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Cellular and Molecular Mechanisms of Tumour Promotion and their Implications for Risk Assessment

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ABSTRACT

The majority of cancers that occur in humans and in experimental animals probably result from the interaction of target cells with many factors, both endogenous and exogenous, and through several processes. Recent studies on the action of tumour promoters suggest that they interact with cells by mechanisms different from those of the initiating carcinogens. Phorbol ester tumour promoters, for example, bind to specific membrane-associated receptors rather than to cellular DNA. This appears to lead to alterations in cell surface properties, inhibition of intercellular communication and modulation of cell differentiation. There is also evidence that several types of human cancer result from a multistep process with steps analogous to those of initiation and promotion. The exposure of humans to tumour promoters (and various cofactors) may, therefore, play an important role in affecting the incidence of specific human cancers. Thus, in assessing the cancer risk in humans from exposure to environmental agents, it is important that we develop reliable assays for such agents. It is anticipated that further studies on the mechanism of action of tumour promoters at the cellular and biochemical levels will provide new approaches for identifying potential tumour promoters and for more rational approaches to risk estimation in humans.

1 INTRODUCTION

The multistep origin of most malignant tumours is probably due to the interaction of target cells with multiple factors, both endogenous and exogenous. These complex aspects of carcinogenesis must be taken into account when one attempts to analyse quantitatively the cancer risk in humans for specific environmental chemicals. Thus, it is unlikely that a single bacterial mutagenesis test, or other short-term tests, will accurately predict the carcinogenicity of

chemicals, especially if we are also concerned with the important questions of relative potency and risk assessment in humans. Furthermore, since there is increasing evidence that tumour promoters and certain other modifiers act on cells through mechanisms different from those of classical carcinogens, it is essential to include in cancer risk assessment assays for tumour promoters and other cofactors.

In general, the validity of our approaches to risk assessment is highly dependent on our understanding of basic mechanisms of various stages of carcinogenesis. In this paper, we shall therefore review promotion and attempt, wherever possible, to relate this knowledge to the more practical problems of assessing cancer risk in humans.

2 TWO-STAGE MODEL OF CARCINOGENESIS

Among various experimental models of multistage carcinogenesis, mouse skin two-stage carcinogenesis is the clearest example in which at least two distinct stages, 'initiation' and 'promotion', are well defined (Berenblum, 1975). This model, shown schematically in Figure 1, indicates several fundamental properties of 'initiators' and 'promoters'. A single application of a low dose of an 'initiator' or repeated applications of a 'promoter' alone do not induce tumours, yet tumours appear when the 'promoter' is applied repeatedly after a single application of an 'initiator'. This indicates that two apparently non-carcinogenic agents can induce tumours when applied together in the proper order. Since during their lifetime humans are exposed to a variety of environmental factors, and often at low doses, it is conceivable that similar synergistic interactions occur in the causation of many human cancers. Thus the cancer 'risk' for a given substance is highly dependent on what other substance(s) the individual is exposed to.

It is also evident from Figure 1 that the action of an initiator is essentially irreversible. When an initiator, for example, 7,12-dimethylbenz(a)anthracene (DMBA) is painted as a single dose on mouse skin, the exposed mouse can lead a normal life and not develop skin cancer. Yet if as long as one year later the mouse is exposed to repeated doses of a tumour promoter, tumours will appear (Boutwell, 1964). Thus, there exists a long-term 'memory' of the action of an initiator on mouse skin. In one sense, the subsequent exposure to tumour promoters determines whether tumours appear or not. It is important to stress this point in risk assessment for various environmental chemicals, since if initiators are ubiquitous in our environment then the extent of exposure to tumour promoters could determine the incidence of specific human tumours.

In contrast to the irreversible action of the initiators, the action of promoters, at least during the early stages of carcinogenesis, appears to be reversible (Figure 1). For example, when the prolonged application of a promoter is discontinued, some of the papillomas that have already appeared on mouse skin

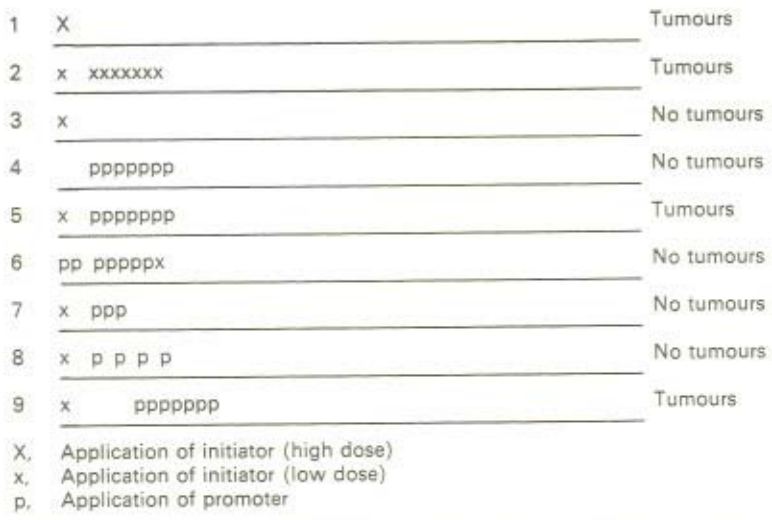

Figure 1 Schematic diagram of multistage mouse skin carcinogenesis

may actually regress. This difference with respect to reversibility may be due to the fact that initiators or their metabolites generally bind covalently to cellular DNA, RNA and proteins and produce heritable changes, whereas the action of tumour promoters appears to be via epigenetic mechanisms, and they are usually not mutagenic (Weinstein *et al.*, 1977, 1979b, and see below).

Until recently, one could wonder whether the model of two-stage carcinogenesis applied only to mouse skin and was not a general principle in carcinogenesis. However, there are now several other experimental models of two-stage carcinogenesis (Table 1). There is, for example, accumulating evidence that liver carcinogenesis in rats occurs by a multistep process with initiation and promotion stages similar to those seen on mouse skin (Peraino *et al.*, 1977; Shinozuka *et al.*, 1979; Farber and Cameron, 1980; Pitot *et al.*, 1980).

The agents that act as tumour promoters in various tissues display considerable chemical diversity (Table 1). They include phorbol esters, phenols, saccharin, phenobarbitol, DDT, etc. Much of our recent knowledge about the mechanism of action of tumour promoters has come from studies of the phorbol esters and their derivatives (Weinstein *et al.*, 1977, 1979a; Slaga *et al.*, 1978; Blumberg, 1980). The mechanism of action of the non-phorbol ester tumour promoters is much less well understood and it seems likely that not all of them act via the same mechanisms as phorbol esters. However, once their action is fully understood, it is possible that they will all share some common final mechanism. Thus, only recently has it become apparent that DNA is a common and critical target for a variety of diverse initiators including radiation, nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, vinyl chloride, etc. It is of

Table 1 Examples of two-stage carcinogenesis in experimental animals

Tissue	Species	Initiator	Promoter
		Phorbol ester-related examples	
Skin	Mouse	Polycyclic hydrocarbons MNNG, β-propiolactone, urethane	Croton oil, phorbol esters teleocidin
Ovary	Mouse	DMBA	TPA
Fore-stomach	Mouse	DMBA	TPA
Stomach	Rat	MNNG	Croton oil
Liver	Mouse	DMN	Phorbol
Lung	Mouse	DMN	Phorbol
		Non-phorbol examples	
Skin	Mouse	Polycyclic hydrocarbons	Anthralin, iodoacetate, tweens, citrus oil, surface-active agents
Liver	Rat	AAF, DEN, 3′-methyl-*N*,*N*-dimethyl-4-aminoazoben-zene	Phenobarbital, DDT, BHT, TCDD
	Mouse	DEN	PCB
Lung	Mouse	Urethane	BHT
Colon	Rat	MNNG	Bile acids
Bladder	Rat	MNU	Cyclamate, saccharin, normal urine
Mammary gland	Rat	Neutron, γ-radiation	Prolactin
Thyroid	Rat	*N-bis*(2-hydroxypropyl) nitrosamine	3-amino-1,2,4-triazole
Intestine	Rat	1,2-Dimethylhydrazine	Sodium barbiturate

See Slaga *et al.* (1978), Hecker *et al.* (1982) and Greenebaum and Weinstein (1981) for individual references.

interest that two new, recently described classes of tumour promoters on mouse skin, the indole alkaloid teleocidin and the polyacetate aplysiatoxin, appear to act by mechanisms very similar to that of phorbol esters despite their quite different chemical structure (Fujiki *et al.*, 1981, 1982; Sugimura *et al.*, 1982).

3 MECHANISM OF ACTION OF TUMOUR-PROMOTING PHORBOL ESTERS

It is not our aim to review all of the known effects of the phorbol esters on cells (the number of such effects is enormous), but we would like to discuss certain general types of effects, particularly those that appear to be relevant to the mechanism of tumour promotion. This subject has been reviewed in detail in the

proceedings of two recent symposia on tumour promoters (Slaga *et al.*, 1978; Hecker *et al.*, 1982).

3.1 Specific Cellular Receptors for Phorbol Esters

Studies with cell culture systems have indicated that the primary action of tumour-promoting phorbol esters takes place at the cell surface. Thus the exposure of cells to phorbol esters can cause increased membrane fluidity, increased uptake of 2-deoxyglucose and ^{32}P and $^{86}Rb^{+}$, inhibition of EGF binding to cellular receptors, synergistic interaction with growth factors, altered cell adhesion and uncoupling of β-adrenergic receptors, and increased turnover of membrane phospholipids (see Weinstein *et al.*, 1977, 1979a). In general, there exists a good correlation between the tumour-promoting activity of phorbol esters on mouse skin and their effects on membrane structure and function (Lee and Weinstein, 1978; Driedger and Blumberg, 1979; Yamasaki *et al.*, 1981).

It has previously been postulated that the phorbol esters may usurp the function of a cell surface receptor whose normal function is to mediate the action of a yet-to-be identified growth regulator or hormone (Lee and Weinstein, 1978; Weinstein *et al.*, 1979b). Consistent with this hypothesis are:

(1) the low concentrations at which the tumour promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and derivatives act in cell culture (10^{-8} to 10^{-10} M);
(2) the remarkable similarity in structural requirements seen when a variety of phorbol esters and related macrocyclic diterpenes are tested in diverse systems;
(3) the highly pleiotropic and reversible effects of these compounds; and
(4) indirect evidence that the putative TPA receptor, like several other receptors, displays down regulation.

Specific receptors for phorbol esters have indeed been demonstrated recently in crude membrane fractions or on intact cells using ^{3}H-phorbol-12, 13-dibutyrate (^{3}H-PDBU) (for review, Blumberg *et al.*, 1982). Their studies and those of others have shown that phorbol ester receptors have a high affinity for phorbol esters (K_d varies from 10 nM to 50 nM, depending on the cells) and that each cell has about $1-2 \times 10^5$ such binding sites. The binding of ^{3}H-PDBU can be inhibited by various tumour-promoting phorbol esters and their inhibitory ability generally correlates with their tumour-promoting activity on mouse skin, and with their biochemical or biological activity exerted on cultured cells (Driedger and Blumberg, 1980; Shoyab and Todaro, 1980; Blumberg *et al.*, 1982; Yamasaki *et al.*, 1982a,b). These specific high-affinity phorbol ester receptors have been detected in a variety of cultured cells and tissues, with the exception of mature red blood cells (Shoyab and Todaro, 1980; Horowitz *et al.*, 1981; Blumberg *et al.*, 1982). The recent findings that phorbol ester binding sites also

exist in lower animals, such as nematodes (Ouazana *et al.*, 1981), hydras and sponges (unpublished observations) suggest a high degree of phylogenetic conservation of these receptors.

These findings suggest that such receptors are normally used by certain endogenous agonists or ligands. Several attempts have already been made to identify the putative physiological ligands from body fluids or tissue extracts but thus far without success (Horowitz *et al.*, 1981, 1982a; Blumberg *et al.*, 1982). If such substances do exist they could exert biochemical and biological effects similar to those of phorbol esters and thus could play an important role as endogenous tumour promoters or as modifiers of carcinogenesis. It is important to note that TPA and related compounds are potent modulators of various programmes of cell differentiation (Weinstein *et al.*, 1979b; Diamond *et al.*, 1980; Yamasaki, 1980; Yamasaki *et al.*, 1982a). It is conceivable that the putative endogenous ligand for phorbol ester receptors may be a factor (or factors) which controls normal cell differentiation, proliferation and development. Recent studies indicate that despite their different chemical structures, two new classes of tumour promoters, teleocidin and aplysiatoxin (Fujiki *et al.*, 1981, 1982), bind to the same cellular receptors as the phorbol ester tumour promoters (Umezawa *et al.*, 1981; Horowitz *et al.*, 1982a,b). Thus the action of these receptors may not be confined to the phorbol esters and could extend to a broad range of naturally occurring compounds. On the other hand, not all classes of tumour promoters act through this receptor system. The effects of tumour promoters on cell differentiation and the possible relevance of these effects to the mechanism of tumour promotion are discussed below (3.3).

3.2 Membrane Effects and Cell–Cell Communication

The binding of phorbol esters to their cellular receptors is followed by a number of membrane-related effects (for review, see Weinstein *et al.*, 1979a; Blumberg, 1980). Among these effects, the recent finding that tumour-promoting phorbol esters inhibit cell–cell communication may provide an important clue to the process of tumour promotion, since cell–cell communication is thought to play a crucial role in the control of cell proliferation and differentiation (Loewenstein, 1981).

Evidence that tumour promoters can inhibt cell–cell communication was first independently obtained by Yotti *et al.* (1979) and Murray and Fitzgerald (1979). These investigators found that TPA and related phorbol ester tumour promoters inhibit metabolic cooperation between cells in culture. Although the effects were striking, the metabolic cooperation technique for the study of cell–cell communication has several limitations:

(1) since metabolic cooperation is the transfer between cells of relatively large molecules, the absence of this process is not in itself a demonstration of complete communication incompetence (Loewenstein, 1979);

(2) metabolic cooperation is a relatively complex process involving several biochemical reactions, and
(3) the inhibition of metabolic cooperation may reflect inhibition of previously established and/or *de novo* formation of pathways of cellular communication in growing cultures, and the procedure used does not distinguish the difference.

An assay that overcomes these limitations is to pass pulses of electric current through intracellular microelectrodes from the interior of a cell to an external medium and to measure the corresponding electrical potential in this cell and in a neighbouring cell (Loewenstein, 1979). By means of such a method, it has recently been demonstrated that tumour-promoting phorbol esters reversibly inhibit both the formation and maintenance of electrical coupling of cultured human epithelial cells (Figure 2). Tumour-promoting phorbol esters did not change the membrane potential, membrane resistance, or growth rate of the cells, suggesting that tumour promoters can inhibit cell–cell communication without affecting the general properties of the surface membrane. However, it is important to emphasize that tumour promoters may inhibit cell–cell communication through indirect effects on membrane structure or function since they are capable of exerting a variety of such effects. Among various phorbol esters and

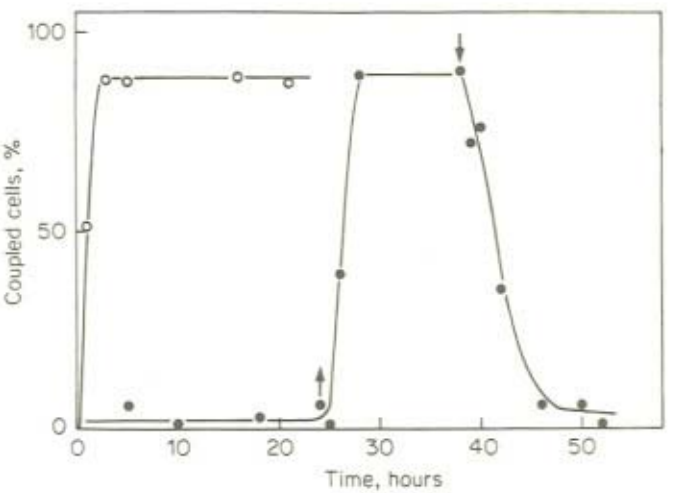

Figure 2 Effect of addition, removal and readdition of 100 ng/ml 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) on coupling between FL cells. Arrows indicate times of addition or removal of TPA. TPA was removed by carefully washing the cell layers 3 times with culture medium containing 10% calf serum. ○, control, ●, TPA treated cells. Reproduced with permission from Enomoto *et al.* (1981)

related compounds tested, there was a good correlation between their ability to inhibit intercellular transfer of electrical current, tumour-promoting activities and the ability to inhibit PDBU binding to the cells (Enomoto *et al.*, 1981; Yamasaki *et al.*, 1983).

The fact that certain tumour promoters can completely inhibit electrical coupling of cells implies that they might totally inhibit various types of cell–cell communication. This block in intercellular communication might in a complex tissue produce disturbance in proliferation and cell differentiation, and thus enhance tumour formation. *In vivo* experiments described by Raick (1973) are consistent with this hypothesis. He examined by electron microscope morphological changes in mouse skin exposed to TPA and found that TPA appeared to induce the appearance of intercellular spaces between epidermal cells. These changes could be associated with the loss of cell–cell communication. The same author also reported that TPA impaired epidermal cell differentiation. However, when ethylphenylpropiolate, a hyperplasmiogenic agent without tumour-promoting activity, was used there was no widening of the intercellular spaces and the inhibition of cell differentiation was not observed (Raick and Burdzy, 1973). These *in vivo* results are consistent with the hypothesis that tumour promotion is related to aberrant cell–cell communication and aberrant cell differentiation (see 3.3 and 3.4), although other explanations have not been excluded.

More recent studies have shown that metabolic cooperation is also inhibited by a variety of non-phorbol tumour promoters (Williams *et al.*, 1981; Trosko *et al.*, 1982). On the other hand other investigators have found that not all tumour promoters inhibit metabolic cooperation (Umeda *et al.*, 1980; Kinsella, 1982). The possible use of this phenomenon for screening of tumour promoters is discussed in section 5.

3.3 Modulation of Cell Differentiation

Since it has long been postulated that aberrant differentiation is involved in the process of carcinogenesis, the modulation of a variety of programmes of cell differentiation by tumour promoters has drawn considerable attention (Weinstein *et al.*, 1979a; Diamond *et al.*, 1980; Yamasaki, 1980). The phorbol ester tumour promoters can inhibit differentiation of many types of cells but it should be emphasized that with certain cell types they enhance differentiation (Table 2). At first sight, these dual effects of tumour promoters appear confusing. On the other hand, they may explain certain tissue specific effects. We believe that the inhibitory effect is directly related to tumour promotion. We presume that in those tissues in which phorbol esters induce terminal differentiation, they do not act as tumour promoters. The latter effect may partially explain why some of these compounds have an antileukaemic action (Kupchan *et al.*, 1976). In those tissues in which terminal differentiation is inhibited, there would be a rapid

accumulation of undifferentiated cells, which could favour the outgrowth of the initiated cells (Weinstein *et al.*, 1979a; Yamasaki, 1980). A similar hypothesis was presented by Berenblum (1954) from earlier studies on mouse skin. Figure 3 illustrates his view of the mechanism of two-stage mouse skin carcinogenesis; we consider that the results obtained *in vitro* strongly support this hypothesis (Yamasaki *et al.*, 1982a). A similar hypothesis has been proposed to explain the origin of hereditary retinoblastomas in persons whose genetic predisposition (a mutation) is considered to be responsible for initiation, and a factor controlling the differentiation of retinoblasts has been postulated to play a role in the promotion stage (Matsunaga, 1979; see also section 4).

The inhibition of cell differentiation by tumour promoters is not due to toxic

Table 2 Modulation of cell differentiation by tumour-promoting phorbol esters. Reproduced by permission of Raven Press, New York, from Yamasaki *et al.* in Hecker *et al.*, *Carcinogenesis: Cocarcinogenesis and Biological Effects of Tumor Promoters*, 1982

Cell type	Phenotype affected
A. *Inhibition or delay of differentiation*	
Murine erythroleukaemia (Friend)	Haemoglobin synthesis
Murine 3T3 fibroblasts*	Lipid accumulation
Murine neuroblastoma	Neurite formation
Murine ganglia*	Neurite formation
Murine melanoma*	Melanin synthesis
Murine myeloid leukaemia†	Morphology, phagocytic activity
Murine C3H 10T1/2 cells	Morphology
Mouse epidermal cultures	Keratinization
Chick embryo myoblasts	Myotube formation
Chick embryo chondroblasts	Sulphated proteoglycan synthesis
Chick embryo melanogenic cells	Melanin synthesis
Rat mammary carcinoma	Dome formation
Hamster epidermal cultures	Keratinization
Nematode (*C. elegans*)	Early development
Sea urchin	Embryogenesis
B. *Induction or stimulation of differentiation*	
Murine erythroleukaemia (Rausher)	Hemoglobin synthesis
Murine myeloid leukaemia†	Morphology, phagocytosis etc.
Chick myeloid leukaemia	Morphology, phagocytosis
Human myeloid leukaemia	Morphology, phagocytosis, etc.
Human melanoma	Dendrite formation
Human T lymphoblasts	E-rosette formation

* Differentiation is delayed, but not completely blocked, by phorbol esters.

† Inhibition was observed when calf serum was used in culture medium; however, TPA induces differentiation in the presence of fetal calf serum (Kasukabe *et al.*, 1981).

See Yamasaki *et al.* (1982a) for individual references.

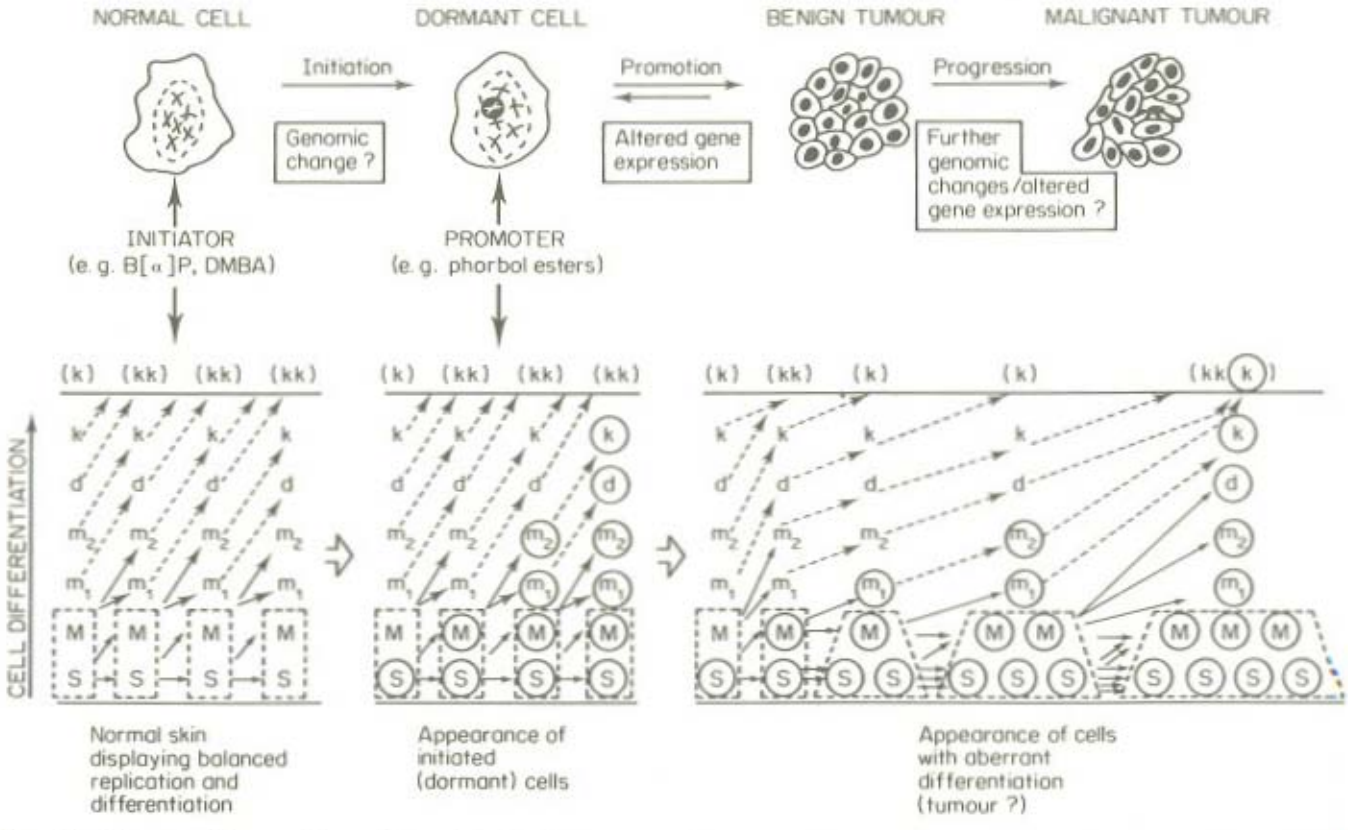

Figure 3 Schematic diagram of a possible mechanism for two-stage chemical carcinogenesis. Modified with permission from Berenblum (1954). ●, initiated cells; ⬚, cells with divising capacity; ⟶ differentiation by division; ⟶ differentiation by movement to the surface; S_1, stem cell; M, m_1, m_2, d, differentiating cells; k, keratin

effects, and the inhibition is generally reversible after removal of the compound from culture medium. For example, with continuous exposure, TPA can inhibit terminal differentiation of cultures of murine erythroleukaemia cells for longer than 2 years with about 250 serial passages. However, the cells never lose their identity as erythroids since after subsequent removal of TPA, the cells differentiate and lose their proliferative capacity after several cell divisions (Yamasaki *et al.*, 1980, 1982a, and unpublished results). The terminal differentiation of murine erythroleukaemia cells is preceded by the accumulation of cytoplasmic globin mRNA (Marks and Rifkind, 1978). As long as TPA is present in culture medium, globin mRNA does not accumulate in the cytoplasm but after removal of TPA a rapid accumulation of globin mRNA occurs (Yamasaki *et al.*, 1977 and unpublished observation). It is, therefore, evident that TPA does not damage but suppresses the expression of globin genes, which is considered to be a key event in the induction of erythroid differentiation. On the other hand, cells divide and proliferate in the presence of TPA at a similar rate as in the control medium (Yamasaki *et al.*, 1980). We can therefore conclude that TPA does not affect the functions of most of the 'house-keeping' genes which are necessary for normal cell proliferation but selectively modulates the expression of genes that are involved in cell differentiation.

Although the TPA-induced modulation of gene expression is selective, various programmes of cell differentiation are affected. These include haemoglobin synthesis, neurite formation, lipid synthesis, melanin synthesis, myosin synthesis and proteoglycan synthesis, all of which involve the expression of different types of genes. How can TPA modulate the expression of this wide variety of genes without affecting most house-keeping functions? Presumably TPA influences a process that is common to a variety of programmes of differentiation. One possible process might be the cell–cell communication (see 3.2 above). On the other hand, since TPA can influence the differentiation of haematopoietic and leukaemic cells that exist as single cells, other mechanisms must also be involved.

3.4 Model of TPA Action

Besides the effects described above, TPA and related tumour promoters have a number of other biological and biochemical effects (see Slaga *et al.*, 1978; Hecker *et al.*, 1982). Among these, the synergistic interaction of TPA with viruses in enhancing cell transformation (Fisher and Weinstein, 1980) and modulation of immunological systems (Keller, 1979) are of particular interest with respect to the mechanism of carcinogenesis.

Figure 4 illustrates a possible model of how TPA enhances the conversion of initiated cells into tumour cells. The interaction of TPA with cellular receptors results in the alteration of a variety of surface membrane properties. These include altered adhesion, altered uptake of nutrients, altered response to growth factors, altered function of cell surface receptors and impaired cell–cell

communication. These events could stimulate the outgrowth and inhibit the differentiation of pre-existing initiated cells. Presumably initiated cells respond to the tumour promoter preferentially. Yuspa and Morgan (1981) have provided evidence that initiating carcinogens alter cellular commitment to differentiation. If tumour promoters act epigenetically, then it is not clear how promotion eventually leads to the outgrowth of autonomous tumours. There is evidence that after prolonged growth of erythroleukaemia cells in the presence of TPA, differentiation is inhibited by agents (such as dimethylsulphoxide or hexamethylene bisacetamide) that would normally act as inducers, suggesting that TPA

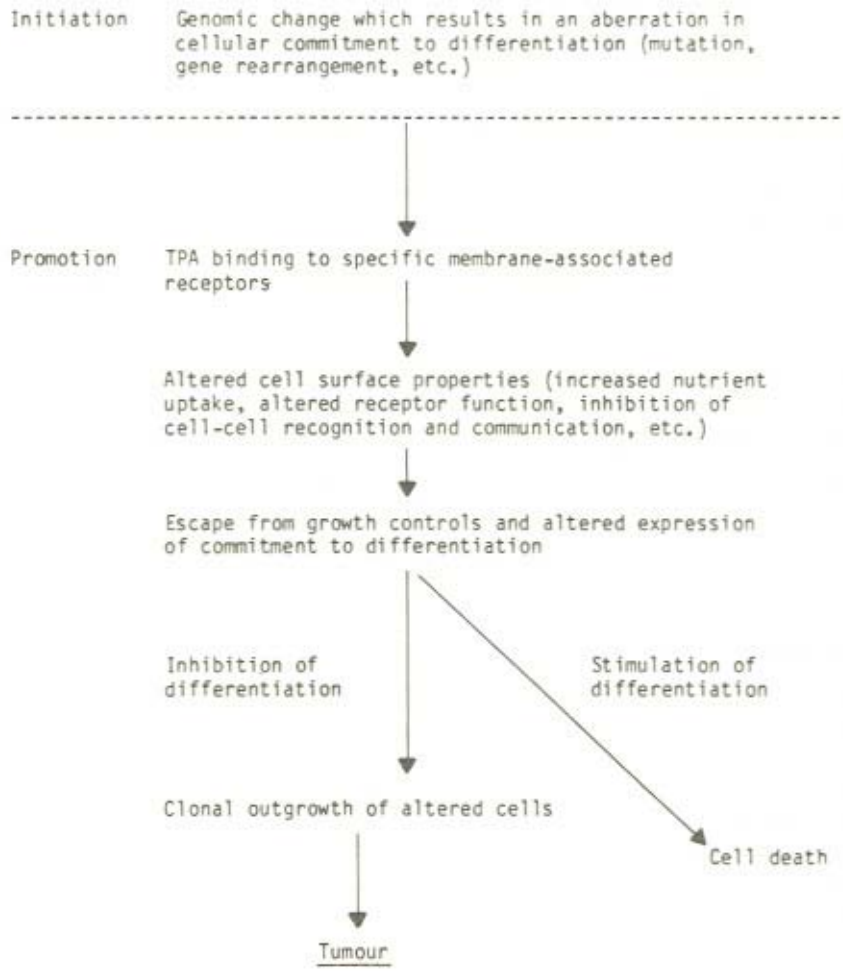

Figure 4 Cellular model of TPA action

can induce permanent changes in differentiating cell systems (Yamasaki *et al.*, 1980, 1982a). Similarly, Marks *et al.* (1978) have suggested that cells in TPA-treated mouse skin respond to a G1-chalone in a different manner than the control skin cells. These results suggest that 'lock-in' of TPA-effect may be achieved by changing cell response to a factor which normally controls cell differentiation. In addition, TPA irreversibly induces anchorage independent growth in adenovirus transformed rat cells (Fisher and Weinstein, 1980) and in certain mouse epidermal cell lines (Colburn *et al.*, 1979). Further studies are required to determine whether these irreversible effects of TPA involve epigenetic or genetic events.

4 EVIDENCE OF MULTISTAGE CARCINOGENESIS IN HUMANS

The latent period of many human cancers, i.e. the time elapsed between exposure to carcinogens and the appearance of cancer, appears to be too long to be explained by a single step event and is more consistent with a multistage process. Epidemiological studies have identified at least 30 chemicals, industrial processes or occupations that are causally related to human cancer (Table 3; IARC, 1982).

Clinical and epidemiological data also provide evidence that certain human cancers are caused by the interaction of several factors and a multistage process. Table 4 summarizes examples of such human cancers. Although the available data do not provide direct evidence of a two-stage model similar to that seen in experimental animals, the evidence is in several cases consistent with the initiation–promotion model (Greenebaum and Weinstein, 1981).

The fact that there is a rapid decline in lung cancer risk in heavy cigarette smokers when they give up smoking has been interpreted as evidence that smoking enhances a late stage as well as an early stage of the carcinogenic process (Peto, 1977; Doll, 1978). In fact, animal studies indicate that cigarette smoke contains numerous substances, including both initiators and tumour promoters (Van Duuren *et al.*, 1973a). Although the evidence is less extensive than in the case of 'cigarette smoking–lung cancer', a similar analysis indicates that conjugated oestrogens act at a late stage of endometrial carcinogenesis in humans; there is a relatively short lag period between onset of postmenopausal oestrogen use and occurrence of endometrial cancer, and of reduction of oestrogen prescription and reduction in new cases of endometrial cancer (Jick *et al.*, 1979). There is suggestive evidence that in certain endemic regions of the world phorbol esters may play a role in the aetiology of human oesophageal and nasopharyngeal cancer. The high incidence of oesophageal cancer in Curacao has been associated with the use of a herbal tea made from the plant *Croton flavens* (Morton, 1968). Weber and Hecker (1978) have isolated a phorbol ester from this plant and suggested that this compound may play a role in the disease. Nasopharyngeal cancer (NPC) in Asia is associated with Epstein–Barr (EB)

Table 3 Established human carcinogenic chemicals and industrial process (IARC, 1982)

Processes

1. Auramine manufacture
2. Boot and shoe manufacture and repair (certain exposures to dusts)
3. Furniture manufacture (certain exposures to dusts)
4. Isopropyl alcohol manufacture (strong-acid process)
5. Nickel refining
6. Rubber manufacturing industry (certain occupations)
7. Underground haematite mining (radon)

Occupational exposures

1. 4-Aminobiphenyl (aromatic amine)
2. Arsenic and arsenic compounds
3. Asbestos
4. Benzene
5. Benzidine (aromatic amine)
6. *Bis*(chloromethyl)ether (BCME) and technical CMME
7. Chromium and chromium compounds
8. Mustard gas
9. 2-Naphthylamine (aromatic amine)
10. Soots, tars and certain mineral oils
11. Vinyl chloride

Iatrogenic exposures

1. Analgesics containing phenacetin
2. Azathioprine
3. Chlorambucil
4. Chlornaphazine
5. Combined chemotherapy including MOPP
6. Conjugated oestrogens
7. Cyclophosphamide
8. Diethylstilboestrol
9. Melphalan
10. Methoxsalen with UVA (PUVA)
11. Myleran
12. Treosulphan

virus, but EB virus infection alone appears to be insufficient to explain the cause of NPC. Hirayama and Ito (1981) have suggested that phorbol esters contained in Euphorbiaceae might serve as tumour promoters in NPC causation, because in South China where the incidence of NPC is high, medicines and nasal balms prepared from these plants are extensively used. It is interesting to note that tumour–promoting phorbol esters enhance *in vitro* replication of EB virus (zur Hausen *et al.*, 1979) and they also enhance *in vitro* transformation of human lymphocytes by EB virus (Yamamoto and zur Hausen, 1979).

The geographical distribution of oesophageal cancer in China shows a very high incidence in Linxian County, Henan Province. The use of tobacco was

Table 4 Examples of multistage and/or multifactor carcinogenesis in humans

	Probable initiator	Probable promoter	References
Lung cancer	Cigarette smoking	Cigarette smoking	Peto, 1977; Doll, 1978
Oesophageal cancer	?	Phorbol esters	Weber and Hecker, 1978
	Nitrosamines?	Pickled vegetables (Roussin's red?)	Cheng *et al.*, 1982
Nasopharyngeal cancer	EB virus	Phorbol esters + n-butyrate	Hirayama and Ito, 1981
Burkitt's lymphoma	EB virus	Malaria infection	Dalldorf *et al.*, 1964
Endometrial cancer	?	Conjugated oestrogens	Jick *et al.*, 1979
Colon cancer	?	Dietary fat	Reddy *et al.*, 1978
Breast cancer	?	Hormones, dietary, fat	Weinstein, 1981; Day, 1982
Prostate cancer	?	?	Akazaki, 1973
Bilateral retinoblastoma	Hereditary	?	Matsunaga, 1979
Liver cancer	Aflatoxin B_1	Hepatitis B	Linsell and Peers, 1977
Lung cancer	Cigarette smoking +	Asbestos exposure	Selikoff and Hammond, 1979
Oesophageal, larynx and oral cavity cancers	Cigarette smoking +	Alcohol consumption	Tuyns, 1979

excluded as a possible aetiological factor since chickens in this region also have an unusually high incidence of oesophageal cancer (Li *et al.*, 1980). Epidemiological studies have shown a direct relationship between the incidence of oesophageal cancer, in both humans and chickens, and the consumption of pickled vegetables. Cheng *et al.* (1982) have obtained evidence that an extract of pickled vegetables has promoting activity in the mouse C3H10T1/2 cell transformation system. They have further suggested that dimethylthiotetranitrosodiiron, or Roussin's red, which was isolated from the pickled vegetables may act as a promoter in the causation of this disease (Cheng *et al.*, 1982). At the same time, it should be emphasized that specific nitrosamines and mutagens have also been identified in extracts of pickled vegetables (Lu *et al.*, 1981).

On the basis of geographical epidemiological studies and experimental evidence from animal studies, several investigators have implicated fat and other dietary factors in the pathogenesis of bowel cancer (for review, see Reddy *et al.*,

1978). Migrant studies show a rapid rise in the incidence of colon cancer among individuals who immigrated to the United States from Japan, Norway or Poland (Haenszel and Kurihara, 1968). An increase in colon cancer mortality rate was observed even in the first generation of immigrants. Thus environmental factors, presumably dietary, contribute to colon cancer causation. Further studies are required to determine to what extent this reflects the occurrence of initiators or promoters or both in the American diet.

Human breast cancer is an important example of multistage carcinogenesis and one in which several physiological factors may play a role as tumour promoters. These aspects are discussed in detail by Weinstein (1981) and Day (1982). With respect to prostatic cancer, in addition to the disease that is expressed clinically and that may kill the patient, there is a latent form of the disease which is often observed only microscopically at the time of autopsy and does not contribute to mortality. While the age-adjusted mortality rate from clinical prostatic carcinoma shows distinct patterns of geographical variation (Segi *et al.*, 1969), the prevalence of the latent form of prostatic carcinoma does not vary between geographical regions (Akazaki, 1973). This suggests that the tendency for latent prostate cancer to develop into clinical carcinoma is strongly influenced by environmental factors. Haenszel and Kurihara (1968) showed that the mortality rate of prostate cancer among the first generation of Japanese immigrants to Hawaii is 3 to 5 times higher than that of native Japanese. This rapid rise in risk supports the role of environmental factors. Since they presumably act on the later stage of the disease they may be more similar to tumour promoters than to initiators.

Matsunaga (1979) has proposed a two-stage mechanism for the causation of bilateral hereditary retinoblastoma. The initiation step is presumed to be due to genetic predisposition, because bilateral retinoblastoma cases usually show autosomal dominant inheritance. According to his analysis of the age-specific incidence rate from 244 bilateral cases of retinoblastoma, there is a high intraclass correlation between the age of patients at the time of diagnosis of the disease in both the right and left eyes. This result argues against the two-mutation theory of this disease proposed by Knudson (1971) in which case one might not expect the occurrence of both the right and left eye cancers within a short time interval, since somatic cell mutation is a random and rare event. He suggested that it was more reasonable to assume that the 'expression of retinoblastoma genes' was largely determined by host factors common to both eyes. Matsunaga (1979) believed that the promotion stage in bilateral retinoblastoma was an error in the process of differentiation.

In Table 4, we have listed multiple factors that have been implicated in the causation of other forms of human cancer. Further studies are required to determine which of these actually act as initiators or promoters or via other mechanisms. We must also emphasize that some of these factors may simply act synergistically as initiators.

5 PROSPECTS FOR SHORT-TERM ASSAYS FOR TUMOUR PROMOTERS

At the present time, the most reliable test for the detection of carcinogenic agents is the rodent carcinogenesis assay. However, more than 4 million chemicals are known and about 1000 new chemicals enter our environment each year (Fishbein, 1977); it cost over $400 000 and takes at least two years to test a single compound in rodent assays. Thus, it is essential to develop more rapid short-term tests as preliminary screens for potential carcinogens. Encouraging progress in developing a battery of such tests for predicting potential carcinogens and for application to human cancer risk assessment has been made (see Hollstein *et al.*, 1979; IARC, 1980; de Serres and Ashby, 1981).

It is well recognized that most currently available short-term tests are based on the fact that certain carcinogens bind covalently to DNA and damage it, and thus can be assayed for their effects on mutation and chromosomal damage. As we emphasized earlier, however, tumour promoters and certain other modifying factors do not appear to bind to DNA and are not mutagenic in conventional assay systems. Thus, these agents escape detection by most currently used short-term assays. It is also important to emphasize that many tumour promoters and other modifying factors may not be detected when tested alone in rodent assay systems, since these agents, by definition, are not strong carcinogens by themselves.

Thus, it is essential to develop short-term assays for tumour promoters and some progress has already been made in this area. For example, a short-term assay for phorbol esters and related tumour promoters was developed using the ability of tumour-promoting phorbol esters to induce the adhesion to the culture dish of murine erythroleukaemia cells which usually grow in suspension (Yamasaki *et al.*, 1979; 1981). It is possible to assay quantitatively 10–20 compounds in this system within a few days. A quantitative study has shown that there is, in general, good correlation between the ability of these types of tumour promoters to induce cell adhesion and to promote mouse skin carcinogenesis. Although this type of assay does not detect all classes of tumour promoters, it does detect the alkaloid tumour promoter teleocidin whose structure is different from that of phorbol esters (Sugimura *et al.*, 1982). Thus assays for alterations in cell adhesion may be part of a battery of tests for tumour promoters.

The assay for inhibition of metabolic cooperation (see 3.2) for the screening of tumour promoters was suggested by Trosko *et al.* (1980) based on their own demonstration that not only non-phorbol esters but also certain non-phorbol ester tumour promoters, such as saccharin, were positive in this assay. Subsequently, it has been shown that a variety of known and putative tumour promoters can inhibit metabolic cooperation (Umeda *et al.*, 1980; Trosko *et al.*, 1982). Therefore, this appears to be a promising screening test for both phorbol and non-phorbol type tumour promoters. Certain precautions, however, must be

taken when this assay is used. It is likely that agents that cause non-specific toxic effects at the cell surface may give false positive results. In addition, certain known tumour promoters or cofactors are apparently negative in this type of assay (Kinsella, 1982).

Two-stage *in vitro* transformation has been demonstrated with mouse fibroblast C3H/10T1/2 cells (Mondal and Heidelberger, 1976), rat embryo fibroblasts (Lasne *et al.*, 1974), Syrian hamster cells (Poiley *et al.*, 1979) and BALB 3T3 cells (Hirakawa *et al.*, 1982). Thus far these systems have been used mainly to study mechanisms of cell transformation by initiators and promoters and have not been extensively explored or validated as screening tests for tumour promoters. A variety of tumour promoters, such as phorbol esters, Tween 60, iodoacetic acid, anthralin and saccharin, do promote transformation of C3H 10T1/2 mouse embryo fibroblasts (Heidelberger and Mondal, 1982). On the other hand, saccharin was reported to be negative as a promoter in the BALB 3T3 cell transformation system (Sivak and Tu, 1980). Since the endpoint in a transformation assay is thought to be directly relevant to carcinogenesis (Ts'o, 1981), this type of approach should be encouraged and further developed. It might also detect a broader range of tumour promoters than assays for specific membrane effects.

Although we have described only a few examples of possible short-term tests for tumour promoters there are many other cell systems in which one can show a good correlation between *in vitro* effects and tumour-promoting activity *in vivo*, thus offering the possibility of developing other types of short-term tests for tumour promoters (Weinstein, 1980). We would like to emphasize, however, that at the present time none of these assays has been sufficiently validated so that it can be used routinely as a screening test. Furthermore, a major difficulty in attempting to devise a universal assay for tumour promoters may be the apparent tissue and species specificity of different types of tumour promoters. These arguments emphasize the importance of further studies on the mechanisms of action of tumour promoters in order to design a reliable short-term test for tumour promoters on a more rational basis (Weinstein, 1980). Another difficulty is that for many tumour promoters there are few quantitative dose–response data in experimental animals, thus making it difficult to make a quantitative comparison between results obtained in short-term tests and the whole animal assay (Yamasaki *et al.*, 1981). This is a major limitation in our approach to develop short-term tests for tumour promoters and more quantitative 'dose–response' studies are required in animal systems.

6 IMPLICATIONS WITH RESPECT TO CANCER RISK ASSESSMENT

If we wish to interpret laboratory assays for environmental carcinogens, tumour promoters and various other modifiers in terms of risk in humans, we cannot

avoid the problem of quantitative extrapolation of these results. The recognition that many human cancers are caused by a multistage process, as described in this paper, considerably complicates this task. This is particularly true for tumour promoters since, as emphasized above, there are very few animal data on tumour promoters for which effects have been studied over a wide range of doses, tissues and species.

One of the major issues pertinent to the problem of risk assessment for tumour promoters is the question of threshold or no-effect-dose level. It appears that, in contrast to initiators, tumour promoters are more likely to have a threshold for effects (Weinstein *et al.*, 1977; Boutwell *et al.*, 1982). To our knowledge, however, actual evidence from animal experiments for the existence of threshold dose levels for tumour promoters is scarce and not definitive (Van Duuren *et al.*, 1973b; Burns and Albert, personal communication). We believe that this is a crucial issue and merits much further study before we can accept this as a principle in quantitative risk assessment.

Although it is true that tumour promoters, in contrast to initiators, require repeated and prolonged application to exert their effects, this does not necessarily provide a wide margin of safety. Many substances that we are concerned with will be used by humans for prolonged periods of time (for example, food additives and pesticides), and many of them are either not biodegradable and will, therefore, persist in the environment, or they may be stored in tissues for prolonged periods of time (for example, PCBs and asbestos).

With respect to the potency of tumour promoters in cancer induction, it is important to emphasize that many known animal tumour promoters are extremely potent. For example, phorbol esters and related agents maximally promote DMBA-initiated mouse skin when repeatedly applied at a dose of 3–4 nmoles per application. Boutwell (1977) emphasized that 'DMBA + TPA' application is at least 10 times more potent than 'DMBA + DMBA' painting in terms of tumour yields in mouse skin. While a total dose of DMBA plus TPA of only 380 nmoles resulted in 75% incidence of papillomas at 18 weeks, to achieve a similar tumour yield at 18 weeks by twice weekly applications of DMBA, a total of 7600 nmoles of DMBA were required. In addition, the phorbol esters tumour promoters and teleocidin are effective at extremely low concentrations in cell culture, i.e. 10^{-8} to 10^{-11} M (Weinstein *et al.*, 1979a; Umezawa *et al.*, 1981). The compound 2,3,7,8-tetrachlorobenzo-*p*-dioxin (TCDD) promotes rat hepatocarcinogenesis initiated by dimethylnitrosamine when used at extremely low doses, i.e., 0.14–1.4 μg/kg twice weekly for 7 months (Pitot *et al.*, 1980). As discussed earlier in this paper (see also Weinstein, 1981), there are several indications that certain hormones, either endogenous or exogenous, can act as tumour promoters. Hormones also act at extremely low doses, i.e. in the range of 10^{-8} to 10^{-10} M. Thus certain tumour promoters are extremely potent and it would be wrong in risk assessments to assume that because a substance is a tumour promoter, higher doses can be tolerated.

Tumour promoters may display dose-schedule effects that are different from those of initiators or complete carcinogens. For example, DMBA-initiated mouse will not develop skin tumours if TPA is applied only once at monthly intervals, whereas applications at weekly intervals are effective (Boutwell, 1964), even if the mice receive, in both cases, the same total dose of TPA. In other words, the effects of repeated doses of tumour promoters do not appear to be simply cumulative and in this sense below threshold exposure situations may exist. However, since total dose of TPA is the same in two experiments, we have a 'dose-schedule threshold' rather than conventional 'dose-threshold'. This aspect of tumour promotion could complicate quantitative risk assessment in humans. As discussed above, epidemiological studies indicate that the cessation of exposure to certain environmental factors can lead to decreased cancer risk in a relatively short period of time (section 4), suggesting that the action of certain late stage carcinogens (tumour promoters) in humans may be reversible. While this factor also complicates the task of risk assessment, this is a comparatively optimistic finding with respect to cancer prevention, since it appears that elimination of tumour promoters from an environment could reduce the cancer risk in a relatively short period of time, even in individuals previously exposed to the agents in question.

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