Short-term Toxicity Tests for Non-genotoxic Effects
Edited by P. Bourdeau *et al.*

CHAPTER 11

The Skin: Predictive Value of Short-term Toxicity Tests

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11.1 INTRODUCTION

The skin is an important interface between man and his environment. It is a significant portal of entry of hazardous agents and a vulnerable target organ system. It is also a uniquely accessible model system for detecting hazards and for studying mechanisms of a wide variety of biological functions. It is a target organ, not only for xenobiotics absorbed through the skin itself, but also for those absorbed through the respiratory and gastrointestinal tracts (Occupational Safety and Health Administration, 1978; Suskind, 1977).

The skin is endowed with a versatile group of adaptive and defence mechanisms. These include multiple defences against penetration, fluid loss, solar radiation, physical trauma, penetration and thermal stress (Suskind, 1977).

Epidemiologic information regarding cutaneous involvement in environmentally-induced disease is limited. Disease frequency information, obtained from occupational skin disease statistics, varies considerably in quality and accuracy. Roughly 20 per cent of occupational skin disease involves allergic responses; the remainder consists of non-allergic inflammatory responses to irritants, or involves other cutaneous features (such as pigmentation, pilosebaceous structures and eccrine sweat glands) or skin cancer. Eczematous dermatoses constitute the largest group of skin problems induced by xenobiotic agents. The majority of these involve primary irritant dermatitis, allergic eczematous contact dermatitis and atopic dermatitis. Those of atopic origin are influenced considerably by multiple environmental factors as well. Of irritant problems, the most common and difficult to manage are those induced by marginal irritants to which the human skin responds only after multiple exposure (Occupational Safety and Health Administration, 1978; Suskind, 1977).

11.2 PERCUTANEOUS ABSORPTION

The normal barrier to the passage of water and other chemical penetrants is the

stratum corneum. This is composed of bonded, interdigitating keratinized cells. It is a compact structure except for the outermost layers, which are shed continuously. It is not a uniform membrane but a composite made up of at least ten distinct cell layers. The basal cells are characterized by an 80 Å unit membrane while the stratum corneum cell membrane is about 200 Å in thickness. It is likely that the thickened cell membrane contributes significantly to the defensive properties of the strateum corneum.

Skin penetration is generally considered to be a process of passive diffusion. The factors which influence the rate of penetration or flux include the lipid partition coefficient, concentration gradients, molecular size of the penetrant, thickness of the horny layer, ambient and skin temperature, ambient humidity, chemical or physical damage to the barrier, pH, duration of exposure, skin area exposed, blood flow in the skin and rate of metabolic transformation within the skin (Malkinson and Rothman, 1963; Rothman, 1954; Scheuplein, 1978; Tregear, 1966).

11.3 CLINICAL AND PATHOLOGICAL PATTERNS OF ADVERSE REACTIONS

Xenobiotics may affect a single tissue component (such as the epidermis, dermis, blood vessels, pilosebaceous unit, melanocytes or eccrine sweat glands), or they may affect several components. Therefore, the type of pathological pattern may vary with the particular stimulus. In order to appreciate the current state of scientific methodologies for hazard assessment and the need for short-term tests, it is essential to identify those pathological patterns which characterize specific clinical reactions to xenobiotics (Occupational Safety and Health Administration, 1978; Suskind, 1977).

11.3.1 Inflammatory responses

The most common pathological pattern is that of an inflammatory response which, except for infection, is of an eczematous nature. The latter is the characteristic response to non-allergic, primary irritants and in antigen-induced cell-mediated hypersensitivity reactions.

11.3.1.1 Primary irritation

There is a lack of scientific information regarding mechanisms of damage to the skin characterized by inflammatory reactions having no immunological basis. These reactions are commonly lumped into the category of primary irritation. McCreesh and Steinberg (1977) distinguish between the following types for which there are predictive short-term animal tests:

(1) Acute primary irritation, which is a local inflammatory response to a single

exposure that does not ostensibly produce cell death and is completely reversible;

(2) A local inflammatory reaction produced after repeated exposure; this group includes the marginal irritant;

(3) Chemical damage leading to irreversible destruction of skin tissue (ulceration) which may result in scarring; this category includes corrosive substances;

(4) Phototoxic reactions, which are characterized by irritation induced by a chemical agent in the presence of ultraviolet light.

11.3.1.2 Allergic reactions

Cutaneous reactions with an immunologic basis can be classified into three types using the criteria of Coombs and Gell (1975):

(1) Type I—anaphylactic reactions. Humoral antibodies are produced with the participation of B cells. The reactions are reagin dependent and the antibody is an IGE protein bound to the cell surface. The urticarial response in the skin is a vascular response to a vasoactive amine, such as histamine, which is present in mast cells and basophils. In the respiratory tract, clinical signs include allergic rhinitis and asthma. In this type of skin reaction, the xenobiotics enter via the respiratory or gastrointestinal tract, but rarely percutaneously. Diagnostic tests, through which hypersensitivity states can be determined, elicit an immediate wheal and flare response from intradermal or scratch tests to the antigen (Coombs and Gell, 1975). There are sufficient incidents to verify the infrequent but definite occurrence of contact uriticaria. This syndrome has been classified into three types (Maibach and Johnson, 1975): (i) non-immunologic or primary urticaria, (ii) immunologic urticaria, and (iii) urticaria of uncertain mechanism. Non-immunologic urticariogens provoke release of vasoactive agents, such as histamine, SRS-A and bradykinin. Known non-immunologic urticariogens include DMSO, a variety of plants such as nettles, the heirs of caterpillars and moths, esters of nicotinic acid, and cobalt chloride. Case studies of immunologic urticaria are often unclear about the major route of absorption involved. This is especially true when cutaneous as well as respiratory reactions—urticaria as well as asthma—are involved. Nevertheless, there are some cases in which cutaneous absorption of a chemical agent alone has induced a wheal-flare type of reaction, along with other anaphylactoid symptoms.

Vasoactive agents which elicit wheal-flare reactions in human skin include the following (in addition to histamine and the kinins): prostaglandins, PGE_1 and PGE_2 and several complement factors including C3a, C5a and C2 peptide.

Urticaria from workplace hazards is rare and usually occurs following respiratory exposure. Chemical agents that have been implicated in this way include drugs (e.g. penicillin), pesticides (e.g. lindane), ammonia, sulphur dioxide, formaldehyde, aminothiazole and sodium sulphide (Key and Withers, 1969).

(2) Type IV—Allergic contact dermatitis. This is the most common skin response; it involves an immunologic mechanism. Simple chemical compounds (e.g. nickel, chromium salts, *p*-phenylenediamine, poison ivy resin) are initially absorbed through the skin and conjugate with protein. This protein antigen is presented by macrophages, which interact with T cells leading to the recognition of the protein antigen by T lymphocytes. Recent studies have demonstrated that Langerhans' cells, which are dendritic cells located above the basal cell layer of the epidermis, bear receptors for the F_c portion of IgG and for C_3 (Stingl *et al.*, 1978; Stingl *et al.*, 1977). The Langerhans' cells subserve the function of macrophages by processing and presenting antigens to T lymphocytes in lymph nodes. The recognition process, on continuous exposure or re-exposure to the allergen, provokes the eczematous response in the skin (allergic contact dermatitis). Allergic eczematous responses are characterized by erythema, swelling and vesiculation, and delayed reaction occurring twelve to forty-eight hours after re-exposure (i.e. challenge). Much new knowledge has been developed recently regarding the interaction between the antigen and the several cell types involved in delayed hypersensitivity (viz. T lymphocytes, B lymphocytes and macrophages (Katz, 1980). This includes interaction between T and B cells in antibody production, in antibody suppression and tolerance; interaction of B cells with other B cells and T cells, where the B cells influence the response of other lymphocytes via a secreted antibody; T cell synergy in graft versus host reaction, and in the suppression of contact dermatitis in the tolerant host. Suppression of hypersensitivity reactions of the delayed type will also occur with B–T cell interaction. Poulter and Turk (1972) have proposed a theory that, under normal circumstances, B cells modulate the effective function of T cells in allergic contact dermatitis.

(3) Type III reactions. These are cutaneous reactions to xenobiotics which involve damage by antigen–antibody complexes (Coombs and Gell, 1975). The antibodies designated are IgG, IgM and, perhaps, even IgA. In the skin, one of the reaction types is known as the Arthus phenomenon; it is seen in reactions to drugs such as penicillin. In this type of reaction, the antigen reacts with precipitating antibodies in and around small blood vessels or in basement membranes. This causes severe local inflammation.

11.3.1.3 Photosensitization

Chemical agents may cause cutaneous reactions in the presence of ultraviolet light (Epstein, 1974). There are two types of such reactions:

(1) Phototoxic reactions induced by coal tar products, essential oils such as bergamot, angelica root, cumin, psoralens, and photoinitiators such as amyl *p*-aminobenzoates. Phototoxic reactions may be accompanied by residual hyperpigmentation. No immunological mechanism is involved.

(2) Photoallergic reactions are allergic contact reactions, the induction of which require exposure to ultraviolet light and a chemical agent. These are usually responses to long-wave ultraviolet light (above 320 nm). Examples of photoallergens are tetrachlorosalicylanilide, tribromosalicylanilide, and bithional. Ultraviolet light may enhance conjugation with polypeptides or may induce photoproducts or photometabolites which are allergenic following conjugation.

11.3.1.4 Pigment changes

Xenobiotics may lead to pigmentary disturbances in the skin, such as localized hypopigmentation or leukoderma, or conversely hyper-pigmentation, as in the case of post-inflammatory reactions. In most instances, the pathologic process can be explained on the basis of changes in the biochemical synthesis of melanin (Fitzpatrick, 1965; Fitzpatrick *et al.*, 1950; Fitzpatrick *et al.*, 1979).

The biochemistry of melanin and the pigmentation system have been studied in great detail (Seiji, 1963; Seiji and Iwashita, 1963; Seiji *et al.*, 1963). In the human epidermis, each melanocyte is associated with about thirty-six viable keratinocytes that transport and, in some cases, degrade the melanin received from the melanocyte. The melanocytes, in association with the keratinocytes, form the epidermal-melanin unit. Human skin contains a very large number of these units, and skin colour is the visual impact of melanin within them. The coloured polymer, melanin, is formed from tyrosine in the presence of the enzyme tyrosinase with the production of dopa, which in turn is converted to dopaquinone. Through a series of subsequent steps, 5,6-dihydroxyindole is formed which is polymerized into melanin. The work of Nicolaus (1962) indicated that melanin is a heteropolymer or a random polymer derived from the linkage of many different indoles.

The range in skin colour among negroids and caucasoids may be accounted for by the interaction of three to four additive gene pairs. The regional frequency distribution of melanocytes are similar in skin of all races. The racial colour differences in man are related to the fine structure of the melanocyte, variation in area are occupied by the rough endoplasmic reticulum and in the development of the Golgi zone, as well as in the relative proportions of melanosomes in the four developmental stages of cutaneous differentiation.

Ultraviolet light may increase pigmentation in several different ways: (1) by catalysing the oxidation of tyrosine to dopa, (2) by decreasing the redox potential of the skin, (3) by thermal enhancement of melanin formation following exposure to actinic radiation, and (4) by darkening of melanin granules already present.

11.3.1.5 Pilosebaceous reactions

Reactions involving the pilosebaceous structure may elicit a variety of damage patterns. They include damage to the hair shaft, damage to the matrix cells which produce the hair, acneform eruptions and folliculitis. Petroleum and cutting oils may

induce a folliculitis (an inflammatory reaction). Occupational and cosmetic acne are common skin problems. Workplace chemicals that are able to induce acne include: petroleum-based cutting oils, coal tar fractions, chlorinated hydrocarbons (e.g. chlorinated naphthalenes, chlorinated biphenyls), and chlorinated phenoxy contaminants such as tetrachlorodibenzo-*p*-dioxin (TCDD) and polychorinated dibenzofurans (PCDF). In the case of chlorinated hydrocarbons, the acneform eruption is the clinical manifestation of changes in the differentiation process of sebaceous cells. The latter are replaced by keratinocytes. Hence, instead of the differentiated acinar cells forming lipid secretions, which is their normal function, the acinar cells become keratin-producing cells. These modifications in differentiation and function may persist for more than thirty years following initial exposure to the acnegenic agent (Suskind and Hertzberg, 1984).

Hair shaft breakage can be induced by exposure to alkaline substances which break the disulphide linkages in keratin. Inorganic agents such as thallium and organic molecules such as chloroprene dimers and a number of therapeutic agents interfere with the metabolism of the hair-forming matrix cells; hair loss may ensue as the result of such exposures.

11.3.1.6 Eccrine sweat gland reactions

Changes in the skin involving eccrine sweat glands are most frequently associated with a combination of high temperature environments and cutaneous irritation. The most common outcome is miliaria, in which epidermal injury produces abnormal keratinization and plugging of the eccrine duct orifices. When the sweat glands are subsequently stimulated thermally, sweat is trapped in a plugged duct. With increased pressure, the duct wall breaks and the collected sweat extravasates in the skin, producing the inflammatory response of miliaria (Suskind, 1977).

11.3.1.7 Other tissue components

Collagen and elastic tissue components of the skin are primarily damaged through the direct effects of radiation, both ultraviolet and ionizing, and only rarely through association with chemical absorption. Blood vessels in the skin are involved in inflammatory reactions. However, they are singularly damaged in prolonged low temperature exposures. Ionizing radiation will also produce irreversible dilatation of capillaries and fibrotic changes in vessels leading to ischaemia, atrophy, necrosis and ulceration.

While cutaneous sensation is informative as well as protective, potential and actual damage may be signalled by several sensory modalities, including itching and pain. Both modalities of sensation are subserved by the same receptors and nerve fibres. Inflammatory reactions provoked by primary irritants as well as allergic sensitizers are characterized by pruritis, which may precede the objective signs of inflammation (Suskind, 1977).

11.4 TESTS FOR THE ASSESSMENT OF CUTANEOUS TOXICITY AND REACTIONS

11.4.1 Dermal toxicity

Following the determination of an oral LD50 on the chemical agent in question, it is appropriate to determine the LD50 using the skin as the route of exposure. This may be carried out in one of several species of animals (rat, guinea-pig or rabbit); ten or more animals are exposed to three dose levels up to 2 g/kg applied for a period of two to 24 hours duration inside a rubber or plastic dam to assure absorption. The necropsy should include gross observations on all vital organs.

For most chemical agents that are absorbed through the skin, subacute percutaneous toxicity can be determined in either the rat, guinea-pig or rabbit. Ten or more animals are exposed daily or every other day for 90 days, using three concentrations up to a minimal effective dose of the dermal LD50. These are applied under a plastic or rubber dam for a period of four to 24 hours duration. Necropsy should include gross observations of the skin and all vital organs, as well as microscopic examinations.

Anderson and Keller (1984) have described the toxicokinetic parameters which determine dose delivered to the skin. They believe that the extrapolation of kinetic data derived from other routes of administration, using *in vitro* permeability constants to predict cutaneous hazard, seems to be a promising application for toxicokinetic simulation.

11.4.2 Methods of assessment of skin penetration

While extensive literature reports *in vivo* and *in vitro* techniques to determine rates of penetration, there is no single method which is standardized for a particular family of chemical agents. One report (Bronaugh and Maibach, 1985) claims good correlation between *in vitro* and *in vivo* human results for several compounds.

The factors involved in cutaneous penetration have been described by Dugard (1977) who indicates that the assessment of hazard in relation to absorption rate must consider the release of the chemical agent from the skin as well as its detoxification and/or elimination. Thus, consideration of the 'dynamic parameters permits an estimate of whether a toxic material accumulates in the body in sufficient quantity to produce adverse effects—measurement of absorption from different formulations may indicate which is the safest'. Dugard also states: 'the comparison of absorption properties of different chemical substances does not indicate the relative safety unless relative potencies and preferably detoxification and elimination kinetics are known'. At least one new *in vitro* method considers rate of penetration in relation to metabolism and biotransformation. This technique uses a multisample apparatus for the kinetic evaluation of skin penetration in relation to viability and metabolic status (Holland *et al.*, 1984; Kao *et al.*, 1984).

The most useful method for the assessment of xenobiotics is the model recently

developed by Wojciechowski and Krueger at the University of Utah. It employs a rat/human skin flap served by a defined and accessible vasculature on a congenitally athymic (nude) rat (Krueger *et al.*, 1985; Wojciechowski *et al.*, 1987). It has been used successfully to quantify transdermal fluxes of drugs such as benzoic acid (Burton *et al.*, 1984), and caffiene and is now being used to study polycyclic aromatic carcinogens and chlorinated dioxins.

Factors which influence passive diffusion through the rate-limiting barrier—the stratum corneum—have already been discussed. Although the stratum corneum is the rate-limiting membrane, other structures of the skin such as the pilosebaceous and eccrine sweat structures are also involved. The relative role of the stratum corneum in relation to these two structures is difficult to measure. The pilosebaceous structure provides a shunt or transient state route of absorption. Other factors which complicate penetration rate measurement methods include the quantity of the penetrant used, the absorption from a solid milieu or whether the penetrant is a liquid, a vapour or a gas (Dugard, 1977).

Recent comparative studies of the absorption of several hydrophobic compounds using *in vivo* and *in vitro* techniques indicate poor correlation between the results using the two types of assessment methods (Bronaugh *et al.*, 1982a and b; Bronaugh and Stewart, 1984).

11.4.3 Primary irritation

A standard irritation test specified by the Federal Hazardous Substances Act (USEPA, 1975) has been in use since 1944 (Draize *et al.*, 1944). It is a simple test in which clipped albino rabbits with intact and abraded skin are exposed to 0.5 ml (in the case of liquids) or 0.5 g (in the case of solids or semi-solids) of the test substance placed under a rubber or plastic dam for a period of 24 hours. Skin reactions are scored after 24 hours of exposure based on the degree of erythema, eschar formation and oedema; reactions are graded again 72 hours subsequently. Modifications of this method have been proposed and the National Institute for Occupational Safety and Health has published interpretations of the skin reaction values with respect to safety for contact with intact human skin (Campbell *et al.*, 1975). Problems encountered with the standard test have been reviewed by McCreesh and Steinberg (1977).

In order to determine the cumulative effect of repeated exposure to a chemical agent, either rabbit or guinea-pig is usually employed, using several concentration levels of the test agent at and below the minimal irritant level. To determine threshold levels, preliminary tests are carried out using six to ten rabbits or guinea-pigs at four concentration levels. The exposure time is 24 hours and the reactions observed after 24 and 48 hours. To assess cumulative effects, animals are exposed daily or every other day for a period of up to three months. Repeated exposures also provide information about accommodation, a phenomenon in which repeated exposure to an irritant (e.g. fatty acids and aliphatic alcohols) results in initial irritation followed by decreased response even to the point of non-responsiveness. Accommodation has been observed in

both guinea-pigs and man but the mechanisms have yet to be determined.

Rabbit skin is more readily irritated by chemical agents than human skin but the reliability of the rabbit model is very limited for predicting the effects of weak irritants in man. Steinberg *et al.* (1975) compared a series of chemicals that were tested for their irritancy in both rabbit and man; the correlation was weak. A useful parameter is the threshold for irritation or the concentration which, after one exposure or a repeated daily 21 day exposure, produces no response in the rabbit. This achieves particular significance where the concentration of a chemical agent intended for topical use is known. It is generally believed that substances which elicit no irritant response or a minimal response in rabbits are unlikely to cause irritation in man.

For chemicals which are likely to contact human skin extensively, either because of intentional contact (e.g. toiletries and cosmetics) or because of inadvertent exposure (such as may occur in certain occupational situations), it is useful to investigate whether the concentration used will produce a reaction in a small panel of human subjects after single or repeated exposures. This is determined by means of the single patch test using a range of concentrations up to ten times the intended use concentration for a period of four to 24 hours. The substance may be applied at the same site daily or every other day for a period of three weeks in order to determine if cumulative effects occur.

No *in vitro* method for irritancy has yet been developed that is a satisfactory substitute for the whole animal. Although the models using intact animals are relatively short-term, the potential for developing an *in vitro* method is promising since the methods of growing human and animal whole skin in culture have improved significantly. Leighton *et al.* (1983) have recently demonstrated that a method using the chorioallantoic membrane of the chick egg can be considered as a substitute for the Draize eye irritancy test for a limited number of chemicals.

11.4.4 Immunologic reactions

The test model for inducing Type I reactions in animals was first described in 1940 (Jacobs, 1940; Jacobs *et al.*, 1940). In the original investigation, both an immediate urticarial reaction and a delayed reaction were elicited in guinea-pigs. The guinea-pig model for assessing potential urticariogenic agents has not been standardized. A major problem is that some substances which induce urticaria-like reactions in the guinea-pig are known not to be hazardous to man.

Predictive tests in laboratory animals for investigating the delayed hypersensitivity (Type IV) potential of chemical agents have been studied extensively. Using the research model designed by Landsteiner and Jacobs (1935), Draize *et al.* (1944) proposed a so-called predictive test which for many years was the recommended procedure by federal regulatory agencies in the USA. A summary of procedures for identification of contact allergens in guinea-pig is presented in Table 11.1. The original Draize method employs 40 albino guinea-pigs—20 in an experiment group and 20 in a control group. The test substance is injected intradermally as a 0.1 per

Table 11.1 Predictive tests for contact allergenicity in guinea-pigs

Test	Year published	Induction			Challenge	
		Route	Skin	No. of exposures	Route of exposure	Test days (total)
Landsteiner and Jacobs	1935	Intradermal		10	Intradermal	39
Draize *et al.*	1944	Intradermal		10	Intradermal	39
Buehler Griffith and Buehler	1965 1969	Topical	Occluded	1–9	Closed	16–44
Magnusson and Kligman	1970	Intradermal, + topical	Occluded	1	Closed	24
Maguire	1975	Topical	Occluded	4	Closed	23
Klecak *et al.*	1977	Topical	Open	20	Open × 2	40

cent solution, suspension or emulsion in 0.85 per cent NaCl, paraffin oil or polyethylene glycol. The test group receives a series of ten intradermal injections given every other day in the anterior flank over an area of 3×4 cm. The initial injection is 0.05 ml and the remaining nine are 0.1 ml, all injected at different sites. The test animals are challenged on the contralateral flank, corresponding to the site of the first injection, with 0.05 ml of 0.1 per cent of the test solution. Challenges are performed 35 days after the initial induction injection and the challenge sites are observed for reaction 24 and 48 hours after the challenge exposure. Reactions are graded on the basis of the intensity of the erythema and size of the edema. While this is a relatively simple test, the induction concentration is fixed and no consideration is given to the use concentration. The original method is not sensitive enough to identify many allergenic materials.

The most sensitive method using guinea-pigs is the 'maximization' test (Magnuson and Kligman, 1970). In this procedure, the experimental group receives three pairs of simultaneous intradermal injections in the shoulder region, followed by an application of a closed patch over the injection site one week afterwards. The injection consists of : (1) 0.1 ml of Freund's complete adjuvant (FCA), (2) 0.1 ml of the test material, and (3) 0.1 ml of the test material in FCA. Injections 1 and 2 are made close to each other and injection 3 caudal to the first two. The control group also receives three pairs of injections: (1) 0.1 ml of FCA, (2) 0.1 ml of the vehicle alone, and (3) 0.1 ml of the vehicle in FCA. Three weeks after the initial exposure, the experimental control animals receive occlusive applied patches with the test agent as well as the vehicle alone. This test will identify all allergenic agents. It is more sensitive than the 'maximization' test using human subjects and it has been noted that there are chemical substances which are not known to be sensitizing to humans which will sensitize guinea-pigs. For example, humans are not sensitized by several alkyl derivatives of cinnamic aldehyde that induce sensitization in the guinea-pig 'maximization' test.

Buehler (1965) introduced a relatively simple procedure that is used for cosmetic components as well as drug products, fragrances and household products. According to the author, this predictive procedure, when compared with the human repeated insult patch test, missed less than 3 per cent of the materials found to be sensitizing by the latter test.

In the United States, panels of human subjects are still used to test chemical materials for their irritancy as well as sensitizing potential. Methods used include the repeated insult test (Draize, 1959), the Kligman 'maximization' test (Kligman, 1966), as well as the methods of Shelanski and Shelanski (1953) and Schwartz (1957). In the repeated insult test, as well as the 'maximization' test, the concentrations of chemicals applied can be related to intended use concentrations. In the case of fragrances and flavours, for example, the human 'maximization' test is carried out with a concentration of ten times the expected maximum use concentration.

It should be noted that all of the animal or human tests for determining potential allergenicity require a minimum five to six weeks. It is not possible to shorten the induction period.

So far, no standardized method has been developed to sensitize cultured cells *in vitro* so that the sensitization potential of a chemical agent can be measured. However, several cellular phenomena have been used to determine the possible antigenic potential of chemical agents. These include the blastogenic effect to leukocytes of such agents as tuberculin-purified protein, phytohaemaglutinin and antiserum. The lymphocyte transformation phenomenon has been used as an *in vitro* method to determine whether or not an animal or a human has been sensitized. Sensitized T cells can be identified *in vitro* by the use of a test for a migration inhibiting factor (MIF) (Unanue and Benacerraf, 1984). In theory, all antigens which induce delayed hypersensitivity reactions should sensitize T lymphocytes, and peritoneal exudates from a sensitized animal inhibit the migration of macrophages in a capilloary tube. This is the basis of a test which provides a highly specific *in vitro* correlate of delayed hypersensitivity in the intact animal. However, the MIF test cannot be used itself to predict the sensitizing potential of a chemical since the whole animal is necessary to produce MIF-containing lymphocytes.

11.4.5 Assay methods for phototoxicity and photoallergenicity

Tests have been developed to determine potential phototoxic effects of chemical agents absorbed through one of several routes and these have been used to assess the activity of drugs, fragrances, flavours and some industrial materials. The effectiveness of some of the models is summarized in Table 11.2. Phototoxic reactions are probably the best understood drug photosensitivity reactions (Harber, 1981). Responses are dose-related, both with respect to the drug and with respect to the ultraviolet light. Harber (1981) has reviewed the status of mammalian and human models for predicting drug photosensitivity. The development of a simple and effective *in vitro* predictive test to assess phototoxicity remains an ideal goal. The work of Schothorst *et al.* (1973) describing biochemical alterations in aminoacids, glutathione and unsaturated fatty acids indicates promise but currently is not standardized for predictive purposes. The use of cell cultures (Freeman, 1970), red blood cells (Blum, 1941), paramecia (Raab, 1900), fungi (Daniels, 1965) and viral systems (Fowlks, 1959) have been described for assaying phototoxicity. Each of these assays is dependent on the detection of a chemical or biological change in the system when the photoabsorbing and phototoxic agent is introduced and exposed to ultraviolet light. Results obtained with these models do not correlate well with photosensitivity reactions in man. The factors which are responsible for the poor correlation include: (1) failure to account for the variations in percutaneous absorption; (2) failure to account for variations in gastrointestinal absorption, cutaneous storage, and excretion of the photosensitizer; (3) failure to account for the metabolic transformation of the sensitizer in liver and in skin; and (4) failure to account for the inactivation of the photosensitizer by metabolism and detoxification following absorption. Mammalian models have better predictive value than those using non-mammalian species but they still have limitations. As is noted in Table 11.2, several animal models have been used effectively for the assessment of

Table 11.2 Summary of findings in some animal models used for detection of agents with phototoxicity

Author(s) reference	Animal	UV-source	Positive	Negative
Stott *et al.* (1970)	Guinea-pig ears (DMSO vehicle)	Fluorescent blacklights (UV-A)	8-methoxypsoralen chloropromazine prochlorperazine demeclocyclin3	Sulphanilamide Chlorothiazide Griseofulvin Chlortetracycline
Gloxhuber (1970)	Hairless mouse	Filtered Osram (UV-A)	8-methoxypsoralen certain essential oils	Chlorpromazine Chlorothiazide
Morikawa *et al.* (1974)	Rabbit, guinea-pig	Blacklights (UV-A)	Phenothiazine coal tar derivatives acridine, etc.	Sulphanilamide Demeclocycline Griseofulvin
Forbes *et al.* (1977)	Hairless mouse, miniature swine	Xenon arc solar simulator blacklights (UV-A)	Certain fragrance materials 8-methoxypsoralen	

From Kaidbey and Kligman (1980).

phototoxicity including mice, mouse ears, hairless mice, rats, rabbits, miniature pigs and guinea-pigs. Phototoxic reactions used as critera for phototoxicity in mice include cutaneous oedema, erythema and necrosis, observed on the ears and tail. However, the mouse epidermis is relatively thin compared with that in man resulting in greater penetration of UVB to the basal cell layer and connective tissue. Rat skin resembles the mouse in this respect (rat tail is the part of the body that is often exposed). The albino Hartley strain guinea-pig is widely used for assessing topical phototoxicity. Some investigators believe that the rabbit is superior to the rat, the mouse and the guinea-pig with respect to both sensitivity and quantitative inflammatory response. In a comparison study with 21 phototoxic agents applied to rabbits and guinea-pigs, it was found that rabbits were more sensitive qualitatively and quantitatively than guinea-pigs (Morikawa *et al.*, 1974).

Human volunteers can be used for the assessment of phototoxic agents. The procedures used include scotch tape stripping and exposure to a solar simulator. Human subjects can also be exposed by intradermal injections of a 0.1 ml aliquot of the photosensitizer in saline, followed by a ten to 30-minute exposure to UVA or UVB radiation. The simplest technique for assessing topical phototoxic agents requires application of fixed amounts to lumbar or scrotal skin, followed by irradiation with known doses of UV light. A 'simulated-use' test can be employed to assess the phototoxicity of drugs (e.g. methacycline). Volunteers are given a therapeutic dose of the drug in question on a double blind basis, followed by exposure to natural sunlight for up to six hours.

Chemical agents which are known to produce photoallergic reactions in the skin include sulphonamides, halogenated salicylanalides, griseofulvin, Fentichlor, promethazine, blankophores, thiazides, chlorpromazine, cyclamates and chlordiazepoxide (Librium). A very potent sensitizing agent is tetrachlorosalicylanalide. It is now used as a positive control substance when materials are being tested in a guinea-pig model.

The photosensitizing and photoeliciting techniques in guinea-pigs (Buehler, 1984; Harber, 1981; Morikawa *et al.*, 1974) involve topical application of the potential photosensitizer to the shaved nuchal area of guinea-pigs 30 minutes prior to irradiation. Two types of UV radiation are employed: (1) a sunlamp with an emission spectrum of 285 nm to 350 nm, and (2) a blacklight fluorescent tube with an emission of 320 nm to 450 nm. Cutaneous exposure followed by irradiation is repeated three times during a seven-day period. Three weeks after the last sensitizing exposure, three concentration levels of the test material are applied to areas not previously exposed, followed by irradiation with non-erythemogenic doses of UVA or blacklight. The response is scored and interpreted on the basis of degree of erythema, comparing the irradiated sites to the non-irradiated sites. Almost all contact photoallergens appear to require wavelengths greater than 320 nm (Kaidbey and Kligman, 1980).

11.4.6 Methods for assessing the effects of xenobiotics on melanin in the skin

Research methods have been developed to determine the effects of xenobiotics on

melanin pigment. These are short-term tests requiring from one to three hours. They include:

(1) The effect of the xenobiotic on tyrosinase activity *in vitro*. Mushroom tyrosinase is incubated with radio-labelled tyrosine; tyrosinase activity is measured by determining the amount of radio-labelled tyrosine that is oxidized.
(2) Interference with the transformation of tyrosine to dopa and dopaquinone *in vitro* in the presence of tyrosinase. The amount of dopa, dopaquinone and the polymer itself can be measured quantitatively.
(3) The effect of different concentrations of a chemical on a culture of murine melanoma cells.

The whole animal can be used to determine the effect of the xenobiotics on pigmented skin. The DBA or C3H strain of mouse is preferred. The site of exposure is the ear. Three to five days after the animals are first exposed, the increase or decrease in pigmentation is observed and the number of melanocytes per mm^2 is determined microscopically.

In assessing a number of human depigmenting agents using black guinea-pigs and black mice as test animals, Gellen *et al.* (1979) noted various confounding factors which needed to be controlled. These included the choice of vehicle, irritation, and false positive reactions produced by either the vehicle or the test material itself as well as false negative responses to known depigmenting agents.

11.4.7 Assessment of toxic effects on the pilosebaceous structures

Although there are no recognized standard methods for measuring the effects of chemical agents on the keratin of the hair shaft or on the matrix cells which produce the hair itself, relatively simple methods are available to screen both topical agents and agents such as drugs intended for systemic administration.

For effects on the hair shaft keratin, segments of human or experimental animal hair can be immersed in solutions of the test agent for specified periods of time, rinsed with water and examined for structural and chemical changes. Components of cosmetics such as hair straightening or waving agents, hair colouring process components, shampoos and pomades are routinely subjected to such examinations.

Protocols can be designed to determine toxic effects on matrix cells (which result in reversible or irreversible hair loss depending upon the extent of the damage to the matrix cells). Thallium, chloroprene, thyroid antagonists, anticoagulants (heparin, heparinoids, coumarin), antimitotic agents (e.g. colchicine), immunosuppressive agents (e.g. folic acid antagonists), purine antagonists, alkylating agents, antipsychotic drugs (e.g. triparanol), antiepileptic agents (e.g. trimethadione) and vinca alkyloids are known to affect the matrix cells. The test protocol can include the administration of graded doses to rodents with known hair growth cycles (e.g. mice with a cycle of 30 days). The test group includes animals whose hair has been

plucked and a group with intact hair. Following administration of graded doses, the plucked group of animals (test and controls) is observed for dose-related differences in rate of growth of hair and hair structure. The unplucked animals are observed for differences in onset of hair loss.

11.4.8 Chemically-induced acne

The first standardized test, published by Adams *et al.* (1941), is still the most widely used. The material to be tested is applied repeatedly (undiluted and in several concentrations) to the pinna of the inner surface of the rabbit ear using an appropriate solvent (olive oil, paraffin oil, ethanol, propylene glycol, etc.). Applications are repeated daily for four weeks or until a reaction is noted. Assessment is made both by clinical examination as well as histologic examination for epidermal hyperplasia, comedones and epithelial cysts. Further modifications of this technique were made by Shelley and Kligman (1957), and Hambrick (1957). Hambrick and Blank (1956) found that the external canal of the rabbit ear, rather than the pinna, is richest in sebaceous structures. The Hambrick technique requires a minor surgical procedure in order to lay open the ear canal surfaces for testing.

Inagami *et al.* (1969) applied rice oil contaminated with chlorinated biphenyls (that was responsible for the Yusho epidemic in Japan) to the skin of hairless mice. He reported follicular hyperkeratotic changes in the sebaceous follicle. More recently, the use of hairless mice has been explored as a model for testing potential acnegens (Puhvel *et al.*, 1982). The strains used were Skh-HR-1 and the HRS/J strains and the materials were applied to the dorsal skin in volumes of 0.1 ml in different vehicles. The response to known human acnegens was not constant. Polychlorinated naphthalenes, such as Halowax 1014, produced hyperkeratosis, epidermal hyperplasia, sebaceous gland involution and keratin cysts within fourteen days. Polychlorinated biphenyls such as Arochlor 1254, in sublethal concentrations, induced no observable changes either grossly or histologically; however, Phenclor 54 produced the same changes as the polychlorinated naphthalenes. 2,3,4,8-TCDD caused hyper-keratinization of the stratum corneum, epidermal hyperplasia, disappearance of sebaceous glands and follicles and numerous keratin cysts. All of the animals treated with these materials and which developed the cutaneous changes, also developed large intraabdominal fat deposits. The authors commented that it was only the 2,3,4,8-TCDD which produced the changes which were close to human chloracne, and only in one of the strains of mice (Skh-HR-1).

A potentially useful model for the short-term study of human acnegens is human skin which has been transplanted to the skin of athymic rats (Brungger *et al.*, 1984; Krueger *et al.*, 1985; Wojciechowski *et al.*, 1987). These transplants have been successfully grown and they completely retain the characteristics of human skin. Laboratory experience with this model has been limited to studies of percutaneous absorption and metabolism. Its usefulness as a bioassay model for xenobiotics, especially acnegens, deserves further exploration.

11.5 DISCUSSION AND STRATEGY FOR TESTING

The development of a strategy for testing a xenobiotic (or combination of chemicals, as in a product) requires knowledge at the outset about its intended uses and background information on its chemical structure, physical properties and known biological activity. If basic toxicological properties are not known, information on the acute toxicity, oral and dermal, should be obtained.

Studies of skin penetration may be of some value, but there are no *in vitro* models which provide data directly applicable to human beings demonstrated to be useful over a wide range of chemicals. Cutaneous exposure of rodent species or rabbit to the penetrant in an appropriate vehicle can provide estimates of the rate of penetration as measured by amount of material (or its metabolite) transferred per unit time into the dermis blood, or that is excreted. Alternatively, the rate of disappearance from the skin surface can be measured. The Wojciechowski/Krueger model is useful in determining rates of absorption and metabolism of xenobiotics.

The most common clinical consequence of xenobiotic exposure is inflammatory skin reactions that are caused by irritants and by antigens. Similar reactions may be enhanced by ultraviolet radiation. Model test systems to assess potential for irritancy require biologically responsive living tissue. Rabbit and guinea-pig are appropriate test subjects for both single and repeated exposure tests. Both are used to determine the threshold and subthreshold irritant dose(s) as well as for classification of irritants as strong, moderate, or weak. For some substances, there is poor correlation between results obtained in rabbits and in humans after repeated exposure; therefore, tests on panels of human subjects may be indicated. This can be carried out on panels used to appraise the sensitizing capacity using 'maximization' (Kligman, 1966) and repeated insult (Draize, 1959) protocols. Repeated exposure of animals to irritant concentrations of a chemical can provide information regarding accommodation or 'hardening'. The *in vitro* model using the chlorioallantoic membrane of the chick egg shows considerable promise as a substitute for the rabbit eye irritancy test and it may have promise as a substitute for the hole animal skin irritancy test.

Many agents or products intended for skin exposure require assessment for potential immunologic reactions, particularly cell-mediated hypersensitivity. Other agents which may be absorbed from the gastrointestinal tract or respiratory tract may be assessed for humoral antibody-associated reactions (Coombs-Gell Type I) in the guinea-pig. Standardized systems for detecting and measuring potency of antigenic agents which induce cell-mediated hypersensitivity reactions should involve panels of guinea-pigs or humans. The most sensitive involves 'maximizing' induction in guinea-pigs with FCA. The minimum duration of tests is four to five weeks. Assessment for allergenic potential requires the living organism. No *in vitro* tests have been developed which correlate with sensitizing capacity determinations in man.

While *in vitro* models using single cell organisms have been described for measuring phototoxic activity, the results of these tests do not correlate well with

tests in man. Photoallergic potential can be determined with a relatively simple guinea-pig model. The minimum time required is four weeks.

Very simple short-term and useful tests are available for detecting effects of a xenobiotic on melanin synthesis using *in vitro* models. They include measuring effect of tyrosinase activity, the transformation of tyrosine to melanin, and the effect on mouse melanoma cells.

The tests for effects on pilosebaceous structures include simple *in vitro* methods of determining damage to hair shaft and whole animal (mouse) models for determining effects on hair-producing cells in which the end-points are: interruption of hair cycle; loss of hair; change in rate of hair growth; damage to matrix cells; and changes in hair structure.

In tests for acnegenic potential, the xenobiotic can be screened with the rabbit ear model. New procedures have been described using hairless mice which show potential for measuring acnegenicity. The use of human skin transplants to athymic rats may provide a fertile method for assaying agents for acnegenicity.

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