with chemical treated skin: MI = (('solvent' - 'chemical') / 'solvent') * 100%. Basal-Suprabasal Distribution of LCs (BSD) is calculated by dividing bLCs/mm by sbLCs/mm. Basal Accumulation (BA) is calculated comparing the BSD values of chemical with solvent treated skin: BA = ('chemical' / 'solvent') * 100%. Cutaneous Immune Modulating Activity (CIMA) index of a chemical concentration is calculated as follows: CIMA index = 10* MI + BA, with a defined maximum score of 10.

6.3 Results

Assessing the lowest weak irritant concentration (LWIC). Weak irritants are defined as causing epidermal cytotoxicity after 48 hours of hOSEC. This concentration differs considerably depending on the donor skin. Epidermal cytotoxicity, measured as a decrease in epidermal keratinocyte RNA using a modified methyl-green pyronine (MGP) staining procedure was used to detect irritancy [102,127,176]. In Table 6.1, the ranges of cytotoxic concentrations for nine test chemicals are shown, after 48-hours exposure. SDS, nonanoic acid and potassium dichromate show differences in the minimal cytotoxic concentration of at least four-fold. This underlines the need to determine skin irritancy per donor skin. The LWICs are set to 1 for each donor in order to allow comparison of LC migration caused by different concentrations in different donors. Relative irritancy is defined as the concentration relative to the LWICs.

Table 6.1. Lowest weak irritant concentrations (LWICs) of chemicals used in skin derived from three different donors.

Chemical (solvent)	Donor 1	Donor 2	Donor 3
Croton oil (min.)	0.1%	0.05%	0.1%
Nonanoic acid (min.)	2%	1%	≤ 0.5% *
SDS (aq.)	1%	2%	0.2%
CoCl2 (aq.)	2%	2%	2%
DNCB (min.)	0.5%	0.5%	0.2%
Eugenol (min.)	2%	2%	5%
K2Cr2O7 (aq.)	0.2%	0.1%	0.5%
Neomycin sulphate (aq.)	> 40% **	40%	> 40%
NiSO4 (aq.)	5%	5%	10%

Weak irritants are defined by toxicity after 48-hours hOSEC. The lowest concentration being a weak irritant is named the lowest weak irritant concentration (LWIC). * ≤ lowest concentration tested was toxic. ** > highest concentration tested was non-toxic.

Cutaneous Immune Modulating Activity (CIMA) index. Figure 6.1 shows examples of LC disappearance out of the epidermis (MI), and LC redistribution (BSD); these effects are used to calculate the CIMA index. CIMA indexes are plotted against the relative irritancy compared to the LWICs (Figure 6.2). Concentrations of all nine chemicals that were

assessed as being weak irritant concentrations or higher (LWIC ≥ 1) had a significantly increased CIMA index (Table 6.2). At non-irritant concentration (LWIC < 1), non-sensitizers had CIMA index ≤ 1.4. Sensitizers had a CIMA index ≥ 4.4, and ≥ 3.5 at LWIC = 0.5 and 0.2, respectively (Table 6.3). The threshold for CIMA index was set at 3 and the CIMA index at 50% of the LWIC was used to assess allergenicity of compounds tested (Table 6.4).

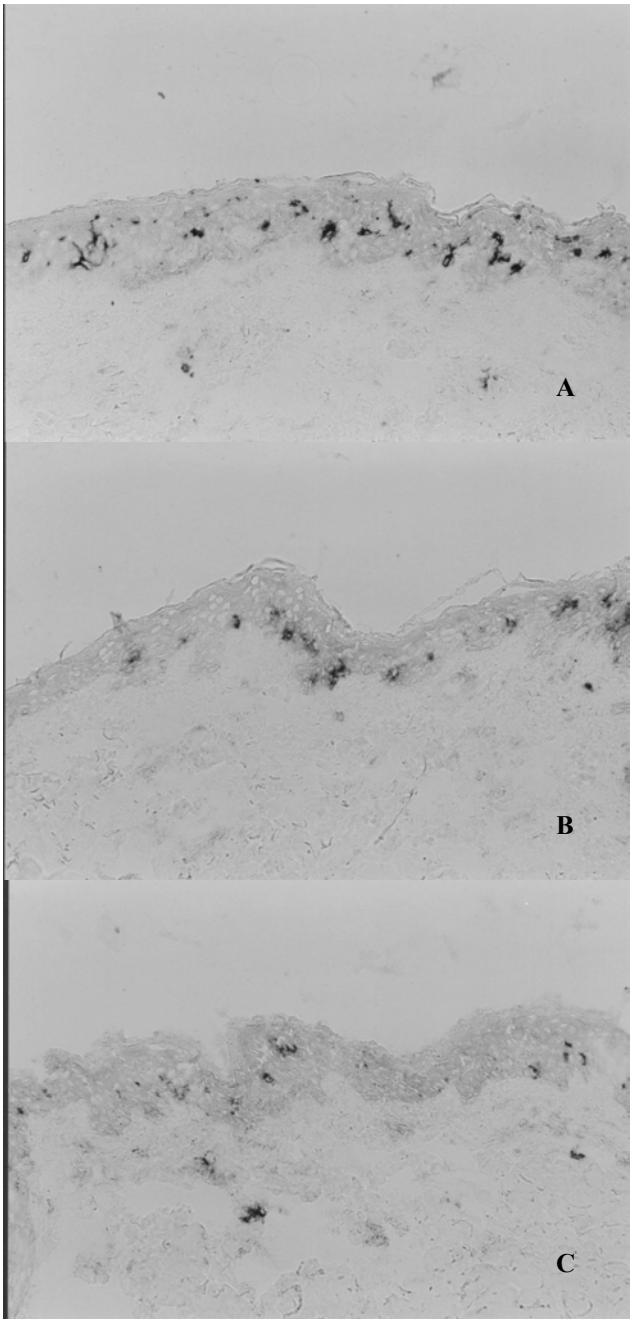

Figure 6.1. Epidermal LCs are double-stained with lag and CD1a, and are shown in hOSEC as examples of (A) no morphological change; (B) basal accumulation of LCs; (C) migration of LCs.

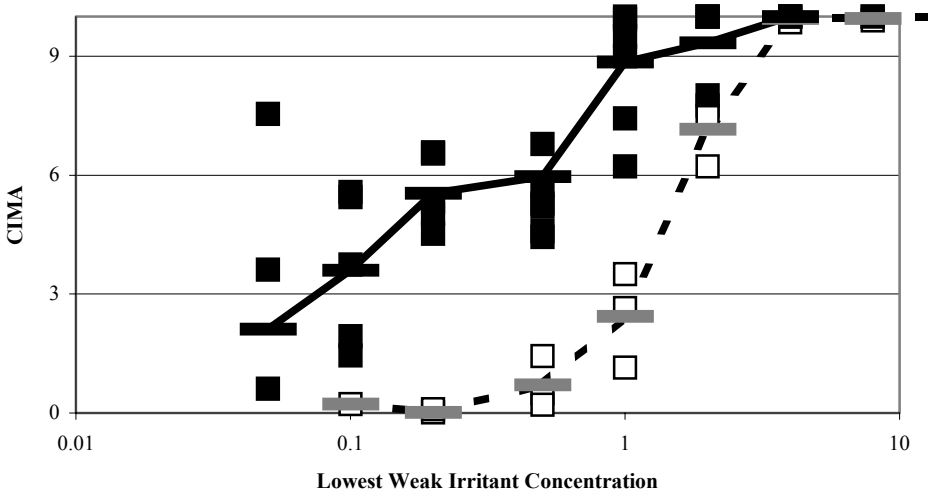

Figure 6.2. Lowest Weak Irritant Concentration versus CIMA index for non-sensitizers (open squares) and sensitizers (closed squares). Group averages are indicated by grey bares connected through a striped line (non-sensitizers) and black bars connected through a closed line (sensitzers).

Table 6.2. CIMA at weak irritant and higher concentrations

	relative irritancy	CIMA	SD	n
Croton oil	20	10.0	0.0	1
	10	10.0	0.0	3
	4	10.0	0.0	3
	2	7.8	2.0	3
	1	2.7	1.9	3
Nonanoic acid	20	10.0	0.0	1
	10	9.9	0.1	2
	4	9.9	0.2	3
	2	7.5	2.4	3
	1	3.5	0.9	3
SDS	10	10.0	0.0	1
	4	10.0	0.0	1
	2	6.2	0.7	2
	1	1.2	1.1	3
$CoCl_2$	1	9.9	0.1	3
DNCB	2	10.0	0.0	1
	1	9.0	0.9	3
Eugenol	2	10.0	0.0	2
	1	9.5	0.4	3
$K_2Cr_2O_7$	10	10.0	0.0	1
	4	10.0	0.0	2
	2	8.0	3.4	3
	1	6.2	2.8	3
Neomycin sulphate	1	10.0	0.0	1
$NiSO_4$	2	10.0	0.0	2
	1	7.4	1.3	2

CIMA index of sensitizers and non-sensitizers at weak and more irritating concentrations. Relative irritancy = concentration relative to the lowest weak irritating concentration, which is set as 1. CIMA-avg. = average CIMA index. CIMA-SD = standard deviation of CIMA. n = number of donors.

Table 6.3. CIMA at non-irritant concentrations

	relative irritancy	CIMA	SD	n
0.5x PBS	*non irritant*	0.0	0.0	3
1x PBS	*non irritant*	1.2	1.2	3
2x PBS	*non irritant*	0.6	0.9	3
Croton oil	0.5	0.2	0.4	3
	0.2	0.1	0.1	2
Nonanoic acid	0.5	1.4	0.2	2
	0.2	0.5	0.0	1
SDS	0.5	0.5	0.9	3
	0.2	0.0	0.0	2
	0.1	0.2	0.3	2
$CoCl_2$	0.5	6.8	2.5	3
	0.2	6.5	3.0	3
	0.1	3.7	1.5	3
DNCB	0.5	5.3	2.1	3
	0.2	3.5	1.2	3
	0.1	1.3	0.8	2
Eugenol	0.5	5.2	2.5	3
	0.2	5.3	0.3	2
	0.1	5.6	2.5	2
	0.05	7.5	0.0	1
$K_2Cr_2O_7$	0.5	5.7	1.1	3
	0.2	5.0	2.4	2
	0.1	1.9	0.0	1
Neomycin sulphate	0.5	4.6	1.8	3
	0.2	6.6	1.2	3
	0.1	1.5	1.3	3
	0.05	0.6	0.9	2
$NiSO_4$	0.5	4.4	1.1	3
	0.2	4.5	1.1	3
	0.1	5.4	1.2	3
	0.05	3.6	0.0	1

CIMA index of sensitizers and non-sensitizers at non-irritating concentrations. Relative irritancy = concentration relative to the LWIC = 1. CIMA-SD = standard deviation of CIMA. n = number of donors used to calculate the average.

Table 6.4. Comparison of contact sensitization potential as determined by different tests

	CIMA index		animal tests		
Chemical	value	classification	human	guinea pig	mouse
Croton oil	0.2	-	-	n.d.	+
Nonanoic acid	1.4	-	-	n.d.	+
SDS	0.5	-	-	-	+
CoCl2	6.8	+	+	+	+
DNCB	5.3	+	+	+	+
Eugenol	4.4	+	+	+	+
K2Cr2O7	5.7	+	+	+	+
Neomycin	4.6	+	+	-	-
NiSO4	4.4	+	+	+	-

Only CIMA of the highest non-irritating concentration is shown, average of n=3, except for nonanoic acid (n=2). Compounds are classified as a sensitizers if the CIMA index ≥ 3. Human data combined from HMT or HPTAs [2]. guinea pig, tested with GPMT or Buehler Test (BT) [2]. Mouse, tested with LLNA, data from [2,38,39].

Analysing skin emigrants. In order to show that disappearing LCs had migrated, and to analyse the skin emigrants, we used larger pieces of skin. These pieces were painted with a solvent (aqua), an allergen (1% $NiSO_4$) or an irritant (10% SDS). After 24 hours hOSEC, the skin was removed, attached cells were loosened with collagenase, and the medium was collected. After collection, the cells in the medium (skin emigrants) were analysed using flow cytometry. Skin emigrating cells were stained for CD1a and CCR7, a chemokine receptor involved in cell migration to lymph nodes. CD1a is a unique marker for LCs in the skin. More than 90% of the $CD1a^+$ cells in the medium were also positive for CCR7 (Figure 6.3d-f). CD83 is a maturation marker of DCs. Also more than 90% of the $CD83^+$ cells in the medium were positive for CCR7 (Figure 6.3g-i). Since both CD1a and CD83 are markers of DC subpopulations, all these DCs were $CCR7^+$. Calculating the percentage $CCR7^+$ cells in skin emigrants, a small increase was found in medium below skin treated with either an allergen or an irritant (Table 6.5). A small allergen- or irritant-induced increase was found for the number of $CD1a^+$ LCs (Table 6.5). The number of $CD83^+$ mature DCs was quite low in solvent-treated skin, but was 3.5 and 4.5 times increased for medium from skin treated with an allergen or an irritant, respectively. (Table 6.5).
Initial experiments showed that some $CD83^+$ cells appeared to be $CD1a^-$ (data not shown). In order to distinguish between $CD83^+$ cells that are true $CD1a^-$ and those that are $CD1a^{dim}$, we stained with a higher concentration of CD1a. In these experiments we found no $CD1a^-$ $CD83^+$ cells. Almost all $CD83^+$ emigrants from solvent- and irritant-treated were $CD1a^+$, but in allergen-treated skin many were $CD1a^{dim}$ (Figures 6.3j-l, 6.4d). $CD1a^+$ $CD83^+$ cells were only marginally increased due to an allergen, but greatly increased under irritant-treated skin (Table 6.5). Only a few cells were $CD1a^+$ $CD83^-$ in irritant-treated skin, but a higher number of these cells were found in medium from solvent and allergen-treated skin (Table 6.5). The number of DCs ($CD1a^+$ or $CD83^+$ cells) in the medium was doubled in both allergen and irritant-treated skin compared to solvent treated skin (Table 6.5).

6.4 Discussion

LC migration. LC migration was assessed by the disappearance from the epidermis of the immunohistochemistry markers CD1a and Lag. Both markers can be used to quantify the number of epidermal LCs in a reliable manner [157]. All LCs were $CD1a^+$ Lag^+, and in untreated skin, LCs reside in the epidermis, mostly in between suprabasal keratinocytes. After culture of treated-treated hOSECs, Lag^+ $CD1a^+$ LCs were often found in the basal part of the epidermis and in the dermis, suggesting LC migration. The relocation of the LC markers, Lag and CD1a, within the dermis has also been found after inducing LC migration *in vivo* [134]. $CD1a^+$ LCs have been found in the culture medium due to spontaneous or induced-induced migration (Figures 6.3d-f,j-l, Table 6.5) [80,81]. The number of DCs ($CD1a^+$ or $CD83^+$) in the medium of skin treated with an allergen or an irritant was much higher than the number of DCs in solvent treated skin (Figures 6.3d-i, Table 6.5). These data confirm that LCs disappearing from the epidermis, migrate out of the skin. In the lymph node, mature LCs stimulate or regulate the immune response, while the absence of LCs in the skin causes a local immunosuppression in the skin [178]. Hence, we prefer the terminology Cutaneous Immune Modulating Activity (CIMA). The CIMA index is increased due to skin irritation or the application of a contact allergen.
The presence of CCR7, the chemokine receptor involved in lymph node homing, on virtual all DCs suggests that these are equipped to migrate to the draining lymph node (Figure 6.3a-i). CCR7 is likely to be expressed on all cells migrating to the lymph node. Besides $CD1a^+$ LCs, these cells include $CD1a^-$ non-LC DCs, other skin leukocytes that migrate to the lymph node, and lymphocytes that may be present in the blood vessels of the skin explants. Since CD1a and CD83 are rare markers in blood cells, it is unlikely that blood leukocytes increased the fractions of $CD1a^+$ or $CD83^+$ we found. Most spontaneously

migrating CD1a$^+$ LCs from hOSEC were CD83$^-$, indicating an immature phenotype. Migration of immature CD1a$^+$ CD83$^-$ LCs has recently been shown to occur *in vivo* in human dermatopatic lymphadenitis [179]. Our results confirm the data from Geissmann's key paper that brought a definite resolution to a long-awaited issue, namely is maturation required for LC migration [43]. In skin treated with allergen or irritant, also a large number of

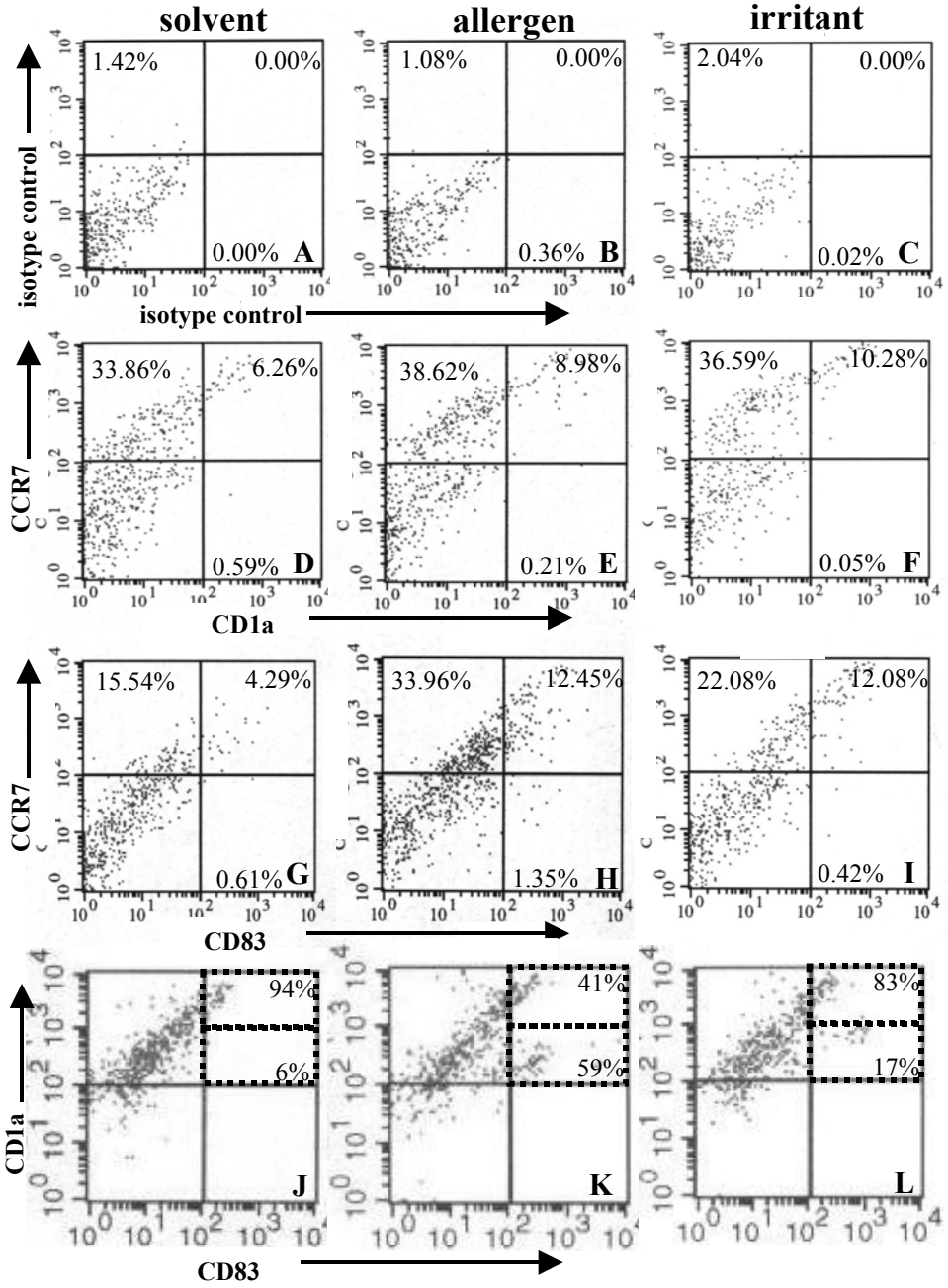

Figure 6.3. FACS staining of skin émigrés from solvent- (A,D,G,J), allergen- (B,E,H,K) and irritant- (C,F,I,L) treated hOSEC. Staining is performed with isotype controls (A,B,C), CD1a and CCR7 (D,E,F), CD83 and CCR7 (G,H,I) and CD83 and CD1a (J,K,L).

Table 6.5. Phenotype of skin emigrants

	$CCR7^+$	$CD1a^+$	$CD83^+$	$CD1a^-83^+$	$CD1a^+83^+$	$CD1a^+83^-$	$CD1a^+/83^+$
Solvent	30.1%	6.3%	2.3%	0.1%	2.2%	4.1%	6.4%
Allergen	43.0%	9.0%	8.2%	4.8%	3.4%	5.6%	13.8%
Irritant	41.0%	10.3%	10.9%	1.8%	9.0%	1.2%	12.1%

Solvent is aqua; allergen is 1% nickel sulphate; irritant is 10% SDS. Percentages are the fractions of all skin emigrants with this phenotype. Clarification of the phenotypes: $CCR7^+$, lymph node homing marker; $CD1a^+$, LCs; $CD83^+$, mature DCs; $CD1a^-83^+$ ($CD1a^-$ $CD83^+$) non-LC (?) mature DCs; $CD1a^+83^+$ ($CD1a^+$ $CD83^+$) mature LCs; $CD1a^+83^-$ ($CD1a^+$ $CD83^-$) immature LCs; $CD1a^+/83^+$ ($CD1a^+$ and/or $CD83^+$) skin emigrants that are either LCs and/or mature DCs.

skin emigrants were positive for the DC maturation marker CD83 (Figures 6.3g-i, Table 6.5). Unexpectedly, we also found $CD1a^-$ or $CD1a^{dim}$ cells that were $CD83^+$ (Table 6.5). We cannot exclude that these cells are mature LCs that have down regulated their expression of CD1a. However, it is not unlikely, that these cells are matured dermal DCs, which stain $CD1a^-$ or $CD1a^{dim}$. The migration of mature dermal DCs has been suggested to play a role in the induction of contact allergy in mice [180].

LC migration at irritating concentration. At moderate irritating concentrations, as assessed by MGP, essentially all LCs disappeared, as is clear from *e.g.* loss of MHC II, CD1a and Lag staining (compare Tables 6.1 and 6.2; data not shown), and in the case of 10% SDS we detected LCs in the culture medium (Figure 6.3; Table 6.5). Moderate irritant concentrations correspond with EC classification R38 [102,176]. Even at most weak irritating concentrations, significant LC migration could be found with all chemicals (Table 6.2). SDS has been shown to induce human LC migration to the draining lymph node [34] and human epidermal LC numbers were decreased after application of nonanoic acid [181]. LC migration is required for the induction of, and directly related to the amount of cell proliferation in the local lymph node assay (LLNA) [182]. Local lymph node cell proliferation is induced by irritating concentration of non-sensitizers, such as SDS, croton oil and nonanoic acid [35-39]. Our finding of $CD83^+$ $CCR7^+$ DCs in the hOSEC medium suggests that these mature DCs can migrate to the draining lymph node and stimulate lymphocyte proliferation. Skin inflammation parameters, such as cytokines and cellular influx, are very similar in case of allergic and irritant contact dermatitis [183]. The immunology of skin inflammation includes the T-helper cell marker CD4 [184] and the costimulatory molecules CD80 [185] and CD28 [186]. Our findings confirm that both irritants and allergens can induce LC maturation and migration, and thus may stimulate immune reactions. However, the precise role of antigen-specific immunity in irritancy is unclear, as irritation-induced inflammation occurs rapidly and similarly in previously unsensitized subjects [183].

LC migration at non-irritating concentrations. Contact sensitizers caused LC migration at non-irritating concentrations, but non-sensitizing compounds did not (Figure 6.2; Table 6.3). The range of dilutions causing LC migration, however, differed between sensitizers. Some sensitizers, like nickel and eugenol, still caused an increase in CIMA at a 20-fold dilutions of the lowest weak irritant concentration, while other sensitizers, like DNCB, potassium dichromate and neomycin, had a smaller window of non-irritating concentration that induced LC migration. But all sensitizers showed LC migration at 0.2 and 0.5 times the LWICs, while no non-sensitizers showed LC migration at these concentrations (Figure 6.2; Table 6.3). Previous studies showed a dose-response relation between contact sensitizer dose and LC migration in hOSEC [84]. However, we could not confirm this dose-response relation for LC migration induced by contact sensitizers at sub-irritating concentrations (Figure 6.2; Table 6.3). But we found a dose-response relation for LC migration at weakly and more irritating concentrations (Figure 6.2; Table 6.2). Comparing Pistoor's data [84] with

our LWICs determined by MGP staining [176], suggests that Pistoor and coworkers most likely used weak irritant concentrations. LC migration is sufficient for sensitization [130] and sub-irritating concentrations of sensitizers can induce sub-clinical sensitization [187]. It is tempting to speculate that sub-irritating concentrations of sensitizers that induce LC migration, also can induce sub-clinical sensitization. The fact that both $CD83^+$ and $CD83^-$ LCs were present suggests that both subclinical sensitization as well as subclinical tolerization might be possible.

Comparison of tests for assessing human contact sensitizers. The CIMA index predicted correctly all six contact sensitizers, identified by the human maximization test (HMT) and in human patch test allergens (HPTAs) (Table 6.4). Only four were detected in the mice (LLNA) and five of these six in guinea pigs (GPMT) [2]. Both these rodents do not react significantly to neomycin, while mice do not react to nickel sulphate too. In the HPTA, both nickel (12.8-14.2%) and neomycin sulphate (2.6-13.1%) are among the most frequent observed contact allergens in Europe and North America [124,188], indicating that some important human sensitizers are not detected by these animal tests, but they are detected by the CIMA indexes determined in hOSECs. All three frequently used irritants that have not been reported to be a sensitizer in HPTAs, had low CIMA index values at non-irritant concentration. SDS was a non-sensitizer in both the HMT and GPMT, while the other two compounds were not reported in maximization assay studies. In contrast, all three non-sensitizers induced significant lymph node cell proliferation in the LLNA [2,38,39]. It is tempting to speculate that LCs migrating to the lymph node due to skin irritation cause the false-positive reactions in the LLNA, since LC migration is directly related to cell proliferation in the LLNA [182].

Potency of contact sensitizers. Allergic contact dermatitis differs from most other immune reactions by its strict dose dependency during the elicitation phase [189,190]. This dose dependency can be circumvented by the addition of an irritant [45]. The dose dependency for allergens is somewhat ambivalent in sensitized humans; some people react to extreme low doses, while other need almost irritating concentrations to develop contact dermatitis [191]. No differences in CIMA indexes were found between compounds known as strong, moderate and weak sensitizers. The potency of sensitizers in the elicitation phase *in vivo* is dose-dependent, *i.e.* a lower concentration is a less potent sensitizer, but also a less potent irritant. Skin irritation can be regarded as epidermal toxicity [13] and is strictly dose dependent, in contrast to most immune-related events. Thus differences in contact sensitizer potency may be due to differences in skin irritation potency. Indeed, MGP staining suggests chemicals known as strong sensitizers in human patch tests (0.5% DNCB; 0.5% $K_2Cr_2O_7$) have stronger irritancy potential than weaker sensitizers (Table 6.6). This is in agreement with primary irritancy data from 0.25% DNCB in unsensitized humans [192]. Sensitizers that are considered to be weaker in human patch test (5% $NiSO_4$, 1% $CoCl_2$, 20% neomycin sulphate, 1% eugenol) showed less or no irritancy effects when applied at hOSEC (Table 6.6). When comparing data for chemicals at human patch test concentrations, we found correlations between the % positive individuals in the HMT and the irritancy of these concentration as assessed by MGP ($R^2 = 0.77$) and also with the CIMA at patch test concentration ($R^2 = 0.85$). No clear correlation between the HMT and any other test was found, including the five true positives in the GPMT ($R^2 = 0.50$) and EC_3 (three-fold cell proliferation index) value of the four true positives in the LLNA ($R^2 = 0.45$) (data not shown). These data indicate that assessment of sensitizer potency is related to potency of irritation.

Table 6.6. Comparison of quantification of contact sensitization potential by different methods

	incidence		potency			hOSEC data		
	HPTA	HPTA	HMT	GPMT	LLNA	CIMA index		MGP
conc. chemical	EC	N-A			EC_3	(LWIC)	(PTC)	(PTC)
Croton oil	(-)	(-)	(-)	n.d.	~1%	0.2	n.d.	n.d.
Nonanoic acid	(-)	(-)	(-)	n.d.	~35%	1.4	n.d.	n.d.
SDS	0%	(-)	(-)	0%	~5%	0.5	n.d.	n.d.
0.5% DNCB	(-)	(-)	100%	100%	0.08%	5.3	9.5	2.0
0.5% $K_2Cr_2O_7$	4.6%	2.8%[#]	100%	10-100%	0.1%	5.7	8.2	1.0
5% $NiSO_4$	12.9%	14.2%[#]	48%	10-60%	-	4.4	6.5	0.6
1% $CoCl_2$	4.7%	9.0%	40%	n.d.**	<0.5%	6.8	6.8	0.0
20% Neomycin	2.6%	13.1%	28%	-	-	4.6	6.2	0.0
2% Eugenol	10.2%[*]	11.7%[*]	n.d.	0-60%	14.5%	5.2	7.8	0.7

Conc., concentration in patch test; HMT, % individuals sensitized in the human maximization test; HPTA, incidence in human patch test allergen; EC = data from European Community [124]; N-A is data from North America [188]; GPMT % animals sensitized in the guinea pig maximization test Assay [2,38,39]; EC_3, minimal sensitizing concentration in the LLNA; PTC, data given for patch test concentration; (-) frequent topically used chemicals for which HMT or HPTA are not formally performed, but negative results are anticipated; #, concentration used is halve of given patch test concentration; *, data from fragrance mix. ** n.d. = not determined.

General impact of our results. The combined screening for skin irritant effects by MGP and compound-induced LC migration provides a novel and powerful tool to assess contact sensitizers. In clinical practice, skin reactions can be due to irritation in all persons, and due to contact allergens in sensitized individuals. Both skin irritants and contact allergens cause LC migration, and induce skin immune responses. Our finding of mature $CD83^+$ DC in the hOSEC medium supports this. Non-immunogenic compounds, that do not cause dermatitis, do not accelerate LC migration. LC migration was not always accompanied by their maturation. Regardless of the cause of migration, LCs in the hOSEC medium, whether mature or immature, were $CCR7^+$. This indicates that these skin emigrants have the potential to migrate to the draining lymph node in vivo.

LC migration and maturation are essential for development of contact sensitization. Assessment of the sensitization potential of a chemical by determining LC migration, in the absence of any irritation, is valid from both an immunological and a dermatological point of view. Therefore, research for sensitizers should rule out irritancy. Our findings may also be important for other tests assessing skin allergens, especially the LLNA, in which skin irritants often produce false positives [2]. Moreover, our results highlight the influence of both immunologic (hapten-mediated) and toxic effects (irritation), on the activation of the immune system (dendritic cells).

Chapter 7
Summarising discussion.

7.1 Project and progress

This project was financed by grant 96-32 of the Dutch platform for alternatives for animal experimentation (*Platform Alternatieven voor Dierproeven, PAD;* now part of *ZON-MW*). The PAD 96-32 project was written in two parts; a part that was to be performed the AMC, and the part described in this thesis, which was to be performed at TNO-PML. The aims for this thesis were: (i) implementation of the hOSEC model to assess contact sensitizers at TNO-PML, (ii) validation of the hOSEC model between the AMC and TNO-PML, (iii) assessing contact sensitizers using blood perfused pig ears, and (iv) study of skin metabolism in blood perfused pig ears.

A major drawback of the hOSEC model as a sensitisation assay was that virtually all irritants scored positive, even if they were not sensitizers. In order to obtain a useful model for screening contact allergens, first a method to determine irritancy in the hOSEC model was developed. This method is described in Chapter 2 and Chapter 3 and may become an alternative test for the assessment of skin irritants [102,127,131]. A second important point is the reproducibility of the assessment of LC migration. In Chapter 4, a software-aided quantification of epidermal LC numbers in immunostained sections is described. This method is more reliable than manual counting of LCs [157].

Before final validation, prevalidation was performed to show that the test system is transferable from one lab to another, and that its results are correct, robust and reproducible (chapter 5) [193]. According to the project, a prevalidation study of hOSEC was done at the AMC and TNO-PML for the assessment of contact allergens; this study was performed before the optimisation of hOSEC by excluding skin irritants (method described in chapter 2 and 3), automated counting of LC migration (chapter 4) and implying these methods for skin irritation (chapter 5). In this prevalidation study, only toxic chemicals (i.e. moderate irritants) were excluded, and the method appeared to be reproducible in different laboratories. However, weak irritant concentrations of non-sensitizers also accelerated LC migration [194]. Determination of the weak irritant concentration per donor eliminates these false positive results as described in chapter 5.

A direct comparison of human OSEC model with a porcine model using perfused ears may reveal differences due to two reasons: different species and different model systems. Thus an intermediate model, the porcine OSEC (pOSEC) was introduced. The pOSEC model was compared with the hOSEC model for the assessment of human skin irritants (Chapter 2 and 3). The differences of porcine and human OSEC models with respect to immunology, spontaneous and compound-induced LC migration were not fully analysed due to lack of time.

In the OSEC models, LC migration is studied after 24 hours of incubation; thus, pig ears need to be perfused for at least 24 hours. Perfusion with blood had,

however, an upper limit of eight hours. Therefore, we decided to perfuse pig ears with a buffer, and this allowed perfusion up to 26 hours, while maintaining skin viability (unpublished results). This work paves the way for studying LC migration in the perfused pig ear, after the method is developed for pOSEC.

7.2 Results and indication of points for discussion

This thesis describes two alternative methods that may replace animal experimentation for skin safety. Both methods have in common that they use fresh cultured skin biopsies, which are named organotypic skin explant cultures (OSECs). The skin can be ear skin derived from a slaughterhouse pig, or human breast skin derived from cosmetic surgery. Skin irritation can be assessed using a simple biochemical toxicity marker (loss of staining of RNA with methyl-green pyronine, MGP) in human or porcine OSECs. Toxicity after 4, 24, or 48 hours corresponds with strong, moderate, and weak irritants, respectively. No toxicity after 48 hours indicates a non-irritant concentration [102,127,131]. At such non-irritant concentrations, the sensitisation potential can be assessed in human OSECs by quantification of epidermal Langerhans cell migration. A software-aided method allowed more reliable quantification of epidermal Langerhans cells than manual counting [157]. After 24 hours of culture, sensitizers accelerate Langerhans cell disappearance from the epidermis, or at least decreased the relative fraction of Langerhans cells in the suprabasal epidermis compared to the basal epidermis (Chapter 5).

The combined screening methods for skin irritant effects by MGP and compound-induced LC migration provides a novel and powerful tool to assess and study contact sensitizers and skin irritants. Toxic and immunologic effects of skin irritants and contact allergens are dissected by these novel *in vitro* methods. Implications for the interrelation of toxic and immunologic effects on the epidermis and its dendritic cells will be discussed in the next sections. This is followed by a brief discussion of contact allergens in the absence of skin irritation.

7.3 Risk assessment of skin irritants

The major cause of non-immunological inflammation of the skin is exposure to skin irritants. Therefore, it is important to identify chemicals or products that can induce skin irritation [55]. In this thesis a new method for assessment of skin irritants is described, using porcine and human OSECs (Chapter 2 and 3). The OSEC method is a replacement alternative, as no animal test or suffering is required. Irritancy was assessed by a toxicity marker, the decrease of epidermal keratinocyte RNA, visualised in frozen sections using a modified methyl-green pyronine (MGP) staining. In contrast to most histological stainings, people not educated in histology can easily interpret this MGP staining. The incubation period before RNA staining decreased correlated with the severity of the skin irritant. In other words, a strong, moderate and a weak irritant decreased RNA staining after 4, 24, and 48 hours, respectively. The chemical was classified as a non-irritant when keratinocyte RNA was still fully present after 48-hours incubation [102,127,131].

The results of OSECs were reproducible. Analysis of duplicate biopsies is sufficient to give a reliable MGP score for any skin donor. Of course, the

distinction between strong, moderate, and weak irritants is arbitrary, but so is the EC directive that considers a 20% SDS solution to be a borderline irritant [104,109,110,112]. Response to skin irritants varies widely among different human volunteers [17,111]. This is reflected by the variation found *in vitro* in both OSECs when using skin from different donors. For this reason, a minimum of three donors was used per chemical.

While most chemicals exert their irritation potential through killing keratinocytes, some (i.e. acids and bases) may be irritating by dissolving the stratum corneum, leading to barrier disruption [13]. Only at high concentrations, possibly when the stratum corneum is sufficiently dissolved, can the acid or base penetrate into the epidermis. Barrier perturbation undermines the most important homeostatic function of the skin, and may lead to irritant contact dermatitis through cytokine release [114]. This suggests that certain irritants, such as dilutions of corrosive compounds, could be tested for their irritating potential by assessing barrier disruption, e.g. by measuring trans-epithelial water loss (TEWL) [115]. The OSEC models are sensitive to these chemicals as corrosive chemicals (R34) are detected in the OSEC model as strong irritants, e.g. causing cell death within 4-hours [102,127,131]. Common sense can be used for the risk assessment of dilutions of strong irritants and corrosives. If these strong irritants are not much diluted, they should be regarded as putative (moderate) irritants (R38). This will circumvent the absence of direct proof for moderate irritancy in the dilutions of strong irritants.

The predictive power of irritancy by the porcine OSEC model is similar to that of the human OSEC model and equal or better than that of the Draize test. These results indicate that the OSEC models can be used for specific, sensitive and reproducible assessment of skin irritants. A classification as R38 or NC based on the MGP staining of hOSEC or pOSEC is robust, sensitive, and specific. Thus both the human and the porcine OSEC–MGP models are promising 'animal-saving' models for screening skin irritants.

7.4 Langerhans cell migration and skin immunology

A reliable, reproducible, semiautomated method was set up to quantify of the number of epidermal LCs [157]. Compound-induced LC migration was quantified by counting CD1a and Lag stained objects (LCs). LC migration was not formally proven, but confirmed by (i) disappearance of LC markers from the epidermis; (ii) detection of LC markers in time on cells in the suprabasal epidermis, followed by the basal epidermis and the dermis; (iii) all cells were either positive or negative for all LC markers; (iv) the presence of LCs in the culture medium [80,81] which are $CCR7^+$ (Figures 6.3d-f, j-l, 6.4b); and (v) *in vitro* generation of LCs takes at least four days.

The presence of CCR7 on the LCs indicates that they are equipped to migrate to the local lymph node [173-175]. It is postulated that immature DCs contribute to tolerance, and mature DCs initiate immune responses [129,130,145]. When LCs do not become activated, low zone tolerance to contact allergens may occur [195]. Thus immunologists are interested in the question "is maturation required for LC migration?" [43]. This question has recently been answered for human dermatopatic lymphadenitis *in vivo* [179]. We found that most spontaneously migrating $CD1a^+$ LCs

from hOSEC were $CD83^-$, thus lacking the marker of mature LCs. Also about halve of LCs, leaving the hOSEC due to accelerated migration caused by a non-irritating allergen were of immature phenotype (Chapter 5). These findings may be related to the mechanism of tissue tolerance and contact tolerance.

7.5 The immunology of skin irritation

Skin irritation is caused by a toxic mechanism [12], such as cell death detected by the MGP-staining [102,127,131]. Toxicological and immunological processes are commonly seen as different and independent processes. However, at least five reasons suggest that immunology plays a major role in contact dermatitis due to skin irritation.

First, all three non-sensitising irritants, induce LC migration in hOSEC, as assessed by the complete loss of MHC II, CD1a and Lag staining in the epidermis (Table 6.1). This decrease of human epidermal LC numbers has been shown in vivo after application of nonanoic acid [181]. That the disappearing LCs migrate is confirmed by the detection of increased numbers of LCs in culture medium of OSEC treated with 10% SDS (Figures 6.3, 6.4). Also *in vivo*, SDS has been shown to induce human LC migration to the draining lymph node [34].

Second, besides inducing LC migration, LC maturation is induced by skin irritants. 10% SDS is even better in inducing LC maturation than 1% $NiSO_4$ (Table 6.5).

Third, skin irritation induces proliferation of lymph node cells. This was already suggested by the direct relation between LC migration and the amount of cell proliferation in the LLNA [182]. Local lymph node cell proliferation is induced by irritating concentration of non-sensitizers, such as SDS, croton oil, and nonanoic acid [2,35-39]. Researchers using the LLNA takes this problem serious and have tried to tackle it by looking for markers that discriminate sensitizers from non-sensitizers. The fraction of B lymphocytes in the local lymph node might be such a marker, which could be higher due to allergen than due to irritant treatment [196,197]. It should be noted however that these authors [196,197] included benzalkonium chloride, a human allergen [2,198,199] as a non-sensitising irritant. Nevertheless, this is also a remarkable finding, in the light of allergic contact dermatitis being a T lymphocyte mediated disease [19].

Fourth, the skin inflammation in irritant contact dermatitis includes T lymphocytes, costimulation molecules (CD80, CD86), and involves the production of cytokines (IL-1α, IL-1β, TNF-α) that are indicative for antigen-specific immune reactions [66,183,185,200-205] (JJLJ, CLL, GRE, PDK, unpublished results).

Fifth, skin irritation critically depends on the T-helper cell marker CD4 [184] and the costimulatory molecules CD80 [185] and CD28 [186].

The findings in this thesis confirm that both irritants and allergens can induce LC maturation and migration, and thus may stimulate immune reactions. All cellular and molecular data indicate stated above indicate that skin irritation is an immunologic disease. However, the precise role of antigen-specific immunity in irritancy is unclear, as irritation-induced inflammation occurs rapidly and similarly in previously unsensitised subjects [183].

7.6 The need to exclude irritation for the risk assessment of contact allergens

The ECVAM stimulates the development of alternative methods for skin sensitisation testing [155]. One main aim of this project was the validation of hOSEC as a model for assessing the allergenicity of novel chemicals, as first reported by Pistoor et al.[84]. A major criticism of this model was that dose-response relations for LC migration was found for many non-allergens at non-toxic concentrations (assayed after a 24 hr exposure). This dose-dependent acceleration of LC migration at non-toxic concentrations of non-sensitizers was confirmed independently at TNO and the AMC/UvA (Tables 5.2 and 5.3). In addition, it is now clear that LC migration can also be induced at even lower concentrations of test chemicals, which are only toxic after a 48 hr exposure (Figure 6.2; Table 6.2). Such concentrations have been classified as weakly irritant [102,131,206]. If we compare our lowest weak irritant concentrations [131] with data of Pistoor *et al.* [84], it is possible that Pistoor et al. used concentrations of test chemicals which were weakly irritant. Thus, while there is a dose response relationship between LC migration and concentration, using concentration ranges similar to those used by Pistoor *et al.*, we found no such dose-response relationship at concentrations of contact sensitizers which were non-toxic after a 48 hr exposure (Figure 6.2; Table 6.3).

Six sensitising and three frequently used non-sensitising chemicals were selected and used in a dose-response to study the correctness of the hOSEC model. The sensitizers were selected because they were among the most frequent sensitizers in the human patch test assays (HPTAs) or the most potent sensitizers in the human maximisation test (HMT). LC migration and relocalisation in the epidermis was combined to the Cutaneous Immune Modulating Activity (CIMA) index. The CIMA index predicted correctly all six contact sensitizers, the guinea pig maximisation test (GPMT) detected four out of five, and the local lymph node assay (LLNA) four out of six chemicals [2] (Table 6.4). Both these rodent tests are false negative for neomycin and the LLNA was also false negative for nickel. Neomycin and nickel are not very potent human sensitizers (28% and 48%, respectively, in the maximisation test), but they both occur quite often as a contact sensitizer in the human population (2.6-13.1% and 12.9%-14.2%, respectively) (Table 6.6) [124,188]. All three non-sensitizers were correctly identified as such by the CIMA index, but all three were false positive in the LLNA.

7.7 Potency of contact sensitizers

In contrast to toxicological reactions, immunological reactions are, in general, not dose dependent. One exception is the elicitation phase of allergic contact dermatitis [189,190]. However, this dose dependency can be replaced by the addition of an irritant; in fact the dose-dependency for contact allergens seems to rely solely on the concentration of the skin irritant [45,207]. Thus it might be that allergic contact dermatitis is not a rare exception in the immunology with its dose dependency, but its dose-dependency might rely only on concomitant irritancy. For this reason, we studied LC migration in the absence of skin irritation. No differences in CIMA indexes were found between compounds known as strong, moderate and weak sensitizers (Tables 6.3 and 6.4). This suggests that there are no inherent stronger or weaker potent contact allergens.

Next, we studied the role of irritancy in the potency of allergic contact dermatitis. Skin irritation can be regarded as epidermal toxicity [13] and is strictly dose dependent [102,127,131]. Thus, differences in contact sensitizer potency found in the maximisation test may be due to differences in skin irritation potency. This was also concluded from the comparison of data obtained for each chemical at human patch test concentrations (Table 6.6). A correlation was found between the % positive individuals in the human maximisation test (HMT) and irritancy as assessed by MGP-score ($R^2 = 0.77$). Both the HMT data and MGP-score correlated with the CIMA index at patch test concentration ($R^2 = 0.85$; Figure 6.2; Table 6.6). A less strong correlation was found between the HMT and the five true positives in the GPMT ($R^2 = 0.50$) and only a minimal correlation between the HMT and the proliferation index (EC_3) of the four true positives of the LLNA ($R^2 = 0.45$). Thus contact allergen potency as determined by the human maximisation test correlates most strongly with skin irritancy.

7.8 Hazard identification at low concentrations

The main problem with hazard identification is to identify the risks of low concentrations. Many sensitised individuals need almost irritating concentrations to develop contact dermatitis, but also quite some people react to extreme low doses [191]. LC migration at non-irritating concentrations is specific for sensitizers (Figure 6.2; Table 6.3). The threshold for accelerating LC migration as measured by the CIMA index differed between sensitizers. Some sensitizers, like nickel and eugenol, still caused an increase in CIMA index at 20-fold dilutions of the lowest weak irritant concentrations (LWICs), while other sensitizers, like DNCB, potassium dichromate and neomycin, had a smaller window of non-irritating concentration that induced LC migration. Nevertheless, all sensitizers showed LC migration at 0.2 and 0.5 times the LWICs, while no non-sensitizers showed LC migration at these concentrations (Figure 6.2; Table 6.3). LC migration is sufficient for sensitisation [130] and sub-irritating concentrations of sensitizers can induce sub-clinical sensitisation [187], but also low-zone tolerance [195,208,209]. The finding of both $CD83^+$ and $CD83^-$ LCs in hOSEC medium (Table 6.5) confirms that both subclinical sensitisation as well as subclinical toleration is possible.

People will attempt to avoid exposure to (weak) irritant concentration to avoid direct clinical skin effects. However, some compounds, like nickel, are very well capable of inducing LC migration at concentrations that do not give direct adverse effects. Thus, people may be exposed more frequently to sensitizing concentrations of compounds like nickel, which induce LC migration at concentrations below those that cause (weak) irritancy. This may explain that some weak sensitizers, e.g. nickel, are weak sensitizers according to the maximisation tests, but are frequent sensitizers as measured by human patch tests.

The indirect measurement of sensitisation makes it difficult to study which concentration is the threshold for sensitisation. However, there are reasons to assume that certain steps in antigen presentation are shared by sensitisation and elicitation reactions. These may include the migration of LC, but also the attraction of T lymphocytes by chemokines produced by LCs [210-214] and keratinocytes [215,216]. Keratinocyte derived chemokines can attract T lymphocytes, but are probably

optimally induced at (weak) irritant concentrations [217-219]. Thus, LCs may play an important role in elicitation reactions to low concentrations. Extreme low concentrations of nickel may induce elicitation reactions [220-223], but higher concentrations are required for an elicitation reaction to e.g. cobalt chloride or potassium dichromate [224]. These data contrast with potency data derived from the human and guinea pig maximisation tests, and the EC_3 value derived of the LLNA [2,38,39,147,151,225-227]. Nevertheless they are in agreement with the frequency of allergic people in the population as assessed by the HPTA [124,188]. The lowest concentrations of a chemical inducing LC migration in hOSECs may potentially correlate with the lowest concentrations causing sensitisation *in vivo*, and possibly also with elicitation *in vivo*. Another putative alternative method for the determination of elicitation threshold would be the measurement of T cell attracting chemokine production in hOSECs by an ELISA assay.

7.9 Mechanism of LC migration

The classic immunological paradigm of self – non-self discrimination [19] has more recently been merged with the danger hypothesis [171]. The awareness of a putative danger, such as cell death or known pathogens, initiates the immune system, while clonal selection avoids most of the autoimmune reaction. Dangers activate dendritic cells [219,228], such as Langerhans cells in the skin, which will maturate and migrate to draining lymph nodes. The danger hypothesis provides an excellent explanation for LC migration induced by skin irritants [229]. Irritancy leads to epidermal cytotoxicity, and dying cells trigger LC migration and maturation [217-219]. However, the danger hypothesis does not predict LC migration induced by non-irritant concentrations of contact sensitizers [229]. Considering the absence of dose-response effects, an alternative explanation would be that LC migration is induced by a limited available biological signal transducer, such as a pattern recognition receptor [230]. In view of this hypothesis, the direct activation of DCs by sensitizers would suggest that contact allergens are recognized as danger [24,76,172,231]. However, almost all identified pattern recognition receptors [230] are not or hardly present on LCs [29].

Cytokines like the three interleukin 1 (IL-1) like molecules (IL-1α, IL-1β, IL-18) and tumour necrosis factor α (TNF-α) can induce LC migration [200,232-238]. These cytokines are induced by contact allergens and different kinds of non-sensitising skin irritation [66,201-205] (unpublished results). In the case of the sensitizer 2,4,6-trinitrochlorobenzene (TNCB), it appears that IL-1α, and not IL-1β, is the causative mediator for priming of antigen-specific T lymphocytes in the lymph node [236]. Contact sensitizers directly induce maturation of monocyte-derived DCs [24,76,231,239], independently of IL-1β and TNF-α [172] But the question remains why do contact allergens induce maturation and migration of LCs? Thus are contact sensitizers dangerous, as migration of Langerhans cells is in general caused by danger [219,228]. More precisely the question arises: are contact sensitizers dangerous besides their ability to induce an allergic reaction?

Contact sensitizers can induce contact tolerance by various mechanisms such as a non-irritating low dose [195,208,209], oral administration [240,241], administration at an LC depleted site [41,178,242-250], or in the absence of dendritic cells [251], and blockade of

cytokines [252]. Tolerance can be associated with suppressor cell activity [253,254] by CD4 [255,256] or CD8 T lymphocytes [209,241]. In humans exposed but not sensitised to nickel, nickel induced T lymphocyte proliferation *in vitro*, also suggesting tolerance to nickel [257]. This tolerance might be induced by immature $CD1a^+$ $CD83^-$ LCs migrating from hOSEC treated with nickel (Chapter 5). Mice and guinea pigs may become orally tolerant to nickel by $CD8^+$ lymphocytes [241,258]. It should be noted, however, that the induction of hypersensitivity in mice requires injection into the skin of oxidised forms of nickel [259], which is different from the sensitisation in human.
The application of several tumour promoters and complete carcinogens at the skin may result in carcinogenesis and concomitant LC migration and toleration [244,254,260]. Local toleration may imply the absence of mature dendritic cells or LCs in tumours [261,262], and may allow developing tumours to escape immune recognition. Toleration and LC migration can also occur after the application of a contact allergen [41,195,208,245]. Moreover, from a chemical point of view, the ability of mutagens to react with DNA is similar to the ability of haptens to react with proteins. In light of common biological and similar chemical pathways of carcinogens and contact sensitizers, many chemicals are toxic by both mechanism [263]. This list includes nickel [222,264-270], chromate [266,271], and polyaromatic hydrocarbons [272]. It is estimated that there could be several thousand contact sensitizers for humans in commercial use that are rodent carcinogens [273].
It can be hypothesised that the biological and chemical resemblance of sensitizers and carcinogens might be sufficient for the body to treat haptens as putative dangers.

7.10 Nickel: a case study of risks.
Here a case study is presented to help understanding of hazards of contact allergens and the reduction of these risks by avoidance of contact. Nickel has been classified as an allergen of moderate potency [151], but is nevertheless the most prevalent contact allergen in the general population of the industrialised world [222]. A specific reaction of nickel with fatty acids in human skin forms a lipophilic nickel soap [274,275], which could penetrate the skin and reach Langerhans cells. Lipid antigens are presented by CD1 molecules [25] which are present on Langerhans cells of humans [91-94], and many other mammals [95-100], including pigs [101] (unpublished data), but not on Langerhans cells of rats and mice.
The most effective way to avoid allergic reactions is to avoid contact with it. This may be hard in the case of nickel, which can be found in food (e.g. 5-10 mg/kg in nuts and cocoa beans) [222]. However, the most important cause of sensitisation and elicitation is direct skin contact. Regulation can strongly reduce contact dermatitis due to nickel by reducing exposure to nickel. The EU nickel directive of 1994 was implemented in Denmark in 1989 and serves as a good example. In Denmark nickel hypersensitivity among children aged 0-18 years decreased from 24.8% in 1986-1987 to 9.2% in 1997-1998 [276]. The nickel directive is, however, not in use for coins, tools, handles and keys as objects that come only into temporary contact with the skin [277]. This leads to the paradox, that the release of nickel from 1- and 2-euro coins in artificial human sweat is a factor 240 to 320 too high according to the

EU Nickel Directive for prolonged exposure [278]. Indeed, prolonged exposure to these nickel containing coins may cause contact dermatitis [277,278]. Sensitisation to coins is not new, but was already reported for decades [279,280].

7.11 The way forward: prevalidation, validation and implementation

Since Russell and Burch's *The Principles of Human Experimental Technique* (1959) the aim is to minimise animal suffering, while maintaining the scientific value of the experiments [6]. Within the three Rs, Replacement is the first alternative, before Reduction and Refinement. The models in this thesis may become replacement alternatives. The way forward is the validation of these methods by the ECVAM or the ICCVAM, which will lead to their acceptance and implementation [281,282].

The research described in this thesis was financially supported by the ZonMw Dutch Programme on Alternatives to Animal Testing (proj.nr 3170.0003).

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Nederlandse samenvatting

Huidkweek voor het screenen van huidirriterende en contact allergene stoffen.

Gepubliceerd met kleine wijzigingen als:
J.J.L. Jacobs (2003). Veiligheid voor de huid zonder dierenleed. www.kennislink.nl

Abstract

In dit proefschrift staan nieuwe proefdiervrije alternatieve testen beschreven die ontwikkeld zijn om na te gaan of een nieuw product wel of niet veilig is voor de menselijke huid. Producten die niet veilig zijn voor de huid, kunnen eczeem veroorzaken. Huideczeem kan zowel veroorzaakt worden door huidirriterende stoffen, als door stoffen die contact allergie veroorzaken. De producten worden getest op stukjes gekweekte huid. Varkenshuid wordt verkregen als slachtafval, mensenhuid als operatie-afval. De reactie van de huid op de teststof vertelt of een stof irriterend dan wel allergeen is. Irriterende stoffen zijn giftig voor de huidcellen. Allergenen stoffen zetten de in de huid aanwezige Langerhanscellen aan tot migratie ook op niet-irriterende concentraties.

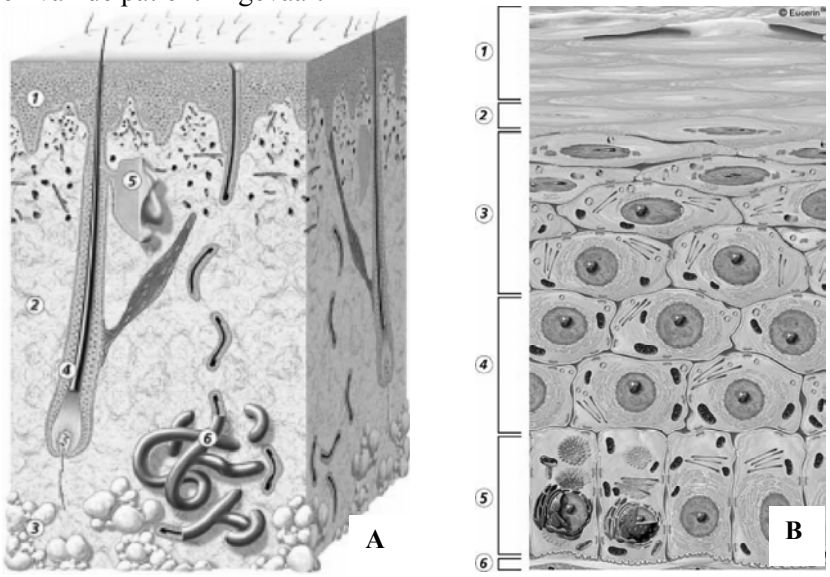
Inleiding.
Producten, zoals cosmetica, die in de EU op de markt komen moeten getest worden op hun veiligheid, waaronder de veiligheid voor de huid. Producten die niet veilig zijn voor de huid, kunnen na contact huideczeem veroorzaken. Eczeem is een huidaadoening die bestaat uit roodheid, schilfering en soms blaasjes, scheurtjes en veelal een hevige jeuk. Eczeem kan veroorzaakt worden door erfelijke aanleg, maar ook door contact met stoffen. Twee soorten stoffen kunnen eczeem veroorzaken: stoffen die de huid irriteren en stoffen die contact allergie veroorzaken. Irriterende stoffen (zoals zepen en zuren) zijn giftig voor de huid, doordat ze huidcellen doden. Blootstelling aan contact allergenen (zoals nikkel en parfums) kan leiden tot overgevoeligheid voor die stoffen. Als een overgevoelig persoon weer wordt blootgesteld aan hetzelfde allergeen ontstaat enkele dagen later huideczeem. In het verleden, en in sommige gevallen nog steeds, werden proefdieren gebruikt voor het testen van de veiligheid van stoffen. Omdat men het proefdiergebruik wil verminderen is dit steeds minder vaak en voor minder stoffen toegestaan. Zo verbiedt nieuwe EU-regelgeving het gebruik van proefdieren voor het testen van cosmetica per 2009. Dus moeten alternatieven worden ontwikkeld die de veiligheid van het product garanderen zonder dierproeven. Voor de veiligheid van de huid zijn alternatieven ontwikkeld die testen of bepaalde stoffen huidirritatie of allergie kunnen veroorzaken. De meest belovende alternatieven maken gebruik van de kweek van stukjes huid, die overblijven na operaties in ziekenhuizen en bij de slacht van dieren.

Achtergrond: Geschiedenis van het gebruik van proefdieren
In 1859 schreef Charles Darwin zijn bekende boek '*On the origin of species*' waarin hij stelde dat de mens biologisch gezien een dier was. Claude Bernard gebruikte dit principe 6 jaar later in zijn boek: '*Introduction â l'étude de la medicine experimentale*' als argument om proefdieren te gebruiken om experimentele medische vooruitgang te boeken. Sindsdien heeft de medische wetenschap grote vooruitgang geboekt, maar is ook het proefdiergebruik gigantisch toegenomen. De wetgeving vereist vaak dat producten op hun veiligheid worden getest, waardoor vele dierproeven noodzakelijk zijn.
In 1959 schreven Russel en Burch hun boek '*The principles of human experimental technique*', een pleidooi voor het terugdringen van het proefdiergebruik. Sindsdien is het proefdiergebruik afgenomen, door bewuster gebruik en wetgeving. In Nederland werd in 1977 de wet op de proefdieren in werking, die in 1999 werd aangescherpt. Verder terugdringen van het proefdiergebruik is afhankelijk van de 3 V's van Russel en Burch: vermindering, verfijning en vervanging. Om de betrouwbaarheid van alternatieve testen te garanderen moeten deze testen worden goedgekeurd op juistheid van de voorspelling en herhaalbaarheid van het resultaat; dit heet validatie. Validatie gebeurt door twee instanties, de ECVAM in de EU en de ICCVAM in de VS, die elkaars validaties erkennen. Indien een alternatieve, dat wil zeggen proefdiervrije test, gevalideerd is, mag deze gebruikt worden in plaats van de dierproef. De wet op de proefdieren regelt dan dat de dierproef niet meer gebruikt mag worden voor testen waar alternatieven voor bestaan. Validatie van alternatieve methoden is dus een belangrijke praktische stap voor vermindering van

dierproeven. Ieder jaar komen ongeveer 2000 nieuwe stoffen op de wereldmarkt. De vraag naar veiligheidstesten is dus enorm groot. Sinds 1999 is het gebruik van proefdieren voor cosmetica in Nederland verboden; het verbod voor de gehele EU volgt onder voorwaarden in 2009. Dit verhoogt de druk voor cosmetica-fabrikanten om alternatieven te ontwikkelen, maar dat geldt niet voor andere stoffen. Daardoor zijn onderzoekers het verst met de ontwikkeling van alternatieve testen voor de veiligheid van cosmetica.

De huid

De huid beschermt het lichaam tegen invloeden van buitenaf, zoals uitdroging en infecties. Hiertoe bevat de huid een aantal barrières (afb. 8.1). De buitenste barrière is de hoornlaag bestaande uit dood celmateriaal, waar deze laag extreem dik is wordt dit eelt genoemd. Daaronder zit een laag van levende keratinocyten (huidcellen), cellen die keratine (hoornstof) produceren voor de hoornlaag. Deze cellen zijn intensief met elkaar verbonden en vormen op die manier de tweede barrière tegen de buitenwereld. Bovenstaande structuren behoren allemaal tot de opperhuid (epidermis). De derde barrière wordt gevormd door het basaalmembraan, dat de opperhuid scheidt van de lederhuid (dermis) en door interactie tussen beiden wordt aangemaakt. De lederhuid is bindweefsel en bestaat uit fibroblasten, bloedvaten en collagene vezels. De bloedvaten zorgen voor de voeding (ook voor de epidermis), en de collagene vezels geven de kracht als de rekbaarheid van de huid. Zonder de bescherming van de huid zijn de gezondheid en het leven van de patiënt in gevaar.



Afbeelding 8.1. (A) Schematische doorsnede van de huid. 1 opperhuid (epidermis) met de verschillende lagen keratinocyten en Langerhanscellen; 2 lederhuid (dermis); 3 onderhuids vetweefsel (subcutis); 4 haarfollikel; 5 talgklier; 6 zweetklier. (B) Schematische doorsnede van de opperhuid (epidermis). 1-2 hoornlaag; de hoornlaag wordt vanaf laag 2 aangemaakt; 3-5 verschillende lagen keratinocyten (huidcellen): De keratinocyten in laag 5 (stratum basale) delen en vermeerderen zich, duwen andere keratinocyten naar boven naar laag 4 (stratum spinosum) en uiteindelijk laag 3 (stratum granulosum) waar de keratinocyten helemaal afgeplat zijn en de korrels bevatten die later de hoornlaag vormen; 6 Basaal membraan. Bron: http://www.ccunix.ccu.edu.tw/~chenmsl/tea/SKIN_910721.htm.

Huidirritatie

Bij huidirritatie is de barrière functie van de huid verminderd. Dit kan zijn doordat de hoornlaag is opgelost door beschadiging, maar ook door zuren, of zepen (bijvoorbeeld door overmatig handen wassen). De afwezigheid van de hoornlaag leidt celdood van de onbeschermde keratinocyten. Vaak gaat het ook om giftige stoffen die door de hoornlaag heen dringen en vervolgens bij de keratinocyten komen. Dit leidt dan tot dood van de keratinocyten, en dus een verlies van de barrière-functies van de huid. Omdat de huid beschadigd is, reageert het lichaam via de lederhuid met een herstelreactie. Die reactie kan bestaan uit een sterkere doorbloeding, hetgeen waar te nemen is als warmte en roodheid. Ook kunnen beschadigingen van de hoornlaag leiden tot een 'droge' huid. Deze verschijnselen noemen we eczeem.

Screenen van stoffen die de huid kunnen irriteren

De eerste test voor het screenen van stoffen op huidirritatie stamt uit 1944: de *Draize test*. In de *Draize test*, worden stoffen op een kaalgeschoren plekje op de rug van een konijn opgebracht. Na 24 uur wordt gekeken of er een huidontsteking is, en hoe erg deze is. Ondanks de vele beperkingen van deze test, mede door verschillen tussen mens en konijn, wordt deze test toch nog veel gebruikt.

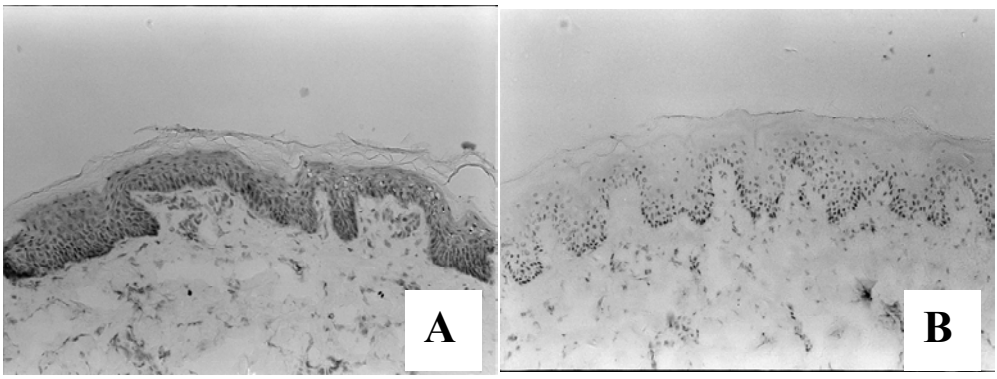
De meest betrouwbare manier om te testen of een stof huidirritatie veroorzaakt bij mensen, is deze op de huid van een mens te smeren. Dit gebeurt onder stringente voorwaarden in testen met vrijwilligers. Zo mag de stof niet kankerverwekkend zijn, geen hinderlijke allergie opwekken, of op een andere manier blijvende schade veroorzaken. Veel stoffen worden dus al bij voorbaat uitgesloten van deze test, en stoffen die wel gebruikt mogen worden, worden heel voorzichtig getest. Eerst wordt gekeken of ze na 30 minuten blootstelling huidirritatie veroorzaken, indien dat niet het geval is na 1 uur, vervolgens 2 uur, 3 uur en uiteindelijk na 4 uur. Stoffen die na 4 uur geen huidirritatie veroorzaken worden beschouwd als niet-irriterend voor de mens. Ondanks de vele beperkingen van deze test, is deze toch erg belangrijk, omdat hij voor, weliswaar een beperkt aantal stoffen, de echte uitkomst geeft: wel of niet irriterend. Deze uitkomst kan gebruikt worden voor de validatie van nieuwe testen, die dan in hun voorspellende waarde vergeleken kunnen worden met de *Draize* test. In de *Draize* test geeft bijvoorbeeld decanol, een organisch alcohol, een positive uitslag, terwijl deze stof niet irriterend is voor mensen.

Op dit moment is er nog een andere test goedgekeurd die de meest agressieve irriterende stoffen, de zogenaamde corrosieve stoffen, kan aantonen. Hierbij wordt gebruik gemaakt van een laagje collageen waar de teststof wordt opgebracht. Collageen is een stof die veel voorkomt in de huid, en de huid zijn stevigheid geeft. Stoffen die collageen oplossen zijn naar alle waarschijnlijkheid corrosieve stoffen. Dat is het principe van de test. Vele stoffen, zoals zepen zijn niet in staat collageen op te lossen, en zijn dus geen corrosieve stoffen; ze kunnen echter wel huidirritatie veroorzaken. Zulke stoffen moeten dus op een andere manier worden getest.

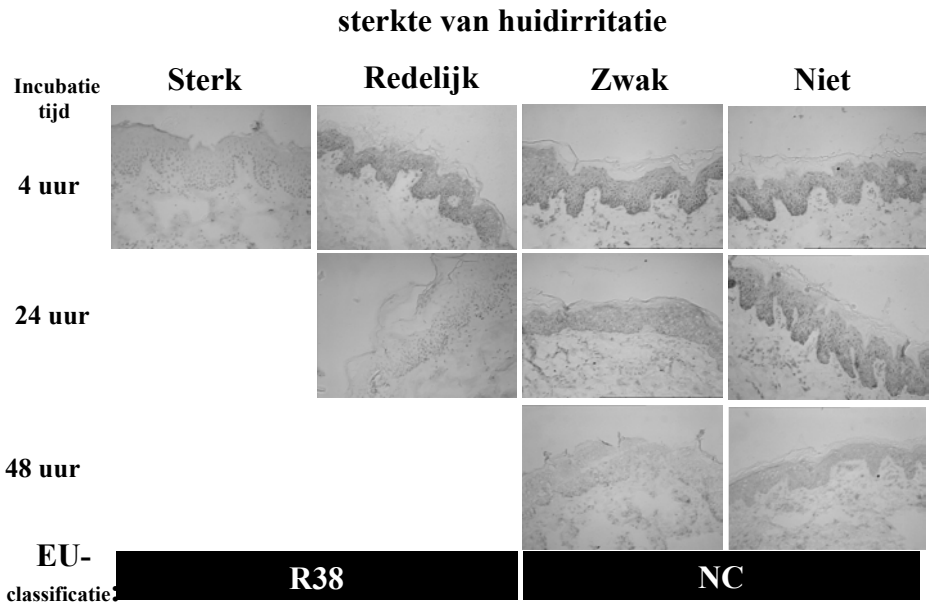
Screenen van irriterende stoffen in huidkweek

Huidirritatie is het gevolg van giftige stoffen die celdood veroorzaken in de opperhuid. Dus irriterende stoffen kan men screenen met levende mensenhuid. De mensenhuid wordt voor een deel verkregen bij ziekenhuis-operaties, zoals borst- en buikverkleiningen. Na het schoonmaken worden de stukjes huid in leven gehouden, door ze te kweken. Dit kweken gebeurt zo natuurgetrouw mogelijk: de lederhuid ligt in kweekmedium en krijgt voedingsstoffen, de opperhuid en hoornlaag zitten boven het kweekmedium in de vrije lucht. Tijdens het kweken wordt de te testen stof boven op de huid aangebracht; indien de stof irriterend is, zal dit leiden tot celdood van de keratinocyten. Cellen kunnen echter op vele manieren sterven en ook binnen deze manieren bestaan er vele variaties. Omdat celdood op verschillende manieren kan beginnen, kan men alleen celdood aantonen als men wacht totdat de cel dood is.

Om celdood betrouwbaar aan te kunnen tonen is een test nodig die alle gevallen van celdood kan registern. RNA, de werkkopie van het DNA, is noodzakelijk voor het maken van eiwitten en daarmee noodzakelijk voor het blijven leven van een cel. RNA is echter ook heel instabiel, en wordt snel afgebroken door RNAses. Indien een cel doodgaat zal binnen enkele uren geen RNA meer aanwezig zijn. Dit kan worden aangetoond met de methylgroen-pyronine kleuring (Afbeelding 8.2). De tijd van blootstelling die nodig is om celdood te krijgen is een goede maat voor de sterkte van de irriterende stoffen gebleken. Stoffen die in 4 uur de keratinocyten doden zijn sterk irriterend, in 24 uur redelijk irriterend, in 48 uur zwak irriterend, en stoffen die de keratinocyten niet doden na 48 uur zijn niet irriterend. Redelijk irriterende stoffen vallen onder de EU regelgeving, die daar een veiligheidscode R38 aan geeft, welke staat voor "irriterend" (afbeelding 8.3). Deze testen voor irriterende stoffen staan beschreven in dit proefschrift in hoofdstuk 2 voor varkenshuid en hoofdstuk 3 voor mensenhuid.



Afbeelding 8.2 Aantonen van celdood in de huidkweek met behulp van de methylgroen pyronine (MGP) kleuring. Methylgroen kleurt de kernen (DNA) van de cellen groen-blauw aan (donkere puntjes) en pyronine kleurt het cytoplasma van de cellen paars aan. RNA is alleen in levende cellen aanwezig. Duidelijk is te zien dat foto A een grijze band met veel RNA bevat terwijl foto B een dode epidermis zonder RNA laat zien.



Afbeelding 8.3. Deze afbeelding laat zien hoe aan de hand van het tijdstip van celdood in de huidkweek na het opbrengen van de stof, de sterkte van de huid-irriterende stof kan worden bepaald. R38 is de Internationale risico-aanduiding voor irriterende stoffen; NC betekend *not-classified*, dus niet aangeduid als een irriterende stof.

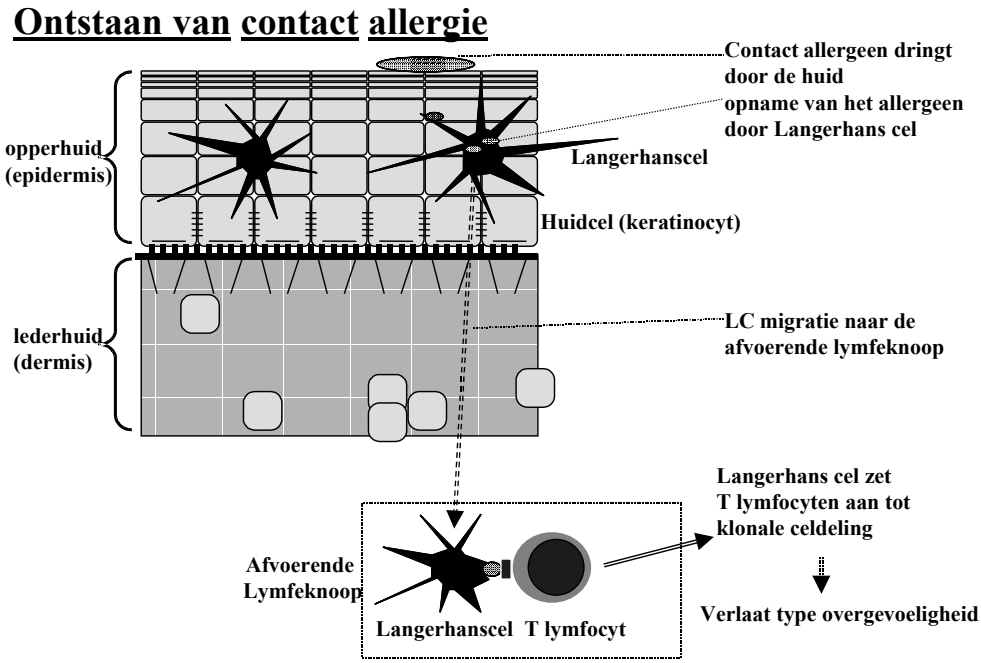
Het afweersysteem in de huid

De huid beschermt ook tegen infecties. Dit gebeurt door de fysieke barrières, maar ook door het aanzetten van het afweersysteem. Het aanzetten van het afweersysteem gebeurt door speciale cellen in de huid, de dendritische cellen. De dendritische cellen in de opperhuid heten Langerhanscellen, genoemd naar Paul Langerhans. De Langerhanscellen hebben niets te maken met de eilandjes van Langerhans die insuline maken in de alvleesklier, maar zijn wel naar dezelfde ontdekker genoemd. Dendritische cellen vinden hun oorsprong in het beenmerg als witte bloedcellen, en gaan vandaar naar het bloed. Vanuit het bloed komen ze in alle weefsels en organen van het lichaam (dus ook in de huid). In deze weefsels worden ze 'onrijpe' dendritische cellen genoemd, en wachten ze op activatie door een ontsteking. Indien de 'onrijpe' dendritische cel wordt geactiveerd dan rijpt de cel, verlaat de huid en gaat via het lymfevat naar de afvoerende lymfeknoop. In de lymfeknoop geeft de, inmiddels rijpe, dendritische cel zijn informatie over het type ontsteking en de ziekteverwekker door aan de lymfocyten. De T- en B-lymfocyten die passen bij deze ziekteverwekker gaan vervolgens delen waardoor een afweerreactie ontstaat.

Contact allergie

Contact allergie (type 4 allergische reactie) wordt veroorzaakt door T-lymfocyten, die normaal niet in de huid zitten. Daardoor duurt het 2 tot 3 dagen eer een contact allergie reactie zich ontwikkelt. De bekendste contact allergenen zijn nikkel (in sierraden en euromunten), rubber, conserveermiddelen (formaldehyde, zit in

shampoo), geneesmiddelen (antibiotica, zoals neomycine) kleurstoffen en vele parfums (in cosmetica).
Contact allergie kent twee stadia, het sensibilisatie (het overgevoelig worden) en het elicitatie stadium (de overgevoeligheidsreactie). Tijdens het overgevoelig worden komt een lichaamsvreemde stof in contact met de huid en dringt door de hoornlaag heen de opperhuid in. Daar nemen de Langerhanscellen deze stoffen op en worden geactiveerd. De geactiveerde Langerhanscellen migreren vervolgens naar de lymfeknoop om de T-lymfocyten te activeren (afbeelding 8.4). Als je T-lymfocyten hebt ontwikkeld tegen een allergeen dan ben je allergisch. Van het allergisch worden en allergisch zijn merk je niets totdat je in aanraking komt met het allergeen waarvoor je allergisch bent.
Als een allergisch iemand in aanraking komt met het specifieke allergeen volgt de overgevoeligheidsreactie (elicitatie). Aangezet door geactiveerde Langerhans-cellen, komen T-lymfocyten de huid binnen. Als deze T-lymfocyten hun allergeen herkennen maken ze meer ontstekingsfactoren, waardoor een huidontsteking, oftewel huideczeem ontstaat. Dit proces vergt 2 tot 7 dagen om op te komen en vaak een of enkele weken om weer te verdwijnen.



Afbeelding 8.4 Contact allergie is een verlaat type overgevoeligheidsreactie. Contact allergie ontstaat doordat een lichaamsvreemde stof op de huid komt, waarna die stof door de huid heendringt en Langerhans-cellen activeert. Vervolgens migreren de Langerhanscellen uit de huid naar de afvoerende lymfeknoop, alwaar ze witte bloedcellen (om precies te zijn T-lymfocyten) aanzetten tot celdeling, waardoor allergie ontstaat. Bij blootstelling van een allergisch persoon, zullen de specfieke T-lymfocyten naar de plaats van blootstelling gaan, waar ze de ontstekingsreactie en dus de huiduitslag veroorzaken.

Een bijzonder geval van een overgevoeligheidsreactie is de zogenaamde *recall* of herhalingsreactie. Deze reactie ontstaat indien hetzelfde allergeen op een plaats komt waar eerder een overgevoeligheidsreactie heeft plaatsgevonden. Na de eerdere reactie blijven een aantal specifieke T-lymfocyten achter in de huid. Indien op diezelfde plaats weer hetzelfde allergeen komt, gaat de allergische reactie veel sneller. Bij veelvuldige herhaling kan een allergische reacties binnen seconden, minuten of uren ontstaan. Een voorbeeld hiervan is de herhalingsreactie op nikkel, bijvoorbeeld in de Euro-munten. Deze munten geven 200 keer zoveel nikkel af dan de EU toestaat voor langdurig contact. De EU gaat er echter vanuit dat mensen niet langdurig worden blootgesteld aan euro-munten. Sommige mensen krijgen herhalingsreactie van het eten van de stof waarvoor ze allergisch zijn. Zo komt het voor dat mensen huiduitslag krijgen na het eten van nikkelhoudende voedingsmiddelen zoals pinda's en chocola.

De relatie huidirritatie en contact allergie

Vrijwel alle afweerreacties zijn onafhankelijk van de hoeveelheid antigeen (of allergeen). Dit in tegenstelling tot reacties op giftige stoffen die juist altijd afhankelijk zijn van de hoeveelheid giftige stof. Contact allergie is een belangrijke uitzondering, doordat de afweerreactie meestal strikt afhankelijk is van de dosis van het allergeen. In hogere concentraties zijn contact allergenen ook huid irriterende stoffen. Hoe dichter de concentratie van het allergeen komt bij de concentratie die huidirritatie veroorzaakt, hoe sterker de reactie. Toch blijkt het niet zo te zijn dat altijd meer allergeen nodig is. De benodigde dosis allergeen kan laag worden gehouden als een irriterende stof wordt toegevoegd. Klaarblijkelijk is een zekere mate van huid irritatie nodig voor een allergische reactie. Als een dermatoloog naar huideczeem kijkt kan deze niet zien of het gaat om huidirritatie of om contact allergie. Beide ziekten hebben een groot aantal overlappende verschijnselen. De risico's tussen beide stoffen verschillen echter sterk; een huidirritant is veilig zolang je onder de irriterende concentratie blijft, maar een contact allergeen kan al bij zeer lage concentraties een schadelijke reactie veroorzaken.

Mogelijkheden om contact allergenen te screenen

Tot voor kort werd met de cavia maximizatie test bepaald of een stof een contact allergeen is. In deze test wordt getracht om cavia's overgevoelig te maken voor de teststof. Net als bij mensen kun je alleen zien of een cavia overgevoelig is door de stof op de huid op te brengen en te kijken naar de huidreactie. Deze test kost zeer veel cavia's. Recent is er een alternatief om proefdiergebruik te verminderen gevalideerd waarbij muizen gebruikt worden in plaats van cavia's: de lokale lymfeknoop test (*local lymph node assay*). Deze test is ook anders van opzet: men kijkt niet naar huideczeem maar naar celdeling in de lokale lymfeklier. Een verhoogde celdeling in de lymfeklier geeft aan dat de stof een allergeen is. Door het verschil in testmethode zijn voor de muizentest minder dieren nodig per teststof dan bij de caviatest (ongeveer 15 tegenover ongeveer 30). De voorkeur gaat echter uit naar een alternatief waarbij helemaal geen proefdieren meer gebruikt worden.

Net zoals bij testen voor de huid-irriterende stoffen, is de mens de meest betrouwbare bron van informatie. Het is echter ethisch onverantwoord om te proberen mensen overgevoelig te maken voor een stof waar ze in hun dagelijks leven mee in aanraking kunnen komen. Dus op deze manier testen (de zogenaamde humane maximisatie test) kan alleen met stoffen, waar mensen normaal niet mee in aanraking komen. Een voorbeeld hiervan is DNCB, dinitrochlorobenzeen, een chemische stof die buiten het lab nooit wordt gebruikt. Een tweede bron van informatie zijn de zogenaamde, *humane patch test* allergenen. De patch test is de plakproef die wordt uitgevoerd om te kijken of iemand voor een stof allergisch is. Alle stoffen waarvoor mensen allergisch zijn noemen we patch test allergenen. Bij patch test allergenen zijn mensen overgevoelig geworden door het dagelijks gebruik van allergische stoffen. Deze test is dus alleen geschikt voor stoffen die al een tijdje op de markt zijn, en waar mensen in hun dagelijks leven mee in aanraking komen. Nikkel uit de sieraden en (iso)eugenol uit parfum zijn daar de bekendste voorbeelden van. De gegevens van de humane maximizatie test en de humane patch test allergenen kunnen worden gecombineerd om een totaalbeeld van de menselijke allergenen te krijgen. Als men dit doet, dan blijkt dat de testen in cavia en muis slechts in ongeveer 72% van de stoffen correct voorspellen of ze een allergeen zijn of niet. Een voorbeeld van deze 'missers' is het menselijke allergeen neomycine, waar muis en cavia ongevoelig voor zijn. De muizentest mist zelfs nikkel als allergeen.

Screenen van allergenen in huidkweek

Het model van de huidkweek dat wordt gebruikt om irriterende stoffen te screenen kan ook worden gebruikt bij het testen van allergenen. Zoals boven staat, migreren de actief geworden Langerhanscellen bij een overgevoeligheidsreactie uit de epidermis naar de lymfeknoop. In de huidkweek zit weliswaar geen lymfeknoop, maar de Langerhanscellen verdwijnen wel uit de opperhuid. Het aantal Langerhanscellen in de huid kan worden geteld door dunne plakjes (coupes) te snijden van de huid. Die coupes worden vervolgens gekleurd met behulp van een antilichaam tegen Langerhanscellen. Zodoende kan het aantal Langerhanscellen in de huid worden geteld, en kan dus worden gekeken of een stof Langerhanscellen versneld uit de huid laat migreren (hoofdstuk 4 van dit proefschrift). Dit blijkt het geval te zijn voor contact allergenen en stoffen die de huid (ten minste zwak) irriteren. Met behulp van de eerder beschreven test voor huid irriterende stoffen kunnen deze stoffen worden uitgesloten. Om onderscheid te maken tussen irriterende stoffen en allergenen moet een lage concentratie worden gebruikt die als niet-irriterend bekend staat: dit is een stof die na 48 uur op de huid (in de weefselkweek test) nog geen celdood veroorzaakt. Op deze manier kunnen contact allergenen specifiek worden aangetoond. In tegenstelling tot de dierproeven toont deze test wel aan dat stoffen als neomycine en nikkel contact allergenen voor de mens zijn. Deze test staat beschreven in hoofdstuk 5 van het proefschrift.

Dierenhuid als vervanger voor mensenhuid

De vacht van de meeste dieren oogt anders dan de roze huid van de mens, zowel op het eerste gezicht, als onder de microscoop. Dieren met een harige vacht hebben

vaak een dunne huid bestaande uit 1 of 2 cellagen, terwijl de huid van de mens vele cellagen dik is. Dieren met een vacht reageren dan ook anders en op andere stoffen met betrekking tot huidirritatie en contact allergie. Dit leidt tot vele foute uitkomsten in dierproeven, zoals in de Draize test bij konijnen, de maximisatie test bij cavia's en de locale lymfeklier test bij muizen. Het probleem met mensenhuid is echter de beperkte beschikbaarheid. Huid die overblijft van een operatie mag en kan met toestemming van de patiënt en medewerking van de arts worden gebruikt. Het is echter twijfelachtig of die hoeveelheden voldoende zijn om op jaarbasis 2000 nieuwe stoffen te screenen. Vandaar de vraag naar een diersoort met een vergelijkbare huid als die van de mens. De roze varkenshuid lijkt zowel op het eerste gezicht als onder de microscoop sterk op de menselijke huid. Proeven met tientallen huid-irriterende stoffen hebben laten zien dat varkenshuid voor 95% hetzelfde reageert als mensenhuid. Het kweken van varkenshuid zou dus een goede aanvulling zijn voor het kweken van mensenhuid, omdat varkenshuid in grotere hoeveelheden voorradig is.

De toekomst van alternatieven voor proefdieren

De toekomst van alternatieven voor dierproeven en proefdieren hangt sterk af van de prioriteiten die worden gesteld. Proefdieren zullen waarschijnlijk nog lange tijd nodig blijven voor het ophelderen van mechanismen van ziekten en het daarmee samenhangende ontwikkelen van nieuwe geneesmiddelen. Ontwikkelingen in laboratoria laten echter zien dat vele routine-testen bijvoorbeeld op giftigheid, die nu nog met proefdieren worden uitgevoerd, op den duur kunnen worden vervangen door alternatieve proefdiervrije testen. Er is echter tijd en geld nodig om alternatieve testen eerst te ontwikkelen en vervolgens te valideren. Voor wat betreft de ontwikkeling van alternatieve testen voor huidirritatie en contact allergie is dit proefschrift een goede aanzet.

The research described in this thesis was financially supported by the ZonMw Dutch Programme on Alternatives to Animal Testing (proj.nr 3170.0003).

Dankwoord

Als eerste wil ik Graham Elliott en Pran Das bedanken voor het schrijven van de subsidieaanvraag t.b.v. het projectvoorstel PAD 96-32 en het Platform Alternatieven voor Dierproeven voor het honoreren van deze subsidieaanvraag (ZonMw Dutch Programme on Alternatives to Animal Testing, project nummer 3170.0003). Prof. J.J. Weening wil ik bedanken dat hij mijn promotor wil zijn; Graham en Pran dat ze copromotor willen zijn. De commissieleden wil ik van harte bedanken voor hun zitting in de commissie.

Juist omdat ik in het laatste AIO-jaar (zonder afdeling) heb mogen ervaren hoe weinig je alleen kunt bereiken, en dus ook hoe belangrijk steun van medewerkers is, wil ik een aantal mensen bedanken voor hun steun, hulp en interactie. De plastisch chirurgen van het Reinier de Graaf Gasthuis in Delft en het West Einde ziekenhuis in Den Haag wil ik bedanken voor hun medewerking m.b.t. het leveren van humane huid als ziekenhuisafval. Slagerij Hoogenboezem in Pijnakker wil ik bedanken voor de vele varkensoren die ik van hun heb mogen gebruiken. Ook wil ik vele collega's bedanken voor het doneren van antilichamen Mark Pescovitz (varkens CD1), Mark de Boer (humaan CD40), Sem Sealand (Langerin), Myltinii biotec (BDCA-1, -2, -3 en -4), Hitoshi Hasegawa (CCR7), Kozo Yoneda (lag) en eenieder die ik vergeten ben hier te noemen.

Karen Lammers wil ik bedanken voor lessen voor het opzetten van hOSECs hetgeen ook nuttig is geweest bij het opzetten van de pOSECs. Cynthia Lehé voor het uitvoeren van de cytokine ELISA's op hOSEC media en het overleg m.b.t. de interlab studie. Kees Poot wil ik bedanken voor het mij leren perfunderen van varkensoren. Kees, Jan de Lange en Henk Goedoen wil ik bedanken voor de hulp bij de proeven met het geperfundeerde varkensoor. Henk wil ik ook bedanken voor de hulp bij de TNF-α experimenten. Arnold Hammer bedankt voor het delen van de zitkamer en labruimten, en de goede interactie m.b.t. (immuno)histologische werkzaamheden en gitaarspel (ook belangrijk). Ook mijn mede AIO's Tjerk Bueters, Erwin Claassen en de stagiaires wil ik bedanken voor de gezellige kamer. AnneMarie de Vries - de Ruit wil ik bedanken voor de interacties m.b.t. immunohistochemie. Martien Broekhuizen wil ik bedanken voor de hulp m.b.t. de uitgevoerde Phage Display experimenten, helaas zonder het gewenste resultaat van fagen gericht tegen varkens Langerhanscellen. Gerrit Koopmans en Piet Bruijnzeel voor hun beider belangstelling voor het model en het meehelpen zoeken naar antistoffen voor varkenslangerhanscellen. Herman van Helden wil ik bedanken voor de uitstapjes naar de longimmunologie en het indienen van subsidie-voorstellen.

Graham, Pran, Martien Kapsenberg, Karen en Cynthia wil ik bedanken voor het tweemaandelijkse overleg in het eerste jaar. De medewerkers van de afdeling Farmacologie op TNO-PML wil ik bedanken voor de prettige werksfeer en de collega's van Pathologie op het AMC voor het gevoel welkom te zijn. Ook wil ik alle (inter)nationale collega's bedanken voor de broodnodige feedback op o.a. de congressen. Natuurlijk wil ik mijn vroegere (stage-) begeleiders bedanken die mij zoveel geholpen hebben bij mijn wetenschappelijke vorming, met name Han Vreeswijk, van wie ik zeer veel geleerd heb, maar ook John Voermans, Linda Everse, Wim Den Otter en Jan Bogerd. Verder wil ik Peter Nibbering bedanken voor zijn oogje in het zeil houden namens het ALIFI en de medewerkers van celbiologie & histologie van diergeneeskunde op de UU voor hun mental support in moeilijke tijden.

Voor dagelijkse problemen kon ik altijd de kamer van Graham binnen lopen, die tussen de dagelijkse beslommeringen door altijd tijd vond voor een kort antwoord. Zo zal ik niet snel het moment vergeten dat ik teleurgesteld binnenkwam om mede te delen dat ik, zelfs in herhaalde experimenten, minimaal een 5-voudige spreiding kreeg in de uitkomsten voor huidirritatie. Zonder te stoppen met bellen èn emailen, deelde Graham mij mede dat zelfs een 10-voudige spreiding normaal was voor het doordringen van stoffen in de huid. Ook wil ik Graham bedanken voor de wekelijkse werkbesprekingen op een rustige vrijdagmiddag in de kroeg, onder het genot van een paar biertjes. Speciaal wil ik Graham bedanken voor zijn bijdrage aan met name de artikelen m.b.t. huidirritatie en Langerhanscel tellen.

Grote dank gaat uit naar 'mijn' drie' MLO-stagiaires: Keith Cammans, Sandra van der Borst en Paul van Beers. Keith bedankt dat je in mijn eerste jaar als een wetenschappelijke feedback en stimulans was voor het opzetten van methodes, welke later geperfectioneerd zijn. Mede door jouw harde werk kende het project een snelle en goede start. Sandra bedankt voor de vele coupes en kleuringen waardoor de huidirritatie testen voor mens en varken voltooid konden worden en een proefdiervrije test voor irriterende stoffen is opgezet. Paul bedankt voor je hulp bij de interlab-validatie-proeven en je goede inzet bij de varkensoorproeven, waarmee ik zonder jouw hulp lang niet zoveel proeven had kunnen doen; helaas is nog lang niet alles van dit werk gepubliceerd. Ook wil ik Marjan Jongsma bedanken voor haar hulp bij de interlab validatie, en Susan van der Eeden en Maria van der Sluis bedankt voor hulp bij diverse experimenten.

Dankwoord

Grote dank gaat uit naar Wim Den Otter, wiens waardevolle adviezen van grote waarde waren en zijn. Ook zijn werkmotto *ora et labora* hetgeen zich mede uit in afspraken die gehouden worden en tegelijkertijd klaar staan met liefdevolle hulp is een goede stimulans. Samen met zijn voortvarende, doeltreffende en nauwgezette correcties zijn deze van doorslaggevend belang geweest voor de afronding van dit proefschrift.

Ter afsluiting wil ik nog vertellen dat misschien wel mijn vertrouwen in sommige mensen is aangetast, maar dit zeker niet het geval is voor mijn vertrouwen in God. In die zin, heeft, om met Johan Cruijff te spreken, ook dit nadeel zijn voordeel. Speciale dank voor mijn ouders die mijn studie al die jaren liefdevol ondersteund hebben. Verdere wil ik mijn familie en vrienden bedanken voor al hun steun en liefde. In het bijzonder wil ik Cees Tuijn herinneren die 20 september j.l. na een geestelijk slopende ziekte is heengegaan. *We fall down, we get up*, we vallen allemaal neer, maar komen omhoog dankzij Gods genade.

Zeer speciale dank voor Marleen, met wie ik in de liefde diepere niveaus heb bereikt. Charles Darwin beweerde dat de mens ontstaan is door de selectie door liefde. In ieder geval is door de Liefde een geschenk gegeven aan Marleen en mij: Jonathan, en er is goede reden om aan te nemen dat het daar niet bij blijft ....

Liefde verheugt zich in de waarheid. Daarom wil ik ook nog zeggen dat ook ik een nieuwe schepping ben door Gods Liefde.

Moge jullie allemaal Gods Liefde, vol van waarheid en vergeving ervaren in jullie leven,

John

Curriculum Vitae

De schrijver van dit proefschrift werd op 05-08-1968 geboren te Arcen en Velden als J.J.L. Jacobs, roepnaam John. Na het voltooien van de H.L.O., medisch laboratorium research in 1991 (ing.), heeft hij in 1994 de studie medisch gerichte biologie voltooid (drs.).

Sinds 1990 heeft hij stage en werkervaringen opgedaan op verschillende labs in Lelystad (ID), Utrecht (UU), Amsterdam (AMC) en Rijswijk (TNO-PML). Hij liep stage tumor immunologie op de afdeling pathologie van het AZU (nu UMC) bij Linda Everse en Prof. Wim den Otter en stage moleculaire endocrinologie bij de afdeling experimentele dierkunde van faculteit biologie aan de UU bij Dr. Jan Bogerd. Hij heeft eiwit-chemisch onderzoek gedaan op de afdeling biochemie van het AMC, AMC, Amsterdam. In drie verschillende periodes heeft hij onderzoek in de moleculaire virologie en immunologie gedaan bij de Mond- en klauwzeer vaccin-afdeling van ID-DLO (voorheen CDI) in Lelystad o.l.v. van Dr. Han Vreeswijk.

Zijn promotie-onderzoek was in de research groep huidfarmacologie op de afdeling farmacologie van TNO-PML, Rijswijk bij Dr. Graham Elliot van 1 juli 1997 tot 30 juni 2001. Dit onderzoek was in samenwerking met de groep van Dr. Pran Das binnen de afdeling Pathologie van het AMC. In september 2000 werd de research groep huidfarmacologie opgeheven door TNO-PML waarna echter het promotie-onderzoek mocht worden afgerond.

Na zijn promotie-onderzoek was hij tot 1 september j.l. in dienst als post-doc tumor immunologie bij de afdeling celbiologie en histologie van de faculteit diergeneeskunde, UU onder leiding van Prof. Wim Den Otter.

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