

Session 1

INFLAMMATORY MEDIATORS APPLIED TO *IN VITRO* TOXICOLOGY: STUDIES ON MEDIATOR RELEASE AND TWO-CELL SYSTEMS

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Abstract—Release of mediators is a manifestation of tissue stimulation or damage applicable to *in vitro* toxicological investigations. Intercell mediators, or cytokines, are released on tissue stimulation or as a result of mild damage. Intracellular mediators, or enzymes of respiration and metabolism, released from damaged cells, participate in inflammatory change and are the usual mediator determinants in *in vitro* tests. Cells examined for *in vitro* mediator release are mast cells, which are applicable to anaphylactic allergy and acute inflammation, macrophages, which are particularly applicable to toxicity of inhalable dusts, and fibroblasts/fibrocytes, which are widely used as a general model for cytotoxicity. In tests on organs, lungs may be perfused to identify cytokines released by test substances, which may be used to distinguish between effects due to allergy and those resulting from chemically-induced inflammation. Skin, both human and animal, as full depth or keratome slices of epidermis, has an application in the detection of irritant and corrosive substances. Weak irritants are identified by release of enzymes, for example acid phosphatase, neutral protease and lactate dehydrogenase. Strong irritants and corrosive substances inhibit release or destroy the enzymes. There are inconsistencies in the identification of moderate irritants, both chemicals and bacterial toxins, in which the rank order of severity of effect does not correspond to other *in vitro* features or *in vivo* potency. Detection of cytokines (prostaglandins, leukotrienes and interleukin-1) is possible with appropriate conditions of culture, but has been little applied to toxicology. Studies on two-organ/cell systems are briefly reported in which biological mediator release is detectable without specific identification. Cultured skin preparations were treated *in vitro* with irritants. The culture media were transferred to cultures of normal skin (examined for stimulus to DNA synthesis), to fibroblasts (examined for DNA and polysaccharide/heparan sulphate synthesis) and to suspensions of neutrophils in Boyden chambers (for chemotaxis). The media were also examined for histamine (none found), acid protease cathepsins, acid phosphatase and lactate dehydrogenase. As before, weak chemical irritants were detectable by stimulation of normal skin and fibroblasts to DNA synthesis, fibroblasts to increased heparan sulphate-like synthesis, and analytically by release of enzymes. Moderate irritants and *Clostridium perfringens* type C toxins depressed these features. Reference is made to the possibility to identify substances likely to induce progressive pulmonary fibrosis, by transfer to fibrocyte cultures of media from macrophages ingesting the dusts or co-culturing macrophages and fibrocytes, to detect a sustained stimulus to fibrogenesis.

Introduction

Every response of the body to injury is induced, enhanced or diminished by endogenous mediators, of which at least fifty influence inflammatory change. Inflammation refers to the changes that occur in living tissue when it is injured, provided that the injury is not so severe as to immediately kill all the cells. Depending upon the nature, intensity and duration of the damage, several types of cell are mobilized to infiltrate, degenerate or proliferate, or are stimulated to secrete mediators.

The primary event or response of the first exposed, perturbed or damaged cells is the release of one or both of two groups of mediators: (1) the mediators

regulating internal cell function, mainly enzymes of respiration or metabolism; and (2) cytokines, whether preformed or newly generated on stimulation, with intercell stimulating properties. This group comprises the true mediators of inflammation. A third group of mediators will not be considered; these are the enzymes that activate humoral substrates, to form bradykinin and kallikrein, or that activate complement.

The initial harmful stimulus, following mild or moderate irritants applied to skin, may cause little observable damage to the keratinocytes; it is the subsequent inflammatory process, mainly infiltration by leucocytes, that induces much of the tissue damage (Parish 1986a,b). The mediators released on first contact with the irritant attract and activate other cells, for example neutrophils and macrophages, which release further mediators or activate plasma components. Furthermore, some mediators potentiate the activity of others; leukotrienes C4 and D4 enhance smooth-muscle contraction by histamine,

Abbreviations: EGF = epidermal growth factor; ETAF/IL-1 = epidermal cell derived thymocyte activating factor; IL-1 = interleukin-1; LT = leukotriene; PG = prostaglandins; SRS = slow reacting substance; TNF = tumour necrosis factor.

and stimulate formation of prostaglandins. The intricate cascade of mediator activation increases or amplifies the inflammatory event, and an initial mild injury in surface epithelial cells results in infiltration by neutrophils and macrophages, changes in dermal mast cells, vascular endothelium and plasma pharmacology, followed by proliferation of fibroblasts and of contiguous keratinocytes. These changes reflect the sum of the activities of the mediators promoting or inhibiting each feature at the time of observation. Though over fifty mediator cytokines are known there are few that, like histamine, exert a dominant independent effect. The great majority are interdependent in their formation and for their clinical effect, and their identification has not shown one mediator to be wholly responsible for a particular cellular change. Nevertheless, identification and assay of mediators are valuable determinants for *in vitro* toxicological tests for irritants to lung and skin, for phototoxic irritants, and to contribute to studies on allergic responses.

This review of the examination of cell mediators applied to *in vitro* predictive toxicological tests is presented as follows: (1) the sources and activities of the major mediators of inflammation; (2) assays of mediator release as evidence of toxicological change; and (3) the use of two-cell or tissue systems in which mediators from one cell type influence the activities of other cells. A consideration of products of activated lymphocytes or lymphokines is omitted because there is no *in vitro* technique to detect induction of allergy, and assay of lymphokines is rarely applied to toxicological investigations.

Sources and activities of pro-inflammatory mediators with potential as toxicological assay tests

Various cytokine mediators released from one cell type influencing the activity of other cell types have been used as determinants of tissue activation or damage for routine or experimental toxicological tests for irritation or allergic responses (Table 1). Mediators that may prove to have future relevance to *in vitro* tests are tumour necrosis factor (TNF α),

epidermal growth factor (EGF) and fibroblast activating factors.

Assay of mediator release as evidence of toxicological change

Methods to detect the first stage, or primary event, when an irritant chemical damages susceptible cells without immediately destroying them are most applicable to *in vitro* toxicological techniques (Table 2). Changes in mediator function or release reflect different features of cell damage or stimulation, and assays expressed either as units of the amounts released, or as a proportion of the total cell content, indicate the intensity of the change as observed in the particular *in vitro* system, which enables ranking of similar chemicals without, however, direct extrapolation to risk for man.

Table 2 indicates methods to detect the primary irritant event using *in vitro* tests for mediators. The following examples of the application of the assay of mediators in toxicological *in vitro* tests are selected from studies on mast cells, macrophages, fibroblasts and lung and skin tissue.

Mast cells. Connective tissue mast cells as obtained in suspension from the peritoneal cavity of rats and mice, or as spreads of mesentery of guinea-pigs, may be used in tests to represent acute inflammation or anaphylactic-type allergy.

Mast cells of rodents are stable during laboratory manipulation and are easily purified, whereas those of man, guinea-pig and other species, are difficult to separate and become modified in culture media. Mast cells contain many preformed mediators and are the chief source of tissue histamine in man and the guinea-pig, and 5-hydroxytryptamine in addition to the histamine in rat and mouse. Other mediators include prostaglandins D and E, leukotrienes, platelet-activating factor, eosinophil and neutrophil chemotactic factors, in addition to the enzymes chymase, arylsulphatase and β -glucosamidase (see Pepys and Edwards, 1979).

Mast cells undergo characteristic changes when tissue is mildly damaged by irritants, either as a

Table 1. Pro-inflammatory cytokine mediators

Cytokine	Cell origin	Preformed/newly generated	Methods of assay
Histamine (H)	Mast cells, basophils, platelets	Preformed	Bioassay, fluorimetric, radioimmunoassay
Prostaglandins (PG)	Most cells, including mast cells, macrophages and keratinocytes	Generated	Radioimmunoassay
Leukotrienes (LT)	Most cells, including macrophages and keratinocytes	Generated	Bioassay, radioimmunoassay
Platelet-activating factor (PAF)	Activated leucocytes	Generated	Radioimmunoassay
Eosinophil chemotactic factor (ECF-A)	Mast cells, basophils, neutrophils (ECF)	Preformed	Bioassay
Neutrophil chemotactic factor (NCF)	Lung mast cells	Preformed	Bioassay
Interleukin-1 (IL-1)	Most cells, including macrophages and keratinocytes	Generated	Radioimmunoassay
Interferon (IFN)	Most cells, including macrophages and fibroblasts (α and/or β) lymphocytes (λ)	Generated	Bioassay (λ), viral inhibition, radioimmunoassay

*More detailed information is available from Houck (1979), Lee (1982), Parish (1986a), De Maeyer and De Mayer-Guignard (1988) and Camp (1989).

Table 2. Methods to detect the primary irritant event using *in vitro* tests for mediators

Nature of change	Detection
Cell morphology/tissue histology	Morphology and staining properties Histochemistry Enzymes of respiration and metabolism
Release of mediators of intracellular function	
Release of mediators of intercellular activity (cytokines)	
(i) Preformed	e.g. histamine
(ii) Newly generated	e.g. prostaglandins; leukotrienes

result of direct contact with the irritant or when oedema fluid from damaged tissue, almost certainly containing mast-cell active mediators, perfuses the cells. Similar changes occur when the mast cells sensitized by anaphylactic antibody are exposed to antigen.

The stability of the rodent cell and the relatively large amounts of the mediators promote its use for *in vitro* tests, but with limited success despite the large amount of fundamental work on its relevance to inflammation and allergy. In an attempt to use rat peritoneal mast cells to assay the amount of a substance that causes the release of all available histamine (Prottey and Ferguson, 1976) as a prediction of the effect on the rabbit eye, 150 soap shampoos and toilet preparations were examined, but there was no reliable correlation with *in vivo* activity. Rat mast cells were also examined by an *in vitro* cell perfusion technique in which continuous monitoring of changes in cell morphology by video photography concomitant with analysis of the culture media continuously perfusing the cell chamber for release of histamine, β -D-glucuronidase and lactate dehydrogenase provided information on the kinetics of mediator release (Parsons *et al.*, 1982). It even detected the two types of mast cell change, degranulation or granular rupture with biphasic histamine release (Parsons *et al.*, 1986), but it was not a technique applicable to predictive examination of substances, though it proved useful to reduce the number of animals for additional *in vivo* tests when a routine test required further examination for a particular feature.

Macrophages. These are long-lived phagocytic cells, both mobile and tissue-bound (histiocytes) found throughout the body. They are the first cells in the lung to respond to inhalation of dust particles by ingesting them and by synthesis of pro-inflammatory mediators, and they participate in the stimulation to fibrogenesis (discussed under two-cell techniques). This cell has been widely used in many investigations of pulmonary toxicity and in the development of *in vitro* tests. Most studies have been made on primary isolates, cells obtained from the peritoneal cavity of rats and mice. A possible alternative to primary isolates is the laboratory adapted macrophage-like P388DI cell line originally derived from a mouse lymphoid tumour and which developed several properties of macrophages, including phagocytosis, on culture (Koren *et al.*, 1975). When cell morphology and death have been used as determinants of toxicity, P388DI cells have been found not to reflect *in vivo* changes (Pigott and Judge, 1980) or to correlate well with pleural sarcoma induction (Wade *et al.*, 1980). We have used P388DI cells as a routine screen for toxicity of particulates, using lactate dehydrogenase release as evidence of potential for pulmonary irritation. If this enzyme is not released,

the test is repeated using unstimulated mouse primary peritoneal macrophages as a second screen before a decision to do an *in vivo* test.

Macrophages synthesize many mediator cytokines following phagocytosis, or stimulation by mediators from other cells or by humoral substances, for example complement C3 (van Furth, 1975). They are capable of transformation into epithelioid cells, losing phagocytic but much increasing secretory activity. Among the many mediators synthesized are the arachidonic acid derivatives and associated substances, prostaglandins, leukotrienes, interferons, platelet-activating factor and interleukin-1. On appropriate stimulation lysosomal enzymes that degrade tissues, including collagenase, elastase, several cathepsin acid proteases and neutral proteases, may be released (Allison and Davies, 1975). Other enzymes, for example lactate dehydrogenase, β -glucuronidase, β -galactosidase, which normally regulate the internal function of the cells, may be released if harmful or non-digestible substances are ingested, or if the cell is otherwise damaged by a toxic substance, for example surfactant or bacterial toxin. The release of each organelle source of enzyme, for example cytoplasmic or lysosomal, is selective depending upon the stimulus (Badenoch-Jones *et al.*, 1977; Davies, 1980; Davies *et al.*, 1974; Lock *et al.*, 1987; Morgan and Allison, 1980; Schorlemmer *et al.*, 1977). Three critical features of macrophage tests are: (1) that primary cultures obtained by peritoneal wash-out should not be stimulated by paraffin oil glycogen or casein; (2) the duration of exposure in culture, because after 17–24 hr there is non-selective leakage; and (3) the use of serum in the media. Serum protects the macrophages, stabilizes the culture, provides opsonins, and enhances mediator synthesis and release of enzymes from damaged cells. Heat-inactivated, acid-treated foetal calf serum is effective. Release of enzymes is a more objective criterion of macrophage damage than are morphological observations, or loss of the following properties—mobility, chemotaxis, adhesion to glass or phagocytosis. As applicable to other *in vitro* techniques, macrophages exposed to toxic dusts may be examined for release of cytokine intercell mediators, or intracellular enzymes. Assays for cytokines have not been generally applied to toxicology, partly because they may indicate macrophage stimulation rather than damage. Rat alveolar macrophages treated with silica or asbestos *in vitro* were stimulated to release IL-1, and showed a distinct difference between the test and control cultures (Oghiso, 1987; Oghiso and Kubota, 1987).

Most studies on selectivity of enzyme release report assays on two or three lysosomal enzymes, and one from the cytoplasm. The validity of the selection has been tested in one study in which eight enzymes were

Table 3. Release of macrophage enzymes examined after exposure to non-toxic and toxic particulates *in vitro**

Enzyme (source)	Discrimination non-toxic/toxic
Leucine aminopeptidase (membrane)	Poor
Lactate dehydrogenase (cytoplasm)†	Good
Malate dehydrogenase (mitochondria)	Good
Acid protease	Nil
Acid phosphatase‡	Poor
β -Galactosidase	Weak
β -Glucuronidase†	Good
<i>N</i> - β -D-glucosaminidase (lysosomes)	Good

*Based on assays of culture media at intervals up to 17 hr after treatment. †Selected for routine test evidence of toxicity. ‡In other tests by the author release of acid phosphatase was detected.

assayed reflecting damage to cell membrane, cytoplasm, mitochondria and lysosomes. The results of these tests (Table 3) confirmed the selection of lactate dehydrogenase (cytoplasmic damage) and β -glucuronidase (lysosomal release) as determinants of toxicity (Lock *et al.*, 1987).

As reported by several other laboratories, different patterns of enzyme release, depending upon the toxicity of the particulates, were observed from cells whose phagocytic ingestion was confirmed by stained preparations or chemical analysis.

Non-toxic. No significant release of either enzyme. Macrophages may become loaded with particles of titanium dioxide, sodium metaphosphate and dicalcium phosphate with no release of enzymes (Lock *et al.*, 1987), yet indigestible non-toxic latex particles induced release of β -glucosaminidase and β -glucuronidase (Keeling and Henson, 1982) and β -glucuronidase and cathepsin (W. E. Parish, unpublished observations).

Non-toxic and toxic. No release of lactate dehydrogenase but slow release of β -glucuronidase. Examples are chalk, asbestos, yeasts and IgG complexes. This may reflect an overlap between a stimulation to chronic inflammation, and removal of harmless material equally able to stimulate the cells. It is possible that in some instances the ingested substance stimulates synthesis of complement C3, auto-stimulating the cell receptors activating the lysosomes and releasing enzymes.

Toxic. Slow release of 70–80% of both enzymes over 17 hr. An example is crystalline quartz DQ12.

Toxic. Rapid release of 70–80% of both enzymes over 6 hr. An example is amorphous silica dusts.

There are discrepancies in reports of some tests, which may or may not be due to technique. The example of non-toxic substances either releasing no enzyme, or releasing lysosomal enzymes indicative of phagocytosis but not of cell damage, is cited above. Asbestos UICC chrysotile was found to induce selective release of lysosomal enzyme, for example β -glucuronidase, but no lactate dehydrogenase (Davies *et al.*, 1974; Lock *et al.*, 1987), whereas Davies (1980) reported release of both enzymes.

In our laboratory macrophage tests have been used routinely to screen dusts for their potential to damage the lung, and if subsequently tested *in vivo*, a close relation between *in vitro* and *in vivo* results was obtained for a very wide range of substances, silicas, clays, metals, polymers and resins. Though the

macrophage *in vitro* test indicates harmful potential, it cannot differentiate between the changes induced *in vivo*, for example transient acute inflammation, chronic inflammation and progressive fibrosis, or indicate if the changes are persistent or reversible.

Fibroblasts/fibrocytes. These cells maintain the integrity of normal connective tissue, and replace many of its elements in repair after damage, or substitute scar tissue. Though capable of responding to direct exposure to exogenous stimuli, activation *in vivo* is usually indirect through mediators from stimulated epithelial cells, macrophages and platelets. On stimulation fibrocytes secrete: (1) tissue elements and ground substance, for example collagen, elastin (by myofibroblasts), proteoglycan/glycosaminoglycans (hyaluronic acid, heparan sulphate and others) and fibronectin; (2) cytokine mediators, interleukin-1, interferon, colony stimulating factor, transforming growth factor and prostaglandin E₂; and (3) mediator enzymes (on stimulation or damage), for example neutral protease, collagenase and acid phosphatase, which modify surrounding tissues, and the intracellular enzyme, lactate dehydrogenase, which is widely used as a determinant of cytoplasmic damage.

Primary cultures of fibroblasts are prepared without difficulty, and several laboratory-adapted transformed lines are available. They are therefore widely used in toxicological *in vitro* tests, but most techniques rely on features other than cytokine generation or enzyme release as determinants of damage. Scaife (1985) found mouse fibroblasts to be as effective as epithelial Hep2 cells to detect irritation potential of detergents, using alkaline phosphatase as an endpoint. Duffy *et al.* (1986) examined mouse fibroblasts for leakage of hexosaminidase and lactate dehydrogenase. Parish (1985 and 1986b) exposed human primary fibroblast cells in culture as monolayers without and with an agar-keratin overlayer, to irritant chemicals and *Clostridium perfringens* toxins, and assayed the media of surviving cultures for release of acid phosphatase and lactate dehydrogenase correlated with uptake of tritiated thymidine, and release of ⁵¹chromium.

Toxicological *in vitro* tests on the fibroblast as a secretory cell are mainly concerned with pulmonary fibrosis, which is discussed below as a two-cell system.

Mediator release from organs by superfusion of lung and skin

Continuous perfusion of the vascular system of isolated lung with examination of the biological properties of the perfusate was much used to identify the mediators released from anaphylactic or irritated lung and skin, and prepared the way for identification of the leukotrienes comprising slow reacting substance (SRS-A or SRS). The techniques may still be usefully applied to toxicological investigations.

Isolated guinea-pig lung from freshly exsanguinated animals, with a continuous flow of warm Tyrode solution through the cannulated arterial system, and a constant pressure of air applied to the trachea, provides the test system (Brocklehurst, 1960). Tests for the mediators of anaphylactic allergy, irritant chemicals, drugs or finely particulate material, may be made by way of the vascular system

and by analysing the perfusate for its pharmacological agents. Bronchospasm is detectable by obstruction of the air flow. The superfusing fluid was originally examined for its ability to contract or relax a series of different smooth muscle preparations in a cascade (Moncada *et al.*, 1978; Vane 1964). Now it is possible to identify substances in the perfusate by radioimmunoassay.

There have been few applications of this procedure to *in vitro* toxicology, but J. F. Parsons (unpublished observations) applied it to differentiate between the allergic and pharmacological or irritant properties of enzyme dusts of concern in occupational hygiene.

The isolated rabbit ear, cannulated and perfused through the arterial system, was found to be of limited application as an *in vitro* organ procedure. Topical application of mild irritants, or of allergens to sensitized ears, or inducing mild trauma by tape stripping, did not release inflammatory mediators into the perfusate after 2 hr (J. F. Parsons, unpublished observations). Injection of antigen into the perfusate of sensitized ears, or of histamine-releasing compound 48/80, released small amounts of histamine and prostaglandins.

The necessity to kill normal animals to obtain organs for mediator release tests is only a half-way stage to *in vitro* test development. Thus far, superfusion of intact organs remains an effective means to study release of preformed or newly generated exogenous mediator/cytokines, but there is no comparable *in vitro* culture system.

Enzyme release from skin slices as evidence of irritation or corrosive potential

The epidermis *in vitro* synthesizes IL-1, also known as epidermal cell derived thymocyte activating factor or ETAF/IL-1 (Sauder *et al.*, 1982), PGE₂ (Jonsson and Anggard, 1972), LTB₄ (Fincham *et al.*, 1985) and messenger RNA for IL-1 extracted from keratinocytes (Bell *et al.*, 1987), being some of the several leucocyte chemotactic substances from damaged skin. Whole skin containing mast cells also releases histamine.

Skin slices may be cultured for 24 hr in suitable condition for tests to detect irritants; the culture technique determines which mediators can be assayed. There are two main culture methods: (1) supporting keratome slices, or full depth skin, on stainless-steel grids, with or without agarose to anchor them, in open dishes; or (2) sealing the slices in, or on, a clamped tube (Oliver *et al.*, 1986; Parish, 1985). In both methods, the epithelial surface is exposed to air or to the test chemical, and the dermis

is bathed in culture media, which can be withdrawn for analysis for mediators released from the skin. There are advantages and disadvantages for both techniques (Table 4). Several determinants of tissue change have been used to detect evidence of damage, but mediator release has been confined to assay of cell enzymes, and not to the cytokines. Sealing or clamping skin in closed tubes induces synthesis of the cytokines (prostaglandins, leukotriene, interleukin-1, chemotactic factors), which results in a high background in control, untested skin, making it difficult to determine significant responses to irritants. Supporting the skin preparations on steel mesh without trauma avoids excessive cytokine synthesis. Strong irritants and corrosive substances inhibit formation of these cytokines.

Release of cytokines *in vitro* from human epidermis treated with irritants was reported by Bloom *et al.* (1987) and De Leo *et al.* (1987). One irritant stimulated release of PGE₂ ten-fold over background in 24 hr from a human skin organ culture, and a similar increase from cultured keratinocytes in 2–3 hr. No LTB₄ was detected (Bloom *et al.*, 1987). In another approach keratinocytes treated with tritiated arachidonic acid were tested with one of three irritant surfactants, and the media examined for arachidonic acid release and its metabolites, which included prostaglandins. The rank order of effect of the three chemicals was the same as that observed *in vivo* (De Leo *et al.*, 1987).

There have been few studies using release of enzymes from skin as a determinant of potential for irritancy of chemicals. Gibson and Teal (1983) assayed release of acid phosphatase, lactate dehydrogenase and *N*-acetyl glucosaminidase from rat skin slices exposed to two surfactant irritants compared with a non-irritant similar chemical. The two irritant chemicals released more of the three enzymes than did the non-irritant surfactant. However, in further tests (W. T. Gibson, unpublished data, 1983) there was no reliable correlation between irritant potency of surfactants and other chemicals *in vivo* and the release of the three enzymes from skin slices. The technique was unreliable in the detection of irritants or in ranking different intensities of irritation.

In studies by Parish (1985 and 1986b) in which the effect of clostridial toxins was compared with those of irritant and non-irritant chemicals on human and rabbit skin slices *in vitro*, histochemical staining for acid phosphatase and lactate dehydrogenase, and assay of the culture media for release of acid phosphatase, neutral protease and lactate dehydrogenase were two of the several determinants of cell damage investigated. A summary of the results relevant to

Table 4. Advantages and disadvantages of two culture methods for skin full depth or keratome slices to study release of intracellular enzymes or of cytokines

Steel mesh in open dish	Clamped in tube
Preparation easily dislodged	Preparation stable
Difficult to retain test substance on surface	Test substance in any volume stays on surface
Less intracellular enzymes released or detected in media	Cell enzymes detected in greater concentration
Also greater amount of media therefore greater dilution	
Keratome slice preparation initiates synthesis of cytokines. Full depth skin can detect significant changes in PG, LT, IL-1 and NCF between controls and effect of mild irritants	Keratome slices and full depth skin not utilizable. Compression induces constant synthesis of PG, LT, IL-1 and NCF. Therefore high background in non-test control preparations

NCF = neutrophil chemotactic factors

Table 5. Results from 24-hr *in vitro* epidermal keratome preparations of man and rabbit, related to irritation and corrosion *in vivo**

Test substances	Enzyme release	Histology/histochemistry	
	Acid phosphatase Neutral protease Lactate dehydrogenase	Acid phosphatase Lactate dehydrogenase	
		Man	Rabbit
Weak irritants	++	+	0
Moderate irritants			
Alkalis	+	W	W
Acids	++	+	0
Bacterial toxins	+	++	+
Corrosive chemicals	0	0 to ++	0

0 = no change detected W = weak response +, ++ = degrees of enzyme concentrations and severity.

*Abbreviated from Parish (1985b).

mediator release (Table 5) shows that significant amounts of enzymes were released into the culture fluid in the presence of weak irritants, classified according to their response after 4 hr application to rabbit skin. Human skin yielded much better results, with greater release of enzymes, possibly because of the larger number of epidermal cells than in rabbit skin. Similarly, the histochemical staining was more obvious, and in some tests there was a gradation of effect from the stratum granulosum to the basal layer.

Further attempts with T. C. Williams and I. Slack to apply histochemical techniques to detect early irritant changes in keratinocytes of rat and rabbit have confirmed the earlier observations on rabbit and human skin that histochemistry is too imprecise for this purpose. Test substances (1% sodium hydroxide or 3% sodium lauryl sulphate or 100% ethanol) were applied to the clipped skin of rats and rabbits for 4 hr, after which the animals were killed and the skin was examined immediately, or the animals were killed 20 hr later. These skin sites were compared with those of animals killed just before the 4-hr test application, and skin samples taken immediately after 4 hr, or after 20 hr at room temperature. The tests on the skin of dead animals enabled examination of the epidermis unaffected by infiltrating leucocytes also containing enzymes. The skin samples were examined for succinic dehydrogenase, acid phosphatase, glucose- β -phosphatase, lactate dehydrogenase, non-specific esterase and ATPase. No consistent changes were seen that indicated an early response to the mild irritants, or between the 4 and 24 hr *in vivo* samples when leucocyte infiltration was sparse. There were differences in responses to the irritants, since histochemical reaction products were missing after treatment with one irritant (SLS), but increased by another (NaOH) (Plate 1).

Enzyme release into the culture medium was an unreliable criterion to identify irritants of moderate potency (Parish 1985 and 1986b; Table 5). Substances giving similar responses *in vivo* on rabbit skin showed a widely varying histological, histochemical and enzyme release picture *in vitro*. The failure to release, or release of small amounts of one or two of the three enzymes, did not correlate consistently with necrosis or significant cell damage based on histological features. However, mild irritants released significant amounts of acid phosphatase, neutral protease and lactate dehydrogenase. Corrosive or strong irritants did not release significant amounts of the enzymes, or none were detected, presumably because of destruc-

tion of cells containing the enzymes, fixation of the cell membranes, or even denaturation of the enzymes diffusing towards the culture media. Thus, mild irritants could be identified by the stimulus to release enzyme, and corrosive or strong irritants by their failure to release enzyme, confirmed by histology or by subsequent ultrasonic disruption failing to cause enzyme release indicating destruction by the test substance. The unresolved problem was identification of moderate irritants, but further investigations of the early changes in keratinocytes should reveal an appropriate determinant.

In vitro two-cell systems

Cytokines are identified by analysis of the products formed by one cell type influencing the activity of another. The same principle can be applied to *in vitro* toxicology when cells or pieces of an organ are subjected to a stimulus or damage, and the products released are tested for their activity on other cells or organ tissue. Thus the influence of activated macrophages on fibroblasts, or of damaged skin on normal skin, on fibroblasts or on neutrophils may be examined in defined culture conditions. Two-cell systems have some clear advantages over methods requiring assay of known, defined cytokines, as follows: (1) identification of the mediators influencing the responder cell is not essential; (2) activity of unstable mediators is observed; (3) natural cellular inhibitors balance pro-inflammatory mediators; and (4) they enable identification of the promotor and responding cells.

An example of a two-cell system is described briefly, in which culture medium from skin preparations treated with chemical irritants and *C. perfringens* toxins was transferred to other cell cultures to detect mediator activity. Another example is the possible application of macrophage-conditioned medium, or co-culturing macrophages with fibrocytes to identify pulmonary fibrogenic dusts.

Products from skin tested with irritants *in vitro* transferred to normal skin, fibroblasts or neutrophils

The following studies at the Lister Institute of Preventive Medicine formed part of an investigation on the properties of *C. perfringens* type C isolated from the sheep and chicken (Parish 1961 and 1985). Though the results did not provide new evidence of clostridial toxin activity, the concept of the test with

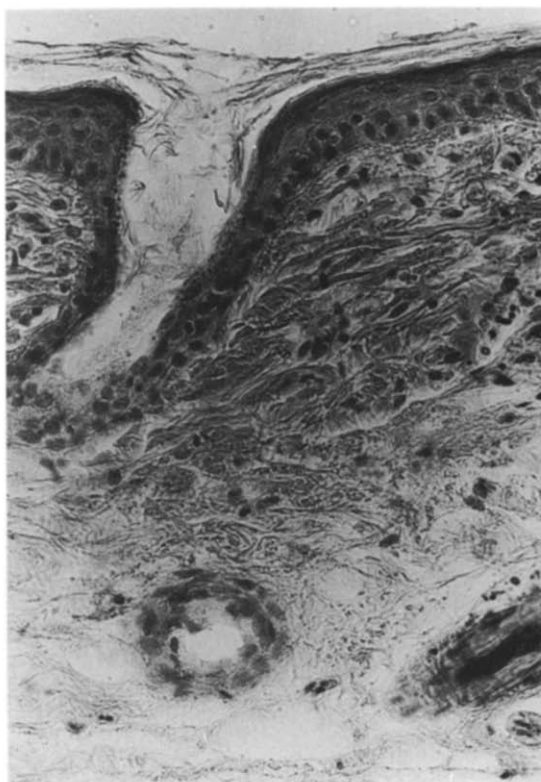


Plate 1. Photomicrograph (magnification $\times 120$) of untreated skin from hairless mice cultured for 44 hr (including the pre-incubation period).

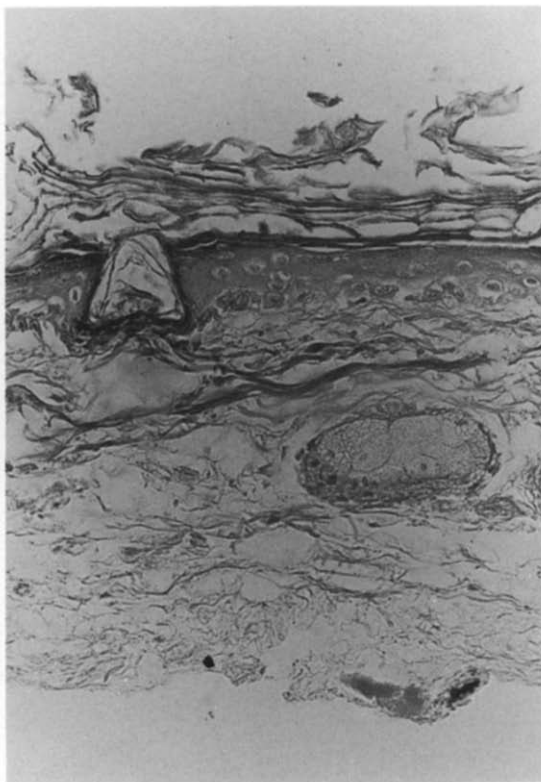


Plate 2. Photomicrograph (magnification $\times 120$) of skin of hairless mice exposed for 24 hr to dodecyl sulphate ($750 \mu\text{g}/\text{cm}^2$ skin). Complete loss of stratum granulosum and all basophilic staining of epidermal and dermal nuclei.

SKIN IN VITRO TOXICITY/STIMULATION

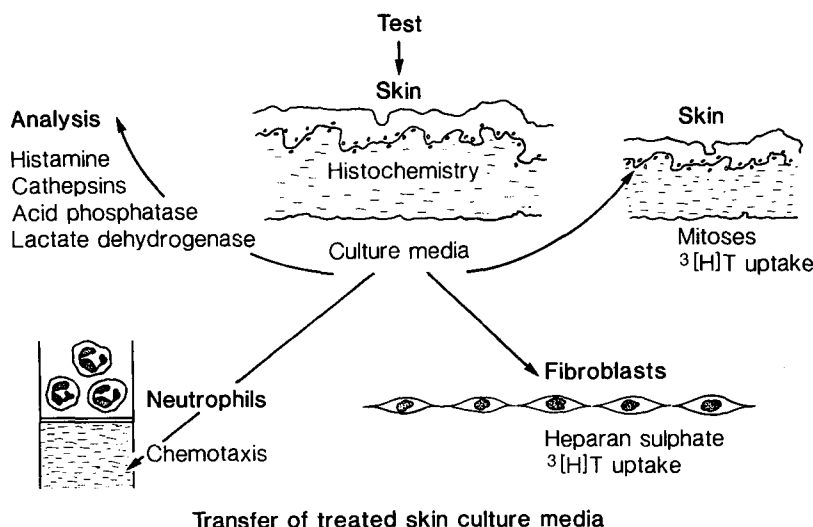


Fig. 1. Diagram of two-cell/organ systems to detect biological activity of cytokines or enzymes released from full depth skin treated with test substances *in vitro*. The test skin was examined by histochemistry after 4 or 24 hr. The medium was transferred to normal skin or keratome preparations (examined for mitoses and DNA synthesis), to primary fibroblasts (examined for heparan sulphate formation and DNA synthesis) and to neutrophils (for chemotaxis). Samples were also analysed for histamine, Cathepsin D, acid phosphatase and lactate dehydrogenase.

evidence of the chemicals used as irritant controls, are relevant to *in vitro* toxicological techniques.

The principle of the procedure (Fig. 1) was that full depth rabbit skin was cultured in Trowell (1959) type chambers with Eagle's medium without serum in an atmosphere of 5% CO₂ in air. Test substances were applied to the epidermis, and after 5 min the surplus was removed with filter paper, leaving the surface wet with the test substance. Samples of the medium were withdrawn after 4 or 24 hr and transferred to cultures of normal skin, or to fibroblasts, or to Boyden (1962) chambers containing neutrophils, and were also analysed for the presence of histamine by the Schultz-Dale technique on guinea-pig ileum, Cathepsin D on haemoglobin, pH 3.5 and 37°C (Barrett, 1967), acid phosphatase and lactate dehydrogenase. As in the tests reported previously (Parish, 1986b) the weak irritants tested were 0.01 and 0.1% NaOH, 0.1% HCl and 5% sodium lauryl sulphate. Moderate irritants were 1% NaOH and 1% HCl. For concentrations of *C. perfringens* type C toxins, see Table 6.

The results of tests on normal whole skin and

fibroblasts, and the analytical findings are summarized in Table 6.

No consistent changes in histochemical examination for acid phosphatase and lactate dehydrogenase were found that are applicable to detection of irritants.

The tests for neutrophil chemotaxis in the Boyden (1962) chambers were ineffective in differentiating irritant activity. The excised skin, without treatment, released chemotactic substances, not identified, and the application of irritants did not always increase the effect determined by the dilution of the culture media attracting a two-fold increase in the number of neutrophils penetrating the filter greater than the control. If 10% unheated rabbit serum was added to the medium the chemotactic activity was greatly enhanced. The damage induced by excising and culturing the skin was sufficient to stimulate release of chemotactic factors, and though later investigations showed that it was possible by culturing full depth skin to show differences in generation of cytokines, for example leukotrienes from untreated and irritated skin, it was not possible to make the

Table 6. Result of transfer of culture medium from skin treated *in vitro* with chemicals or *Clostridium perfringens* toxins, added to cultures of normal skin and primary fibroblasts, with an analysis for histamine and enzymes in the medium*

Test substance	Skin ³ [H]T† %	Fibroblasts		Mediator release			
		HEP SO ₄	³ [H]T† %	H	C	AP	LD
Nil control	2.9	Base	9.1	—	—	—	—
Weak irritant	7.3	+	14.9	—	+	+	+
Moderate irritant	1.4	+	9.2	—	—	—	+
<i>C. perfringens</i>							
Sheep α 12 U/β22 U	0	—	0	—	—	—	—
Chicken ∇ 11 U/β32 U	0	—	0	—	—	—	—

Hep SO₄ = heparan sulphate-type polysaccharides H = histamine C = Cathepsin D AP = acid phosphatase LD = lactate dehydrogenase

*All tissues derived from rabbits.

† = % ³[H]T incorporation.

same distinction using neutrophil chemotaxis as the determinant.

Culture media from the treated skin was transferred to preparations of normal skin for 20 hr, followed by a 4-hr incubation with tritiated thymidine. Weak irritants stimulated DNA synthesis above the nil control percentage, moderate irritants decreased DNA synthesis, but the Clostridium toxin completely abolished DNA synthesis (Table 6). It was thought unlikely that the toxins had penetrated the test skin to reach the culture medium, but this was not verified by experiment.

Samples of the test skin medium added to primary cultures of rabbit fibroblasts similarly stimulated DNA synthesis, or in the test with moderate irritants, the mean result showed no change from the control, untreated culture value (Table 6). Similarly, in a crude test for stimulation to form ground substance polysaccharide, for example heparan sulphate, the cultures were stained with alcian blue and separately with Hale's colloidal iron, and any change from the control base value was observed by a quantimet. Irritants stimulated greater formation of this stainable material than seen in the control cultures.

On analysis of the culture fluid no histamine was detected, but weak irritants induced release of Cathepsin D, acid phosphatase and lactate dehydrogenase; moderate irritants induced release of lactate dehydrogenase (Table 6). This is consistent with all our previous studies on enzyme release with weak and moderate irritants.

The two-cell/organ system showed that weak irritants induced release of one or more substances that stimulated DNA synthesis in normal skin and in fibroblasts. This may have been IL-1, which was not a known substance when these tests were done. Furthermore, the IL-1-like activity in media from test skin treated with moderate irritants, when transferred to normal skin, reduced DNA synthesis. This is similar to the effect on enzyme release from skin treated with moderate irritants. It was further shown that formation of ground substance polysaccharides, for example heparan sulphate stainable by alcian blue, by the treated fibroblasts was increased by media from the skin treated with irritants. Such evidence is not available from single-cell systems and may have relevance for test procedures for dermal changes following irritation.

The possible application of macrophage-fibrocyte interaction to identify fibrogenic dusts

The interaction by transfer of macrophage-conditioned medium to fibrocytes, or co-culture of macrophages and fibrocytes, shows potential as a technique to identify those dusts inducing progressive pulmonary fibrosis. Macrophage phagocytosis and enzyme release, described earlier, is a technique to identify particulates likely to be irritant when inhaled. The result does not indicate the nature of the inflammatory change or identify those dusts likely to be fibrogenic. There is strong evidence that macrophages stimulate fibrocytes to secrete collagen (Heppleston, 1984; Heppleston and Styles, 1967), and quartz and asbestos ingested by macrophages release one or more substances that on disrupting the macrophages,

or harvesting the medium in which the cells are cultured, will stimulate incorporation of [³H]proline in fibroblasts (e.g. Aalto and Heppleston, 1984).

Reference was made earlier to the several mediators secreted by macrophages. Of these, some stimulate fibrocyte collagen formation, particularly transforming growth factor- β (TGF- β) (Pellegrino *et al.*, 1988; Varga and Jimenez, 1986), tumour necrosis factor- λ (TNF- λ) (Tracey *et al.*, 1989) and IL-1 (Postlethwaite *et al.*, 1988). Some of these cytokines are not completely selective for collagen synthesis, or act independently of other agents. Furthermore, the macrophage also secretes regulating substances that decrease synthesis of collagen (Clark *et al.*, 1983; Jordana *et al.*, 1987). TNF- λ stimulates secretion of collagenase and therefore contributes also to collagen degradation (Scharffetter *et al.*, 1989). It is too early to predict if techniques now available will enable identification of one or more cytokines stimulating fibrogenesis, and provide an *in vitro* technique to predict the fibrogenic potential of dusts. It is also possible that the long-term stimulus depends upon macrophages loaded with the ingested particles persisting at the site continually synthesizing cytokines, or that the stimulus results from messenger RNA coding the fibrocyte for progressive collagen synthesis. These possibilities should be considered for future investigation and reproduced *in vitro*.

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