Organotypic skin explant cultures to identify skin irritants and contact allergens

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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. 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.

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). 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. 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. At such non-irritant concentrations, the sensitisation potential can be assessed in human OSECs by quantification of epidermal Langerhan cell migration. A software-aided method allowed more reliable quantification of epidermal Langerhan cells than manual counting. After 24 hours of culture, sensitizers accelerate Langerhan cell disappearance from the epidermis, or at least decreased the relative fraction of Langerhan 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. 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. 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
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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. Response to skin irritants varies widely among different human volunteers. 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. 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. 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). 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. 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. 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 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. It is postulated that immature DCs contribute to tolerance, and mature DCs initiate immune responses. When LCs do not become activated, low zone tolerance to contact allergens may occur. Thus immunologists are interested in the question “is maturation required for LC migration?” This question has recently been answered for human dermatopathic lymphadenitis in vivo. We found that most spontaneously migrating CD1a+ LCs
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from hOSEC were CD83', thus lacking the marker of mature LCs. Also about half 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, such as cell death detected by the MGP-staining. 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. 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.

Second, besides inducing LC migration, LC maturation is induced by skin irritants. 10% SDS is even better in inducing LC maturation than 1% NiSO₄ (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. Local lymph node cell proliferation is induced by irritating concentration of non-sensitizers, such as SDS, croton oil, and nonanoic acid. 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. It should be noted however that these authors included benzalkonium chloride, a human allergen, 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.

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 (JLJ, CLL, GRE, PDK, unpublished results).

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

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.
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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. 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. 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. If we compare our lowest weak irritant concentrations with data of Pistoor et al., 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 (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). 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. 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. 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 and is strictly dose dependent. 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. 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 and sub-irritating concentrations of sensitizers can induce subclinical sensitisation, but also low-zone tolerance. 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 and keratinocytes. Keratinocyte derived chemokines can attract T lymphocytes, but are probably
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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 mature 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 \(^{24,76,172,251}\). 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-1\(\alpha\), IL-1\(\beta\), IL-18) and tumour necrosis factor \(\alpha\) (TNF-\(\alpha\)) 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-1\(\alpha\), and not IL-1\(\beta\), 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-1\(\beta\) and TNF-\(\alpha\) \(^{172}\). 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. Tolerance can be associated with suppressor cell activity by CD4 or CD8 T lymphocytes. In humans exposed but not sensitised to nickel, nickel induced T lymphocyte proliferation in vitro, also suggesting tolerance to nickel. 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. It should be noted, however, that the induction of hypersensitivity in mice requires injection into the skin of oxidised forms of nickel, 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. Local toleration may imply the absence of mature dendritic cells or LCs in tumours, and may allow developing tumours to escape immune recognition. Toleration and LC migration can also occur after the application of a contact allergen. Moreover, from a chemical point of view, the ability of mutagens to react with DNA is similar to the ability of haptons 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. This list includes nickel, chromium, and polyaromatic hydrocarbons. It is estimated that there could be several thousand contact sensitizers for humans in commercial use that are rodent carcinogens.

It can be hypothesised that the biological and chemical resemblance of sensitizers and carcinogens might be sufficient for the body to treat haptons 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, but is nevertheless the most prevalent contact allergen in the general population of the industrialised world. A specific reaction of nickel with fatty acids in human skin forms a lipophilic nickel soap, which could penetrate the skin and reach Langerhans cells. Lipid antigens are presented by CD1 molecules which are present on Langerhans cells of humans and many other mammals, including pigs (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). 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. 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. 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
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EU Nickel Directive for prolonged exposure. Indeed, prolonged exposure to these nickel containing coins may cause contact dermatitis. Sensitisation to coins is not new, but was already reported for decades.

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. 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.

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