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Current Concepts and Controversies in Chemical Carcinogenesis

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When I was invited to present the Keynote Address at this Keystone Conference I was quite flattered, but I wasn't certain what my responsibilities were. So I consulted Webster's dictionary, which said "keynote address: an address (as at a political convention) intended to present the issues of primary concern to the assembly but often concentrated upon arousing unity and enthusiasm." This is, actually, an appropriate assignment since the subject of chemical carcinogenesis is truly an exciting one for at least three reasons. One, it deals with a disease of major magnitude, since cancer is the second leading cause of death in the United States and the Western World. Two, it probes some of the most fundamental questions in contemporary biology, ie, questions related to DNA structure, chromatin and the control of gene expression, membrane structure and function, the very basic problem of growth control, and the specificity and stability of cellular differentiation. Three, it brings together investigators from such diverse disciplines as epidemiology, toxicology, cell biology, and molecular genetics. Thus, in these days of specialization to the point of boredom, the investigator in carcinogenesis can remain a renaissance person!

Table I presents, in general terms, the challenge that confronts us at this meeting. Our colleagues in epidemiology have provided persuasive evidence that a major fraction of human cancer is due to environmental (ie, exogenous) rather than endogenous factors [1]. This is an optimistic message, since it means that if we could identify the exogenous causative factors we could hope to prevent a major fraction of human cancer, either by reducing human exposure or somehow protecting the host. Significant progress has already been made through the identification of cigarette smoking as the major cause of lung cancer. In addition, over 20 chemicals or chemical processes have been implicated in various forms of human cancer [2]. However, the specific causes of other major cancers (ie, cancers of the large bowel and breast) have not been identified with certainty. In addition, several important general questions remain unresolved. These include the current controversies concerning the extent to which human cancers are due to naturally occurring

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TABLE I. The Challenge

Known
60–80% of human cancers are due to environmental factors
Unknown causative factors
Natural or synthetic chemicals
Initiators or promoters
Chemicals or viruses
Nutritional and other cofactors
Multifactor interactions

versus man-made chemicals, the relative contribution of initiators and promoters, the roles of chemical versus viral agents, the role of general nutritional factors (ie, fat, fiber, and vitamins) and the role of multifactor interactions.

It is unlikely that these issues will be resolved by more high-level committees, polemic editorials, or public debates; nor do I think they will be resolved by conventional epidemiologic approaches, more routine rodent feeding studies, or even the recently developed short-term tests. It is my conviction that solutions to these questions will come primarily from two areas: 1) fundamental research at the cellular and molecular levels on mechanisms of chemical carcinogenesis, and 2) a new type of human clinical study which my colleagues at Columbia University and I call "molecular epidemiology" [3]. During this presentation I stress these two themes, with emphasis on what I believe are the major known facts and unanswered questions related to the mechanism of action of chemical carcinogens. Later I stress the more applied aspects and the concept of molecular epidemiology.

THE ACTION OF INITIATING AGENTS; METABOLISM AND COVALENT BINDING TO MACROMOLECULES

Metabolic Activation

The principle that many carcinogens undergo metabolic activation to electrophiles that bind covalently to DNA, as well as RNA and protein, has become an axiom in our field [4,5]. In a sense carcinogenesis induced by these agents is an error in drug detoxification. Metabolic systems whose purpose is to detoxify certain xenobiotic substances can generate highly reactive intermediates which, if not rapidly further metabolized, may react with cellular macromolecules to form covalent adducts.

There is some evidence, but it is not conclusive, that covalent binding to DNA is a critical event in the carcinogenic process [5]. Thus factors that limit or inhibit metabolic activation generally inhibit carcinogenesis, and factors that limit or inhibit DNA excision repair appear to enhance carcinogenesis. The evidence, however, is indirect and certainly does not exclude RNA and protein binding as important events in the action of some carcinogens. Later I discuss the question of the functional consequences of covalent modification of nucleic acids by chemical carcinogens.

Carcinogen Potency

At present we know very little about what determines carcinogen potency, organ specificity, and species specificity. In some cases differences in metabolic activation or detoxification, or differential rates of repair of specific DNA adducts (ie,

O^6 methylguanine), have been demonstrated and associated with organ and species specificity [1,5]. However, detailed comparative biochemistry and the actual mechanisms underlying these differences are poorly understood. In addition, I suspect that other mechanisms such as those related to tumor promotion and progression and cofactor interactions underlie a number of these aspects of carcinogen potency and specificity.

In the case of the polycyclic aromatic hydrocarbons, it appears that the major determinant of carcinogenic potency relates to the substrate specificity of the cytochrome P-450 monooxygenase system [6]. Although the Bay Region theory [6] predicts certain aspects of the reactivity of diolepoxides once they are formed, it does not predict to what extent a given parent PAH will be metabolized by the P-450 system; and the latter aspect may be the most critical with respect to carcinogen potency. Thus benzo(e)pyrene and benz(a)anthracene are noncarcinogenic or only weakly carcinogenic, not because they lack a Bay Region but because they are poor substrates for the P-450 system [6]. At present we have no insights into why this is so. Obviously, the biochemical determinants of carcinogen potency, and of species and organ specificity, must be understood in greater detail if we are to make intelligent risk extrapolations to humans with data obtained from animal bioassays and short-term tests.

Structures of Carcinogen-DNA Adducts

With several carcinogens the chemistry and steric aspects of their interactions with nucleic acids are now understood in exquisite detail [4,5,7–9]. The simple alkylating agents can methylate or ethylate any of the nitrogen or oxygens in all four bases in DNA, as well as the sugar residues and phosphates of the DNA backbone [7]. Although the N-7 position is generally the most extensive site of modification, current evidence indicates that the O^6 position is the most significant site of attack with respect to mutagensis and carcinogenesis [10]. The major DNA adducts produced by certain polycyclic carcinogens are shown in Figure 1. Activated derivatives of certain aromatic amines attack the C-8 position of guanine, as well as the N-2 position; the epoxide derivative of aflatoxin attacks the N-7 position of guanine; and the dihydrodiol epoxide derivatives of benzo(a)pyrene (BP) and dimethylbenz(a)anthracene (DMBA) attack the N-2 position of guanine [8,11], and to a lesser extent the BP metabolite forms adducts with adenine and cytosine [11]. Thus, the theme that emerges is that different carcinogens can attack different sites on the DNA, and that even a single carcinogen can form multiple types of adducts. This considerably complicates attempts to formulate a unified or simple theory relating specific types of DNA damage to the mechanism of carcinogenesis. There is evidence, however, that the major DNA adducts formed by activated derivatives of BP, aflatoxin, and certain nitrosamines are generally the same in diverse species and tissues [11, 12], thus providing some unity to the comparative chemistry of carcinogen-DNA adducts. Studies with benzo(a)pyrene have emphasized the stereoselective aspects of carcinogen metabolism and DNA binding [for review, see 6,11], and this theme is likely to apply to other polycyclic carcinogens as well.

Although I have emphasized carcinogens that form covalent adducts with DNA, it is likely that certain carcinogenic agents (ionizing radiation, free radicals, activated oxygen, etc.) produce their effects by a"hit-and-run" attack on the DNA. The detection and quantification of these effects is much more difficult, but is obviously an important area for further research.

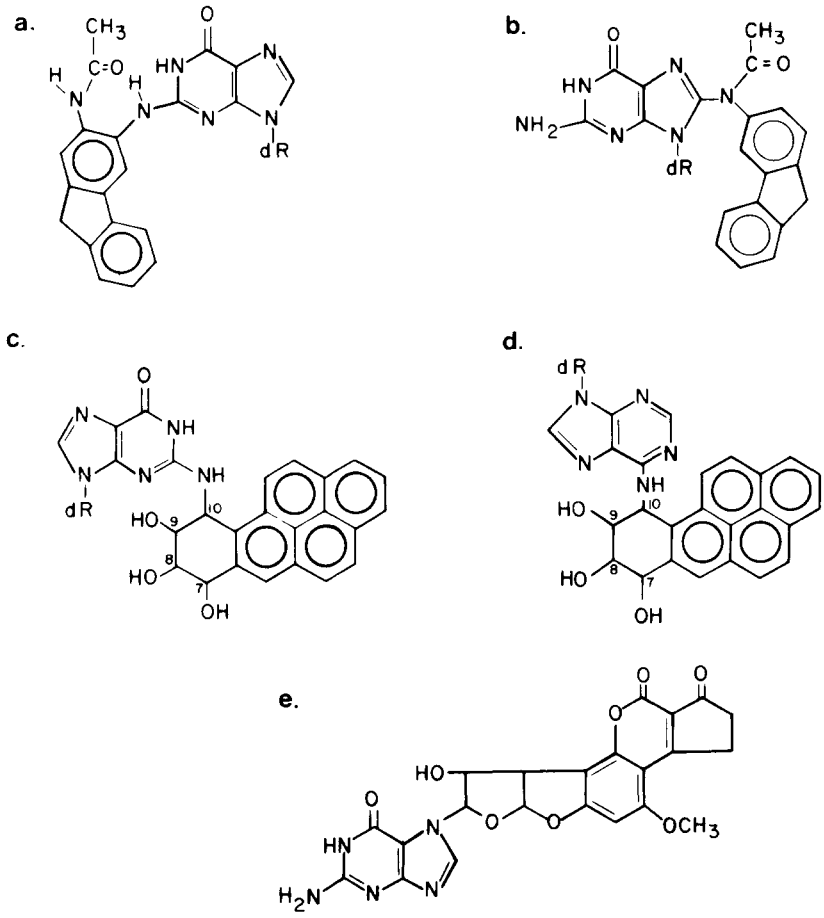

Fig. 1. The structures of several carcinogen-nucleoside adducts. (a) 2-Acetamido-3-(2′-deoxy-N^2-guanosyl)fluorene. (b) 3-[N-(2′-Deoxy-8-guanosyl)acetamido]fluorene. (c) 2′-Deoxy-N^2-(7,8,9,10-tetrahydro-7β,8α,9α-trihydroxybenzo[a]pyren-10-yl)guanosine. (d) 2′-Deoxy-N^6 (7,8,9,10-tetrahydro-7β,8α,9α-trihydroxybenzo[a]pyren-10-yl)adenosine. (e) 7-Guanyl-dihydrohydroxyaflatoxin B_1. Reprinted by permission from [9].

Conformational Changes in DNA

What is the conformation of DNA at local sites of carcinogen modification and what is the orientation of the carcinogen residue with respect to the DNA bases? Because of their small size, methylating and ethylating agents do not present major steric problems, but this is not the case with the bulkier polycyclic compounds. There is considerable evidence that AAF modification of the C-8 position of guanine in double-stranded DNA produces a distortion of the structure termed "base displacement," whereas N-2 modification of guanine by BPDE produces little or no denaturation of the DNA helix (Fig. 2) [8,9,11]. In the former case the AAF

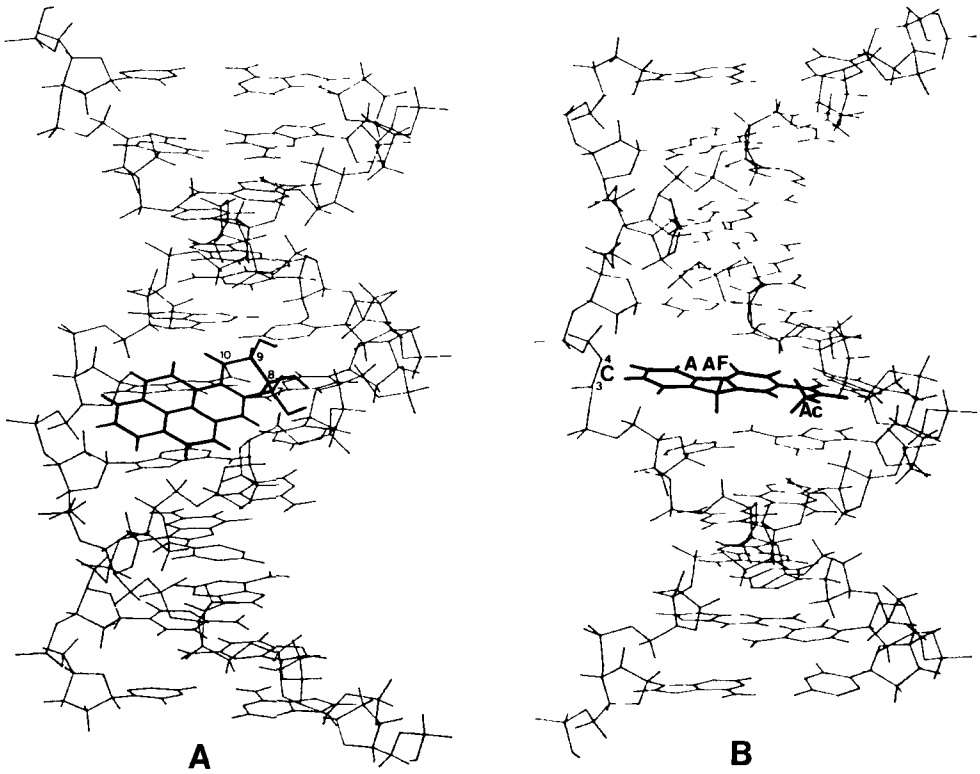

Fig. 2. (A) A computer graphics-generated display of BPDE covalently bound to B-DNA with the BPDE residue in the minor groove of the DNA helix. In this model the conformation of the guanine and the coplanarity of the N^2 amino group and the base to which the BPDE is attached are retained. The 7- and 8-hydroxyl groups and the 9- and 10-hydrogens of BPDE are quasiequatorial. The angle between the plane of the pyrene and the axis of the DNA is 28°. This results in some slight steric hindrance, based on van der Walls radii, which is presumably relieved by slight distortions of the helix. (B) AAF covalently bound to the C-8 position of guanine in B-DNA; the "base displacement model." The guanine to which the AAF is attached has been rotated out of the helix, and the AAF moiety is inserted into the helix and stacked with the bases above and below. AC designates acetyl group of AAF. The cytosine (marked C) residue on the opposite strand would overlap with the AAF residue, therefore it has been removed and the 3′ and 4′ carbon atoms of the corresponding deoxyribose in the DNA backbone are indicated. In reality, this C probably rotates out from the helix to accommodate the AAF; its exact conformation is not known, although there is evidence that this region of DNA is "single-stranded." For additional details see [11].

residue is inserted into the DNA occupying the position of the displaced guanine to which it is covalently bound. In contrast, studies from our laboratory and from Geacintov's group on BPDE-modified calf thymus DNA suggest that the BPDE residue lies in the minor groove of the DNA double helix [8,9,111]. Other investigators have suggested, however, that the BPDE residue is intercalated between base pairs in supercoiled closed circular DNA [13]. The current physicochemical methods used to examine these questions are indirect and are subject to considerable interpretation. In the future it would be desirable to obtain more direct information on the conformation of carcinogen-modified DNA by x-ray crystallography of defined synthetic DNAs containing specific covalently bound carcinogen residues.

An additional complexity is the emerging evidence that, although the B DNA helix of Watson and Crick is the predominant conformation of DNA, alternative conformations can exist [14]. Sage and Leng [15], and our group in collaboration with A. Rich [11, 16], have obtained evidence that the modification of poly(dG.dC), (dG.dC) by AAF induces it to flip from the conventional right-handed B helix to the left-handed Z DNA. If similar events occur in vivo, then unusual base sequences of DNA might be preferential targets and/or undergo specific conformational changes in response to carcinogen modification. This could be particularly important when one considers the likelihood that in the chromatin structure regions of the DNA might be held in specific conformations because of their association with proteins.

Carcinogen Modification of Mitochondrial DNA

Most of the studies on carcinogen–DNA interactions have focused on nuclear DNA. We have found, however, that when cell cultures are exposed to either radioactive BP or BPDE there is also very extensive modification of mitochondrial DNA (mtDNA) [17]. In fact, the specific activity of mtDNA is 50–100 times that of nuclear DNA [17]. Allen and Coombs [18] have also seen this with a variety of PAH carcinogens [18]. Extensive modification of mtDNA by alkylating agents has also been seen in vivo [19]. We have obtained evidence that the preferential modification of mtDNA by BPDE reflects partitioning of the lipophilic carcinogen into the lipid-rich mitochondria, as well as the high lipid to DNA ratio of the mitochondria [17]. We believe that this is an important consideration since, although during in vitro studies on DNA modification the carcinogen is often in an aqueous medium, it is likely that hydrophobic carcinogens are carried in the lipid phases of the cell in vivo. The functional significance of carcinogen attack on mtDNA is not known. This could, of course, cause disturbance in energy metabolism, although we favor the possibility that perturbations of mitochonrial ion flux, particularly intracellular Ca^{2+} homeostasis, might be more important, and thus contribute to alterations in growth control in carcinogen-exposed cells. Obviously, the relative roles of carcinogen modification of nuclear and mtDNA in cell transformation require further study.

DNA Repair

With respect to the repair of carcinogen-DNA adducts, it is of interest that enzyme purification studies are revealing a multiplicity of excision repair mechanisms, including N-glycosylases, phosphodiesterases, and even enzymes that simply remove an alkyl residue from the O^6 of guanine, thus restoring the normal structure [20]. Very little is known, however, about the enzymatic mechanisms by which polycyclic carcinogens like AAF, BP, or aflatoxin are removed, although in vivo studies indicate that excision does occur in rodent and human cells [11, 21]. The multiplicity of excision mechanisms and enzymes that nature has evolved to protect our DNA from the onslaught of environmental chemicals is somewhat reassuring, but obviously we cannot allow our environment to become so polluted that it overtaxes this defense mechanism.

Much less is known about the consequences of persistent lesions—ie—those that elude excision repair, and the consequences these have on DNA replication and gene transcription, particularly in eukaryotes. The level and duration of persistence of some of these adducts is appreciable [20, 21]. As I discuss later, I think that it is

likely that complex host responses to such lesions, analogous to those controlled by the Rec A system in bacteria [20,22], do exist in higher organisms and may play a key role in carcinogenesis. I suspect, however, that the host responses to DNA damage in higher organisms are qualitatively different than the "SOS-response" system of bacteria. This is an important area for future genetic and biochemical studies.

Carcinogen-DNA Modification and the Mechanism of Initiation

There is considerable evidence that the modification of DNA, RNA, and synthetic nucleic acids by carcinogens impairs their template activities during in vitro replication, transcription, and translation [5,7,9]. Although in some cases mispairing errors can be demonstrated, I am struck by the fact that the most predominant effects are usually inhibition of template function and, often, arrest of synthesis at the site of carcinogen modification. I wonder, therefore, whether complex host responses to the latter events in vivo might be more important in carcinogenesis than simple errors in base pairing. We have, for example, demonstrated that BPDE-induced mutagenesis in E coli is dependent on the Rec A gene-"SOS" type system [22], and this is also the case for certain other bulky lesions [20,22].

Table II lists several possible mechanisms by which covalent modification of DNA by carcinogens might initiate the carcinogenic process. I have argued elsewhere [5,23] that it is unlikely that the process involves random point mutations. Evidence against this includes 1) the above-described effects on template function, 2) the fact that the in vitro transformation of rodent cells by chemical carcinogens and radiation can occur with a much higher efficiency than random mutation [for review, see 23], 3) the fact that, although human and rodent cell cultures are equally susceptible to mutagenesis, human cells appear to be much more resistant to cell transformation, 4) the long lag between carcinogen exposure and tumor formation, and 5) the striking parallels between differentiation and carcinogenesis [23]. Of the possible mechanisms listed in Table II, I believe that the induction of gene rearrangements and/or alterations in the state of methylation of specific genes are likely to be the key events in carcinogenesis. The latter mechanism is of considerable interest because of the increasing evidence that activation of gene expression is often associated with a decrease in 5-methylcytidine content of the expressed gene, although other factors must also play an important role [24]. Later I consider the possible role of gene rearrangements in carcinogenesis.

TABLE II. Possible Molecular Mechanisms of Initiation of the Carcinogenesis Process*

With permanent changes in DNA sequence
Random point mutations
Direct: base substitution, frame shift, deletion in structural or regulatory gene.
Indirect: induction of "SOS-type" error-prone DNA synthesis.
Ordered gene rearrangements: transposition, amplification, deletion, integration of exogenous sequences, etc
Without permanent changes in DNA sequence
Altered chromatin structure, altered feedback loops, altered DNA methylation, etc

*For a detailed discussion, see text and [23].

TUMOR PROMOTION AND MULTIFACTOR INTERACTIONS

Two-Stage Skin Carcinogenesis

There is increasing appreciation of the fact that in the intact animal carcinogenesis is a multistage process that can proceed over a considerable fraction of the life-span of the individual, and that the evolution of a fully malignant tumor is subject to a variety of promoting, as well as inhibitory, factors [23,25]. These basic phenomena are becoming apparent during the transformation of cells in culture, whether the process is induced by chemical carcinogens or certain oncogenic viruses [23,26]. Indeed, it seems likely that a full understanding of the molecular mechanisms of the initial events in the carcinogenic process will also require an understanding of the later events.

The most powerful paradigm for understanding these complex phenomena has been the model of 2-stage carcinogenesis on mouse skin, where at least 2 stages, initiation and promotion, have been clearly defined [25,27]. Each of these stages is elicited or inhibited by different types of agents, and the 2 stages have different biologic properties. As stressed elsewhere [23,28], the major difference is that, whereas initiation appears to involve DNA damage, this is not the case for promotion. The 2-stage mouse skin carcinogenesis system has also served as a paradigm for studies on the multistage aspects of carcinogenesis in several other tissues and species. Evidence that hepatocellular cancer, bladder cancer, colon cancer, and breast cancer also proceed via processes analogous to initiation and promotion has been reviewed elsewhere [25,29]. The concept of promotion appears to be particularly relevant to the causation of human breast cancer [29].

Whereas just a few years ago there were very few specific cellular or biochemical markers for the action of tumor promoters, exciting advances have recently been made through studies on the biochemical effects of the phorbol esters in cell culture systems and on mouse skin [for review see 23, 25, 28]. Indeed, there has been such a plethora of findings that it will now require a considerable scientific effort to determine which of them are relevant to the mechanism of tumor promotion, and which are epiphenomena. It will be important, for example, to distinguish effects peculiar to the particular specialized cells examined (ie, macrophages or lymphocytes), or in vitro toxic effects, from those that actually occur during tumor promotion on mouse skin.

In an attempt to rationalize the numerous effects elicited by the phorbol ester tumor promoters, I have classified them into three categories [23,28], all of which mysteriously begin with the letter M: 1) mimicry of transformation, 2) modulation of differentiation, and 3) membrane effects (Table III).

Mimicry of Transformation

Perhaps the most intriguing capacities of the potent tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) and related phorbol esters are their abilities to induce in normal cells the expression of several phenotypic traits characteristic of tumor cells, and to enhance the further expression of some of these traits in cells that are already transformed [23,25,28]. These findings provide a clear demonstration of a recurring theme in cancer biology—ie, that the phenotypic properties of tumor cells preexist but lie dormant in the normal tissue of origin. The phorbol esters provide potent pharmacologic agents for studying the cellular mechanisms that control the expression of these genes.

TABLE III. Effects of TPA on Cell Surfaces and Membranes in Cell Culture

Altered Na/K ATPase
Increased uptake 2-DG, ^{32}P, ^{86}RB
Increased membrane lipid "fluidity"
Increased phospholipid turnover
Increased release arachidonic acid, prostaglandins
Altered morphology and cell-cell orientation
Altered cell adhesion
Increased pinocytosis
Altered fucose-glycopeptides
Decreased LETS protein
"Uncoupling" of β-adrenergic receptors
Inhibition of binding of EGF to receptors
Decrease in acetylcholine receptors
Synergistic interaction with growth factors
Inhibition of metabolic cooperation

For specific references, see [23, 25].

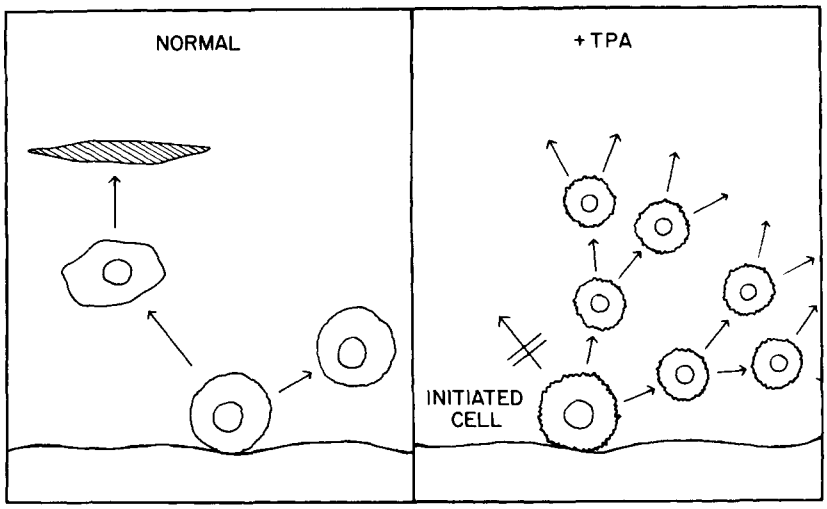

Fig. 3. Schematic representation of the normal mode of asymmetric stem cell division in epidermis and of the hypothesis that TPA induces exponential growth of an initiated stem cell, thus yielding a clone of such cells from which tumors can arise.

Modulation of Differentiation

Since it is likely that carcinogenesis involves major disturbances in differentiation, it is of interest that TPA is a highly potent inhibitor or inducer of differentiation in a variety of cell systems [23,25,28]. The examples include a variety of programs of differentiation and cells from such diverse species as bird, rodent, human, and even echinoderm. We have previously stressed that the ability of tumor promoters to inhibit terminal differentiation may be central to their action as tumor promoters [23,25] (Fig. 3). The basal cells in the adult epidermis are continually dividing, yet the tissue is in a state of balanced growth because of asymmetric division of stem cells. One daughter cell remains a stem cell and the other is committed to keratinize and terminally differentiate, thus, irreversibly losing its growth potential.

If an "initiated" stem cell were restrained to this mode of division, it could not increase its proportion in the stem cell pool. If this mode of tissue renewal were interrupted by a tumor promoter, the initiated cell could undergo exponential division, this yielding a clone of similar cells. Since TPA can also induce phenotypic changes in cells that mimic those of transformed cells, the microenvironment of a clone of such cells might itself enhance their further outgrowth and development into a tumor. In addition, clonal expansion of the population of initiated cells would provide a larger population from which variants that have undergone progression to later stages of neoplasia might emerge. I assume that in those tissues in which TPA induces rather than inhibits terminal differentiation it would not be a tumor promoter. This could provide one explanation for the tissue specificity of the phorbol esters as tumor promoters [23]. Elsewhere we have suggested that it might be possible to design phorbol analogues that induce the terminal differentiation of certain neoplastic cells yet lack significant tumor-promoting activity and that such compounds would offer a novel approach to cancer chemotherapy [10,28].

Phorboid Receptors

Our studies on the cell culture effects of the phorbol ester tumor promoters led us to suggest that they act by binding to and usurping the function of membrane-associated receptors that are normally utilized by an endogenous growth factor [23]. Indirect evidence for this hypothesis includes (1) the fact that these compounds act in a concentration range similar to that of several hormones and growth factors (ie, $\sim 10^{-8} - 10^{-10}$M), (2) the fact that these compounds display very similar structure–function requirements on cells from diverse species and tissues, and (3) the fact that, like known hormones, they induce highly pleiotropic effects, which vary considerably depending upon the target cell. Since the earliest cellular responses to these agents occur at the cell surface membrane [23,25,28], we suggested that the putative receptors were associated with the plasma membrane.

By using ^{3}H-phorbol dibutyrate (PDBu), which is much less hydrophobic than TPA, to overcome the problem of nonspecific binding, Blumberg et al have obtained direct evidence for specific high-affinity, saturable receptors in crude membrane preparations of chick embryo fibroblasts [30] and mouse epidermis [31].

A Scatchard analysis of ^{3}H-PDBu binding to intact rat embryo fibroblasts suggests that there are at least two classes of specific binding sites, a high-affinity class with K_D of about 8 nM and 1.6×10^5 sites per cell, and a low-affinity class with a K_D of 710 nM and about 3×10^6 sites per cell [32] (Fig. 4). Our values for the high-affinity ^{3}H-PDBu receptors in intact rat fibroblasts agree with the values obtained with crude membrane preparations from avian fibroblasts or mouse epidermis [30,31]. Specific high-affinity phorbol ester receptors have been detected in a variety of both normal and transformed cell cultures and in a variety of normal tissues, with the exception of mature red blood cells [30–34]. It is curious that Blumberg et al have found that the highest number of receptors is detected in brain tissue [35]. Since with prolonged or repeated exposure cell cultures can become refractory (or desensitized) to the action of TPA [23], it will be of interest to see if this reflects down-regulation of the phorboid receptors. This phenomenon could play an important role in tissue-specific effects of TPA.

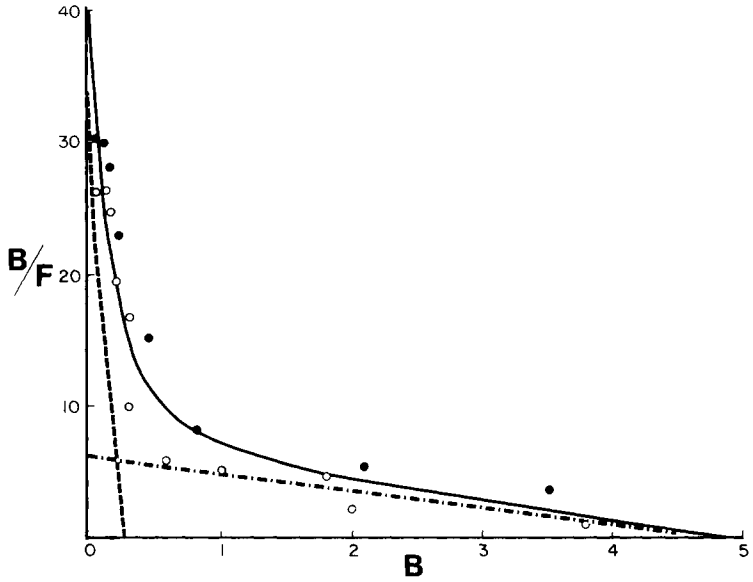

Fig. 4. Scatchard plot of specific binding of ^{3}PDBu to CREF cells. Each point is the average of duplicate plates. The results from two separate experiments are shown (○, and ●). Binding assays were performed on approximately 2 × 10^6 cells/dish at 37° C for 30 min. Bound ^{3}H-PDBu (B) is expressed as picomoles per 10^6 cells, and is corrected for nonspecific binding. The concentration of ^{3}HDBu in the binding solution (F) is in μmoles/L. For additional details see reference 32. The data are consistent with two binding sites, with K_{D_1} = 7.6 nM, at 1.6 × 10^5 sites/cell; and K_{D_2} = 710 nM, at 2.8 × 10^6 sites/cell. Binding to the first site is shown by a dashed line; and to the second, by a dotted line.

In general, the abilities of a series of TPA analogs to compete with ^{3}H-PDBu for binding to cell surface receptors correlates with their known potencies in cell culture and with their activities as tumor promoters on mouse skin [30–35]. These results provide evidence that the ^{3}H-PDBu receptors mediate the biologic action of the phorbol esters. In collaborative studies we have found that the indole alkaloid teleocidin B is also a potent inhibitor of ^{3}H-PDBu binding [36]. This result is of particular interest since, although this compound is structurally unrelated to the phorbol esters (Fig. 5), Sugimura and his colleagues have found that it shares with these compounds a number of similar cell culture effects [37,38], and it is also as potent as TPA as a tumor promoter on mouse skin [39]. We have also found that, like TPA, nanomolar concentrations of teleocidin B and dihydroteleocidin induce a rapid increase in 2-deoxyglucose uptake, induce arachidonic acid release and prostaglandin synthesis, and inhibit EGF receptor binding [36]. The results obtained with the teleocidins suggest that the phorboid receptors mediate the effects of both the phorbol esters and the teleocidin tumor promoters, thus explaining their similar, if not identical, effects on cells.

It has been found that a large number of polypeptide growth factors and various hormones fail to inhibit ^{3}H-PDBu-receptor binding [30–33]. We have found that sera from a variety of species, amniotic fluid, and various tissue extracts do inhibit specific ^{3}H-PDBu binding, and this factor has been partially purified from

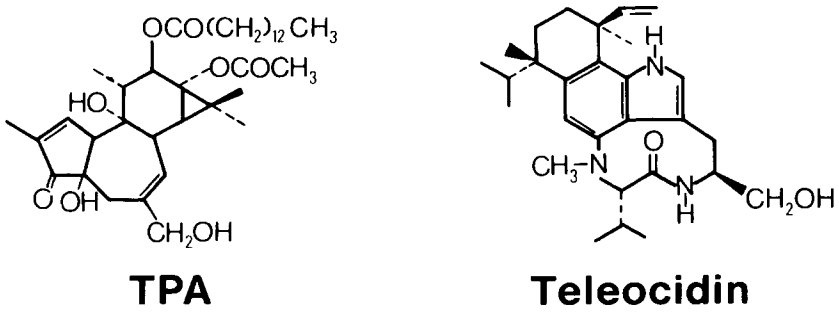

Fig. 5. Structures of 12-0-tetradecanoyl-phorbol-13-acetate (TPA) and teleocidin.

human serum [32]. Studies are in progress to determine whether this substance merely inhibits binding or whether it binds directly to the phorboid receptors and serves as an endogenous agonist.

What might be the normal function of phorboid receptors and their putative endogenous ligand? We postulate that this effector system could play a role during embryogenesis by enhancing the outgrowth of new stem cell populations. In the adult this same system might enhance expansion of stem cell populations during hyperplasia, wound healing, and regeneration. In all of these situations it might be necessary to transiently inhibit terminal differentiation so as to expand the proliferative population, and then at a later time turn off this effector system to allow terminal differentiation to proceed and to return to a stable state of tissue renewal. Thus, the inhibitory effects on differentiation and growth stimulation produced by the phorbol esters in experimental systems might occur, under host control, via the phorboid affector system during normal development and physiologic states. During tumor promotion aberrant stem cells (generated during initiation) might undergo preferential clonal expansion as a result of excessive stimulation of the phorboid receptor system (see Fig. 3). This model has obvious implications in terms of the normal control of proliferation of stem cell populations and the possible role of endogenous host factors as promoters of the carcinogenic process. If our hypothesis is correct, then it might be possible to develop analogues of the phorbol esters that could be used as pharmacologic agents to enhance normal tissue repair and to enhance the repletion of tissues with stem cells following trauma, radiation, or drug toxicity. Alternatively, it might be possible to design agents that would block the phorboid receptors and thus protect the host from certain endogenous or exogenous promoters.

Membrane Effects and Phospholipid Metabolism

The earliest responses of cells to the phorbol ester tumor promoters involve alterations in membrane function [23,25,28] (Table III), and this also appears to be the case with teleocidin [36]. The amphipathic polypeptides melittin [4] and delta hemolysin [41], which act by insertion into membrane lipid bilayers, thereby disturbing membrane structure, mimic some of the biologic effects of TPA. Some of these membrane effects occur within minutes and are not blocked by inhibitors of protein or RNA synthesis; therefore, they are mediated directly at the level of the cell membrane, presumably through the activation of preexistent enzymes or other

membrane-associated proteins. As discussed later, it is not yet clear whether the primary effect is exerted at the level of ion influx, allosteric effects, or post-translational activation of proteases [67], esterases [45], protein kinases, phospholipases, etc.

Exposure of cells to the phorbol ester tumor promoters causes a rapid increase in membrane phospholipid turnover. TPA stimulates the incorporation of ^{32}P and choline into membrane phospholipids; it also induces deacylation of phospholipids with release of arachidonic acid and an increase in prostaglandin synthesis [for review, see 23,25,28]. Recently. we discovered that within 5 min TPA induces the release of choline from the phosphatidyl choline fraction of C3H 10 T½ cells [28,42]. TPA did not induce the release of inositol from phosphatidyl inositol, and it did not enhance the methylation of phosphatidyl ethanolamine, although these effects have been seen with other agonists [43,44]. We also found that, although the calcium ionophore A23187 and EGF enhanced (^{3}H)-arachidonic acid release from prelabeled 10 T½ cells, and also enhanced prostaglandin synthesis, neither of these compounds enhanced choline release [28,42]. Thus different membrane-active compounds can produce different effects on phospholipid metabolism.

Figure 6 is a schematic representation of the effects of TPA and other compounds on phosphatidyl choline metabolism. Our results [23,42] suggest that TPA-

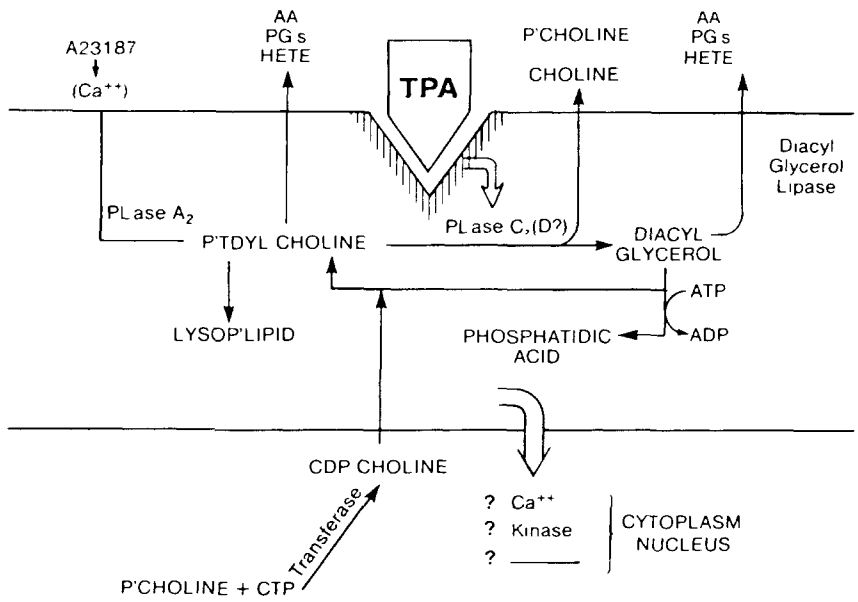

Fig. 6. Schematic diagram of effects of TPA and related compounds on phosphatidylcholine turnover. We postulate that the binding of TPA to specific cell membrane receptors activates phospholipase (PLase) C and/or D, resulting in the conversion of phosphatidylcholine (P'TDYL Choline) to diacylglycerol plus choline. Arachidonic acid (AA) is then released by diacylglycerol lipase; prostaglandins (PGs) and other AA metabolites are also formed. AA also may be released by the direct action of PLase A_2 on P'TDYL Choline. The calcium ionophore A23187 and melittin may induce AA release via the latter mechanism. P'TDYL Choline may be resynthesized via CDP choline, as shown. Presumably, during these biochemical transformations, a transmembrane signal to the cytoplasm and/or nucleus is generated ie, increased Ca^{2+} uptake or redistribution, activation of a protein kinase, or some other mediator. Reprinted from [23].

induced choline release is due to activation of an endogenous phospholipase C or D, although other mechanisms have not been excluded. TPA-induced arachidonic acid release and prostaglandin synthesis could be due to the subsequent action of a diacylglycerol lipase. Alternatively, TPA could have an independent effect on phospholipase A_2. The phorbol ester tumor promoters are the only known compounds that specifically induce the turnover of phosphatidyl choline. Thus it would appear that the turnover of this phospholipid plays an important role in the action of these tumor promoters. The studies of Wertz and Mueller [45], indicating that TPA enhances the activity of CDP choline transferase in bovine lymphocytes, are consistent with this conclusion.

Several questions remain unresolved with respect to the effects of the phorbol esters on phospholipid metabolism. Are these effects due to perturbation in the phospholipid substrate or to activation of membrane-associated phospholipases? Since it is known that these phospholipases are activated by Ca^{2+}, does TPA produce its effects on phospholipid metabolism by altering Ca^{2+} flux or intracellular distribution? (See below.) Does the phorbol ester-induced degradation of phosphatidyl choline generate metabolites that mediate subsequent events in this cascade? These mediators could include arachidonic acid metabolites (cyclooxygenase or lipoxygenase products), lysopholipids, phosphatidic acid, or diacylglycerol (see Fig. 6). Phosphatidic acid is of interest since it has been shown to be a calcium ionophore [46]. Diacylglycerol is also of interest since it has recently been shown that a protein kinase, which is Ca^{2+}-activated and phospholipid-dependent, is markedly stimulated by diacylglycerol [47]. Do the effects of the phorbol esters on phospholipid metabolism produce a generalized change in the physical properties of the lipid matrix of cell membranes, for example increased fluidity [23], which then alters the activities of a number of membrane-associated proteins and functions? Elsewhere, we have suggested that the role of dietary lipid in enchancing colon and breast cancer might be mediated by changes in membrane lipids in the target tissue, changes that are similar to those produced by tumor promoters [29]. Thus, much more work is required to clarify the role of lipid metabolism in tumor promotion.

Ion Flux

Within minutes of exposure of cells to the phorbol ester tumor promoters there are also rapid changes in Na^+ and Rb^+ (an analog of K^+) flux [48,49]. These findings have taken on increased significance with the accumulating evidence that several mitogens and growth factors have similar effects on Na^+ and $^{86}Rb^+$ flux [49,50]. Since the enhancement of $^{86}Rb^+$ flux is ouabain sensitive, it is apparently mediated via the membrane-associated Na/K ATPase. This is intriguing in view of the recent evidence of Spector and Racker [51] that phosphorylation of the β subunit of this enzyme may be altered in tumor cells, and that certain viral "sarc" genes code for protein kinases that may be part of a cascade reaction that leads to phosphorylation of the same subunit [52].*

Several findings suggest that alterations in Ca^{2+} uptake and/or intracellular distribution may play a role in mediating the cytoplasmic and nuclear events induced by TPA. The calcium ionophore A23187 mimics some but not all of the actions of TPA [53]. As discussed above, changes in Ca^{2+} flux might be associated with the effects of TPA on phospholipid turnover. TPA induces rapid effects on Ca^{2+} influx in chick embryo myoblasts [54]. TPA is mitogenic to lymphocytes, but only in the presence of Ca^{2+}, and this mitogenic effect is synergistic with the calcium

*Recent findings have questioned the validity of these results.

ionophore A23187 [55]. Changes in the flux of Ca^{2+} and other ions appear to play an important role in modulating differentiation [for review, see 56]. Ca^{2+} also plays an important role in modulating the growth of normal cells. In general, normal cells exhibit negligible or limited growth in media containing low Ca^{2+} concentrations (0.001–0.01 mM rather than 1.25 mM), and transformation by chemical carcinogens or viruses enhances the ability of cells to grow in low Ca^{2+} media [for review, see 57–59]. It is of interest that TPA can enhance the growth of cells in low Ca^{2+} medium [57–59]. Possible mechanisms by which TPA might alter Ca^{2+} metabolism include effects on Ca^{2+} flux [54,59]; increased release of mitochondrial Ca^{2+} stores into the cytosol, perhaps in response to increased Na^{+} uptake [49]; or increased levels of calmodulin. With respect to the latter possibility, it is of interest that there is evidence that cells transformed by viruses or carcinogens have increased levels of calmodulin [60].

Possible Role of Protein Kinases

It is apparent that much more work is required to determine the precise temporal sequence and the possible cause-and-effect relationships between TPA-receptor binding, alterations in ion flux, and stimulation of phosphatidyl choline turnover; and the relationship between these early membrane-associated events and the subsequent cytoplasmic and nuclear effects of TPA. In view of the current evidence that the viral "sarc" genes are protein kinases [for review, see 61,86], an attractive unifying mechanism to explain the mimicry of transformation by tumor promoters is that they activate somewhat similar endogenous protein kinases. Direct tests of this hypothesis with respect to the avian pp60 sarc protein kinase have been negative [62,63], but because of the complexities of the "sarc" gene system, these results do not rule out the general hypothesis. The search for novel protein kinase activities in cells treated with tumor promoters and various growth factors will, no doubt, be an intensive area of future research.

Tumor-Promoting Activity of Polycyclic Aromatic Hydrocarbons

Although the application of a single low dose of BP or other PAH carcinogen to mouse skin does not induce tumors unless this is followed by repeated applications of a tumor promoter, repeated applications of a PAH carcinogen will induce skin tumors [27]. This suggests that PAHs can have tumor-promoting activity. Therefore, we have recently studied the possibility that PAH carcinogens might induce cell membrane changes that are similar to those induced by the phorbol ester tumor promoters [64]. We have indeed found that the exposure of C3H 10 T½ cells to BP and certain other PAHs leads to a loss of EGF-receptor binding [64]. These and other results have led us to propose that binding of certain compounds to the cytosolic "Ah" receptor induces a pleiotropic program that includes not only increases in certain drug metabolizing enzymes but also changes in membrane structure and function [64]. Thus certain PAHs might be complete carcinogens because they not only are converted to metabolites that bind covalently to cellular DNA, but also they induce membrane changes that alter growth and differentiation.

Chromosomal Effects and Activated Oxygen

It has been suggested that tumor promoters act by inducing chromosomal aberrations and/or segregation thus converting a heterozygous recessive mutation established by the initiator to be expressed [65]. A few studies have shown that TPA can

induce sister chromatid exchange (SCE) as well as various chromosomal aberrations [65,66]. These effects have not, however, been highly reproducible between laboratories, often require high concentrations of the TPA, and sometimes are seen only with certain batches of TPA (raising the question of a contaminant). Moreover, we know that when a population of cells is exposed to TPA the entire population responds within minutes or hours and the effects are usually reversible when the agent is removed [23,25]. In addition, the papillomas on mouse skin often regress when application of the tumor promoter is stopped [27]. Thus, chromosome aberrations or segregation could not account for the early effects of tumor promoters. It is true that the malignant tumors that appear much later on mouse skin are autonomous and often show chromosomal abnormalities. Thus if the in vitro chromosomal effects of TPA have any significance, I suggest that they relate more to late stages in the process such as tumor progression rather than to tumor promotion. Consistent with this possibility is the fact that, whereas the early stages of neoplasia and benign tumors are often associated with a diploid karyotype, tumor progression and highly malignant tumors are often associated with chromosomal abnormalities [93,94].

Troll et al [67] have demonstrated that TPA and teleocidin induce oxygen radicals and peroxides in polymorphonuclear leukocytes. The stimulation of arachidonic acid metabolism [45] and lipid turnover [67] by tumor promoters could generate highly reactive forms of activated oxygen [88] that could lead to lipid peroxidation and other toxic effects, including chromosomal damage. It is essential to determine whether such effects are confined to only certain cell types, whether they occur only under conditions of toxicity, and whether they can actually be associated with tumor promotion or progression on mouse skin.

Chemical-Viral Interactions

There are several examples in which initiating carcinogens, tumor promoters, or other chemical and physical agents interact synergistically with viruses in the carcinogenic process, both in vivo and in cell culture [for review, see 26,28]. Indeed, it seems likely that certain human cancers may be due to interactions between chemical agents and types of viruses that alone would have little or no oncogenic potential. This appears to be the case for liver cancer in Africa, nasopharyngeal cancer in Asia, and Burkitt's lymphoma [29,68].

We discovered that the transformation of rat embryo (RE) cells by an adenovirus is enhanced when the infected cells are grown in the presence of phorbol ester tumor promoters; EGF and melittin also enhanced adenovirus transformation [68]. TPA and EGF also induce the growth in agar of morphologically altered adenovirus-transformed RE cells [68], which may provide a useful in vitro model for studying the process of tumor progression.

Subsequent studies have revealed that TPA also enhances cell transformation induced by EBV virus [69], polyoma virus [70], and SV40 virus [71]. In addition, it accelerates the replication and cytopathic effects of adenovirus in human cells [72], enhances EBV replication and antigen expression in lymphoblast cell lines [69], and enhances the replication of mouse mammary tumor virus [73] and an endogenous murine xenotropic type-C retrovirus [74]. TPA also enhances the expression of markers of transformation in chick embryo fibroblasts transformed by RSV [23,62,63].

As emphasized previously in this review, it appears that the primary effects of the phorbol ester tumor promoters result from alterations in cell surface membrane structure and function. It is of considerable interest, therefore, that current evidence suggests that the transforming proteins coded for by several tumor viruses act at or near the cell surface membrane. Thus, the pp60 "sarc" protein of Rous sarcoma virus and the Harvey MSV p21 protein are located at the inner surface of the plasma membrane [75,76]. The Abelson MuLV p120 protein [77] and the polyoma middle T antigen [78] also appear to be membrane associated. These findings suggest that alterations in membrane function play a central role not only during the process of chemical carcinogenesis, but also during maintenance of the tumor cell phenotype in cells transformed by oncogenic viruses.

It is unfortunate that in the past cancer research has been polarized into two camps, those in search of viruses as causes of human cancer and those in search of human chemical carcinogens. I suspect that much greater progress will be made if one takes the view that certain human cancers result from complex interactions between viruses and chemicals and that the final pathways by which both classes of agents produce cell transformation are quite similar.

A UNIFIED THEORY OF INITIATION AND PROMOTION

I would now like to return to the question of the mechanism of initiation and describe a unified theory of initiation and promotion. This theory attempts to explain carcinogenesis within the framework of normal development and differentiation and also provides a bridge to current theories about the origin and mechanism of action of certain tumor viruses.

Earlier in this paper I presented several reasons why I think that initiation of carcinogenesis does not involve simple random point mutation resulting from errors in replication at the sites of DNA damage. It should be stressed that several laboratories have found that the frequency of transformation of rodent cell cultures induced by radiation or carcinogens can be 10 times to several hundred times that obtained for the induction of mutations to specific markers such as drug resistance, even when both types of phenomena are scored in the same cell culture system [for review, see 23]. This discrepancy is even greater when one considers the likelihood that cell transformation occurs via a multistep process that is limited, therefore, by the joint probabilities of each of the successive steps. Thus the initial step induced by the carcinogen may occur with an even greater frequency than the net transformation frequency. Indeed, there is evidence that when exposure to chemical carcinogens [79] or radiation [80] occurs at low cell densities almost 100% of the exposed cells are capable of giving rise to progeny that are transformed. This result provides powerful evidence against random mutation, which usually occurs with a frequency in the range of 10^{-4}–10^{-8}.

We have previously postulated that the establishment of normal populations of stem cells involves gene rearrangements [for review of gene rearrangements, see 81,82] and that DNA damage by initiating carcinogens might induce, with high frequency, aberrant stem cells [23]. The casette model for the control of mating type in yeast [82] is a particularly attractive model for studying how gene rearrangements might be involved in differentiation and carcinogenesis. Some of the evidence favoring gene rearrangements rather than random mutations in the action of initiating carcinogens is summarized in Table IV.

TABLE IV. Mechanism of Action of Initiating Carcinogens: Evidence Against Random Mutation and Favoring Gene Rearrangements*

A. High efficiency of cell transformation in vitro speaks against random mutation
1. With chemicals or x-ray; in 10 T½ or hamster embryo cultures
2. Transformation frequency > > random mutation
3. "Initiation" of transformation can approach 100% efficiency
B. Characteristics of gene transposition
1. Occurs in prokaryotes and eukaryotes (ie, cassette model in yeast, immunoglobulin synthesis)
2. High specificity and efficiency
C. Models suggested from the action of retroviruses
1. Multiple "sarc" genes exist in normal vertebrate cells
2. Transformation by retroviruses resembles gene transportation

*For details related to points A and B, see text and [23]. For reviews on gene transposition, "sarc" genes, and retroviruses, see [23, 81, 82, 86].

The subsequent role of tumor promoters could be to enhance the outgrowth of these aberrant stem cells, as well as to "switch on" their abnormal programs of differentiation, just as normal growth factors might induce normal stem cells to grow and express their specialized functions. As already discussed, presumably the phorbol ester tumor promoters accomplish this by binding to and usurping the function of receptors normally occupied by endogenous factors that control stem cell replication and differentiation. Following repeated exposure of initiated cells to a tumor promoter, a neoplastic population might eventually emerge that grows autonomously in the absence of the promoter, perhaps due to further changes in genome structure. It is also possible that the mechanism by which the transformed phenotype is eventually "locked in" with respect to constitutive expression occurs by mechanisms (yet to be discovered) similar to those that provide stability to normal states of differentiation. Recently, Cairns [83] has also proposed that carcinogenesis involves gene rearrangements, although he arrived at this conclusion on the basis of somewhat different evidence.

A major challenge to future research in carcinogenesis is to identify the specific host genes involved in the transformation of cells by chemical and physical agents and to utilize recombinant DNA techniques to analyze the state of integration and/or expression of these genes in normal and carcinogen-transformed cells. Recent studies from the laboratories of R. Weinberg [84] and G. Cooper [85] suggest that the techniques of DNA transfection may prove to be extremely useful for such studies. Studies of the RNA sarcoma viruses have led to the concept that they arose by the recombination of retroviruses with specific "onc" genes (also called "sarc" genes or proto-oncogenes) endogenous to normal vertebrate species [86]. Infection of cells by these viruses leads to the integration of these genes into aberrant sites in the host genome, where they are expressed at high levels and thus lead to the transformed state [86]. It is possible, therefore, that the same "onc" genes are involved in the transformation of cells by chemical carcinogens, but that in this case DNA damage triggers rearrangements and/or switch-on of these genes in the absence of a virus vector. In collaboration with Dino Dina's laboratory, our research group is testing this hypothesis in carcinogen-transformed rodent cells [87].

MOLECULAR EPIDEMIOLOGY AND CANCER PREVENTION

Limitations of Current Approaches

From the preceding discussion it is apparent that, although major progress has been made, there are still important gaps in our understanding of the fundamental mechanisms involved in the carcinogenic process. But several concepts and methods have already emerged that provide new approaches to identifying potential human carcinogens and to improve the science of risk extrapolation.

Although conventional approaches in cancer epidemiology have provided a wealth of information, they have serious limitations in terms of identifying specific causal factors, particularly in human cancers that result from multifactor interactions. In addition, such studies are largely retrospective rather than predictive and, unless very large numbers of individuals are studied, they are not highly sensitive [1,3,89]. At the same time, although animal bioassays and the newly developed short-term tests are extremely sensitive and useful for detecting potential human carcinogens [1,3,89], there is a paucity of information on how such data can be extrapolated to humans. Indeed, the large amount of data now emerging from such laboratory studies has led to what I refer to as a "crisis in risk extrapolation" (Fig. 7).

Molecular Cancer Epidemiology

Obviously the solution to this problem will not come simply from more of the same types of studies. We believe that it is necessary to develop an entirely different type of methodology in which studies on human cancer causation combine epidemiologic methods with laboratory techniques in which specific biochemical and molecular parameters are measured in humans, in human tissues, and in various biologic fluids. We refer to this approach as "molecular epidemiology" [3]. The importance of this combined approach has also been stressed by others [12,91] and is discussed in greater detail elsewhere [3]. A variety of highly sensitive and specific laboratory procedures are now available that can be used as markers to assess specific human factors related to 1) genetic and acquired host susceptibility, 2) metabolism and tissue levels of carcinogen, 3) levels of covalent adducts formed

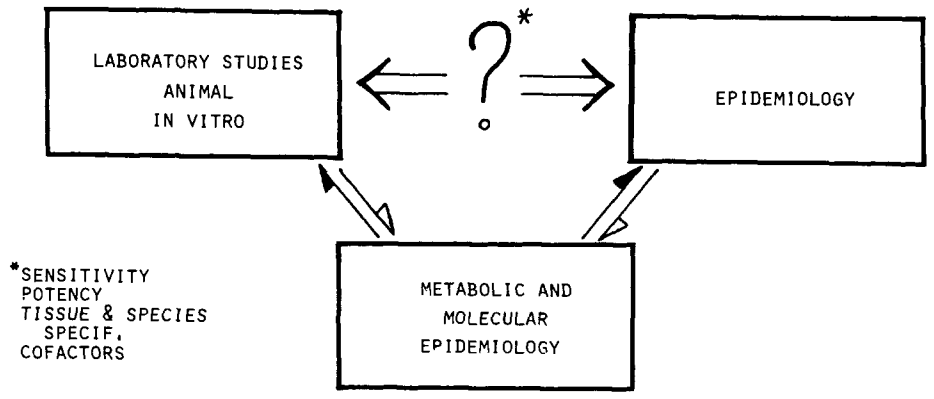

Fig. 7. The problem of risk extrapolation.

between carcinogens and cellular macromolecules, and 4) markers of early cellular responses to carcinogen exposure (ie, SCE, DNA repair, altered gene expression, etc) [3,91].

A particularly promising tool for studies on molecular cancer epidemiology has been provided by the recent development of highly sensitive immunoassays for carcinogen-DNA adducts [for review, see 3, 89, 92]. This approach might actually provide a tissue dosimeter of carcinogen exposure that would be much more valid than assays of ambient levels and total dosage, since it would take into account complex pharmacodynamic parameters, including metabolic activation of the substance in question. The BPDE-DNA adduct can be detected at the level of a few femtomoles in a few micrograms of DNA (or ~ 1 residue/10^7 nucleosides), a sensitivity that should be sufficient for epidemiologic studies. Possible sources of material for study include surgical specimens, autopsy material, skin biopsies, and peripheral blood white cells. It should also be possible to detect and quantitate the carcinogen-DNA adducts in the urine, as a result of their excision from DNA, thus greatly facilitating epidemiologic studies.

CONCLUSION

Thus, it is apparent that advances in basic research related to carcinogenesis have provided not only new insights into cancer causation but also potential tools for cancer prevention. Webster's dictionary defines "keystone" as, "a wedge-shaped piece at the crown of an arch: such a piece inserted last and locking the other pieces in place." Obviously, we have a long way to go before we can fit the keystone into place in our knowledge of carcinogenesis. I am confident, however, that the papers and discussions presented at this conference will put into place essential building blocks in the arch that constitutes our knowledge of cancer causation and prevention.

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