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Experimental Models in Skin Pharmacology

MARTINE BOUCLIER*, DANIEL CAVEY, NICK KAIL, AND CHRISTOPHER HENSBY

Centre International de Recherches Dermatologiques (C.I.R.D.), Valbonne Cedex, France

* To whom reprint requests should be addressed: Experimental Pharmacology and Toxicology Department, C.I.R.D., Sophia-Antipolis, 06565 Valbonne Cedex, France.

I. Introduction

The pharmacology of skin is increasingly being studied as a fascinating new way of exploring human physiology and pathology in addition to being an important step in the development and study of new drugs, useful to the improvement of currently existing therapies of dermatological disorders. In recent years, there has been increasing interest in the use and development of experimental models in vitro and in vivo for the investigation of the pathophysiology of skin diseases as well as responses to potential therapeutic agents.

For most dermatological disorders, none of the existing models fully reflect the whole set of symptoms and mechanisms normally encountered in clinical disease in human skin. Therefore, although attempts have been made to simulate disease characteristics in animals and to use these similarities to investigate different aspects of the human disease counterpart, mimicking of the whole disease process requires the use of a battery of different in vitro and in vivo animal models conducted in parallel. For these reasons, the dermatopharmacologist must be careful when extrapolating experimental results from animals to humans.

It is not possible in a single paper to review every test model. However, we will attempt to provide the reader with a current overview and references for further reading about the various models used in different areas of dermatopharmacology. For a detailed and comprehensive review concerning a large number of models, the interested reader could refer to the excellent 4 volumes of *Models in Dermatology* edited by Maibach and Lowe (1985a, 1985b, 1987, 1989).

The first parts of this review will address and discuss animal models that can be currently used in cutaneous pharmacology. However, because the establishment of alternative complementary in vitro models for the prediction of pharmacological and/or toxicological activities of drugs is currently a great matter of public and scientific concern, we have also included a review of a number of such models that are of interest in cutaneous pharmacology.

II. Animal Models Used in Cutaneous Pharmacology: A Review

The major symptoms observed in most dermatoses are related to syndromes of chronic inflammation, disorders of proliferation, differentiation, and infections. These different symptoms, in general, characterise the major classes of current animal models in use or under development.

A. Animal Models of the Acute and Subacute Cutaneous Inflammatory Response

1. Introduction. The capacity for survival of an organism resides, in part, within its ability to recognise foreign or mechanically inflicted trauma. It is this recognition that triggers a series of events culminating in a host defence or inflammatory reaction. This observation prompted John Hunter to describe inflammation as "a salutary process." Acute cutaneous inflammation is characterised by a distinct vascular response consisting of transient vasoconstriction followed by a dilation and accompanied by enhanced vascular permeability. This gives rise to extravasation of plasma proteins, salts, and water, leading to oedema formation. These changes are associated with the release of a variety of inflammatory mediators, such as histamine and prostaglandin E_2, and account for the redness, heat, swelling, and pain that all types of skin inflammation exhibit. This type of vascular dominated acute inflammation is clinically referred to as a urticarial response.

In extreme circumstances, loss of function of the inflammed tissue may occur. Subsequently, leucocytes commence to adhere to the endothelial cells lining the local blood vessel walls and migrate into the extravascular connective tissue. PMNs† predominate in the early stages (acute phase), followed by mononuclear leucocytes as the reaction progresses. Whether or not the reaction subsides depends very much on the nature of the stimulus, removal of which usually leads to resolution and healing. Failure to do so results in a continuous influx of leucocytes, particularly mononuclear (monocytes, lymphocytes), in an attempt to remove the offending agent. The participation of lymphoid cells is not obligatory. The increasing army of mononuclear phagocytes may be amplified by local division of these cells as well as by differentiation into cells that are more specialised for the task at hand (e.g., secretory epithelioid and multinucleate giant cells). Activated fibroblasts undergo mitosis and lay down collagen encapsulating the lesion, which now exhibits the features of a typical chronic inflammatory reaction (for review see paper by Dunn and Willoughby, 1989).

† Abbreviations: PMN, polymorphonuclear leucocyte; UV, ultraviolet; NSAID, nonsteroidal anti-inflammatory drug; DNA, deoxyribonucleic acid; MSLR, mixed skin cell-lymphocyte culture reaction; IL, interleukin; ICAM-1, intercellular adhesion molecule 1; IFN, interferon; MHC, major histocompatibility complex.

Although the most severe and debilitating inflammatory disease states are generally chronic in nature, the available animal models generally reflect acute or subacute reactions. This is because of three principal reasons: (*a*) acute models are simple and reproducible; (*b*) these models are useful for studying the biochemical and cellular mechanisms involved in the transition from acute to chronic inflammation; and (*c*) these models permit the investigation of the biochemical, vascular, and cellular components of inflammation as a function of time as well as the evaluation of the action of known and potential anti-inflammatory agents.

The cardinal signs of inflammation have long been used to establish methods for the detection and definition of anti-inflammatory drug actions in models of cutaneous inflammation induced either chemically, physically, or mechanically. Thus, inflammation is characterised by one or more of the following symptoms which can be used as end points for the measurement of anti-inflammatory drug action: erythema (rubor), hyperthermia (calor), oedema (tumor), or exudation of circulating leucocytes which can be observed, scored, and analysed. The various animal models that have been used to evaluate anti-inflammatory drug actions in the skin are listed in table 1.

2. Animal models of vascular type acute inflammation. The majority of the animal models used in cutaneous inflammation research are related to vascular type acute inflammatory responses and have oedema and/or erythema as their end points. Inflammation is induced by chemical agents such as croton oil, arachidonic acid, or carrageenin or by physical methods such as IR and UV irradiation. The classical Williams technique for determining vascular permeability changes is well established (Williams, 1981; Rampart and Williams, 1986). Local plasma exudation in rabbit skin (or from another suitable species) is measured by the accumulation of intravenously injected radiolabeled proteins such as ^{125}I-serum albumin at the inflammatory or control sites.

All of these models, which probably correlate better with the urticarial phase (or oedema phase) of inflammatory skin responses, can be used to evaluate anti-inflammatory activity after either topical/local or systemic administration.

3. Animal models of cellular type inflammation. With the increasing recognition of the role of leucocytes in both the acute and chronic inflammatory cutaneous reactions and with the discovery of agents that can modify cell movement, particular attention has been focused on acute models of inflammation which allow quantitative estimates of cell accumulation to be made.

The subcutaneously implanted sponge and the air pouch models are used by a number of laboratories to investigate cell migration in a variety of animal species.

TABLE 1
Animal models of skin inflammation

Inducer	Pharmacological end point	Species	References
Vascular type acute inflammation			
UV	Erythema	Guinea pig	Winder et al., 1958; Snyder, 1978; Peters et al., 1977; Law et al., 1977
Heat	Ear oedema	Rat	Bronaugh et al., 1978
Croton oil	Ear oedema	Rat, mouse	Weirich et al., 1977; Schlagel 1975; Lorenzetti, 1975; Tonelli et al., 1965; Swingle et al., 1981; Tubaro et al., 1985
Cantharidin	Ear oedema	Rat, mouse	Swingle et al., 1981; Boris and Hurley 1977; Tarayre et al., 1984.
Arachidonic acid	Ear oedema	Mouse, rat, rabbit	Young et al., 1984; Humes et al., 1986; Opas et al., 1985; Arner et al., 1985; Carlson et al., 1985; Crummey et al., 1987; Bouclier et al., 1990; Aker et al., 1986
Carrageenin	Paw oedema	Rat	Winter et al., 1962
Mediator-induced vascular permeability	Plasma exudation	Rabbit	Williams, 1981; Rampart and Williams, 1986
Cellular type inflammation			
Air-pouch + challenging stimulus	Exudate, mediators	Rat	Konno and Tsurufuji, 1983; Gordon et al., 1986; Sedwick and Lees, 1986; Tsurufuji, 1986
Implanted sponge + challenging stimulus	Exudate, mediators	Rat	Ford-Huchison et al., 1971; Clarke et al., 1975; Lackie and Brown, 1982; Gilbertsen et al., 1980; Higgs, 1984; Mellor et al., 1986
Implanted cotton pellet	Granuloma	Rat	Moore and Swingle, 1982
Immune type inflammation			
Sensitization + challenging stimulus	Ear oedema	Mouse	Crowle and Crowle, 1961; Tarayre et al., 1984; Tarayre and Lauressergues, 1982
Passive Arthus reaction	Skin lesion	Rat	Goldlust and Schrieber, 1975
Passive cutaneous anaphylaxis	Skin lesion	Rat, guinea pig	Baldo et al., 1980; Taichman et al., 1971.

In these models, the inflammatory response is evaluated as a function of time by measuring exudate volume, leucocyte number and types, other cell types (i.e., red blood cells, platelets), putative inflammatory mediator concentrations, or relevant enzyme activities. Data reported in a recent paper (Sedwick and Lees, 1986) have shown that the air pouch model is likely to be superior to the implanted sponge model. It was reported to be the most satisfactory and most sensitive model for assessing the potency of glucocorticosteroid-type anti-inflammatory agents (e.g., systemic administration of betamethasone and dexamethasone).

4. Animal models of acute immune-type inflammation. Recent attempts to produce animal models of allergic contact hypersensitivity have been successful for both lesion induction and evaluation of treatment. Animals are sensitised by painting the abdomen with the sensitiser (oxazolone, DNCB, etc.). Several days later, the sensitised animals are challenged on one ear by topical application of the same sensitiser. The contact-delayed hypersensitivity reaction can be quantified by measuring the swelling of the ear with a micrometer or by weighing. Micrometer measurement is superior to weighing in that it is noninvasive and the same animal can be used for different measures.

Mouse models of acute immune-type inflammation have been used to investigate the immunosupressive properties of certain anti-inflammatory drugs such as corticosteroids (De Sousa and Fachet, 1972). The experimental passive Arthus reaction mimics certain elements of the chronic inflammatory reaction, e.g., protein complex deposition, complement activation, infiltration of PMNs, release of lysosomal enzymes, and tissue damage. Nonsteroidal anti-inflammatory compounds such as aspirin, phenylbutazone, indomethacin, and naproxen are weakly active or ineffective in this model, whereas corticosteroidal anti-inflammatory drugs (e.g., hydrocortisone, betamethasone) produce a significant inhibition of the above responses (Goldlust and Schrieber, 1975).

Passive cutaneous anaphylaxis can be elicited by an intravenous injection of antigen in animals previouly sensitised intracutaneously with homologous or heterologous antibodies. The measurement of the passive cutaneous anaphylaxis reaction is generally quantified using either Evan's blue dye alone or in combination with radioisotopes as indicators of capillary permeability. The passive cutaneous anaphylaxis reaction has been of great value in in vivo studies of immediate type hypersensitivity reactions. The release of vasoactive substances, such as histamine, prostaglandin D_2, platelet-activating factor, etc., from mast cells and basophils, plays a prominent role in this reaction in both human and animal skin.

5. Correlation between animal models and clinical results. Acute rodent models of inflammation are reviewed in table 1. These have been instrumental in the discovery and development of NSAIDs for systemic use. However, the utility of these models to predict a therapeutic activity in clinical dermatology can be questionable because most of the NSAIDs active in a large number of animal models are therapeutically ineffective against inflammatory skin disorders. Exceptionally, bufexamac remains one of the few therapeutically useful topical NSAIDs that is used clinically, although its dermatological efficacy has not clearly been demonstrated (Trancik, 1977; Christiansen et al., 1987).

It is evident that cutaneous inflammation is only one of the pathological components of dermatological diseases. The frequent association with epidermal hyperproliferation also should probably be the focus of additional attention in the search for new topical or systemic agents that could be used to treat the wide variety of corticosteroid-responsive dermatoses.

A rational approach to discover new anti-inflammatory drugs for dermatology would be to establish a pharmacological profile showing significant activity in a battery of assays which mimic several components of dermatological disease (e.g., erythema, oedema, infiltration of inflammatory cells, proliferation). Ideally, a drug with a broad spectrum of action that demonstrated significant activity against oedema, inflammatory cell migration and proliferation would have a better chance of therapeutic success than would a more selective drug which is only active in one of the above systems.

6. New models of inflammation adapted to dermatology. Because subchronic animal models should more closely mimic the different pathological components of inflammatory skin disorders, they may represent an improved tool for the development of new drugs and/or new therapeutic agents for use in dermatology. Therefore, we and several other groups decided to place some efforts in this scientific direction. We have recently reported (Bouclier et al., 1989) some preliminary results obtained in a subchronic mouse model presenting the main characteristics of chronic inflammation in human skin.

Skin inflammation developed in response to repeated applications of arachidonic acid to the mouse ear and was maintained for several weeks. In practice, a solution of arachidonic acid in acetone was applied to the inner face of the right ear each day (25 μl of a 4%, w/v, solution), 5 days/week for 3 weeks. The left ear received the vehicle alone (25 μl acetone). Ear thickness was measured each day using a micrometer (Oditest) 4 h after arachidonic acid application. Animals were killed on days 1, 3, 5, 12, and 19, and the resulting histopathological changes were evaluated microscopically.

The acute vascular phase of inflammation (dermal oedema) was observed during the first day of treatment (day 1) followed by a reduced oedema response on day 2. From day 3, ear thickness increased and reached a plateau on day 10. Histopathological examination of the various events occurring as a function of time were as follows. First event (day 1): dermal oedema; second event

(first week), vascular congestion, erythrocyte extravasation, PMN infiltration, weak epidermal hyperplasia; third event (second week), general symptoms of skin inflammation with PMN and mononuclear cell infiltration, microabscesses, and marked epidermal hyperplasia; fourth event (third week), regression of inflammation and marked epidermal hyperplasia.

Daily topical application of betamethasone-17-valerate in acetone (0.1%, w/v) was able to markedly reduce the clinical and histopathological signs of this chronic inflammatory response, namely, epidermal hyperplasia and, in parallel, ear thickness. In addition, the systemic side effects of the corticosteroid were demonstrated on the untreated ear as a reduced thickness associated with skin atrophy. These systemic effects were not observed with a daily dose of 0.01% (w/v), whereas inflammation and epidermal hyperplasia were still significantly reduced on the treated ear. The above observations illustrate the superiority of a subchronic animal model, i.e., the possibility to evaluate both a pharmacological activity and potential side effects (e.g., skin atrophy on an untreated site). A meaningful biological index may be established, taking into consideration both the pharmacologically active dose and the toxic dose.

These preliminary results lead us to believe that this model will be a useful tool for the preclinical evaluation of anti-inflammatory drug actions in the skin. We are currently assessing a number of reference drugs known to be active or inactive in the treatment of chronic cutaneous inflammation. Preliminary data suggest that a number of classical NSAIDs are inactive in the subchronic phase of this model.

B. Animal Models of Proliferation and Differentiation

1. Proliferation. Another key pathological component of a variety of dermatological diseases associated with inflammation is epidermal hyperproliferation. Epidermal proliferation can easily be stimulated in animal skin (mice, rat, and guinea pig) using topical applications of a chemical such as either *n*-hexane or phorbol esters (Bouclier et al., 1988; Delescluse et al., 1981; Bourin et al, 1982). As an example, an epidermal hyperplasia induced in hairless rat skin 48 h after a single topical application of the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate is shown in fig. 1.

It appears that epidermal hyperplasia is frequently associated with increased quantities of polyamines, prostaglandins, and other arachidonic acid metabolites. Ornithine decarboxylase activity, the rate-limiting enzyme in polyamine biosynthesis, and levels of polyamines are known to be elevated in various proliferative states (Pegg and Mac Cann, 1982). In skin, these parameters were corrrelated with continuous growth of the epidermis and dermis (Lowe, 1980).

Recently, we (Bouclier et al., 1988) studied several biochemical parameters, including ornithine decarboxylase activity, polyamine levels, and DNA biosynthesis,

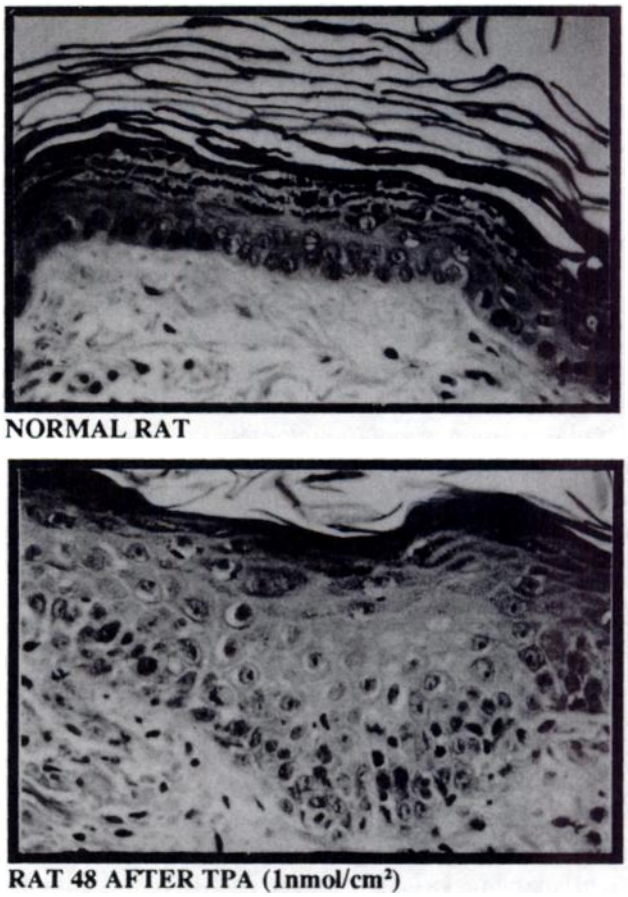

FIG. 1. Epidermal hyperplasia induced in the hairless rat skin by topical application of 12-O-tetradecanoyl-phorbol-13-acetate (*TPA*).

during the *n*-hexadecane-induced epidermal hyperplasia in female hairless rat skin. A succession of biochemical events takes place before development of a hyperplasia: (*a*) induction of ornithine decarboxylase activity (10–12 h after the stimulus), (*b*) increase of polyamine levels (12 h), (*c*) increase in de novo DNA biosynthesis (24 h), and (*d*) epidermal hyperplasia (48–72 h). These data support the hypothesis that, in skin, ornithine decarboxylase induction and polyamines are one of the early signals for de novo biosynthesis of DNA and epidermal cell proliferation (Marks et al., 1983). Thus, epidermal ornithine decarboxylase activity induced by various stimuli such as 12-O-tetradecanoyl-phorbol-13-acetate application, UV irradiation, and cellotape stripping can be used as an index of cellular hyperproliferation. A number of models in both rats and mice using ornithine decarboxylase activity as a marker of epidermal proliferation have been described (Verma et al., 1978; Lowe and Breeding, 1982; Bouclier et al., 1986).

Some years ago we proposed the assay of the induced ornithine decarboxylase activity in the hairless rat epidermis after cellotape stripping for the rapid evaluation of new regulators of epidermal proliferation such as retinoids and anthranoids (Bouclier et al., 1986). This model was successfully used to evaluate activity after both topical and oral administration of retinoids which

are useful agents for the control of both cellular differentiation and cellular proliferation (Sporn and Roberts, 1983).

2. *Differentiation.* To form the tough outer covering layer known as the stratum corneum, epidermal keratinocytes undergo terminal differentiation and keratinisation. Parakeratosis refers to the production of a stratum corneum in which the keratinised cells retain their nuclei. The majority of skin differentiates in an orthokeratotic fashion in which, late in the keratinocyte's life, cellular organelles are degraded by hydrolytic enzymes which are released and/or activated in the granular layer region. Among these organelles, the nucleus is destroyed, and when plasma membrane toughening and keratin synthesis are complete, the stratum corneum (end point of differentiation) is devoid of much of its previous internal structure and, hence, is a cell with considerable flexibility.

Parakeratosis is an abnormal form of keratinisation occurring in the disease psoriasis in which lesional epidermal keratinocytes undergo an accelerated and disturbed terminal differentiation process. In psoriasis, increased epidermal cell turnover and the inherent abnormal differentiation cause a failure of the granular layer phase of keratinisation. The resultant inflexible horny cells stick together, and associated with additional defects in desquamation, the psoriatic patient sheds large unsightly squames. To identify agents that will change a parakeratotic differentiation back to the orthokeratotic type requires a stable animal model.

The mouse tail epidermis displays a regular and highly ordered pattern of clearly parakeratotic scale regions which alternate sharply with orthokeratotic interscale regions. This virtually unique morphological differentiation pattern is supplemented with two high molecular weight type II keratin proteins of 65 and 70 kD. Drugs able to modulate epidermal differentiation, such as retinoids, lead to a complete orthokeratotic conversion of the scale regions and a suppression of the two 65- and 70-kD keratins. The resulting epidermis acquires the morphological and biochemical features of normal skin (Schweizer et al., 1987). This model, named the mouse tail assay by the authors (for review see paper by Wrench, 1985), is highly suitable for the histological evaluation of the influence of drugs on epidermal differentiation. As an illustration, the orthokeratotic conversion induced by repeated oral administration of all-*trans*-retinoic acid to the mouse is shown in fig. 2.

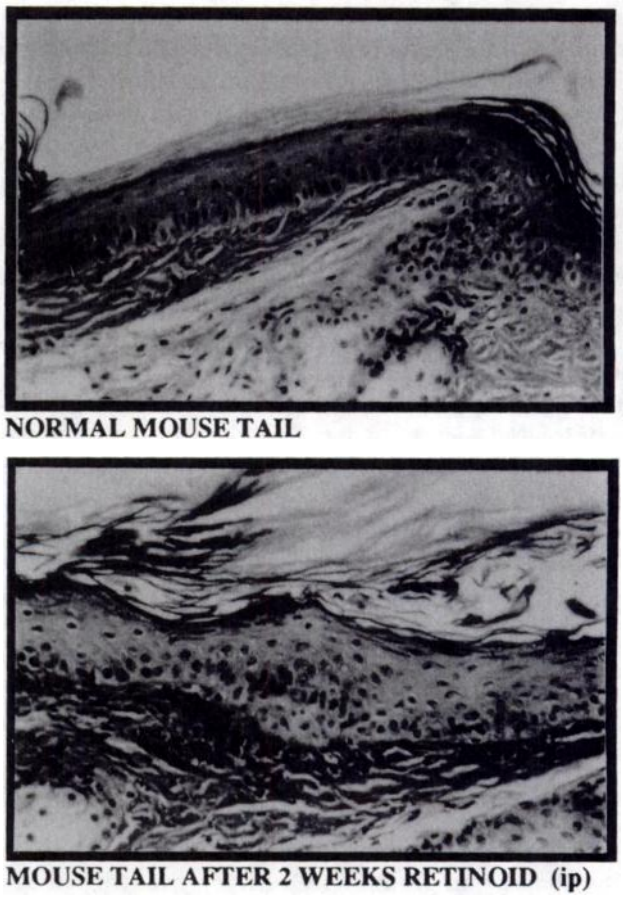

FIG. 2. Orthokeratotic conversion of the mouse tail epidermis induced by repeated intraperitoneal (ip) administration of all-*trans*-retinoic acid.

C. Animal Models of Infection

A large number of microbial diseases (bacterial, fungal, herpes virus) involve the skin, either directly or indirectly, in their etiology (for review see paper by Weinberg and Swatz, 1987). Although specific animal models do not exist for mimicking all of the manifestations of cutaneous infections, a large number of in vivo tests have been described. The most useful models are for fungal infections, i.e., *Candida albicans* and *Dermatophytes* (Ray, 1985; Hay et al., 1985; Jones, 1975). Acute infection of the skin and mucous membranes with the dimorphic fungus *C. albicans* and related species is a common affliction in humans. Dogs (Schwartzman et al., 1965), guinea pigs (Sohnle et al., 1976), rabbits (Wildfeuer, 1972), and rodents (Ray and Wuepper, 1976) have been used sucessfully to develop models of infections on keratinised epithelium. Experimental candidosis in mice and rabbits is essentially initiated by innoculation using either the intravenous route, the topical route on skin and mucosae, or after oral gavage. *Candida* infections developed in animals closely approximate human infections and reproduce most of the histological features of the human disease (for review see paper by Ray, 1985). Experimental infections with *Dermatophytes* are generally induced in the skin of various animal species after clipping, shaving, and/or abrading with or without occlusion (Hay et al., 1985; Jones, 1975). *Dermatophytes* subsequently invade keratinised layers of skin, hair, and nails.

D. Animal Models Relevant to Acne: Sebaceous Gland and Comedone Models

Although the basic etiology of acne remains unknown, considerable information concerning the various factors

involved in its pathogenesis have accumulated in recent years. Acne is a multifactorial self-limiting disease seen primarily in adolescents and involving the pilosebaceous unit and sebum secretion activity. Clinically, there are usually a variety of lesions consisting of comedones, papules, pustules, nodules, cysts, and, as sequelae to active lesions, pitted or hypertrophic scars (for review see papers by Strauss, 1987; Downing et al., 1987). The lesions may be either noninflammatory or inflammatory. The noninflammatory lesions are comedones. The inflammatory lesions vary from small papules with an inflammatory areola to pustules and, subsequently, to large tender fluctuant nodules and cysts. All of these lesions demonstrate an inflammatory infiltrate in the dermis. The inflammation of the pilosebaceous unit can be attributed, at least in part, to the anaerobic microorganism *Propionibacterium acnes* present in the enclosed follicle. Sebaceous gland and comedone models afford a useful tool in the development of drugs for the treatment of acne. Available evidence indicates that the development and secretory activity of the sebaceous gland are essential factors in the formation of the inflammatory lesions of this disease and, possibly, in the induction of the abnormal follicular keratinisation process that precedes them.

1. Sebaceous gland. Various methods can be used to assess sebaceous gland activity in animals. They include different procedures for evaluating glandular size and measuring sebum production (Pochi, 1985). The hamster models are the most useful. These models use either the flank organ (Weissmann et al., 1984) or the ear sebaceous glands (Motoyoshi, 1988).

2. Comedone models. Comedones are the result of a complex pattern of changes in differentiation of the epithelial cells lining the ducts of sebaceous follicles. The comedonal "plug," in fact, is made up of multiple layers of abnormally keratinised cells packed together in a compacted mass in which sebum, proliferating intrafollicular bacteria, and remnants of hairs are interspersed. At least two experimental animal models have been used for evaluating the comedolytic properties of different substances: the rhino mouse and the rabbit ear assay (Puhvel, 1985).

a. THE RHINO MOUSE. Adult rhino mice are hairless mutants with a rhinoceros-like appearance which carry the rhino gene, a recessive allele of the hairless gene ($hr^{rh}hr^{rh}$, Howard 1941). At birth, rhino mice are indistinguishable from their nonmutant littermates. Soon afterward, and prior to the end of their first hair growth cycle, a defect in catagen results in irreversible hair loss. The pilary canals widen, accumulate keratin, and undergo a transformation into horn-filled utriculi which resemble human microcomedones (fig. 4). Compared with the smooth-skinned hairless allele, the skin of the rhino mouse is extremely wrinkled and contains many horn-filled utriculi (Mann, 1971).

The comedolytic effects of various antiacne agents in rhino mice skin were first reported by Van Scott (1972) and Kligman and Kligman (1979) using qualitative histological methods. Other investigators assayed comedolytic activity by the whole mount epidermal technique to quantify the size of horn-filled utriculi (Mezick et al., 1985). We have used quantitative histological and image analysis methods for measuring the number of epidermal comedones, the number of dermal cysts, the transformation of closed comedones to open comedones (comedone profile), and the epidermal thickness. We now routinely use this model to evaluate the potential comedolytic activity of newly synthetised drugs after topical and/or oral administration (Chatelus et al., 1989).

b. THE RABBIT EAR ASSAY. This procedure was described by Mills and Kligman (1975). Sebaceous follicles in the skin of the inner surface of rabbit ears are sensitive to many substances called comedogens, which, when applied topically, induce comedone formation. This comedone induction takes place after about 2 weeks of repeated topical application of a chemical comedogen such as 1% coal tar, 50% oleic acid, or 50% tetradecane. Evaluation of the comedolytic effect of a given drug is performed after 2 weeks of treatment by histological examination of punch biopsies taken from treated and untreated ears.

c. INTRADERMAL INJECTION OF *PROPIONIBACTERIUM ACNES* TO THE EAR OF RAT. Intradermal injection of killed *P. acnes* into the rat ear induces a chronic acne-like inflammation characterised by oedema and cell infiltration of several months' duration, formation of comedones, hypersensitization, and transepithelial elimination. Details of this method have been reported by De Young et al. (1984, 1985).

E. Animal Models of Photodamage/Photoaging and Repair

Recent studies have suggested that changes accompanying the aging process are quite different in sun-exposed areas such as the face and hands versus those observed in protected skin such as the buttocks (Smith et al., 1962; Braverman and Fonkerko, 1982). Protected skin, even at an advanced age, is usually smooth, unblemished, and without neoplastic growths and is only somewhat looser and less elastic. Physiological decrements such as reduced blood flow are not remarkable (Kligman, 1979). In actinically damaged (photoaged) skin, many of the visible alterations are epidermal. They include hyper- and hypopigmentation, dryness, scaling, and a variety of benign, premalignant, and malignant neoplasms. Profound structural changes occur in photoaged dermis and account for the visible yellowing, wrinkling, sagging, and thickening of facial skin. Additionally, it takes many decades for actinically damaged dermal connective tissue to reach its ultimate, amorphous degeneration. The evolution of such a long process cannot easily be studied

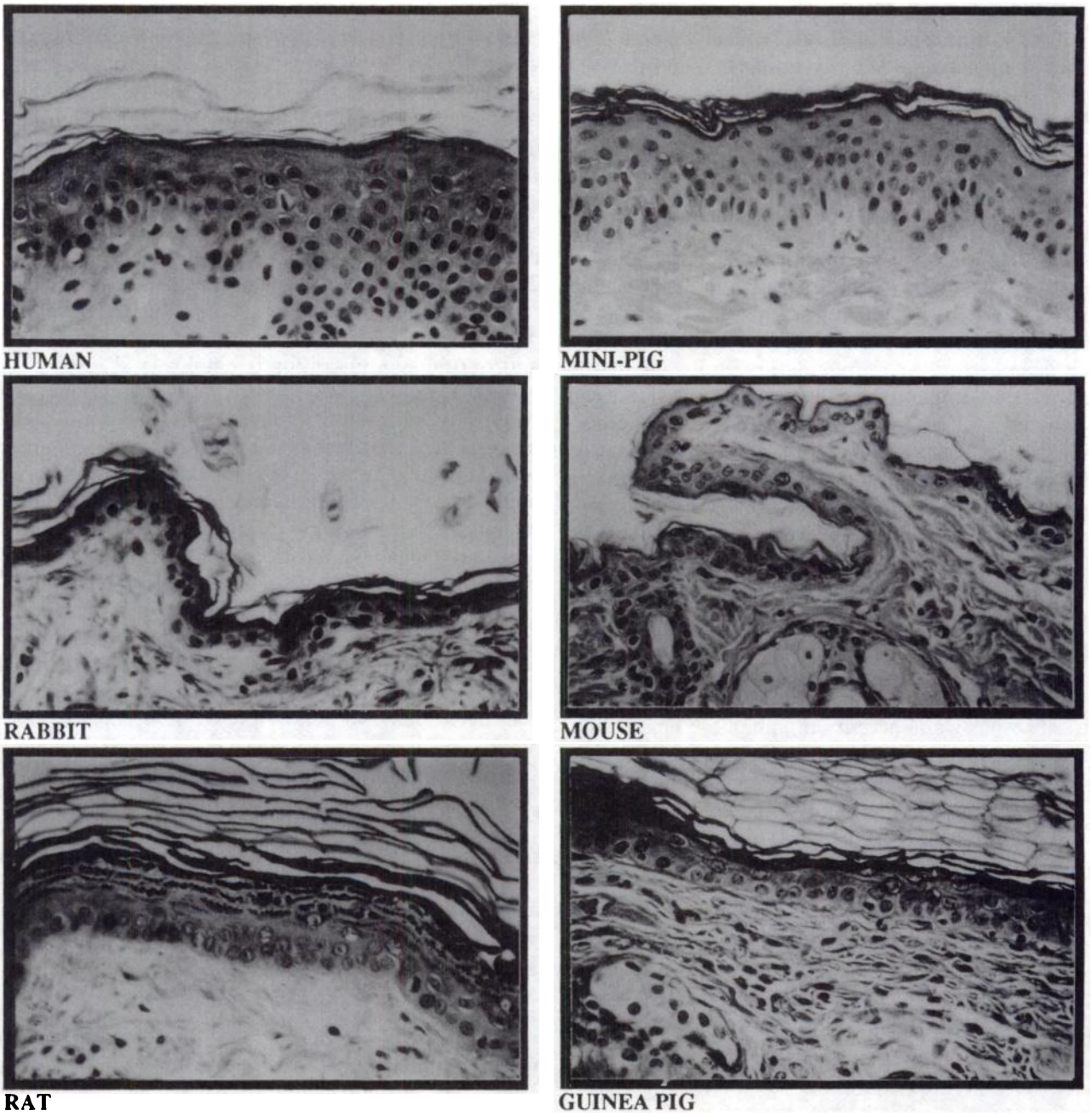

FIG. 3. Histological aspect of the back skin from various species.

in humans. Systematic, sequential studies require animal models.

Investigations of various murine species have unequivocally placed the hairless mouse in a prominent position. Hairless mice have proved valuable in various photobiological investigations ranging from phototoxicity (Forbes et al., 1976), photoimmune effects (Degruijl and Van Der Leun,1982), carcinogenesis (Epstein, 1965), and UV-induced DNA damage (Ley et al., 1983). Furthermore, these animals occur in two varieties, albino and lightly pigmented. The presence of pigment, albeit in small amounts, adds another dimension to the experimental possibilities. Not only are hairless mice convenient and hardy, but the UV-induced changes are comparable to those found in human skin and, hence, have high relevance. The action spectrum and time course for the acute responses to UV radiation, oedema in the hairless mouse, and sunburn erythema in humans are comparable (Cole et al., 1983). It has been shown that the range of UV-induced epidermal tumors is similar in mice and humans with the single exception of basal cell cancers on the back in mice (Kligman and Kligman, 1981). UV-induced connective tissue damage is largely analogous to that which occurs in humans (Kligman, 1982). Kligman and her collaborators have utilised the hairless mouse to (*a*) identify the separate and combined effects of UVB, UVA, and infrared radiation on the dermis (Kligman et al. 1985); (*b*) show that sunscreens are highly protective

against this damage (Kligman et al., 1982; Kligman et al., 1983); (*c*) demonstrate postradiation repair of UV-irradiated skin (Kligman et al., 1982; Kligman et al., 1983); and (*d*) determine that topical all-*trans*-retinoic acid enhances the repair of photodamaged skin (Kligman, 1986; Kligman et al., 1984). This latter point is of particular interest, and recently Weiss et al. (1988) demonstrated, in a clinical double-blind vehicle controlled study, that topical tretinoin (all-*trans*-retinoic acid) significantly improves photodamaged skin.

F. Induced Dermatoses

1. Magnesium-deficient rats. Dermatosis in magnesium-deficient hairless rats has been described as a reproducible model of skin inflammation (Barbier et al., 1986; Saurat et al., 1985). This dermatosis is characterised by the development of a cyclic rash taking the form of erythematous plaques without vascular necrosis and is accompanied by biological and immunological abnormalities. Corticosteroid anti-inflammatory drugs are able to abolish the rash, whereas NSAIDs such as indomethacin and phenylbutazone are inactive.

2. Essential fatty acid-deficient rats. When the essential fatty acid, linoleic acid (C18:2), is removed from the diet of mammals, a deficiency state characterised by widespread pathologic findings in several organ systems ensues. Cutaneous abnormalities, including hyperproliferative epidermal changes and impaired barrier function, are two components of essential fatty acid deficiency in rodents (Elias, 1985; Lowe and Stoughton, 1977).

3. Ichthyosis. Ichthyosis is a scaling dermatosis associated with abnormal lipid metabolism. In an attempt to develop appropriate models of ichthyosis, two approaches were successfully used (Elias et al., 1985): (*a*) systemic administration of 20,25-diazacholesterol to hairless mice. This drug interferes with cholesterol metabolism and has been associated with ichthyotic abnormalities in humans and (*b*) topical application to hairless mice of lipids (cholesterol sulfate, *n*-alkanes) which are known to accumulate in the stratum corneum in human ichthyotic skin.

Genetic ichthyosis has been described in the mouse, cow, and dog (Goldsmith, 1985), but the relationship between human and animal forms of the disease remains to be established.

4. The Azebia mouse. The homozygous Azebia mouse displays chronic epidermal hyperplasia, dermal inflammation, and a conveniently sparse hair coat. It might be a good model for studying chronic epidermal hyperproliferation (Brown and Hardy, 1985). However, extensive and long-term drug testing are needed to appreciate the potential usefulness of this model for either drug evaluation or for studying the pathophysiological aspects of hyperproliferation.

G. Athymic Nude Mice and Human Skin Grafting (Black and Jederberg, 1985)

The nude mouse is functionally athymic but has a rudimentary thymus. The mouse is immunodeficient and accepts xenografts of human skin. The grafts can remain viable for the lifetime of the recipient mouse. The skin-grafted nude mouse is an in vivo model for use in the study of skin function, metabolism, and diseases, including those occurring in some immunocompromised hosts. However, because the animals are immunodeficient, data obtained in studies intended for the extrapolation to the normal human state require careful validation. Nevertheless, the model has a lot of potential for use in dermatology as a tool for the study of human skin which may be maintained on the mouse for many months.

H. Matching the Animal to Research Objectives

1. Various animal species: criteria of selection. A large number of animal species and strains are used by skin pharmacologists. The more useful of them are listed in table 2. The hairless animals are undoubtly the most adapted for skin pharmacology and topical application of drugs. Small animals such as the rat and mouse are currently used because of availability, convenience, and low cost. However, as far as selecting an animal species that will give the same desired pharmacological response as that observed in humans, the rat and mouse are probably not the best choice. The histological aspect of the back skin from various laboratory animal species and humans is shown in fig. 3

The epidermis of humans and minipigs, on the one hand, and rabbits and mice, on the other, present a similar morphological aspect. Surprisingly, rat epidermis is very different from that of other rodents. The markedly developed stratum granulosum would indicate an unusual differentiation process in this species.

Thus, the large morphological differences existing in the skin from one species to another would probably lead to differences in respective pharmacological responses.

TABLE 2

Animal species used in cutaneous pharmacology

Mouse
Traditional strains
Nude
Hairless
Rhino
Rat
Traditional strains
Hairless
Nude
Guinea pig
Traditional strains
Hairless
Rabbit: Traditional strains
Minipig and micropig
Dog: Mexican (hairless)

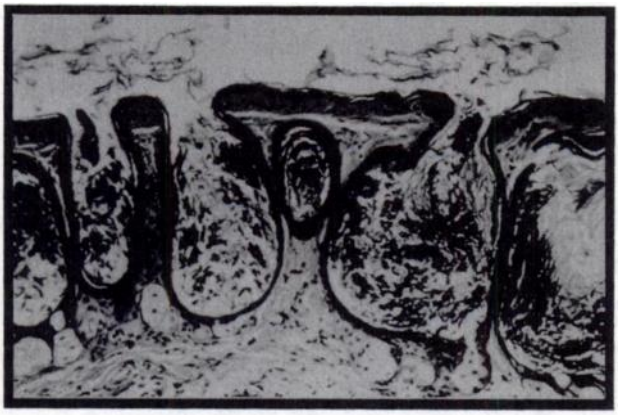

FIG. 4. Histological aspect of adult rhino mouse skin.

For example, the extent to which a drug or stimulus penetrates the skin would determine its efficacy. What should be one of the most important criteria, namely, comparability of relative skin permeability between animal models and man, rarely has been addressed. Permeability properties of dog, monkey, and pig skin are closer to those of humans than are those of the skin of rodents such as rabbits, mice, and rats (Barteck et al., 1972; Marzulli et al., 1969; Mac Creesh, 1965). Pig skin shares many biochemical, physiological, and morphological characteristics with human skin. Like human skin, pig skin may have a sparse pelage and a surface carved by fine lines that form characteristic patterns (Montagna and Yun, 1964). Furthermore, the thickness and general morphologic appearance of epidermis and dermis (Montagna and Yun, 1964), tritiated thymidine-labeling pattern, and index of epidermal cells (Jurnovoy et al., 1975), epidermal cell turnover time (Weinstein, 1965), and size, orientation, and distribution of vessels in skin (Forbes, 1969) are similar in the two species. Thus, pigs, and especially various strains of minipigs, appear to be a suitable nonprimate animal for the evaluation of drug activity for potential human therapy. Nonetheless, availability, convenience, size, and cost are major problems for routine work with an animal weighing 20–100 kg. This is why few data are available concerning the skin pharmacology of the pig and, as discussed in the first part of this review, rodents remain the most routinely used species. In skin pharmacology today, extensive use is made of rats and mice. Thus, in view of the marked morphological differences observed in the skin of these two species, extrapolation of results obtained from one to the other and also to humans must be tempered with prudence. An illustration is given in table 3 in which data obtained in our laboratories (Bouclier et al., 1990) showing the pharmacological modulation of the arachidonic acid-induced ear oedema in four strains of rats and mice are summarised.

The pharmacological responses obtained with various strains within the same species did not show any major differences. In our experimental conditions and at the doses tested, mice were sensitive to NSAIDs, including

TABLE 3

Pharmacological modulation of the arachidonic acid-induced ear oedema formation in 4 strains of rats and mice

Drug tested*	Inhibition of the ear oedema	
	Rat (Wistar, OFA, ICO and Fisher)	Mice (OF_1,* CD_1,* NMRI,* and C_3H)
Indomethacin	Yes	Yes
Naproxen	Yes	Yes
5,8,11,14-ETYA	Yes (except Wistar)	Yes
BW 755C	Yes	Yes
Nordihydroguaiaretic acid	No (except OFA)	Yes
Hydrocortisone	No	Yes (weak)
Betamethasone-17-valerate	No	Yes

* The maximum dose evaluated was 5% (w/v) in acetone (1.25 mg/ear).

TABLE 4

Criteria for choosing the animal species

- Macroscopic state of the skin
- Histological profile of the skin
- Ability of the animal species to produce a given pharmacological response
- Ability of the species to be considered as giving a pharmacological response similar or identical to that obtained in human skin
- Comparable pharmacological activity of drugs in the animal model with those observed in human pharmacology/therapeutics
- Others: cost, size, availability, stability of strain, difficulty of breeding, etc.

selective cyclooxygenase inhibitors (indomethacin, naproxen), double-cyclooxygenase and lipoxygenase inhibitors (ETYA, BW 755C), and predominantly lipoxygenase inhibitors (nordihydroguaiaretic acid), in addition to corticosteroidal anti-inflammatory drugs (hydrocortisone and betamethasone valerate). However, in rats, a sensitivity to the cyclooxygenase and double inhibitors tested was observed, whereas the lipoxygenase inhibitors (nordihydroguaiaretic acid) or the corticosteroidal anti-inflammatory agents were inactive. These results clearly demonstrate a difference in the pharmacological responses obtained in rats and mice. In the various strains of rats, the arachidonic acid-induced oedema would appear to be predominantly cyclooxygenase dependent, whereas in the mouse, it would appear to be both cyclooxygenase and lipoxygenase dependent. In addition, because of distinct morphologic properties of the two skin types, a difference in skin penetration and bioavailability of drugs can also be suspected between the two species.

Thus, one must carefully consider a number of criteria when choosing an animal to establish a pharmacological model. These criteria are summarised in table 4. Regarding these criteria, the pharmacologist must try to reach the best compromise when selecting an acceptable relevant species. The ability of the animal species to produce (*a*) a given pharmacological response, (*b*) a pharmaco-

logical response similar to that obtained in human skin, and (*c*) a comparable pharmacological activity of drugs with those obtained in human pharmacology and clinical therapy would be the essential points.

2. Biological and environmental parameters that can influence the pharmacological response. It is clear that the choice of animal to be used in any given research project depends upon many factors including size, species, sex, and suitability for the nature of the investigative technique to be used, as well as the cost and the facilities available for housing (table 5).

After these factors have been taken into account, however, a number of biological parameters can also influence the biological response. Among these parameters (which include strain, sex, and reproduction cycle in females), the skin and the *quality* of both the animal and the experimental design (tables 5 and 6) are certainly major factors influencing the production of reliable and reproducible results. The following examples will further illustrate some of the these points.

Some years ago we developed a model using a mutant hairless Sprague-Dawley rat to evaluate the capacity of retinoids to inhibit the epidermal ornithine decarboxylase activity induced by cellotape stripping (see "Proliferation"; Bouclier et al., 1986). To minimise variability introduced by the animals in this model, we thought it necessary to validate the hairless rat used in our laboratory, i.e., to define animal specifications closely after experimental determination of the principal factors having a potential influence on our results. The major parameters considered capable of introducing variability and, therefore, affecting the reproducibility of the test were (*a*) the characteristics of the animal used (sex, age, etc.) and (*b*) the characteristics of the skin at the test site, which can influence the stripping efficiency and, therefore, the induction of ornithine decarboxylase activity (the stripping efficiency depends on the morphologic characteristics of the stratum corneum and on the presence or absence of hair follicles). These parameters were chosen on the basis of a review of a large number of studies conducted in our laboratory during the past years. We used a single batch of 4- to 11-week-old males and females acclimated to controlled laboratory conditions to study the effect of age and sex on physiological parameters (hair growth, stratum corneum, and epidermal thickness), biochemical parameters (epidermal ornithine decarboxylase activity with and without stripping), and procedural parameters (histological determination of stripping efficacy). Other factors (technician variability, analytical procedures, etc.) were controlled by the standard procedures currently used in our laboratories. Results obtained in this study (Bouclier et al., 1987) demonstrated that hair development was more pronounced in males; two "regrowth" periods were observed in both sexes; the epidermal thickness was higher in 7- to 9-week-old males; the thickness of the stratum corneum was not significantly changed either between males and females or as a function of age; stripping efficiency was highest in 7- to 11-week-old females; and the induction of ornithine decarboxylase activity in the epidermis was much more reproducible in females. These results, as well as general observations of the animals, led us to the conclusion that female skin characteristics are more homogeneous and, therefore, would give more reproducible results than would male skin. From these data we decided to specify 8-week-old female hairless rats for all tests using cellotape-stripping induction of epidermal ornithine decarboxylase.

The results obtained with three models we are using routinely in our center are given in fig. 5. With strict standardization of each experiment, we have been able to obtain a good to excellent reproducibility of the data obtained from one experiment to another with both negative and positive control groups, for a period of more than 1 or 2 years. These results are part of the evidence demonstrating the acceptable quality of our models.

Another essential tool to obtain reliable results and to avoid wrong interpretations of data is the calibration of the model with known reference drugs potentially active in the model. From our expertise with a number of inflammation models, we would strongly recommend the calibration of each model with drugs known to have an anti-inflammatory activity (corticosteroidal, nonsteroidal, and miscellaneous anti-inflammatory agents). Data obtained under controlled conditions with a number of topically applied reference drugs in the 3 models of inflammation reported in fig. 5 are summarised in table

TABLE 5

Biological and environmental parameters that can influence the pharmacological response

- Strain
- Sex
- Skin
- Reproductive cycle
- Food
- Genetic stability
- Health Status
- Environment (temperature, relative humidity, air quality, air movement, light, sound)

TABLE 6

Parameters that can influence the quality of experiment design "managing the design"

- Communication
- Literature searches/personal contact
- Ethical supervision/ethical committee
- Standardized protocol
- Standardized methods and practices
- Availability/quality of animals
- Statistical design
- Adapted controls
- Interpretation of results

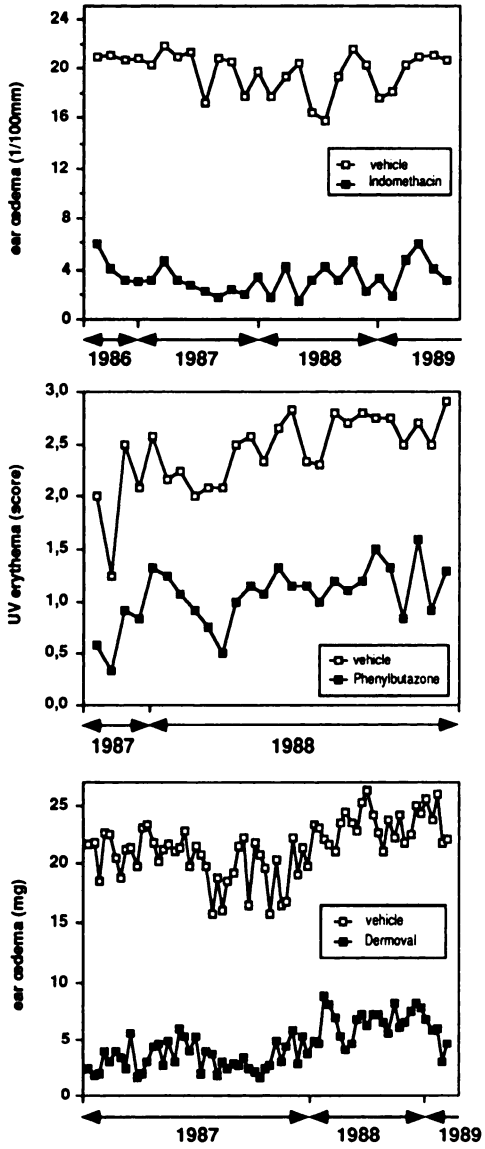

FIG. 5. Curves show the mean results obtained in a large number of experiments between 1986 and 1989. □, responses obtained with the negative control groups (inducer alone). ■, Inhibitory responses obtained with the positive control groups (inducer + reference drug). *Top,* Arachidonic acid-induced ear oedema in the mouse (model 1). Effect of topical application of indomethacin; 3.5 μmol indomethacin was coapplied with 1 mg arachidonic acid in 25 μl tetrahydrofuran/methanol (1/1, v/v) solution. Oedema was measured 1 h after the application. *Middle,* UV-induced erythema formation in the guinea pig (model 2). Effect of topical application of phenylbutazone. Phenylbutazone was applied 1 h after irradiation [100 μl of a 3% (w/w) solution in dimethylacetamide/acetone/ethanol (20/40/40, w/w/w)]. Erythema was scored 3 h after irradiation. *Bottom,* Croton oil-induced ear oedema formation in the rat (model 3). Effect of topical application of dermoval cream; 100 μl of dermoval cream (clobetasol-17-propionate, 0.05%, w/v) was applied 1 h before croton oil. Oedema was measured 6 h after croton oil application.

7. Corticosteroids are potent inhibitors of croton oil-induced ear oedema in the rat (model 3), but they do not show a significant activity in the arachidonic acid-induced ear oedema in the mouse (model 1) or in the UVB irradiation (290–320 nm) induced erythema in the guinea pig (model 2). In contrast, cyclooxygenase inhibitors, double inhibitors, and nordihydroguaiaretic acid reduce oedema induced by arachidonic acid (model 1) and the erythema induced by UVB irradiation (model 2), but they do not show any significant activity against the oedema induced by croton oil (model 3). These exploratory experiments are part of our quality assurance effort and confirm the need to carefully choose suitable animals and strains when developing animal models.

I. Conclusion

If animal models are mostly used for the evaluation of drug action, they are also necessary tools for mechanistic studies leading to knowledge of the various events occurring during skin inflammation. The increasing need for the proof of ethical use of animals has already resulted in the establishment of a variety of regulations for the control and type of animal models used in pharmacology. If it is obvious that in vitro assays using cultured cells, isolated cells, or isolated enzymes can resolve certain ethical problems, it is also obvious that in vivo methods are indispensable for establishing dose ranges and potential clinical utility. Nevertheless, the vulnerability of animal models means that we must be cautious when making casual extrapolations to humans. This problem is compounded when active agents are added, especially when application is made to the entire dorsum of the mouse, for example, in contrast to limited areas of human skin. In addition, the skin barrier function is modified in most dermatoses leading to an altered bioavailability of topically applied drugs. Thus, in most cases, animal studies have to be interpreted carefully. If acute models are useful, they are far from the clinical situation. New subchronic animal models will be essential tools for both a better understanding of many skin diseases and the design of new drugs for skin therapies.

III. In Vitro Models of Interest in Cutaneous Pharmacology

The establishment of in vitro test systems for the prediction of pharmacological and/or toxicological activities of drugs is currently a great matter of public and scientific concern. It has to be acknowledged, however, that in many instances in vitro pharmacological approaches suffer from serious scientific and practical limitations, and indeed, the present goal of in vitro drug testing is not yet at a stage whereby it can replace the existing in vivo assessment but rather can only complement conventional procedures. Nevertheless, in skin pharmacology we are facing a situation in which discovering and evaluating potential drugs for the treatment of skin diseases is quite unique: in most cases, topically applied substances are, by definition, targeted to the desired organ. Hence, it is conceivable that, if this organ can be "reconstructed" artificially, particularly using human cells, there is no major theoretical reason why pharmacological treatment of this artificial system might

TABLE 7

*Calibration of 3 animal models of acute inflammation with reference anti-inflammatory agents applied topically**

Drug tested	Inhibition of:		
	Arachidonic acid-induced ear oedema in mouse (model 1)	UV-induced erythema in guinea pig (model 2)	Croton oil-induced ear oedema in rat (model 3)
Strong cyclooxygenase inhibitors			
Indomethacin	Potent	Potent	No
Naproxen	Mild	Potent	No
Phenylbutazone	Mild	Potent	No
Piroxicam	No	Weak	No
Ibuprofen	No	Mild	No
Double inhibitors			
Benoxaprofen	No	Weak	No
BW 755C	Mild	Potent	Weak
ETYA	Potent	Potent	Weak
Lipoxygenase inhibitor: Nordihydroguaiaretic acid	Mild	Mild	No
Corticosteroids			
Hydrocortisone	Very weak	No	Potent
Betamethasone-17-valerate	Weak	No	Potent

* Model 1: Inhibition of arachidonic acid-induced ear oedema formation in the mouse by topical application of drugs (CIRD standardized protocol). The vehicle used was tetrahydrofuran, methanol (1/1, v/v). Arachidonic acid (4%, w/v) was applied with the drug. Oedema was measured 1 h after the arachidonic acid application. Model 2: Inhibition of UV-induced erythema formation in the guinea pig by topical application of drugs (CIRD standardized protocol). The vehicle used was N-N-dimethylacetamide/acetone/ethanol, 20/40/40, w/w/w. The drug was applied 1 h after UV irradiation. Oedema was measured 2, 3.5, 5, and 24 h after irradiation. Model 3: Inhibition of croton oil-induced ear oedema formation in the rat by topical application of drugs (CIRD standardized protocol). Drugs were evaluated in 6 different vehicles (hydro alcoholic gel, eucerin emulsion, anionic emulsion, oil-water emulsion, vaseline/lanoline, vaseline). It should be stressed that no significant difference between results obtained with the various vehicles was observed. Oedema was evaluated 6 h after croton oil application.

not yield more relevant data than do existing animal models. Recent advances in this field indicate that such in vitro models may represent quite powerful tools for pharmacological studies and for our understanding of fundamental processes in the skin involving cellular interactions and dermal-epidermal interactions.

Yet, the advent of sophisticated skin models should not make us neglect more "traditional" approaches, such as conventional cultures of epidermal cells, for the study of such basic processes as cell proliferation and its pharmacological modulation. There is evidence showing that, with these simple cultures, accurate prediction of clinical efficacy of certain classes of drugs, e.g., certain antipsoriatics, can be obtained. Conversely, there is by no means any chance that existing reconstructed models of skin can reproduce all symptoms of skin diseases, chronic inflammation being a good example in this respect. Inflammation is an extremely common and disabling aspect of skin diseases. In vitro models aimed at studying inflammation usually represent a mechanistic approach to the problem and commonly involve cells that strongly participate in the reaction while being usually present in fairly low quantities in the skin, e.g., leucocytes, lymphocytes, or platelets. However, we should not ignore the importance of the skin in these processes. There is accumulating experimental evidence to demonstrate that skin is not a simple passive participant of the inflammatory reaction. In vitro models that combine skin cells and inflammatory cells could help improve our understanding of the basic processes involved and their pharmacological control.

A. Organ and Three-Dimensional Cultures

1. Organ cultures. A great advantage of cultured organ systems is usually the retention of cell-to-cell interrelationships as found in vivo. In practice, split thickness fragments of skin are laid dermal side down on various substrates which may or may not allow epidermal cells to migrate out. In the first case, peripheral outgrowth is obtained, thus giving rise to an epidermal cell culture (fig. 6). In the second case, epidermal cells proliferate vertically, thus retaining the original organization of cells in the skin. The result is a true organ culture. From a pharmacological viewpoint, such a true organ culture is preferable because drug effects upon epidermal cells may be influenced and/or mediated by dermal elements. For further details of skin explant cultures, the interested reader is referred to the paper by Prunieras and Regnier (1987). Because the dermal part of the explant cannot be strictly controlled in terms of matricial and cellular components, these authors stress that such cultures "represent a useful but 'dirty' experimental design."

2. Three-dimensional cultures. Three-dimensional cultures of human keratinocytes on a "dermal equivalent" have gained increasing interest during the last few years. A living skin equivalent was developed by Bell et al. (1979, 1981). It consists of two well-defined entities: a dermal equivalent and an overlying epidermis. Dermis is essentially made of collagen (approximately 90%) and fibroblasts; therefore, it was logical to reconstitute a dermal equivalent by mixing skin fibroblasts with a collagen solution (usually type I collagen). After the

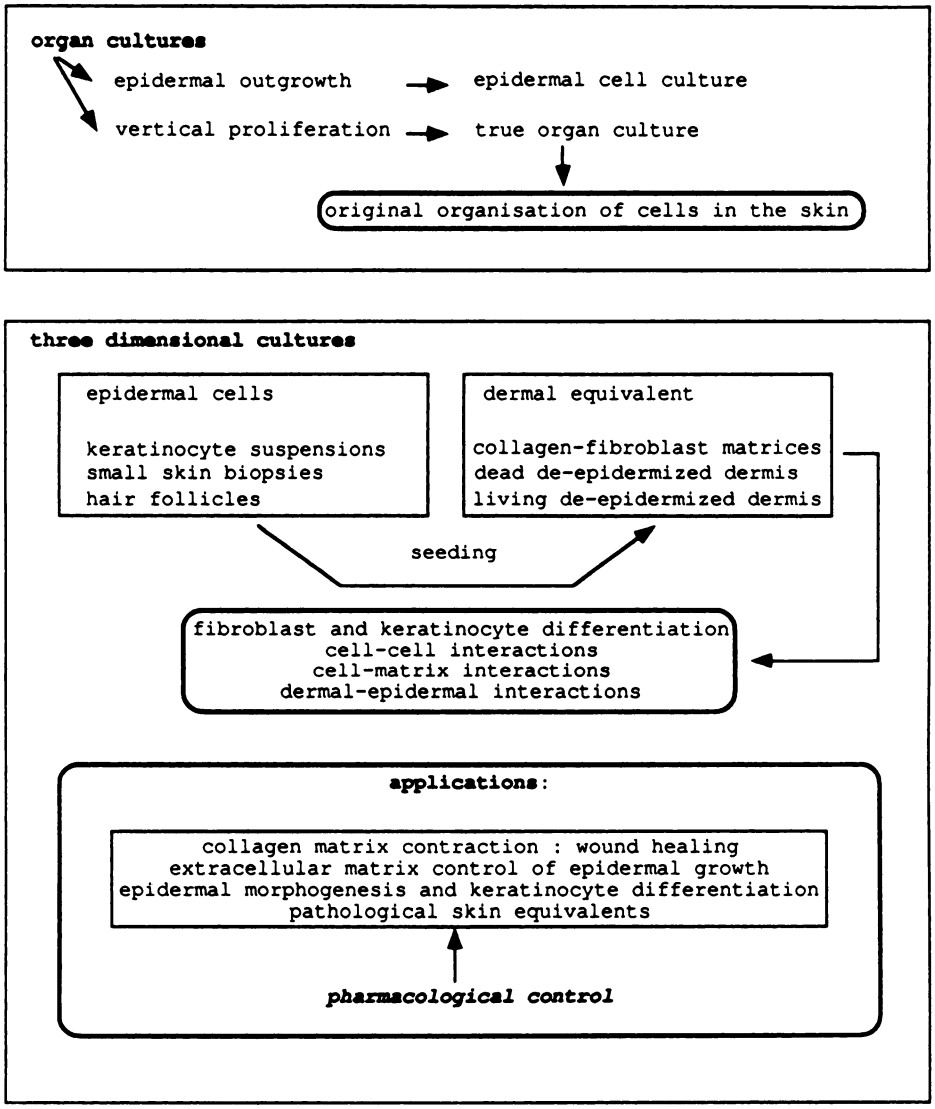

FIG. 6. Organ and three-dimensional cultures.

mixture is made, collagen polymerises rapidly with fibroblasts being uniformly dispersed in the gel. The fibroblasts extend cytoplasmic processes and collect and arrange collagen fibrils, thereby resulting in gel contraction, which is stabilised within a few days.

Keratinocytes obtained from human or animal skin can be cultured on such collagen-fibroblasts lattices, resulting in the reconstruction of a "simplified skin" (fig. 6). Alternatively, Prunieras et al. (1979) used dead human deepidermised dermis to reconstruct epidermis in vitro. Deepidermization was performed under conditions that preserved the basement membrane, the natural physiological substrate of epidermal cells. In addition, the fact that dermal cells are dead in this system (e.g., by irradiation) makes it possible to investigate extracellular matrix-epidermal interactions. Using a similar approach, Mackenzie and Fusenig (1983) reconstituted whole skin in vitro using living deepidermised dermis.

The potential advantages of cultures of reconstituted skin, as opposed to conventional cell cultures or organ cultures, are numerous. When cells are placed in a defined extracellular matrix environment like the collagen-fibroblast matrix, fibroblast and keratinocyte differentiation close to the in vivo situation can be obtained, and cell functions and responses to pharmacological agents can be investigated at the tissue level. Cell-cell, cell-matrix, and dermal-epidermal interactions can be quantitatively studied, which is indeed more difficult to perform in vivo. Because these tissues can be made with cells derived from normal or pathological skin, the pathophysiology of skin can also be approached, along with the development of potential models for pharmacology. The following examples illustrate these points.

Fibroblasts in monolayer behave differently than fibroblasts in a dermal equivalent with respect to enzyme expression, membrane permeability, collagen synthesis, and processing, all of which, in the dermal equivalent, closely resemble the in vivo situation (reviewed by Cou-

lomb et al., 1989a). These differences may be of importance when considering pharmacological control of fibroblast activity and differentiation.

The capacity of fibroblasts to contract the collagen matrix is closely related to the condensation of collagen fibrils during wound healing in vivo. Hence, when this three-dimensional culture is used, it is possible to investigate the pharmacological control of fibroblast-induced contraction by drugs such as the corticosteroids (Coulomb et al., 1984).

When epidermal cell suspensions are seeded onto plastic or collagen-coated culture dishes, epidermal cell proliferation (mostly keratinocytes) ensues. However, despite serious improvements to the original culture method (Rheinwald and Green, 1975), a stratified but poorly differentiated epidermis is obtained. In contrast, when epidermal cells are cultivated either on dead deepidermised dermis or on dermal equivalents and the culture is grown at the air-liquid interface, epidermal differentiation is complete (i.e., formation of cornified layers), thus closely resembling that observed in vivo (Regnier et al., 1986; Asselineau et al., 1985), although the topology of some differentiation markers may differ slightly (Asselineau et al., 1986). Using the human living skin equivalent, Coulomb et al. (1989b) investigated dermal-epidermal interactions and confirmed the importance of an extracellular matrix on epidermal growth. These authors further demonstrated the involvement of fibroblasts as modulators of epidermalization through the remodeling of collagen fibers and the secretion of diffusible factors. Under these conditions, it is expected that drug action on epidermal cell proliferation might be influenced somewhat by the presence of dermal fibroblasts. Indeed, Sanquer et al. (1989) recently showed that in a human living skin equivalent model, repeated exposure to all-*trans*-retinoic acid and 13-*cis*-retinoic acid results in an increased epidermal growth in the absence of living fibroblasts in the dermal equivalent; however, an inhibition of epidermal growth is obtained when living fibroblasts are present.

Detailed investigation of the control of epidermal morphogenesis and differentiation by retinoids have been performed by Asselineau et al. (1989), using human keratinocytes cultured on an emerged dermal equivalent. This work shows that optimal epidermal morphogenesis seems to be achieved in the presence of a critical retinoic acid concentration (10^{-9}-10^{-8} M). In the absence of retinoic acid, morphogenesis is altered, leading to a hyperkeratotic epithelium (as characterised by an excess production of horny layers). Conversely, in the presence of retinoic acid concentrations $>10^{-8}$ M, a parakeratotic epithelium is obtained (as characterised by the absence of granular layers and the presence of nuclei throughout the whole depth of epidermis). From a pharmacological viewpoint, these studies open up new possibilities for the investigation of the modulation of epidermal morphogenesis and differentiation by new synthetic retinoid analogues. In addition, this model offers the possibility of dealing with a "pathological" i.e., hyperkeratotic or parakeratotic, skin equivalent.

Abnormal or pathological skin equivalents can also be obtained using pathological cells. Dermal diseases are currently under investigation, e.g., dermatosparaxis, recessive epidermolysis bullosa dystrophica, and progressive systemic scleroderma. In all instances, the capacity of diseased fibroblasts to contract the collagen matrix is altered (reviewed by Coulomb et al., 1989a). Sunlight-induced ageing of keratinocytes is another example: human epidermal growth in a living skin equivalent is considerably reduced when keratinocytes are isolated from sun-exposed skin (Coulomb et al., 1989c). Pharmacological manipulation of such pathological skin equivalents will undoubtedly be of great interest.

Pharmacological studies with human living skin equivalents may require large quantities of epidermal cells, thus raising the important problem of finding a convenient source. Coulomb et al. (1986) developed a method for epidermalization in vitro which consisted of inserting a small biopsy specimen into a freshly prepared dermal equivalent. Such small biopsy specimens provoke small wounds which rapidly heal without leaving any scar at the donor site. When the gel contracts into a tissue, the specimen is held firmly in place. Epidermal cells migrate out of the explant onto the collagen surface, in a manner identical with that observed during wound healing in vivo, and eventually cover the whole surface of the collagen matrix. This method offers several advantages over the use of keratinocyte suspensions as a source of epidermal cells: basal keratinocytes of the growing epidermis are well organised into a coherent germinative layer of cuboidal cells, like that observed during normal healing in vivo, and this may be of importance for a normal program of differentiation to take place. Along with the analysis of morphological and differentiation parameters, this system makes it possible to measure the area of outgrowth, its DNA content, and the incorporation of radiolabeled thymidine, thus providing an appropriate tool for pharmacological investigations.

Alternatively, in our institute, Lenoir et al. (1988) implanted hair follicles vertically in a freshly cast dermal equivalent and raised the culture at the air-liquid interface. Under these conditions, outer root sheath cells are able to shift their phenotype into interfollicular keratinocytes. A fully differentiated epithelium is obtained with an organisation very similar to that of interfollicular epidermis in vivo. Basement membrane elements are formed, basal cells have a normal cuboidal shape and are polarised, and the distribution of differentiation markers is identical with that observed in vivo. All of these features are usually not seen in vitro after seeding of trypsinised keratinocytes. It is noteworthy that, in this tissue culture system, 1 cm^2 can be covered by epithelial

outgrowth from only one hair follicle within 10–12 days. Therefore, this new culture system raises the possibility of producing large quantities of living skin equivalents by implanting in large lattices a small number of hair follicles obtained from a large number of subjects without producing any trauma.

From this overview, it can be seen that in vitro reconstructed skin may provide pharmacologists with a powerful tool to study drug action on skin cell growth and differentiation and cellular and tissue interactions. It is hoped that in the near future a large body of information will be available to further establish the relevance of such in vitro systems to skin pharmacology.

B. Keratinocyte Cultures

Despite the limited capacity of keratinocyte cultures to differentiate and stratify, they are well suited for studying the effects of drugs on cell proliferation and the expression of certain differentiation markers. Immortilised keratinocytes are particularly well suited to screen for antiproliferative drugs. Different culture methods have been developed for animal and human keratinocytes. Epidermis may be separated from dermis in skin biopsy specimens by enzymic or mechanical means (Medawar, 1941), followed by enzymic dispersion of epidermal cells by proteases such as trypsin (Cruickshank et al., 1960). As previously mentioned, explant cultures or human scalp hair follicles also can be used as a source of keratinocytes.

1. High-density dispersed cultures. Several methods have been used to optimise keratinocyte attachment and growth rates. When epidermal cell suspensions are plated at high density (e.g., 10^5 cells/cm^2) ("high-density dispersed cultures") on plastic- or collagen-coated dishes, in serum-supplemented culture medium, a coherent monolayer is obtained within 1–3 days (Prunieras et al., 1980; Liu and Karasek, 1978). Although high-density dispersed cultures can be subcultured for as many as four passages, the main problem is poor growth, thus requiring large amounts of starting material.

Attempts have been made to increase the proportion of dividing cells in the initial cell suspensions, e.g., enrichment of cell suspension in basal cells by density gradients, cytoflow fluorometry, or, most efficiently, panning on collagen (Skerrow and Skerrow, 1983). Because some differentiation takes place in these cultures when they start stratifying, it is possible to investigate epidermal cell desquamation in vitro and the effects of chemicals on synthesis of epidermal differentiation markers (fig. 7). In addition, keratinocytes grown (third passage) in delipidised serum, i.e., in the absence of vitamin A, were shown to synthesise high molecular weight keratin polypeptides typical of differentiated epidermal cells that were not observed in total serum containing vitamin A (Fuchs and Green, 1981). Using primary cultures of human keratinocytes, Regnier et al. (1989) showed that, similar to vitamin A (Fuchs and Green, 1981), synthetic vitamin A analogues (retinoids) also promote, in a dose-dependent fashion, the synthesis of a simple epithelial keratin K19 (40 kD) which is normally absent in stratified epithelia. Furthermore, in this culture system, retinoids inhibited the synthesis of the cross-linked envelope, a characteristic process of keratinocyte differentiation to mature corneocytes of the stratum corneum. Inhibition of cross-linked envelope formation was clearly more sensitive to retinoids than changes in keratin synthesis and was claimed to represent an accurate, reproducible, and highly sensitive bioassay for retinoid activity. The synthesis of intercellular lipids (acylceramide, cholesterol sulfate) which accompanies terminal differentiation of keratinocytes is also controlled by retinoids (Brod et al., 1989a). When keratinocytes are cultivated on plastic at high density and immersed in medium supplemented with delipidised serum, the synthesis of these lipids normally produced during epidermal differentiation is inhibited by retinoids at concentrations that also inhibit envelope formation. Therefore, lipids can be taken as markers for in vitro assays of the activity of retinoids (Brod et al., 1989b).

2. Low-density dispersed cultures. Low-density dispersed cultures of keratinocytes have also been developed. Cell suspensions are plated at low density (10^2-10^4 cells/cm^2), in the presence or in the absence of a feeder layer and in the presence of growth factors, e.g., epidermal growth factor, hydrocortisone, cholera toxin. The most efficient culture method was developed by Rheinwald and Green (1975). With this method, keratinocytes are plated on a feeder layer of growth-arrested murine 3T3 fibroblasts in the presence of the above mentioned growth factors. A major advantage of low-density dispersed cultures is high cell yield. In addition, after one or two subcultures, keratinocytes can be maintained for 1 or 2 weeks in chemically defined media, thus making such cultures an appropriate tool for in vitro pharmacological investigations (fig. 7). Because of its immortilised nature, the SV40-transformed line SV-K14 (Taylor-Papadimitriou et al., 1982) has proved to be an interesting additional and convenient tool for the screening of antiproliferative drugs.

3. Low-density dispersed cultures: pharmacological studies. Using these SV-K14 cells, Reichert et al. (1985) made a detailed investigation of the molecular mode of action of the antipsoriatic drug anthralin and related compounds. The cytotoxic potential was evaluated by determining cell detachment (protein determination); DNA synthesis was readily assessed by [^{3}H]thymidine incorporation. Like normal epidermal cells, SV-K14 cells have the peculiarity that glutamine constitutes a major respiratory energy source, whereas glucose is essentially oxidised via the pentose shunt. Thus, cellular respiration and the functionality of the cytosolic pentose phosphate pathway could be simultaneously investigated by the comparative measurements of [^{14}C]CO_2 release from

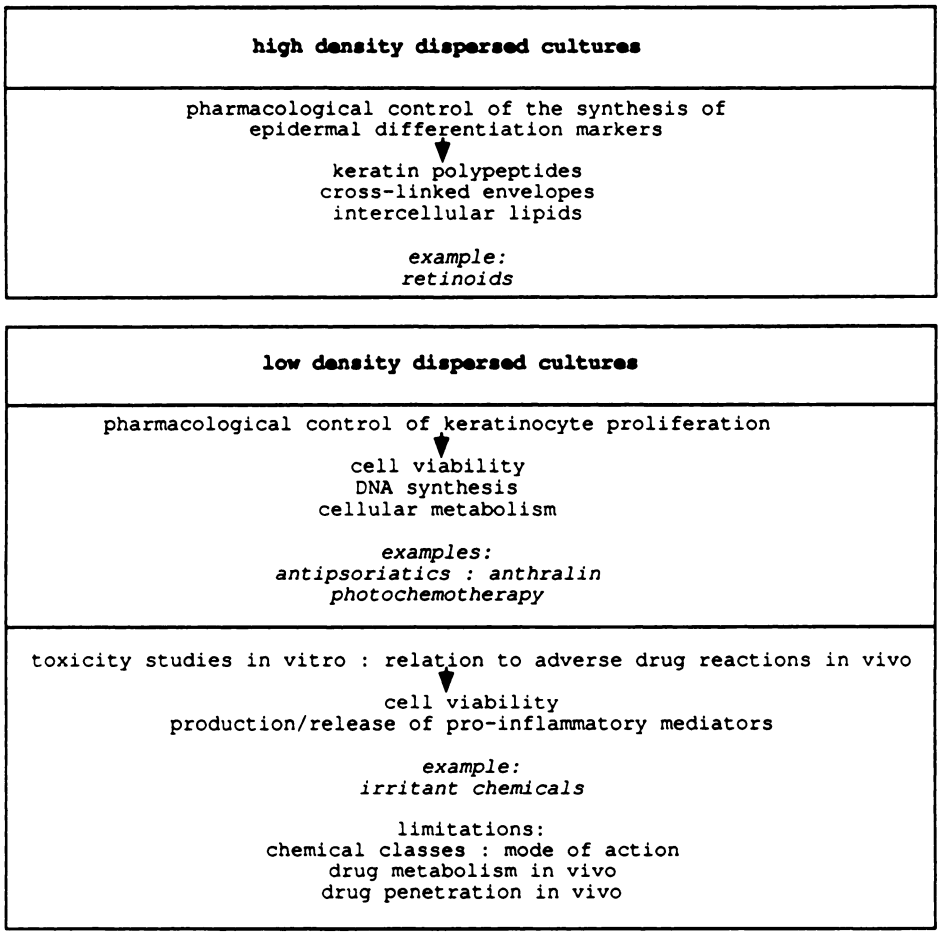

FIG. 7. Keratinocyte cultures.

[^{14}C]glutamine and [^{14}C]glucose, respectively. A comparison of dose-response curves obtained with these various parameters indicated that respiration was the most sensitive target to anthralin treatment and was depressed at concentrations that are likely to be encountered in the skin (Kammerau et al., 1975). As a consequence of diminished energy supply, energy-dependent biosynthetic processes, including DNA replication, were slowed down. Hence, the antirespiratory effect of anthralin appeared to be the main reason for its antiproliferative and antipsoriatic action. Because cell death in vitro may be associated with toxic effects, i.e., irritation in the skin in vivo (see below), the ratio between ID_{50} values for cytotoxicity and inhibition of respiration could be used as an in vitro guideline for a "risk to benefit ratio." Based on these data a drug discovery programme was undertaken in our institute, in which anthralin structural analogues were submitted to a primary screening using the above described assay on SV-K14 keratinocytes. The same system was also successfully used to assess the molecular basis for the antiproliferative action of photochemotherapy (psoralen + UVA, "PUVA treatment") in psoriasis (reviewed by Reichert, 1986).

4. Low-density dispersed cultures: toxicological investigations. Several attempts are currently being made to use conventional keratinocyte (and other cell types) cultures as in vitro models for the prediction of adverse drug reactions in the skin, e.g., mutagenicity, metabolism-mediated polycyclic hydrocarbon toxicity, or irritation. The major goals of such studies are to develop primary screening in vitro tests to identify potential toxicants and rank order toxicities and to gain knowledge about the mechanisms of action of toxicants in terms of direct actions on skin cells. Local irritation is responsible for the bulk of the problems encountered with the use of topical drugs. Hence, major efforts in this field are directed toward the establishment of in vitro methods for the detection of potential skin irritants. Using SV-K14 keratinocytes, we addressed the following questions (fig. 7) (D. Cavey and U. Reichert, unpublished): (*a*) What parameters of cellular activity should be investigated in vitro, the modulation of which is best correlated with the production of skin irritation in vivo? (*b*) Because irritation is usually assessed on the basis of an inflammatory reaction (erythema), does the study of chemically induced production of proinflammatory mediators by cultured keratinocytes contribute to our understanding of the processes? (*c*) Considering that topical drugs are most frequently administered in a repetitive fashion, is it worth investigating drug action in vitro using repeated

incubations? (*d*) What are the limits of such in vitro assays when different chemical classes of products are considered?

To answer these questions, we selected representative molecules with a known profile of skin irritation: irritant chemicals such as sodium lauryl sulfate or phorbol myristate acetate, nonirritant chemicals such as indomethacin, and drugs of current use in dermatology: anthralin (psoriasis), all-*trans*-retinoic acid (acne), along with several new synthetic retinoid analogues. Our major conclusions were that, in answer to question *a*, more accurate prediction of skin irritation is achieved when at least two parameters of cellular activity are assessed in parallel, e.g., cytotoxicity and release of inflammation mediators (arachidonic acid and metabolites). The same conclusion was reached by Duffy et al. (1986), who developed a two-component in vitro system combining a cytotoxicity component using 3T3 mouse fibroblasts and an in vitro model of a keratinising and stratifying epithelium (XB-2 cell line).

In answer to question *b*, along with improving the accuracy of the in vitro model, the study of the production and/or release of inflammation mediators may help elucidate the mechanism of action of primary irritants. The cell membrane may be a site of interaction for many cutaneous irritants. De Leo et al. (1987, 1989) demonstrated that, after prelabeling with [^{3}H]arachidonic acid, the treatment of murine fibroblasts and human keratinocytes in culture with surfactants and marketed surfactant mixtures results in a dose-related release of tritium in the culture medium. High-performance liquid chromatography analysis of the media revealed the presence of arachidonic acid along with cyclooxygenase and lipoxygenase arachidonate metabolites. The in vitro system rank ordered the compounds in a highly predictive fashion when compared with in vivo data concerning animal skin. Other classes of irritant molecules have been found to produce membrane alterations resulting in the release of fatty acid groups from membrane phospholipids and subsequent metabolism. For example, phorbol ester tumor promoters induce inflammation through mechanisms that include hydrolysis of arachidonic acid and the production of prostaglandins and other arachidonate metabolites in intact skin and cells in culture (Weinstein and Lee, 1979). We also found that anthralin stimulates the release of tritium from [^{3}H] arachidonate-prelabeled SV-K14 keratinocytes.

In answer to question *c*, repetitive exposure of SV-K14 cells to the test compounds was not associated with any marked change in their toxic action on SV-K14 cells as compared to single applications. In addition, this protocol is time-consuming and, therefore, does not offer any real advantage over traditional single exposures.

In answer to question *d*, insofar as different classes of molecules are concerned, it was striking to see in our system that irrelevant in vitro data were obtained when retinoids were considered (poor cytotoxicity of strong irritants), thus indicating a mechanism of action different from a direct effect on keratinocyte viability and proliferation. In contrast, other classes of molecules were accurately detected. Previous work with anthralin and 40 structural analogues of anthralin (Reichert, 1986) had shown that reasonable correlation between in vivo (primary skin irritation in the rabbit) and in vitro data (cytotoxicity on keratinocyte and fibroblast cultures) could be observed, 10% false-negative and 15% false-positive results being obtained in vitro. As previously mentioned, surfactants also can be accurately studied in culture.

Altogether, these data suggest that the relevance of in vitro data in culture to adverse effects in the skin largely depends on the target cell and the mechanism of action of the test compounds. Cytotoxicity assays using cultured fibroblasts or keratinocytes may yield relevant information if irritation in the skin is a direct result of the cytotoxicity of compounds on skin cells. Even in this case, other factors may affect the validity of these in vitro systems. The drug must not be metabolised in vivo or at least must undergo the same conversion in vitro and in vivo. The ability of drugs to penetrate the permeability barrier of the skin may be largely responsible for discrepancies, e.g., a chemical may be toxic to isolated cells but will not be an irritant to intact skin simply because it will not have appreciable tissue penetration. Conversely, barrier function integrity may be altered by drugs, especially corrosive chemicals, which results in skin pharmacokinetics being disturbed and eventually higher sensitivity of the tissue to these drugs.

Thus, in vitro methods using cultures of keratinocytes or fibroblasts can be used for primary screening tests of toxicity for defined chemical classes but cannot be considered so far as "alternative" methods that would fully replace the in vivo assessment.

C. In Vitro Models for the Evaluation of Anti-Inflammatory Drug Action

The search for new anti-inflammatory drugs should be based on our knowledge of the processes of inflammation including the mediators and cellular reactions/interactions involved. In vitro evaluation of new drugs may serve as an initial screening step, in which the presumed action on a particular aspect of an inflammatory process can be investigated in a relatively simple environment. However, in view of the extreme complexity of inflammatory processes in vivo, the ultimate demonstration of a beneficial pharmacological activity necessarily implies that the drug be evaluated with in vivo models.

In vitro models used for the evaluation of anti-inflammatory drug action in the skin have been reviewed in detail (Cavey, 1989). It should be stressed that none of the existing in vitro models has been specifically designed for the study of dermatological drugs. Thus far, much attention has been paid to lipid mediator production by

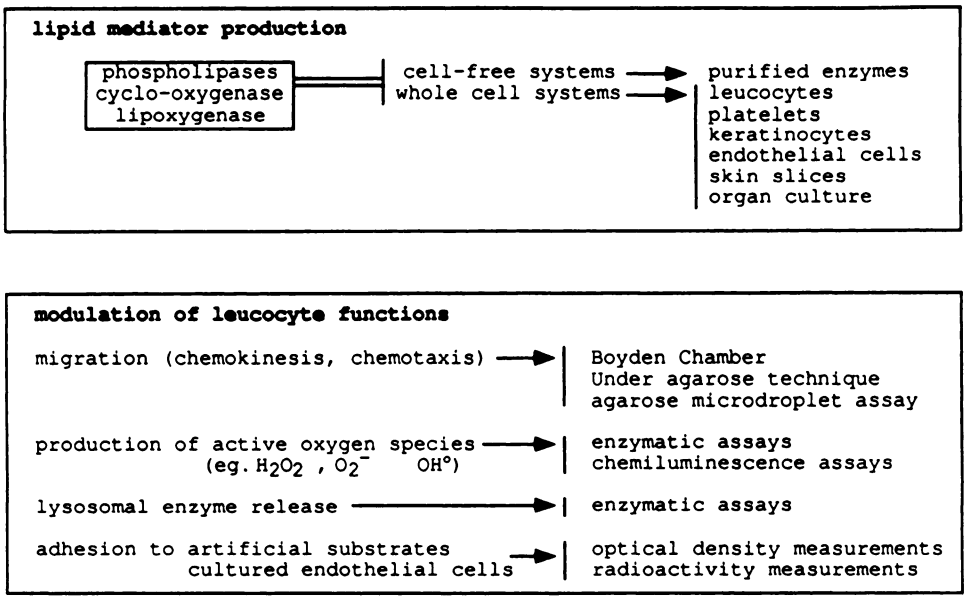

FIG. 8. Some in vitro models for the evaluation of anti-inflammatory drug action.

inflammatory and other cells and to the modulation of leucocyte activities (fig. 8).

1. Lipid mediator production. The release of lipid mediators such as either the cyclooxygenase or lipoxygenase products of arachidonic acid requires a prior liberation of arachidonic acid from cellular lipids. Therefore, pharmacological modulation of mediator release is possible at the level of either of two main steps: an early stage, i.e., arachidonic acid release, or a secondary stage, i.e., formation of pharmacologically active metabolites. These steps can be measured in vitro using either cell-free (e.g., purified enzymes) or whole cell systems (e.g., intact leucocytes, platelets, keratinocytes, endothelial cells) (Ahnfelt-Ronne and Arrigoni-Martelli, 1984; Gryglewski, 1979; Higgs et al., 1985).

Regarding arachidonate release itself, it is striking to realise that, with the exception of glucocorticoids, it is difficult to correlate inhibitory effects on phospholipases and anti-inflammatory properties in the skin (Cavey, 1989). On the other hand, although anti-inflammatory effects of glucocorticoids are not entirely dependent on the induction of lipocortins and subsequent inhibition of phospholipase activity, in vitro models for such cutaneous effects of glucocorticoids may yield relevant data.

For example, using cultures of psoriatic skin slices, Ilcyhshyn et al. (1986) investigated phospholipase activity by measuring the rate of release of ^{14}C-fatty acids from phospholipids and found this in vitro system to rank-order topical glucocorticoids in a manner similar to that observed in vivo. Indeed, there is a specificity of glucocorticoid action, which is largely dependent on the early step of drug-target cell interaction, the binding to specific receptors. In vitro studies performed by Ponec et al. (1981) demonstrated that the binding affinities of a series of corticosteroids for their cytosolic receptor in cultured keratinocytes show a rather good degree of correlation with their known clinical efficacy as anti-inflammatory agents in the skin.

The formation of arachidonate metabolites has also been found to be impaired by corticosteroid treatment of human skin organ culture and cultured human epidermal keratinocytes and dermal fibroblasts (Bloom et al., 1989). However, when nonsteroidal drugs are considered, there is no straightforward relationship between their ability to interfere with arachidonic acid metabolism (cyclooxygenase and/or lipoxygenase inhibition) and their efficacy against skin inflammation. The well-known lack of therapeutic efficacy of nonsteroidal, aspirin-like drugs against inflammatory dermatoses illustrates this point. The idea that benoxaprofen might work against psoriasis through a dual cyclooxygenase and lipoxygenase inhibition has been questioned (Salmon et al., 1984).

It is apparent that an inhibition of lipid mediator production could participate in the mode of action of dermatological drugs, but it is certainly not sufficient to explain entirely their therapeutic efficacy. For a better understanding of this mode of action, a number of other functions and processes known or presumed to be of importance have to be investigated.

2. The modulation of leucocyte functions. This in vitro model has emerged essentially from the study of the mode of action of NSAIDs. The basis for this model is the fact that high doses of NSAIDs exert a dose-dependent inhibition of leucocyte activation (Kaplan et al., 1984). As previously mentioned, it is well recognised that most of these drugs are not of particular help in the treatment of inflammatory skin reactions. Nevertheless, the proinflammatory potential of leucocyte activities in a number of skin diseases (Cavey, 1989) would suggest that such investigations might provide skin pharmacol-

ogists with valuable information as to part of the mechanisms involved in the anti-inflammatory action of these drugs.

The migration in vitro of PMNs or monocytes can be conveniently studied by the Boyden chamber technique, the under agarose technique, or the agarose microdroplet assay (Boyden, 1962; Cutler, 1974; Cunningham and Camp, 1987). A number of drugs have been shown to inhibit leucocyte migration in vitro, including NSAIDs, corticosteroids, retinoids, and other drugs of dermatological interest (table 8). It has to be recognised that data interpretation and extrapolation to an in vivo situation should be made with caution. Results can be largely influenced by technical factors and the pathophysiological state of the cells. Cell migration in vivo does not simply reflect cellular motility but also involves the local generation of chemoattractants, e.g., arachidonate lipoxygenase products, the adhesion to vascular endothelium and passage through basement membranes, and the disruption of dermal or epidermal barriers. These features also are amenable to in vitro investigations.

The production and/or release of oxygen metabolites and lysosomal hydrolases and other enzymes from activated leucocytes may participate in inflammatory cell emigration and tissue invasion and in the establishment of chronic destructive lesions. These activities can be monitored using standardised techniques, such as enzymatic activity measurements or chemiluminescence assays (reviewed by Cavey, 1989). Here again, a number of drugs can modulate these functions (table 8). Leucocyte-endothelial cell interactions can be assessed in vitro with the aid of primary or low passage cultures of blood vessel-derived endothelial cells (see references paper by Cavey, 1989).

Since leucocytes sticking to blood vessels and emigration in vivo certainly involve "facilitatory" or "priming" mechanisms (Movat, 1985) acting on endothelial cells and/or the leucocytes to localize and amplify responses, it may be more pertinent to assess drug action under such circumstances. Lipid mediators such as LTB_4 or platelet-activating factor and cytokines such as IL-1 may participate in these amplification mechanisms. Another potential target for anti-inflammatory therapy may be the production of these factors by inflammatory cells, e.g., macrophages, but also by epidermal cells (see next section).

From studies performed on these various leucocyte activities, part of an "in vitro profile of anti-inflammatory activity" can be defined, which should necessarily better account for the in vivo properties of drugs than can single parameter assays (table 8). A critical issue is that there are no striking differences in vitro between dermatologically inactive agents (e.g., aspirin-like NSAIDs) and potent drugs. This means that the modulation of the cellular activities investigated may be part of the mode of action of active compounds but is certainly not crucial. This also implies that existing tests are not predictive for the situation in the skin and that there is a need for improving these tests and developing more specific models.

Those aspects of cellular activity that may play a pivotal role in the establishment of inflammatory lesions should be recognised, particularly in the case of chronic lesions which represent a major problem associated with inflammatory dermatoses. Our understanding of the mechanisms, cells, and mediators involved in the transformation of an acute response to a chronic lesion should be partly based on in vitro models using not only leucocytes but also, as detailed below, endothelial cells, epidermal cells, and lymphocytes, and the study of drug influence on these various cell types and their interactions.

D. Mixed Cultures and New Models for Investigating the Pharmacology of Immune Responses in the Skin

Immunological processes are known to be involved in about 40%, and possibly more, of skin diseases. There is compelling evidence that the skin is a peripheral lymphoid organ, which contains a number of the cellular constituents involved in the initiation, modulation, and elicitation of immune responses. These include Langerhans' cells and epidermotropic T-lymphocyte subpopulations, keratinocytes, vascular high endothelial cells, mast cells, tissue macrophages, and afferent lymphatic endothelial

TABLE 8

*Pharmacological control of PMN functions in vitro and clinical efficacy in inflammatory dermatoses**

Drug	5-Lipoxygenase activity	Chemotaxis to f-Met-leu-Phe	Lysozyme release	Oxidant production	Clinical use in dermatology
Indomethacin	0	+	+	+	None
Phenylbutazone	0	+	+	+	None
Benoxaprofen	+	+	0	+	Psoriasis (abandoned), + ANIF
Hydrocortisone	0	+	0	0	Inflammation, + ANIF
All-*trans* retinoic acid	±	+	0	+	Acne, ? ANIF
Etretinate	0	+		0	Psoriasis, + ANIF
Anthralin	0 or ±	+	0 or stimulates	+	Psoriasis, ? ANIF

* For experimental details, see paper by Cavey, 1989. Symbols: 0, inactive drug; ±, slight inhibitor; +, good inhibitor. ANIF, anti-inflammatory activity.

cells, which altogether form the "skin immune system" (reviewed by Bos and Kapsenberg, 1986).

Lymphocyte activation is usually an indicator of an immunological component of an ongoing reaction. Observations also exist implicating lymphocytes in the development of acute, nonimmune inflammatory reactions (Sannomiya et al., 1985). Hence, there is increasing interest in the modulation of lymphocyte functions by pharmacological agents. It has been proposed that corticosteroids exert part of their actions through immunomodulatory mechanisms, and it is more and more evident that the same holds true for retinoids and possibly NSAIDs.

1. The modulation of lymphocyte functions. A detailed and complete description of the assays used for the in vitro assessment of immunomodulation can be found in specialised text books (e.g., Urbianak et al., 1978; Di Sabato et al., 1984; Di Sabato et al., 1985). The functional assessment of T-lymphocytes in vitro is usually based on the ability of activated T-cells to proliferate (lymphocyte transformation) and release mediators (e.g., macrophage migration inhibitory factor, INF-γ, IL-2, colony-stimulating factors, B-cell growth factors, etc.).

Anti-inflammatory steroids suppress both immune and nonimmune inflammatory reactions. In vitro, the stimulation of lymphocyte proliferation by phytohaemagglutinin was shown to be depressed by pharmacological concentrations of hydrocortisone and dexamethasone (Mullink et al., 1980). The effect was independent of macrophage concentration, thus suggesting a direct action on lymphocytes. Glucocorticoids also cause an inhibition of IL-2-induced proliferation of IL-2 responder T-lymphocytes (Lewis and Barrett, 1987) and inhibit the production of IL-2 by mitogen-activated lymphocytes (Larsson, 1980).

The idea that aspirin-like NSAIDs might display immunomodulatory effects was proposed on the basis that arachidonic acid metabolites, essentially prostaglandin E_2, might regulate, as a physiological feedback inhibitor, cellular immune responses as well as monocyte and natural killer cell functions. Mullink et al. (1980) studied the effect of varying concentrations of cyclooxygenase inhibitors on the proliferative response to phytohaemagglutinin of guinea pig lymphoid cell cultures, consisting of 60–65% T-lymphocytes, with 20–25% B-lymphocytes and varying proportions of macrophages. A biphasic regulation of lymphocyte proliferation was observed, with a reduction at high, nonpharmacological and presumably cytotoxic concentrations (0.1–1 mM); in contrast, lower concentrations, in the range found in the skin, were associated with an increased proliferative response, the amplitude of which was positively related to the percentage of macrophages present in the cultures. These results were interpreted in terms of inhibition of prostaglandin synthesis by the macrophages. Although extrapolation of these in vitro findings to in vivo may be questionable, it is noteworthy that cellular immune responses, such as delayed skin reactions of guinea pigs to antigens, were found to be amplified by oral administration of indomethacin (Muscoplat et al., 1978).

Several lines of evidence suggest that lymphocyte activation is also regulated by other arachidonic acid metabolites such as thromboxanes and leukotrienes (Kelly et al., 1979; Webb et al., 1982) and 15-hydroxyeicosatetraenoic acid (Bailey et al., 1982). However, the reported effects of anti-inflammatory drugs acting at these various levels of arachidonate metabolism are often contradictory (Cavey, 1989). The same holds true for retinoids. There is a link between retinoids and the immune system: antineoplastic effects of retinoids on cutaneous T-cell lymphoma might involve immune mechanisms (Claudy et al., 1983); retinoids also affect inflammation in chronic dermatological disorders in humans (Bollag, 1983); all-*trans*-retinoic acid and etretinate have been shown to inhibit the delayed hypersensitivity response to methylated bovine serum albumin in the mouse (Ney et al., 1987). In vitro, depending on experimental conditions, an enhancement or, on the contrary, a depression of immune functions have been observed (reviewed in Shapiro and Edelson, 1985).

Because lymphocytes represent a heterogeneous group of cells involved in numerous different functions, a heterogeneity in the influence of drugs is plausible. In addition, pharmacological modulation of accessory, antigen-presenting cell functions (e.g., macrophages, Langerhans' cells) needs to be taken into account. The recognition that lymphocyte and macrophage/Langerhans' cell activities are strongly interdependent, along with the knowledge that such immunologically active cells are normally present in the skin, has led to the establishment of mixed cultures of epidermal cells and lymphocytes, which allow the investigation of lymphoepidermal interactions in a so-called "mixed skin cell-lymphocyte culture reaction" (MSLR).

2. The mixed skin cell-lymphocyte reaction. Lymphoepidermal interactions are operating in a wide variety of dermatological diseases, typically exemplified by allergic contact dermatitis (a special form of delayed-type hypersensitivity), which is quite common and sometimes represents a harmful pathologic condition. The in vitro approach of lymphoepidermal interactions has been provided by in vitro coculture of dissociated epidermal cells with peripheral blood lymphocytes (reviewed by Czernielewski, 1985) (fig. 9).

Human peripheral blood lymphocytes may proliferate upon stimulation with allogeneic as well as autologous epidermal cells, a maximal response being obtained with allogeneic epidermal cells, a peripheral blood lymphocytes to epidermal cells ratio of 1:1, and on days 5–6 of coculture. In this model, allogeneic peripheral blood lymphocytes responses, as well as antigen-specific induced T-cell proliferation, are due to epidermal Ia^+ or HLA-

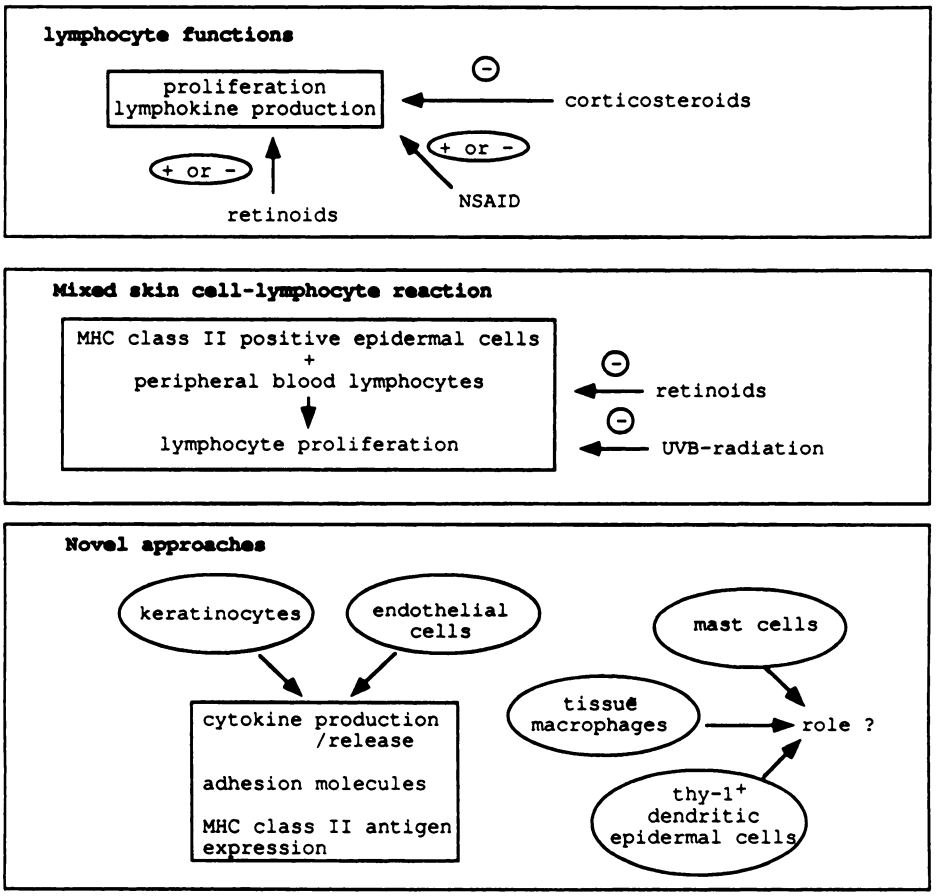

FIG. 9. In vitro models for investigating the pharmacology of immune responses.

DR^+ (class II MHC antigens) Langerhans' cells (Stingl et al., 1978; Czernielewski et al., 1983). Using monoclonal antibodies for total T-cells and helper and suppressor T-cell subsets, Czernielewski et al. (1984) demonstrated that helper T-cells play a major role in allogeneic MSLR.

This MSLR reaction has been shown to be a predictive model in graft-versus-host disease, a pathologic condition that involves epidermal cell-lymphocyte interactions (Bagot et al., 1986). The effect of retinoids on these interactions has recently been investigated (Dupuy et al., 1989). The synthetic retinoids isotretinoin, etretinate, acitretin (the main metabolite of etretinate), and arotinoid-free acid were shown to reduce lymphocyte proliferation in the MSLR, whereas vitamin A (retinol) was inactive. The inhibitory effect was primarily directed toward epidermal cells; however, the precise mechanism of action still remains to be elucidated. It also remains to be established to what extent the modulation by retinoids of lymphocyte activation in this in vitro model reflects the situation in vivo.

The MSLR system has also been used to elucidate part of the mechanisms involved in UVB-induced alterations of normal immunological processes in the skin. For example, a low UVB dose administered to mouse skin causes a loss of induction of contact hypersensitivity; antigen-specific unresponsiveness ensues, instead of sensitization (Toews et al., 1980), a process that may be associated with the generation of suppressor T-lymphocytes (Elmets et al., 1983). Defective antigen presentation by UVB-radiated Langerhans' cells may participate in these phenomena. When epidermal cell suspensions are exposed to UV light, in subsequent cocultures with allogeneic peripheral blood lymphocytes, a markedly decreased or even suppressed stimulatory response is observed (Czernielewski, 1985). In these experiments, Langerhans' cells retain their surface markers (OKT6, HLA-DR), thus suggesting that the defect induced by irradiation is essentially functional and may result from a transient inhibition of antigen-processing functions of Langerhans' cells. However, other mechanisms are operating in UV light-induced immunological disturbances in the skin. In particular, keratinocytes might play an important role (Elmets et al., 1983).

3. Perspectives. There is accumulating evidence that the epidermis contains cellular elements other than Langerhans' cells, especially keratinocytes, which have the capacity to modulate immune responses. Keratinocytes produce a plethora of cytokines which may be operating in both nonimmune and immunologically mediated skin reactions, e.g., ETAF [interleukin (IL) 1] (Luger et al., 1981), an IL-3-like mast cell-activating factor (Luger et al., 1985), granulocyte-macrophage colony-stimulating

factor (Chodakewitz et al., 1988), an inhibitor of lymphocyte proliferation (KLIF) (Brian et al., 1985), a lymphocyte-differentiating factor (Nicolas et al., 1987), a leucocyte-stimulating activity (Danner et al., 1987), IL-6 (Kupper et al., 1988), and a neutrophil-activating protein also chemotactic for T-lymphocytes (IL-8) (Larsen et al., 1989).

Recently, the maturation of murine epidermal Langerhans' cells into potent immunostimulatory dentritic cells in vitro was shown to be mediated by the combined action of IL-1 and granulocyte-macrophage colony-stimulating factor (Heufler et al., 1988). Whether a similar maturation can be observed with human Langerhans' cells remains to be demonstrated. It was hypothesised that Langerhans' cells in the skin are immunologically immature cells which, in response to epidermis-derived cytokines produced by local injury or antigen deposition, become highly reactive immunocompetent cells. Keratinocyte-derived factors may also regulate lymphocyte functions. The influence of arachidonic acid metabolites has previously been described.

In normal human skin, the reported absence of B-cells and the lack of evidence for keratinocyte-T cell interactions (Bos et al., 1987) would argue against an active participation of keratinocytes in lymphocyte "homing" in the skin and the hypothesis that lymphocytes continuously mature in a close spatial relationship with keratinocytes. However, in various lymphocyte-mediated diseases, T-lymphocytes show a specific affinity for the epidermis (epidermotropism) (Streilein, 1983). Recent in vitro data obtained with murine cloned T-cells demonstrated that certain class II antigen-restricted $L3T4^+$ autoreactive and alloreactive clones (which have been found to be epidermotropic in vivo) show specific directional migration to epidermal fragments and to the keratinocyte cell line PAM-212, whereas nonepidermotropic clones and freshly isolated lymph node T-cells do not (Shiohara et al., 1989).

Evidence was given to indicate that the responder T-cells were not specifically attracted to a site of antigen deposition but were more probably chemotactically stimulated by keratinocyte-derived growth factors, the nature of which remains to be clarified (IL-1? granulocyte-macrophage colony-stimulating factor? IL-8? other factors?). Hence, the production and release of cytokines by keratinocytes might be a target for pharmacologically active compounds (fig. 9).

T-cell infiltration of diseased epidermis also closely correlates with ICAM-1 expression by keratinocytes (Nickoloff, 1988). ICAM-1 is a surface glycoprotein of the immunoglobulin family, present on a variety of different cell types, which is a natural ligand for the lymphocyte function-associated antigen 1, a member of the integrin family of molecules (Marlin and Springer, 1987). Although ICAM-1 is not expressed on normal epidermis, the cell surface expression of this molecule has been found to be up-regulated at the site of lymphoid infiltration in a variety of inflammatory dermatoses including atopic dermatitis, allergic contact dermatitis, spongiotic dermatitis, lichen simplex chronicus, psoriasis, graft-versus-host disease, and cutaneous T-cell lymphoma (Singer et al, 1989). Vascular endothelium was also strongly reactive with antibody to ICAM-1. The importance of ICAM-1 in the pathophysiology of skin diseases has been reviewed by Kupper (1989).

Along with a role of ICAM-1 in T-cell infiltration of the epidermis through nonantigen mechanisms involving microvascular endothelial cells in the dermis and keratinocytes in the epidermis, adhesion molecules may also be essential for helper T-cell activation by antigen-presenting cells and cytotoxic T-cell interaction with target cells (cell-cell contact). Emphasis is also given to the fact that cytokines and adhesion molecules are related. It is speculated (Kupper, 1989) that "chronic inflammatory skin diseases like psoriasis or lichen planus involve a biochemical 'dialogue' between T-cells and keratinocytes or other resident skin cells," where "cytokines comprise the vocabulary." Under these conditions, one has to expect that efficient therapies might interrupt these interactions between lymphocytes and skin cells, either by a regulation of adhesion molecule expression or by a regulation of cytokines or their receptors (fig. 9).

Indeed, as previously reported, keratinocyte- (and other resident skin cells) derived cytokines can modulate lymphocyte functions. Conversely, the activation of lymphocytes results in the production of factors that influence skin cells. In particular, IFN-γ has been found to up-regulate keratinocyte ICAM-1 expression (Dustin et al., 1986; Barker et al., 1989). This IFN-γ-induced ICAM-1 expression was found to be unchanged after corticosteroid treatment (prednisolone, dexamethasone, 10^{-9}-10^{-5} M) of cultured human keratinocytes (Sawami et al., 1989). In the same studies, the retinoid etretin (10^{-9}-10^{-5} M) did not induce ICAM-1 expression but augmented the intensity and percentage of ICAM-1-positive cells induced by IFN-γ. This effect was dependent on the state of differentiation of the keratinocytes, because undifferentiated cells grown in low calcium medium had higher levels of IFN-γ-induced ICAM-1 which were not enhanced by etretin. The discovery of new compounds inhibiting adhesion molecule expression would be of great interest.

In keeping with the beneficial effects of psoralen + UVA photochemotherapy against certain dermatoses characterised by an epidermal T-cell infiltration is the finding that, in vitro, psoralen + UVA treatment reduces T-cell lymphocyte function-associated antigen 1 expression and the ability of these cells to bind to ICAM-1 (Mizutani et al., 1989). The expression of ICAM-1 in skin biopsies from patients with psoriasis has been studied before and during the course of psoralen + UVA treatment (Lisby et al., 1989). During the treatment, a

marked decrease of keratinocyte ICAM-1 staining paralleled the clinical improvement and a reduced infiltration of mononuclear cells.

The infiltration of activated T-lymphocytes into the skin is also associated with the expression of MHC class II molecules (e.g., HLA-DR, Ia) by keratinocytes (Volc-Platzer et al., 1984). Recombinant IFN-γ has been shown to induce HLA-DR expression on cultured human keratinocytes (Basham et al., 1984). The functional role of this expression has not been fully elucidated as yet. Although MHC class II-positive keratinocytes are unable to provoke primary antigen specific T-cell responses, they may participate in the amplification of previously sensitised T-cell responses (Tjernlund and Scheynius, 1987). They also may serve as targets for MHC class II-restricted cytolytic T-cell clones (Gaspari and Katz, 1988). However, other experimental data would suggest that, in some cases, MHC class II-positive keratinocytes may be involved in the down-regulation of immune responses. The lower peripheral blood lymphocyte responses obtained with IFN-γ-treated keratinocytes in the MSLR (Czernielewski, 1985) would indicate that HLA-DR expression by keratinocytes might provide a down-regulation signal during a primary contact with nonstimulated lymphocytes as in the case of MSLR. Gaspari et al. (1988) found that incubation of trinitrobenzene sulfonic acid-specific T-cells clones with trinitrobenzene sulfonic acid-modified Ia^+ murine keratinocytes, but not Ia^- keratinocytes, results in unresponsiveness to subsequent stimulation with trinitrobenzene sulfonic acid-modified functional accessory cells.

It has also been proposed that keratinocytes produce tolerogenic signals in UVB-radiated skin (Elmets et al., 1983). Hence, keratinocytes might well be able to release up- and down-regulating signals of the immune response. Investigating the pharmacological modulation of the balance between these opposite activities may be relevant to our understanding of the mode of action of existing treatments and to the discovery of new families of active compounds. In vitro (and in vivo) models for the investigation of drug action in the skin should take these aspects into consideration. The functions of cell types other than those previously alluded to also should be considered (fig. 9). For example, endothelial cells, tissue macrophages, and mast cells participate in the skin immune system (Bos and Kapsenberg, 1986). In the mouse, a distinct population of functional T-cells, termed "thy-1^+ dendritic epidermal cells" (Tschachler et al., 1983; Bergstresser et al., 1983), might function as antigen-presenting cells in the activation of suppression. Whether an equivalent to these cells exists in human epidermis is still unknown.

There is no doubt that in vitro models using conventional cultures, mixed cultures, and reconstructed skin equivalents will bring further basic information to our understanding of skin pathophysiology and will provide appropriate tools for a reduction of the use of animal models in skin pharmacology.

IV. Conclusion

The aim of this review paper has been to give an up to date review in which there was sufficient background details for the understanding of the subject but, of course, with sufficient referencing to serve as a guide to additional reading.

Althrough there is no model, either in vivo or in vitro, identical with any of the predominant types of dermatoses, each model offers characteristics that may be more or less advantageous for the study of the underlying pathophysiology of a disease and its drug treatment. In every model, it is clear that many variables are operative that can affect both the resulting pathology and the quality of the results obtained. Caution is needed in controlling these variables particularly because they can avoid false interpretation and wrong decisions. Choosing an appropriate model is, therefore, critical when attempting to demonstrate either efficacy or the mechanism(s) by which any therapeutic effect is observed. Although most experimental models are usable for pathological analyses, the ability to quantitatively assess functional deficits and their amelioration may well be the most significant index of the efficacy of any therapy used for the treatment of skin pathology.

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