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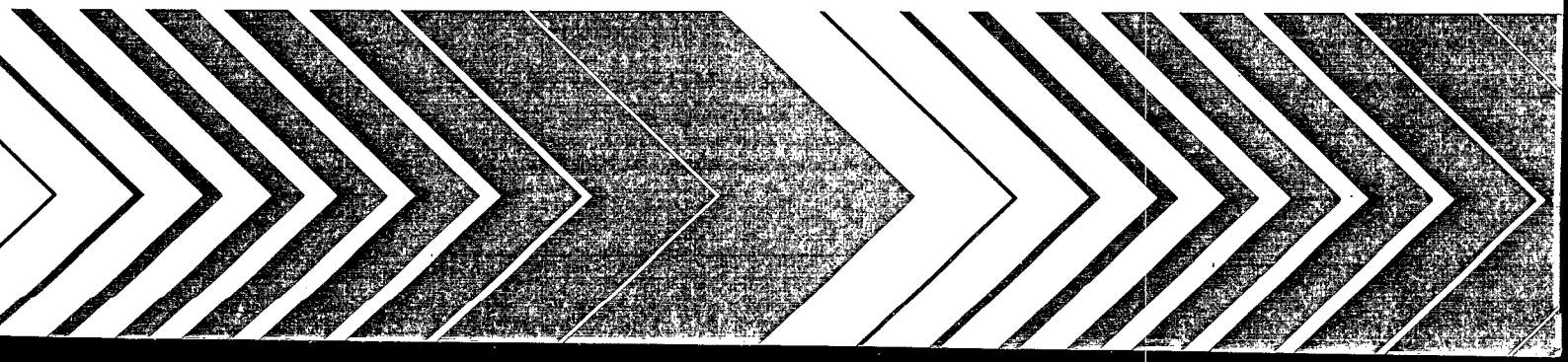
Report of the EPA Workshop on the Development of Risk Assessment Methodologies for Tumor Promoters



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REPORT OF THE EPA WORKSHOP ON THE DEVELOPMENT OF RISK ASSESSMENT METHODOLOGIES FOR TUMOR PROMOTERS

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NOTICE

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PREFACE

At present, relatively little is known about the mechanisms of promotion and the identity of promoters. However, available data suggest that promoters may have very different implications for risk assessment than carcinogens. On February 3-5, 1987, the U.S Environmental Protection Agency (EPA) Office of Research and Development sponsored a workshop in Bethesda, Maryland, on "Development of Risk Assessment Methodologies for Tumor Promoters." The purpose of this workshop was to identify and prioritize research to provide data that could be used in risk assessment of tumor promoters. During the two and one-half days of the workshop, thirteen expert panelists discussed the current state of the art in tumor promotion and developed specific research recommendations. Several observers were present to witness and join the discussion. This report summarizes the proceedings of the workshop.

EXECUTIVE SUMMARY

At a workshop sponsored by the EPA Office of Research and Development in February 1987, thirteen expert panelists discussed research needed to support the development of risk assessment methodologies for tumor promoters. During the two and one-half days of the workshop, the panelists exchanged current data on promotion, identified data gaps, and formulated general and specific research recommendations.

The panelists agreed that available data suggest that there are probably at least three stages of carcinogenesis - initiation, promotion and progression - and that there are agents that are associated predominantly with these three stages. Initiation was described as a sudden change probably involving DNA that is irreversible over a long period of time. There is a growing body of data suggesting that the initiation stage is relatively common and involves nonspecific damage to DNA. There is also evidence that there may be a spectrum of initiated cells that vary in their degrees of initiation and thus in their susceptibility to promotion. Promotion was defined as "the reversible selective clonal expansion of initiated cells and the reversible alteration of gene expression." A list of criteria for chemicals that can only promote was developed. Progression was defined by a majority of panelists as "an irreversible change in DNA towards malignancy."

The panelists agreed that the mechanism of promotion is not currently understood and they suggested that there may be several different mechanisms of promotion. Available data suggest that promotion is substantially different from initiation, and that traditional risk assessment models for carcinogens are not appropriate for promoters.

Promoters appear to show more extreme differences in species and strain responses than carcinogens. The panelists agreed that much more work needs to be done to understand these differences from a mechanistic standpoint. Epidemiological studies should be conducted to obtain human data, and existing epidemiological data on promotion should be examined as a potential source of information on human promoters. Although no agents have been unequivocally classified as human promoters, data indicate that several chemicals may be working as human promoters.

Available data suggest that promotion is reversible in the liver and skin, but currently there are not enough data to ascertain whether reversibility is characteristic of all promoters in all systems. There was concern that there may be synergism among promoters. Research is needed to study this phenomenon and to identify the kinds of promoters that are likely to interact.

There is a need to develop and validate statistical models for promotion and to develop data to test the models. The two-stage birth-death-mutation model, developed by Moolgavkar, Venzon and Knudson, was discussed at the workshop. The panelists agreed that it appears to provide a good theoretical framework from which to propose and interpret studies on promotion. Various approaches to validating the model were discussed, including an initiation/promotion/initiation protocol (Potter, 1981) using multiple doses of both the initiating and promoting agents.

The panelists agreed that not enough data are currently available to assess the risks of promoters, and that substantial research is needed in several areas, including:

- Mechanisms of initiation, promotion and progression, particularly data on dose-response and frequency of response.

- The behavior of promoters in humans. Epidemiological studies of promoters in humans are a high priority. The panelists suggested several populations for epidemiological studies.

- Development and validation of statistical models for initiation/promotion systems.

- The behavior of promoters in organs other than the skin and the liver.

- Interspecies differences in promotion.

- Expansion of the chemical data base for known and potential promoters. The panelists offered several suggestions of chemicals to study.

- Synergism among promoters.

- Development and validation of in vitro screening models for known experimental promoters. If successful, the in vitro approach should expedite the selection of chemicals for in vivo study.

1. INTRODUCTION

In recent years, there has been a growing recognition that risk assessment of tumor promoters is important but is precluded by a lack of data. In 1982, the EPA Office of Toxic Substances held a workshop to examine how information on promoter activity could be incorporated into risk assessment. Participants agreed that such information should be incorporated into risk assessment but could not offer the Agency guidance on how to do this. Recently, both the Science Advisory Board in its review of perchloroethylene and the EPA Office of Pesticides and Toxic Substances' panel on dioxin recommended that the EPA consider integrating promotional activity into the traditional risk assessment.

With regard to promoters, the current EPA Guidelines for Carcinogen Risk Assessment (Federal Register, 1986) state:

> Agents that are positive in long-term animal experiments and also show evidence of promoting or cocarcinogenic activity in specialized tests should be considered as complete carcinogens unless there is evidence to the contrary because it is, at present, difficult to determine whether an agent is only a promoting or cocarcinogenic agent. Agents that show positive results in special tests for irritation, promotion or cocarcinogenicity and no indication of tumor response in well conducted and well designed long-term animal studies should be dealt with on an individual basis.

While this approach was not felt to be wholly satisfactory, there was not enough consensus to develop an alternative approach in terms of either a qualitative judgement of how likely an agent is to be a promoter, or, quantitatively, of how great a cancer risk a promoter might pose for given levels of exposure.

As a first step towards risk assessment for tumor promoters, the EPA Office of Research and Development convened

a workshop on "Development of Risk Assessment Methodologies for Tumor Promoters" on February 3-5, 1987, in Bethesda, Maryland. The workshop provided an opportunity for expert scientists to pool their knowledge and set research goals to improve the scientific bases for risk assessment of promoters. The group was asked not to address specific chemicals, but rather to identify research concerning promoters as a class of substances, and to prioritize this research according to its impact and utility for risk assessment. The workshop was chaired by Dr. Albert (University of Cincinnati Medical Center), Dr. Langenbach (National Institute of Environmental Health Science), and Dr. Farland (EPA Carcinogen Assessment Group).

This report summarizes the discussion at the workshop. The first day of discussion focussed on current knowledge of promotion. Panelists exchanged data and identified data gaps. On the second and third days, general and specific research needs were identified.

The report is organized into 13 sections that reflect the major themes of discussion at the workshop. Each section has been synthesized from many different parts of the discussion that pertain to the topic. A list of the panelists and observers can be found in Appendix A. The agenda is provided in Appendix B, and premeeting comments prepared by the panelists can be found in Appendix C.

The reader should bear in mind that this report is based solely on the workshop discussion and panelist comments. As such, it reflects the opinions and data of a limited number of participants exchanged over a brief period of time, and therefore does not provide a comprehensive treatment of the various subject areas. The amount of information provided on a particular topic in this report does not indicate its relative importance, and there may be important aspects of tumor promotion that are not touched on in this report.

2. DEFINITIONS

Summary

The group agreed that there are probably at least three stages of carcinogenesis: initiation, promotion and progression. Panelists offered various definitions of these stages in their premeeting comments (see Appendix C). Some of these definitions were presented and discussed at the workshop. Discussion focussed on the definition of promotion.

Discussion

The group initially defined promotion as "the reversible expansion of initiated cells." Dr. Huberman questioned this definition. In the in vitro hamster embryo cell transformation system, treatment of initiated cells with tumor promoters such as phorbol esters produces transformed colonies. The majority of these colonies revert to a normal phenotype when the promoter is removed. Dr. Huberman argued that a promoter can directly convert an initiated cell from a normal to a tumor phenotype. Other panelists also thought that promoters may cause changes in genetic expression, so the group agreed to define promotion as "the reversible selective clonal expansion of initiated cells and the reversible alteration of gene expression."

Dr. Slaga cautioned that the definition must pertain to all organ systems. He felt that the interval between initiation and promotion is important in an operational definition. He suggested that the definition include a requirement that the promoter must still be effective after a reasonable time (at

least several months) after initiator application. This proposal was not discussed by the group.

Some panelists presented their definitions of initiation and progression. The group did not develop a definition of initiation, but a majority of panelists defined progression as "an irreversible change in DNA towards malignancy." Dr. Magee pointed out that this definition differs from the original usage of the term "progression" by Dr. Leslie Foulds (Foulds, 1975).

3. MECHANISMS OF INITIATION

Summary

There was a general discussion of possible mechanisms of initiation and characteristics of initiated cells. Several panelists offered different opinions and data. The mechanism of initiation was not clear. Initiation has been described as a sudden change that is irreversible for a long period of time. The commonly held view is that initiation is a relatively rare event in vivo that is best explained by a mutational event. However, recent evidence from several laboratories (Fernandez et al., 1980; Ethier and Ullrich, 1982; Clifton et al., 1984; Gould, 1984; Kennedy, 1985a; Terzaghi and Nettesheim, 1979; Stenback et al., 1981) suggesting that the initiating event is a common one challenges the original concept of an initiated cell as one that is mutated. An alternative mechanism suggested was that initiation may involve some irreversible differentiation in cells. How this can be brought about and what it really means is not clear. Conventionally, initiation is thought to be linked to genotoxic agents. It is not clear what kind of genotoxic event would result in an irreversible change in differentiation. The frequency of the initiation event would affect priorities for research. If initiation is common, then promotion and progression would be rate-limiting steps, suggesting that they should be given priority in research.

Discussion

Both Dr. Hennings and Dr. Slaga said they thought that initiation produces a whole spectrum of initiated cells that vary in their degrees of initiation. Some are more easily

promoted than others. One panelist suggested that initiation may have more than one stage. Another panelist thought that the dose has an effect on the type of initiating event that occurs.

Dr. Kennedy questioned the notion that initiation was caused by a single base mutation. She described research by Mulcahy and others at the University of Wisconsin (Mulcahy et al., 1984; Gould, 1984) who found that as few as twenty carcinogen-treated cells were sufficient to give rise to a cancer in a large percentage of the exposed animals. She also mentioned research by Terzaghi and Nettesheim (1979), Ethier and Ullrich (1982) and Stenback et al. (1981) who gave different doses to mouse skin over orders of magnitude and got approximately the same final tumor incidence when promoters were applied to all the animals. It has been concluded by many researchers that initiation must be a common event, even at low doses of carcinogen. If the initiating event is common, then it cannot be a single base mutation in DNA since this occurs at a very low frequency and would not be expected to occur in a high proportion of carcinogen-treated cells.

Dr. Pitot described research by Japanese investigators who claim they can identify initiated cells by an immunohistochemical marker, glutathione transferase-p. Only 1 in 10 or 1 in 100 of the cells identified as initiated expand if a promoter is applied, so Dr. Pitot questioned the researchers' assertion that a single change in a marker indicates initiated cells. He thought that one important characteristic of initiated cells is the ability to expand in the presence of promoter, i.e., the promoter selects for the expansion of initiated cells.

Dr. Kennedy said her studies at Harvard University's School of Public Health have indicated that initiation in vitro is potentially, though not usually, reversible. When protease inhibitors are given after radiation exposure and are then removed, no transformation develops at a later time. This suggests that some agents such as protease inhibitors can completely revert cells to a noninitiated state. Dr. Slaga said that protease inhibitors have very little effect on the initiation of skin tumors if given at the same time as the carcinogen exposure. The question of whether they can reverse initiation if given later in time has not been looked at.

Another issue that has not been addressed is whether any of the noncarcinogenic mutagens are pure initiators.

Oncogene activation was thought to play a role in initiation, in the sense that an amplification of a mutated c-Ha-ras protooncogene has been demonstrated to push cells toward malignancy. Dr. Slaga mentioned studies showing that activated c-Ha-ras will lead to papillomas in the skin if applied by skin scraping and followed by tumor promotion. Balmain's (Quintanilla et al., 1986) and the Millers' work (Wiseman, 1986) suggest that mutation of c-Ha-ras probably occurs during initiation with some chemicals (including DMBA).

4. MECHANISMS OF PROMOTION

Summary

There was considerable discussion of potential mechanisms of promotion. A number of questions were raised. Panelists offered data from their own experience. The group agreed that there may be several different mechanisms of promotion, and that the same promoter may have different mechanisms of action in different tissues and species. Not only the mechanism, but the type of action may vary. Some agents may act as a promoter in one model, and as a complete carcinogen in another. Promotion in some organ systems may have more than one stage. There were some data to suggest a structure-activity relationship for promotion by phorbol esters. Some panelists concluded, based on limited studies, that promotion might not involve the activation of some known oncogenes, although examination of other oncogenes should be undertaken.

Characteristics of the Lesions Produced by Promotion

The consensus was that the majority of the initial lesions that promoters induce in the skin and the liver are benign tumors. However, research suggests that there may be a big difference in papillomas, i.e., that the promoter brings out a spectrum of transformations ranging from benign tumors to those that have characteristics of carcinomas such as aneuploidy. Dr. Hennings offered data to suggest that different papillomas have very different abilities to progress to cancer. In studies at the National Cancer Institute (Hennings et al., 1985), promotion for only 5 weeks (DMBA initiation, TPA promotion) produced one-fourth as many papillomas as did promotion for 10, 20 or 40 weeks; however, the number of

carcinomas was the same regardless of the duration of promotion. Every carcinoma apparently arose from a papilloma. Dr. Slaga mentioned a recent study (in press, PNAS) that he and Drs. Aldaz and Conti had performed which examined benign tumors induced by promotion. They found that early in tumor promotion, most benign tumors are diploid. However, after about 40 weeks of promotion, with treatment twice a week, every benign tumor was aneuploid with areas that could be called carcinoma - in situ. Thus, even benign tumors could have characteristics of carcinomas (e.g., aneuploidy) if they are analyzed in detail.

Receptor Mechanism

According to Dr. Pitot, in the skin and the liver the major known promoters act through a receptor mechanism. There was a discussion of how one could demonstrate that a promoter is working by receptor binding (which implies the existence of a threshold). Since this mechanism is reversible and does not necessarily involve the tumorigenic process, the ability to demonstrate a receptor mechanism would have an enormous impact on the risk assessment of these agents, because it would imply the use of a completely different extrapolation model than low-dose linear extrapolation. A receptor mechanism would explain why some promoters are tissue-specific. The panelists offered suggestions about how to study receptor mechanisms (see Research Recommendations). Further discussion of this topic can be found in Section 6, Reversibility.

Cell-Cell Communication

Dr. Trosko described data suggesting the promotion may occur by blockage of intercellular communication (see Section 8, The Cell-Cell Communication Model).

Altered Differentiation

Dr. Huberman suggested modulation of differentiation by tumor promoters as a possible mechanism of action of tumor promoters. Studies at the National Cancer Institute (Hennings, Yuspa) showed that treatment of normal epidermal cells in culture with TPA induced terminal differentiation in about half the cells. The other half appeared to be unaffected and could then proliferate. If the initiated cells are among the unaffected population, this could be how TPA works (Yuspa et al., 1982). There are several "initiated" cell lines that give papillomas when put on an animal, none of which give a terminal differentiation response to TPA (Hennings et al., 1987a; Yuspa et al., 1986). These cell lines could provide an opportunity to study particular cell groups that respond differently from other cells.

Oncogenes

The panelists concluded that the data indicate that promotion does not involve activation of protooncogenes by mutation or transcription, although evidence in the skin is incomplete since oncogenes are activated in the promoted lesions but not in the skin itself. Work by Balmain in the skin (Quintanilla et al., 1986) and the Millers and others in the liver suggests that mutation of the c-Ha-ras gene probably occurs during initiation (Roop et al., 1986). Work in mouse liver is also inconclusive. Researchers at the University of Texas System Cancer Center (UTSCC) Science Park did not find any evidence of expression of several different oncogenes by promoters in mouse skin in vivo. Data suggest that oncogenes become activated during progression.

Dr. Pitot reported that studies at McArdle (Beer et al., 1986) did not show any transcriptional activation of the

protooncogenes c-myc, c-Ha-ras or Ki-ras in foci or nodules; however, such activation has been seen in carcinomas. With one exception (Wogan et al., n.d.), mutational activation of protooncogenes in rat hepatocarcinogenesis has been either nonexistent or occasionally transient. But mutational activation of the c-Ha-ras gene does occur in carcinomas of the mouse liver. It has also been shown in some mouse adenomas. Recently, fairly consistent transcriptional activation of the c-raf gene has been shown both in nodules and carcinomas in the rat liver. Some, but not all, foci exhibit transcriptional activation by in situ hybridization. Some foci show a lowering or absence of the gap junction protein by the immunohistochemical technology; others show normal levels. One question to be answered is whether the foci that are expressing c-raf also have low levels of the gap junction protein (D. Beer, M. Nevev and H.C. Pitot, unpublished observations).

Dr. Slaga reported that UTSCC Science Park researchers looked at the expression of several different oncogenes by promoters in mouse skin in vivo and did not find any change except from benign papillomas and carcinomas. He did not know of any studies that suggest that the oncogenes are involved in promotion in vivo.

Behavior in In Vitro Systems

Dr. Kennedy mentioned that in vitro systems provide dramatic evidence of the presence of a promoter through the shape of their dose-response curve. The curve is essentially a quadratic or linear quadratic in the presence of an initiator, but becomes linear in the presence of a promoter. She said that TPA and other agents can promote transformation in cells thirteen generations after initiation.

Stages of Promotion

The group discussed whether promotion may have more than one stage. This was felt to be a possibility in the skin, but Dr. Pitot indicated there was no evidence for it in the liver. (For discussion, see Hennings and Yuspa [1985].)

Spontaneous Initiation and Promotion

There was a brief discussion of evidence for spontaneous initiation and promotion. There is evidence that both phenomena occur, and these are factors that may impact risk assessment. In the absence of an initiating agent, the risk from a promoter will be a function of the background of spontaneous initiation or the initiating and promoting actions of the promoter. Dr. Hennings mentioned NCI data suggesting that spontaneous promotion was occurring. Papillomas were found to appear 2 to 3 weeks earlier if promotion was delayed for 5 to 20 weeks following initiation (Hennings and Yuspa, 1985). Dr. Pitot said that studies at McArdle Laboratory (Pitot et al., 1985) suggest that spontaneous initiation in the liver occurs up to 6 to 12 weeks of age, but not from that point up to a year of age. The number of spontaneous foci is three or four orders of magnitude lower than the number of foci induced by an agent. Spontaneous promotion also appears to be occurring, with a few initiated liver cells expanding in the absence of an exogenous promoter. The possibility of studying spontaneous initiation rates in human liver was dismissed because of the need to serial section the liver.

Memory for Promotion

Dr. Langenbach mentioned German studies (Furstenberger et al., 1983 and 1985) in which pretreatment with TPA followed by

an initiator some weeks later and then a stage II promoter increased the number of tumors. This may mean that there is a memory for TPA treatment. In similar experiments, Dr. Slaga found that such memory does not appear to have anything to do with cell proliferation since the pretreatment time is longer than the proliferative response by TPA (Slaga, unpublished results).

Structure-Activity Relationships

Dr. Rosenkranz mentioned a recently completed study at Case Western Reserve University School of Medicine that suggested some structure among the PAHs that appeared to correlate with promoting ability. Thus, there appear to be structural determinants that contribute to promoting ability. He said a study of the relationship between structure and promoting activity would require at least 50 or 60 chemicals, and recommended the list of chemicals compiled by Upton et al. (1984) as a starting point. However, the lack of established negative chemicals is a problem.

Pure Agents

The panelists discussed whether there are any known agents that act purely as a promoter or an initiator. Such agents would be extremely useful for risk assessment-related research. The group agreed that it is theoretically possible to determine experimentally whether something is acting as a initiator or promoter, but some panelists felt this might be difficult without pure initiators or promoters.

In the liver, phenobarbital and dioxin may be pure promoters since they have shown no evidence of initiation. However, dioxin has a very long half-life, which makes it difficult to study because a single dose is effectively a continuous dose.

In the skin, stage-specific agents can be identified. Urethane is an initiator in the skin, but is a complete carcinogen in other tissues. Chrysarobin and benzoyl peroxide are fairly pure promoters in the skin. Likewise the diol epoxide of benzopyrene can be considered a pure skin tumor initiator.

The group was unable to identify compounds that were pure initiators or promoters in all organ systems in which they had been tested. Likewise, it is difficult to identify nonpromoting chemicals due to organ/species differences. Given the present lack of knowledge about promoters, there is a risk that a nonpromoting chemical in one organ/species may be active in another.

Cytotoxicity

The relationship between cytotoxicity and promotion was discussed. While acknowledging that some promoters probably act without cell killing, Dr. Trosko thought that anything that was cytotoxic in the liver would also be a promoter at high enough doses. He pointed out that any agent that is a mutagen not only damages DNA but also kills cells at the appropriate dose, i.e., is cytotoxic, and any agent that can induce cytotoxicity, which would then force compensatory hyperplasia, can act as a promoter. So he thought it was important to acknowledge cytotoxicity regardless of the mechanism by which an agent kills cells.

There was some question about chemicals that promote because they are highly cytotoxic. The cytotoxic effects might occur before all initiated cells have been expressed, thus the maximal effect may not occur following initiation.

Dr. Hennings said that, in the skin, virtually all promoters produce hyperplasia, perhaps as a result of

cytotoxicity. Some promoters may work by a selective cytotoxicity. But there is no cytotoxicity in the liver with phenobarbital and dioxin, and there probably is no direct cytotoxicity by these compounds in other systems. So cytotoxicity may be promoter-specific.

The effect of substituting a tissue-damaging agent such as turpentine for a promoter in an initiation/promotion/progression protocol has not been studied.

Promoters as Irritants

The panelists discussed the fact that all known skin tumor promoters were found as irritants. They agreed that nonirritant promoters should be identified. Dr. Albert pointed out that irritation is not a characteristic of liver promoters.

Promoters as Anti-initiators

Dr. Trosko mentioned that some of the best studied promoters - PCBs, PBBs, DDT, BHT and phenobarbital - can, in some circumstances, act as anti-initiators also. If given before the carcinogen, they protect the animal; if given after, they promote (Williams and Weisburger, 1986).

Chow Diet as a Promoter

Scientists at McArdle have found that the normal chow diet is an effective promoting agent in the liver. They speculate that this may be due to the plant estrogens in the diet, which vary with the time of year. Semisynthetic diets eliminate much

of this problem (S. Hendrich and H.C. Pitot, submitted for publication).

Promotion by Saline?

The panel discussed whether saline is a promoting agent. Dr. Kennedy provided data to suggest that saline instillations could lead to promotion in the lung (Little and Kennedy, 1982; Shami et al., 1982 - see also Human Studies in Section 11, Species Differences/Human Studies). Dr. Pitot did not think saline could be considered to be a promoter. He thought that the apparent promoting activity of saline was due to an alteration of the hormonal environment which leads to an alteration of gene expression. He said there are many agents that are not considered to be promoters, but that change the internal environment in a way that creates effects very similar to promotion. Dr. Homburger pointed out that the instillation of saline or anything else into the hamster lung cannot be compared with human response. Unlike humans, the hamster takes saline in without any general response - no adrenal enlargement, hormonal change or struggle. He said that it is important not to automatically label something as a promoter just because there is an increased tumor incidence when the substance is administered following exposure to a carcinogen. For example, in recent experiments with guinea pigs (McFadden et al., 1986), the retention of small particles of asbestos was increased by the inhalation of cigarette smoke. This could potentially enhance the carcinogenic effect, but it would not be promotion.

5. CHARACTERISTICS OF PROMOTERS

Summary

The panelists discussed two questions:

1. What data would allow us to determine that a chemical has the ability to promote?
2. What data would allow us to determine that a chemical has the ability only to promote?

Based on this discussion, the panelists developed a list of criteria for chemicals that can only promote. These criteria would constitute the weight of evidence for a finding that a chemical essentially only promotes.

Criteria for Chemicals that Can Only Promote

1. The maximal effect follows initiation.
2. There is an experimentally measurable threshold.
3. The effects at both the cellular and gene level are reversible at early stages.
4. There is no covalent binding to DNA.
5. In many cases, a receptor mechanism mediates the effect.
6. Promotion may occur in the absence of cytotoxicity, but certain forms of promotion may involve a cytotoxic mechanism.
7. There is selective clonal expansion of transformed cells.

Another suggested criterion to add to the list was "Causes decreased gap junction function characteristics." There was concern as to whether the definition for promoters eliminates

other types of epigenetic carcinogens as defined by G. Williams and J. Weisburger, i.e., chemicals that may cause gene amplification or gene rearrangement that also cause cancer but are not really initiators although they may act as progressors.

Dr. Albert argued that another criterion for promoters is the induction of benign tumors that take a long time to go to carcinomas. Dr. Pitot objected on the basis that it is difficult to define a "benign tumor" morphologically. Morphology cannot distinguish between a lesion which is still reversible and one that is permanent.

Are Promoters Carcinogens?

The group discussed whether promoters are carcinogens. Dr. Pitot argued that all known promoters are carcinogens because they cause an age-specific increase in neoplasms. He said that any agent that results in a neoplasm following application likely has promoting action. Dr. Albert disagreed. He said that promoters serve to expand the cell population at any early stage of transformation before the cells are malignant, and it is in that expanded cell population that progression toward malignancy occurs. Dr. Langenbach said that the consensus at an NIEHS meeting in the fall of 1986 was that with the information currently available promoters should be considered as a class of carcinogens. Dr. Trosko said it was a question of whether initiation, promotion and progression are discrete stages, but not enough was known about mechanisms to determine this.

Dr. Slaga pointed out that all promoters that have been studied in detail show some carcinogenic activity; however, they do not generally show a dose-response. This suggests that a finite number of spontaneously initiated cells will saturate

at fairly low doses. Dr. Pitot pointed out that TCDD, phenobarbital and saccharin do show a dose-response over a relatively narrow range of doses.

Do Promoters Cause Genetic Damage?

The panelists discussed whether promoters cause genetic damage. Dr. Krewski thought that the notion of a threshold was inconsistent with a genetic component associated with promotion. However, some panelists thought that the possibility that some promoters can cause genetic damage could not be ruled out unequivocally. Dr. Huberman pointed out that Dr. Peter Cerutti and others still assume that genetic damage is an important component of tumor promotion. Dr. Pitot said that data suggest that the known liver promoters do tend to increase DNA synthesis, at least transiently. The panelists agreed that promoters may cause the reversible alteration of gene expression and included this in the definition of promotion (see Section 2, Definitions).

Are Promoters Initiator-Specific?

The question of whether promoters are initiator-specific was left open. Data suggest that promoters are not initiator-specific in the liver and skin, but are in the lung adenoma model using BHT as the promoter (Witschi and Lock, 1978).

6. REVERSIBILITY

Summary

The panelists discussed whether reversibility - which implies a threshold - is inherent in the definition of promotion. This concept has major implications for risk assessment. If promoters have thresholds, then a no-effect level could theoretically be demonstrated. The panelists agreed that, while some promotion appears to be reversible, there are not enough dose-response data to ascertain whether reversibility is characteristic of all promoters in all systems. It is possible that reversibility may be complete in the liver, but not in the skin, based on the recurrence of promoted lesions.

Existing Data

The panelists discussed the degree to which reversibility had been studied in various organ systems. In the liver, reversibility has not been tested thoroughly with numerous compounds. It has been tested with phenobarbital and the choline-deficient diet, and AAF as a selecting agent. In the skin, reversibility is more difficult to study than in the liver because initiated cells cannot be detected in the skin, which is important for quantification. Of the various skin models, only the CD1 mouse strain has been extensively studied for reversibility. Initially there is complete reversibility; however, if promotion is stopped after about 4 to 8 weeks, some of the initiated cells do not revert but continue to develop into carcinomas (Verma and Boutwell, 1980). Other studies, described by Dr. Slaga, also indicate there may be a residual effect. In these studies, promotion was stopped after about

4 to 6 applications and then restarted 6 months later. Tumors appeared much more quickly following the second promotion, although it was not possible to say whether these were from the same foci. Thus, reversibility may not be complete in the skin. Other organ systems, such as the bladder or the breast, have not been adequately characterized to determine whether promotion entails reversibility.

Mechanisms of Regression

The panelists discussed possible mechanisms of regression. One panelist suggested that perhaps papillomas regress to micropapillomas rather than to initiated cells. Dr. Slaga said that, in his progression studies, about 30% of the benign tumors regressed. After promotion was stopped and a progressor applied, papillomas decreased about 50% in size before becoming squamous cell carcinomas.

In the liver, the mechanism may vary from one system to another. In the Solt-Farber liver model, hyperplastic nodules are produced at the expense of the normal liver, with remodelling and loss of cells when promotion is stopped. In other systems (Pitot's, Shulte-Herrmann's), it appears that cells in the promoted foci die in the absence of the promoter. Thresholds in the liver do not appear to be pharmacokinetically determined, since 80 to 90% of the ingested material reaches the liver.

Dr. Trosko suggested a model for promotion that could account for reversibility. In this model, promotion is not a one-hit event in which the promoter blocks the gap junction, preventing intercellular communication. In the absence of a promoter, normal cells suppress the phenotype of premalignant lesions. When promoters block the gap junction, the

interactive suppressing effect is also blocked, resulting in clonal expansion. (The cell-cell communication model is discussed further in Section 8.) Dr. Trosko suggested that models be developed that consider intercellular phenomena.

Demonstrating the Existence of a Threshold

There was doubt about whether the presence or absence of a threshold can be ascertained experimentally for promoters. Theoretically, if promotion is occurring by a threshold mechanism and the equilibrium constant of the ligand is known, the actual concentration of the threshold level could be predicted. Reversibility might be more easily studied. For example, the biochemical effect on gene expression of the compound is reversible and could be measured.

There was some discussion of how in vitro systems could be used to describe reversibility. Promoters do show a dose-response and reversibility in vitro. In Dr. Huberman's experience, about 80 to 90% of the phenotypic changes observed following promotion in vitro are reversible.

7. THE TWO-STAGE BIRTH-DEATH-MUTATION MODEL

Summary

The panelists discussed a two-stage, birth-death-mutation model, developed by Moolgavkar, Venzon and Knudson (M-V-K), that incorporates the concepts of initiation, promotion and progression (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981; Moolgavkar, 1986). This model can potentially be used to provide quantitative predictions of risk at various doses of initiators and promoters. However, the model must be validated before it can be used in risk assessment. Specifically, data are needed on normal cell growth, cell kinetics at the proliferation stage, and mutation rates and tumor occurrence as a function of dose. At present, the model provides a theoretical framework with which to propose and interpret experiments. The panelists agreed that it would be worthwhile to perform studies to validate the model and test the biological notions of initiation, promotion and progression that it incorporates. Several research suggestions were offered.

Description of the Model

The model was presented by Dr. Krewski. It is a stochastic birth-death-mutation model that involves only two stages relating to mutational events. Unlike the multistage model, the M-V-K model explicitly incorporates information on the kinetics of tissue growth and differentiation. It seems to be consistent with much of the experimental and epidemiological data that are currently available on carcinogenesis.

The model assumes three possible fates for normal stem cells: death, division into normal progeny, or mutation resulting in one normal daughter cell and an intermediate or

initiated cell. Likewise, the population of initiated cells can either divide, die, or undergo a second mutation to produce a fully transformed malignant tumor cell along with another intermediate cell (see figure).

Under this model, the age-specific incidence (I) for cancerous lesions at time (t) is:

$$I(t) = u_1 u_2 \int_{v_o}^{t} x(s) e^{(a_2 - b_2)(t-s)} ds$$

where:

$x(s)$ is the number of normal cells in the tissue at time s.

u_1 and u_2 are mutation or DNA damage rates for normal and intermediate cells, respectively.

a_2 and b_2 are the birth and death rates, respectively, for intermediate cells.

(The birth and death rates of normal cells [a_1 and b_1 respectively] do not appear in I(t) since the number of normal cells [X(s)] is assumed to be sufficiently large so as to constitute a deterministic process.)

The product of the mutational rates determines overall tumor incidence. The factors following the integration sign involve tissue growth, cell proliferation, and cell necrosis; they determine the shape of the curve.

In terms of this model, initiator, promoter and progressor can be defined as follows:

An <u>initiator</u> is a substance that increases the rate at which the first mutation occurs, i.e., it increases u_1. If it is a genetic lesion, it may be reasonable to assume that anything that increases u_1 may also increase u_2, although the second rate of increase may be less than the first. If this is so, then prolonged application with an initiator may result in complete carcinogenesis.

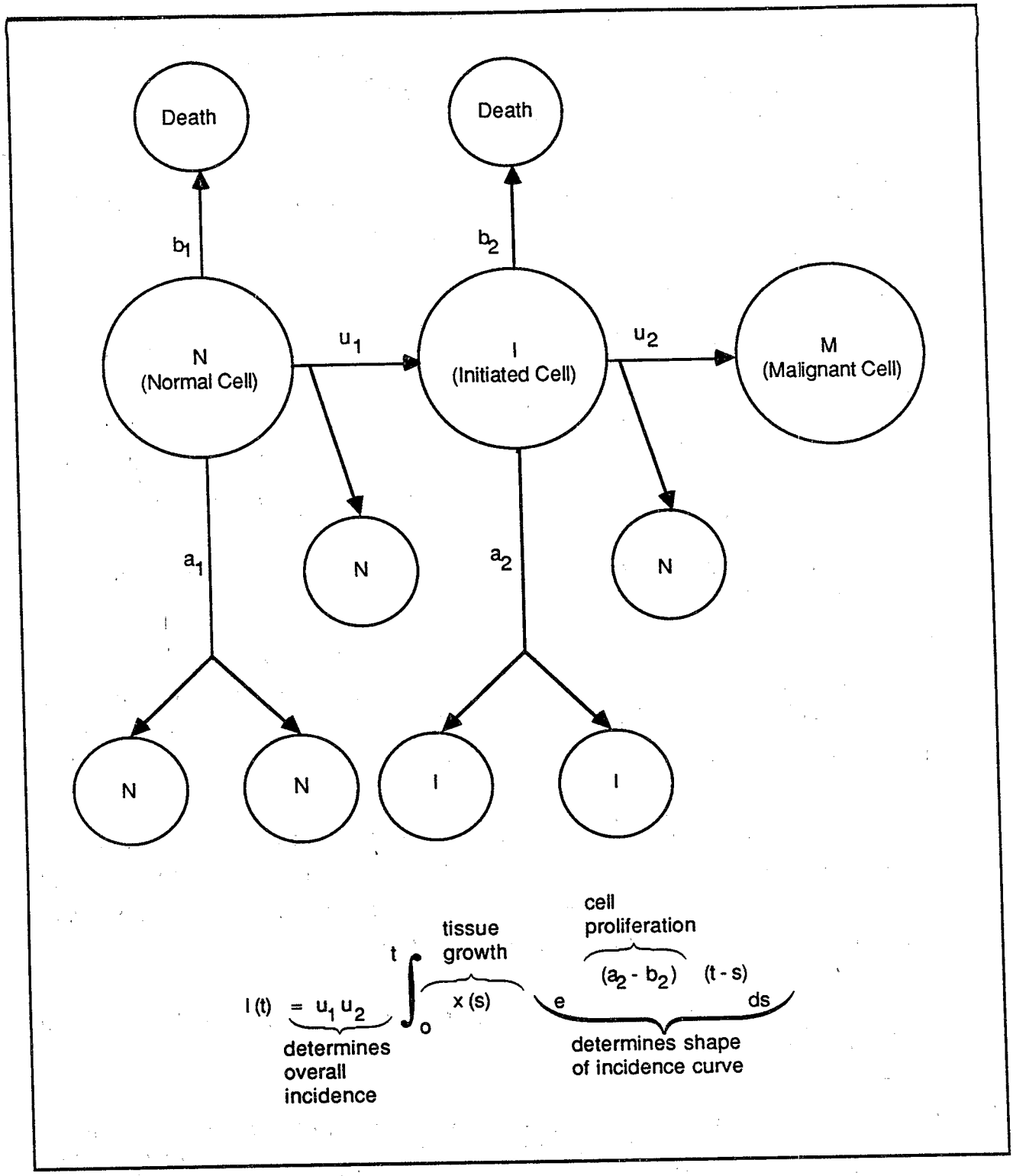

Figure 1. Moolgavkar-Venzon-Knudson Model.

A promoter is a substance that increases the pool of intermediate cells available for subsequent malignant transformation, i.e., it increases the birth rate of the intermediate cells (a_2) and/or decreases the death rate (b_2) so that (a_2-b_2) is positive. Promotion is assumed to involve a reversible nongenetic mechanism such as recurrent cytotoxicity or stimulation of cell proliferation or, possibly, the preclusion of terminal differentiation (i.e., the death rate of intermediate cells would be reduced). Thus, a_2 and b_2 are probably not linear and have thresholds. The group agreed that the intermediate or initiated cell in this model should not be considered as a neoplastic lesion. They did not feel it appropriate to refer to an expanded colony of such intermediate cells as a neoplastic change; however, the group agreed that this expanded colony was not "normal."

The panelists agreed that, in this model, a progressor is a substance that increases u_2.

A complete carcinogen is a substance that increases the rates of both the first and second mutations. The group agreed that complete carcinogenesis does not necessarily involve promotion. Thus, a complete carcinogen could involve either initiation and progression alone or all three stages.

In this model, if the mutation rate per initiated cell division ($u_2/[u_2+a_2]$) is considered to be a constant, then an agent that increases the proliferation of the intermediate cells must also increase the mutation rate for the second stage. If this mutation rate is a constant, then substances that possess promotional activity within the context of this model may also demonstrate some potential for progression.

The age-incidence curve does not involve the birth and death rates of unaltered cells (a_1 and b_1) because the original tissue mass can be thought of as being sufficiently large to be described as a deterministic rather than a stochastic process. All that enters in is the number of cells in that tissue as a function of time.

Validating the Model

Data Requirements

The panelists discussed what data are necessary to validate the model and how they could be obtained. The first data requirement is the growth rate of the normal cells [x(s)] in the tissue of interest as a function of age. This can be obtained fairly readily from studies that are separate from the bioassay. Second, information is needed on the birth and death rate (a_2 and b_2) of the intermediate (or initiated) cells. This could be obtained from laboratory assays for promotion. Third, information is needed on the transformation rates for initiation and progression (u_1 and u_2). This, in conjunction with the previous data, could be obtained from a 2-year rodent bioassay. Possibly, an IPI protocol might be needed to factor out the progression step. To fully examine how mutation rates vary with dose, a bioassay would have to be conducted with various levels of exposure to the initiator. More work needs to be done by statisticians to determine exactly what kind of data are needed to estimate specific parameters in the model and, in particular, how to separate u_1 and u_2.

I(t) is the time rate of appearance of lesions in the bioassay. These data could be obtained by (1) counting lesions in the skin, (2) using serial sacrifices in the liver, (3) assuming that the lesion of interest is rapidly fatal so that the survival time of the animal serves as the proxy for the actual time to tumor induction, or (4) assuming that death as a result of tumor occurrence in a bioassay is independent of death from competing causes, in which case time to tumor could be separated out statistically.

If all the other parameters of the model are known, then the rate of spontaneous initiation can be determined from the rate of spontaneous tumor formation in the unexposed controls.

There was some discussion of how partial hepatectomy in the liver would affect the variables in the model. This was not resolved. Dr. Krewski thought that data on all variables would have to be obtained under similar conditions. This could be a problem since partial hepatectomies are not normally performed in rodent bioassays. Dr. Pitot thought that partial hepatectomy would change only u_1 in his system since many studies have shown that initiation in the liver and other tissues will not occur in the absence of cell division. Dr. Travis argued that, since partial hepatectomy only increases cell division, it would affect the birth rate (a_1 and, probably, a_2) and $x(s)$ but not u_1.

Research

The group discussed past, present and future studies that may help to validate the model. One panelist said that several initiation/promotion/initiation (IPI) studies have shown a dramatic increase in the crop of malignant lesions observed at the end of the study. This tends to confirm the idea that the second initiator enhances the mutation rate, u_2, of the promoted pool of initiated cells. Dr. Krewski mentioned that the effects of changes in the dosing pattern over time on carcinogenic risks are currently being investigated.

Dr. Travis described recent research performed at Oak Ridge National Laboratory to validate the model. In this study, background liver cancer rates in rats as a function of age [I(t)] were obtained from NTP data down to about one-tenth of a percent. The growth rate of the liver as a function of age

[x(s)] was found from the literature. The term (a_2-b_2) was estimated from mitotic rates in the literature. The terms u_1 and u_2 were both assumed to be equal and constant with age. The model was run using these data, and it exactly reproduced the age-specific liver cancer rates in rats. Dr. Travis said the similar studies would be done for mice and humans.

He described plans for a study with tetrachloroethylene, which has been shown to be carcinogenic in mice. Data on the dose-dependency of the increase in cell turnover rate are available from the animal bioassay. Scientists at Dow Chemical Company have measured the increase in mitotic rate as a function of applied dose to the liver. Dr. Travis has estimated the increase in mitotic rate as a function of effective dose to the liver and found that it increases linearly above a no-effect-level threshold. Dr. Travis plans to enter these data into the model. He will assume that tetrachloroethylene has no genotoxic effects, thus u_1 and u_2 will be equal to the transition probabilities associated with the background cancer rate. Using these data, the model should predict the age-specific incidence of cancer from the tetrachloroethylene bioassay. If so, this would suggest that tetrachloroethylene is working solely through a promotional mechanism.

Dr. Travis recommended that similar research be conducted to validate the model, i.e., obtain (from the literature or studies) background cancer rates, mitotic rates as a function of age, and increased cell turnover rates as a function of dose; run the model with these data to see if it predicts the observed cancer bioassay rates. (Cell turnover rates provide a measure of the turnover rates of normal cells and not foci; this approach assumes that these rates are the same.) This model approach could be used, for example, with the hamster lung. Moolgavkar has used this approach for the breast,

although more work could be done on this tissue. Dr. Travis pointed out that the model assumes that the growth rates of foci (a_2-b_2) are time-independent. He felt that the rate of foci growth would increase with time, and suggested that experiments be done to look at livers at different times to see if their volumes increase at a different rate.

Dr. Trosko proposed an IPI protocol (Potter, 1981) to validate the model. According to the model, the pool of promoted cells should vary depending on the duration of promotion. One way to validate it would be to conduct an IPI study in which groups of animals are initiated with the same dose of initiator, promoted for various lengths of time, and then exposed to the same dose of a new initiator. Dr. Trosko suggested using X-rays as the second initiator because this would eliminate complications of metabolism or selective mutagenicity of the cells. Dr. Hennings said this research had been performed at the National Cancer Institute but the data have not yet been published. NCI scientists initiated with DMBA, promoted for 5, 10 or 20 weeks, and then injected i.p. with urethane. Response to urethane was best with the shorter promotion (Hennings et al., 1987b). Similar results were obtained when 4-nitroquinoline-N-oxide was applied topically to papilloma-bearing mice.

Dr. Krewski suggested the following protocol to model dose dependency. Initiation in the chronic bioassay with three doses (e.g., 0, 0.5 and 1, where 1 is the maximum tolerated dose) in a single application, followed by chronic exposure to the promoter at three doses (e.g., 0, 0.5 and 1). There are thus nine possible exposure combinations of initiator and promoter. This would provide dose-response data for the initiator and promoter.

If u_1 and u_2 are the same, an IP protocol would be sufficient to estimate the product of the two rates and hence their common value. If u_1 and u_2 are not the same, a second initiator (or progressor) would be essential to separate out the rates at which the two mutations occur. The IP protocol described above could be expanded by including a single exposure to varying levels of a second initiator following promotion. The second initiator may or may not be the same as the first.

Smaller experiments could also be conducted. For example, if spontaneous initiation is occurring at a sufficient rate for the promoter to be effective, then the initiation step could be omitted.

Saccharin in the bladder was mentioned as a system to study to validate the model. Cohen and Ellwein have been doing work with the saccharin data base. A critical review of their work might help define further research in this area.

8. THE CELL-CELL COMMUNICATION MODEL

Summary

Dr. Trosko presented a model for promotion involving cell-cell communication. He also described a new technology - the scrape loading/dye transfer assay - that has potential for testing mechanisms of promotion at the cellular level (El-Fouly et al., 1987).

Model Description

In the cell-cell communication model, promotion (i.e., selective clonal expansion of initiated cells) occurs as a result of removal of the suppressive contact-inhibiting effects of the normal neighboring cells (Trosko et al., 1983). Removal can be effected in a number of ways, including wounding, surgery, physical irritation, and placement of a solid, such as plastic or metal, next to initiated cells. The cell-cell communication model introduces a higher order of biology into the M-V-K two-stage model because the phenotype and future of the initiated cell depend totally on the communication properties of the normal neighbors.

In this model, promoters may also cause normal cells to proliferate. However, normal cells go into terminal differentiation after proliferation. With initiated cells, once the critical mass of the initiated cells gets large enough, the suppressing effects of the normal cell neighbors are diluted out.

Intercellular communication of molecules below 1,000 daltons is mediated by gap junctions. These structures are

found in virtually all normal cells in every organ. All the critical molecules and ions below 1,000 daltons are in equilibration for cells coupled by gap junctions. Gap junctions are modulated by drugs, food additives, nutrients, endogenous growth factors, biological toxins, pollutants, neurotransmitters, hormones, heavy metals and several oncogenes (Trosko et al., in press).

Scrape Loading Assay

Dr. Trosko described a new technology - the scrape loading/dye transfer technique - which he felt had potential for testing mechanisms such as thresholds, reversibility, synergisms and antagonisms that have been speculated in the animal promotion model. Scrape loading is an extremely simple technology that can be used with any animal or human cell. Human cells and approximately 75 different cell strains and lines have been tested with this technology.

In this technology, cells are grown to confluence, which mimics the normal situation in solid tissues. Then two dyes - lucifer yellow and rodamine red dextran - are applied to the cells. The yellow dye is a small molecular weight dye that can easily pass through gap junctions once it penetrates the cell. The red dye is too large to pass through gap junctions. Normally, the dyes will not pass through the cell membrane. However, the next step is to scrape the cells with a toothpick, which simulates wounding. The dye enters the cells along the wound line where the membranes are temporarily disrupted. The membrane heals within milliseconds, trapping the dye in the cells along the edge. The cells are then washed, immediately put under a fluorescent microscope with two filters, and photographed. At this stage, both dyes can be seen at the edge. The cells are then put back in the incubator with and

without a presumptive modulator of cell-cell communication. Five minutes later the cells are photographed again. If the cells have good gap junction function, the yellow dye will have diffused away from the edge but the red will not. In dose-response studies with this technique, a clear no-effect level can be seen. In cells that communicate well, the rate of communication (i.e., dye diffusion) can be quantitated with a laser machine. It varies between different types of cells. All studies are done at noncytotoxic doses. At cytotoxic doses, dye goes in all the cells.

Current Data

In vitro data indicate that there are at least three classes of promoters: those that have receptors and work at nanogram levels (hormones, TPA, TCDD, etc.); those that do not seem to need receptors (DDT, PBB, etc.) but diffuse into the membrane because they are lipophilic (these agents usually work at microgram levels); and those that do not need receptors but are not lipophilic (e.g., saccharin - these usually work at milligram levels).

Four intercellular chemical messagers seem to be responsible for modulating gap junctions: PKC, calcium, pH and cyclic AMP. The first three close gap junctions. Cyclic AMP has been shown to increase gap junction communication in certain cells (Spray and Bennett, 1985).

There is now direct evidence that many known growth factors work by blocking contact inhibition. A few growth factors have been shown to have promoting properties. Over 100 chemicals have been tested at Michigan State University. For several chemicals that were tested in vitro and in vivo, the in vitro results predicted the in vivo promoting ability of the agent.

Drs. Lowenstein and Borek pointed out about 20 years ago that most cancer cells seem to have defects in their gap junction communication (Kanno, 1985). The scrape loading technique corroborates this. Some tumor cells do not seem to communicate at all. Several oncogenes seem to block cell-cell communication when they are expressed in the appropriate cell.

At Michigan State University, studies were conducted to compare the metabolic cooperation assay using V79 and rat liver WB cells with the scrape loading assay. Over 100 chemicals were tested in the metabolic cooperation assay. It takes at least 3 days in the metabolic cooperation assay before the cooperative donor cell can die or the recipient cell can be rescued. In the scrape loading assay, TPA blocks communication in liver cells, but only for an hour or two. However, in the metabolic cooperation assay, TPA does not appear to block cell-cell communication, because cell communication inhibition was transient and the cooperating cell dies. So these assays measure two different responses: a transient response and a more long-term response. Also, the metabolic cooperation systems use serum which contains growth factors so it may not reflect in vivo conditions. The scrape assay appears to be a better mimic of in vivo conditions because it can use serum-free media.

Dr. Trosko also described research suggesting synergism can occur between promoters that modulate gap junctions (see Section 10, Synergism).

Future Research

The panelists agreed that cell-cell communication should be studied further. Suggestions for future research are provided in Section 13, Research Recommendations.

9. QUANTIFICATION IN THE LIVER

Summary

Dr. Pitot described the liver system studied in his laboratory and how it can be used to quantify the potency of a single chemical for the three stages of carcinogenesis.

System Description

The liver system used at the McArdle Laboratory for Cancer Research is analogous to the skin with one exception: initiation must take place during cell proliferation. Cell proliferation is stimulated using a partial hepatectomy. Then diethylnitrosamine (or another agent) is administered, followed by the promoter usually continuously in the diet, in drinking water or by gavage. Lesions are identified using three different histochemical markers. Oncogene expression is examined using in situ hybridization.

Quantification

The foci can be quantitated using computers. A computer plot is obtained for each of three serial sections stained for three different markers. These are overlaid to determine the phenotype of each focus and the number and volume (or area) of the foci.

The potency of initiation and promotion can be quantified by developing an initiating index and a promoting index. The initiating index is the log of the number of foci (corrected for the background level) per liver per millimole of the

compound given in a single dose. The initiation index for TCDD and phenobarbital is zero. The promoting index is the volume occupied by the foci in the liver in the presence of the promoter divided by the volume of the foci in the absence of the promoter per millimole per week. This index measures the ability of the promoter to expand the population of the progeny of initiated cells. It is dependent on time. The effect of the promoter on the initiation index and of the initiator on the promotion index has not yet been studied extensively in this system.

In studies at McArdle, researchers have found foci within foci. Generally, these are morphologically carcinomas and involve only a small part of the population of the original focus. However, in an IPI experiment, the foci within foci increase by at least an order of magnitude. This has been interpreted as a transition from promotion to progression. Quantification of the foci within foci may provide a means of measuring the transition from promotion to progression.

10. SYNERGISM

Summary

The issue of synergism of promoters was discussed, with the conclusion that very little is known about this possible type of interaction. Synergism has not been considered in risk assessment before and may be an important factor. Research is needed to elucidate the mechanism of synergism among promoters and to identify the kinds of promoters that are likely to interact.

Existing Data

Dr. Trosko described research on potential synergism between two promoters - DDT and TPA - that was recently performed at Michigan State University. These studies have been submitted for publication (Aylsworth et al., submitted). The research was conducted to investigate whether synergism could occur by modulating gap junctions. The researchers postulated that the action of some promoters is mediated by PKC (a phospholipid, calcium-dependent enzyme). If so, then a promoting agent that stimulates the phospholipid component of PKC and a promoting agent that modulates calcium should react synergistically. The study investigated the interaction of DDT, which blocks the efflux of calcium through the membrane, and TPA. Either chemical alone produced a clear dose-response curve. When TPA was held constant and DDT was added over the same dose range, the agents showed synergistic rather than additive effects. Synergism was also found between unsaturated fatty acids and DDT, whereas DDT and aldrin show additive effects. Quercetin, which is an inhibitor of PKC, was found to completely block the TPA effect on cell-cell communication

(unpublished results). Dr. Trosko suggested that synergism of DDT and TPA be investigated in the liver and skin.

Dr. Pitot has found that the lab chow diet appears to have a synergistic effect with phenobarbital as a promoter in the initiation/promotion system in the liver. This could potentially confound studies to identify promoters.

One panelist questioned why cis-retinoic acid and TPA show synergism in the metabolic cooperation system, but cis-retinoic acid is antagonistic to TPA in mouse skin.

11. SPECIES DIFFERENCES/HUMAN STUDIES

Summary

The panelists discussed species differences in response to initiation, promotion and progression. Strong carcinogens are notable for attacking multiple strains and species; however, this is not necessarily the case with some of the promoters that have been studied. Thus, it appears that promoters may show more extreme differences in species and strain responses than carcinogens. This would make risk assessment for promoters even more difficult than for carcinogens. The panelists agreed that much more work needs to be done to determine whether there are species differences and to understand these differences from a mechanistic standpoint. The panelists proposed several ideas for human studies.

Species Differences

Phorbol esters promote in some mouse species or strains but not in others. TPA has different effects in different species. Repetitive treatment with TPA results in sustained hyperplasia in the mouse, but not in the rat or hamster.

Phenobarbital may have different effects in rats and humans. Phenobarbital is known to cause liver tumors in rats. However, there was no evidence of increase in any type of neoplasm in 25,000 patients who were given PB as an anticonvulsant for several years (Clemmesen, 1977). Dr. Pitot pointed out that the human dose in the Clemmesen study was comparable to the threshold for neoplastic effects in rats, so it may be that the human dose was simply not high enough to see an effect.

DDT was mentioned as an example of an agent that has been clearly shown to be a promoter in the liver in rodents, but does not seem to be carcinogenic in humans in at least 30 epidemiological studies. One panelist suggested that maybe the human dose was not high enough to see promotion.

Human Studies

Several panelists emphasized the importance of doing epidemiological studies to elucidate how promoters affect humans. A suggestion was made to study the correlation between human and animal data for known or potential promoters for which epidemiological data are currently available, e.g., cigarette smoke, arsenic and dioxin. Another suggestion was made that animal studies for promoters should focus on agents for which there is human exposure and thus the opportunity to collect epidemiological data.

Dr. Kennedy described several human populations that could be studied for promotional effects (Kennedy, 1985b). These populations have been exposed to an initiator and, subsequently, to a potential or known promoter.

Two such populations are individuals who have received occupational exposure to uranium mine dust or asbestos and are now having their lungs gavaged with saline at regular intervals to remove the material. In these populations, asbestos or alpha radiation is the initiator and saline instillations could lead to promotion. Studies conducted at the Harvard University School of Public Health several years ago suggest that saline instillations can result in promotion (Little et al., 1978; Shami et al., 1982). In these studies, a relatively low dose of polonium 210 to hamster lungs produced few lung tumors, whereas treatment with seven instillations of saline 5 months

later resulted in lung tumors in 22 to 44% of the exposed animals. Saline instillations alone did not lead to cancer. Other panelists questioned whether saline could be considered a promoter (see Section 4, Mechanisms of Promotion).

Uranium miners who smoke versus those who do not smoke are another population that could be studied. The curve for the induction of lung cancer in white uranium miners who smoke is linear (Committee on Biological Effects of Ionizing Radiation, 1972). The nonwhite miners (American Indians) who do not smoke (or who smoke very little) had a nonsignificant incidence of cancer (Lundin et al., 1971), although the incidence of cancer has been increasing recently (Archer et al., 1976; Gottlieb and Husen, 1982; Samet et al., 1984). Occupants of many houses and other structures in the United States are exposed to levels of radon comparable to those which are known to exist in uranium mines. This could be another population to study.

Other human populations that could be studied for promotion with radiation as the initiating agent include individuals who were exposed to radium occupationally or were given radium for medical problems and who are now at high risk for the development of bone cancer. Many people were exposed to X-rays in the 1940's and 1950's for various benign disorders such as eczema, acne and thymus enlargement. Dr. Kennedy suggested that, in the X-irradiated population, individuals who have contracted thyroid cancer might be very appropriate to study. Approximately 20,000 cases of thyroid cancer are expected to result from therapeutic X-radiation treatments in the United States. Women, individuals with a Jewish ethnic background, and emigrants from Tunisia and Morocco appear to be at higher risk of developing cancer (Committee on Biological Effects of Ionizing Radiation, 1980; Ron and Modan, 1982); these unusual risk groups suggest that promotion plays a role in the development of this disease. Mortality from thyroid cancer is

extremely low (1 to 3%), so that affected individuals are available for interviewing. At many hospitals, thyroid cancer is diagnosed by scanning with iodine 131 at approximately yearly intervals. This gives a dose of approximately 200 rads to the adult thyroid, which is considered the optimal dose for the induction of cancer in some systems and could be an excellent progressor.

Many of the different cancers resulting from radiation exposure show a dose-response relationship. The thyroid and the female breasts have the greatest sensitivity to radiation-induced cancer, and the dose-response relationship for both types of cancer is linear (Committee on the Biological Effects of Ionizing Radiation, 1980; U.N. Report, 1977; Maxon et al., 1977). Both the breast and the thyroid are under strict hormononal controls and these hormones may act as built-in promotional agents (Troll, 1976). Both thyroid hormones and those affecting the breast act as promoters or cocarcinogens in several systems: in vivo (Berenblum, 1974; Foster, 1975; Doniach, 1974; Hall, 1948; Suss et al., 1973) and in vitro (Blumberg, 1980 and 1981; Weinstein et al., 1979; Guernsey et al., 1980; Fisher et al., 1983; Borek et al., 1983). As promotion in the laboratory results in linear curves, the linear dose-response relationships for radiation-induced breast and thyroid cancers suggest that promoting agents may be important in the genesis of both thyroid and breast cancers in human populations.

One potential source of data on the levels of potential promoters in tissues from human populations could come from samples of breast tissue from reduction mammoplasties and mastectomies. Samples from reduction mammoplasties are currently maintained in liquid nitrogen by Dr. Michael Gould at the Department of Human Oncology, Wisconsin Clinical Center, Madison, Wisconsin (608-263-6615). A study could be performed

to compare levels of potential promoters in the breast tissue samples with known breast cancer rates in the areas of the country from which the tissue originated.

The Moolgavkar-Venzon-Knudson model was developed based on patients with retinoblastoma. This human model may offer a opportunity to study progression. Tumors (which also appear in many sites other than the eyes) are caused by an inherited gene mutation, where all the somatic cells in the embryo are initiated. Presumably promotion occurs in the eye because of differentiation of the eye tissue. Chemotherapy is inducing very high cancer rates in other tissues in the survivors. The survivors of therapy for retinoblastoma could be studied for progression. The advantages of this model are that the mechanism is fairly well understood and it seems to fit well with the M-V-K model. If retinoblastoma proves to be a model of IPI, then animals that are genetically susceptible to organ site cancers could also be studied to determine whether they have inherited an I state or a P state.

Another human model mentioned was xeroderma pigmentosum. This recessive disease predisposes the individual to initiation in the skin by UV light. Most, but not all, tumors form in the skin. Dr. Trosko mentioned that Dr. Kraemer at the National Cancer Institute had been studying nonskin tumors in this model as evidence of progression (Kraemer, 1980). Since UV cannot penetrate internally, these tumors must result from exposure to chemical initiators. Thus, this model could potentially be used to study IPI. Nevertheless, it is a good example of a mutant that might have an unusually high background initiated state.

Two other human models are patients who receive PUV-A (psoralen plus near-UV light [360 nm]) therapy and then X-ray therapy later; these individuals rapidly get tumors.

Individuals with psoriasis, having high rates of cell proliferation, might also be a possible population to study.

Epidemiological data should be obtained for chemicals that are known to be promoters in animals. For example, saturated fatty acids are associated with breast tumors in rats. The change in the U.S. diet in the last few years may offer an opportunity to see whether there is a similar association in humans. In addition, existing epidemiological data should be examined as a potential source of data on promoters.

12. PROGRESSORS

Summary

The concepts of progression and progressors were introduced. There was general agreement that progression may be a distinct stage in carcinogenesis (Hennings et al., 1983) and that there are probably agents that act predominantly as progressors. It is likely that promotion and progression can be separated experimentally in the liver as well as in the skin. (The liver has the advantage that single initiated cells and their early clonal progeny can be identified to provide a quantitative measurement of the effectiveness of the progressor.) The mechanism for progression was not understood; however, a majority of the group agreed that progression is probably related to additions in DNA. A proposed definition for progression was "an irreversible change in DNA towards malignancy." The issue of whether progression can occur by other mechanisms was left open.

Mechanisms of Progression

The group agreed that there was some evidence suggesting that progression is related to DNA damage, and they considered other possible mechanisms. Dr. Hennings suggested that progression indicates all the changes that occur after the development of a benign lesion, and might be divided into at least two stages: "malignant conversion" (i.e, the conversion of the papilloma to a squamous cell carcinoma), and metastasis (the spread of this malignant tumor to other organs) (Nowell, 1986). Dr. Pitot proposed that oxygen radical effects (which are indirect) may take a cell in the reversible stage of promotion and place it into progression. He said that benzoyl

peroxide and hydrogen peroxide are probably the best current examples of progressors. He also thought that gene amplification is involved in progression.

Oncogene activation was thought to play a role in progression. Dr. Hennings said that studies in progress at the National Cancer Institute indicate that c-Ha-ras activation can be either the initiating step (Roop et al., 1986) or the progressing step. Dr. Pitot said that most studies demonstrating oncogene activation indicate the activation of oncogenes during progression (Nicolson, 1987; Nowell, 1986; Klein and Klein, 1985).

The relationship between cytotoxicity and progression was discussed. Studies at UTSCC Science Park indicate that, in some cases, progression appears to be a process that selects more aggressive cells through cytotoxicity and leads to cancer. Initial studies by Slaga and coworkers suggested that there is a fairly strong association between progression and cytotoxicity. However, Dr. Slaga expressed some doubt that cytoxicity was the mechanism by which all progressors worked, even though all the compounds are cytotoxic. Several experiments were performed at UTSCC Science Park in which the dose of TPA was raised to the point of cytoxicity. Even at these doses, it did not act as a progressor. On the other hand, the antidiol-epoxide of benzo(a)pyrene, which is a noninitiator in the skin, is extremely potent as a progressor when applied to benign tumors.

In the skin, application of an initiator followed by a progressor (i.e., no promoter application) gives different results depending on the agent. Urethane gives no malignant tumors and 4NQO gives very few (Hennings et al., 1986), whereas complete carcinogens such as MNNG do give tumors.

Many of the progressors in the skin also progress in the liver. Dr. Pitot mentioned a study by Scherer et al. (1984) that showed that ENU acted as a progressor in the liver following initiation with diethylnitrosamine and promotion with phenobarbital. Research in the liver has focussed on classical initiating agents - alkylating agents - all of which are clastogenic. Other types of agents need to be studied.

Promoters as Progressors

The ability of promoters to act as progressors was discussed. Several panelists offered data from their studies. It appears that the ability of promoters to cause progression varies with different agents. Dr. Hennings mentioned studies where application of TPA following initiation and promotion caused no increase in the progression of papillomas to carcinomas (Hennings, 1983).

Studies at UTSCC Science Park with promoters (including benzoyl peroxide and chrysarobin) and cytotoxic agents (such as acetic acid and hydrogen peroxide) suggest that some promoters can act as progressors; however, cytotoxic agents can be just as effective.

13. RESEARCH RECOMMENDATIONS

Summary

The nature of promoters has important implications for risk assessment. Data suggest that promoters may have very different characteristics from complete carcinogens. However, the panelists agreed that not enough data are currently available to perform risk assessment for promoters. Probably less than 30 to 40 tumor promoters have been studied. Most tumor promotion studies have not been designed to consider risk assessment.

The panelists identified many different general and specific areas of research and made specific suggestions for studies. Basic research is needed to elucidate the identity and mechanism(s) of promotion, initiation and progression. More chemicals need to be studied for their promoting capability, and much more data are needed on the behavior of promoters in organs other than the skin and the liver. Epidemiological studies should be conducted to provide human data on promotion. Models to quantitate initiation and promotion should be developed and validated. In vitro screening models for promoters should be developed. Several questions of particular significance for risk assessment are: Is promotion reversible and does it have a threshold? How does the action of promoters vary from one organ to another and from one species to another? To what degree do agents that have been identified as promoters also possess initiating and progressing abilities? How can the potencies for these actions be quantified?

Many scientists are concerned that promoters may pose as great or greater an environmental hazard than complete

carcinogens. The panelists agreed that long-term research on promotion was needed and is important. However, they felt that, even with a relatively high level of funding, it may take 5 to 10 years to generate sufficient data to formulate a risk assessment policy for promoters.

Organ Systems

The skin is less attractive for purposes of modelling because skin lesions are less of a human health concern than lesions in some other organs. (Although skin cancers are the most numerous of human cancers, they are not as life-threatening as some other malignancies.) The liver has the disadvantage that a partial hepatectomy is required in the adult (but not in the neonate [Peraino model]). This might make it difficult to get in vitro cell transformation data under comparable circumstances.

The panelists agreed that a major priority is to find out whether the promoting characteristics that are true of the two most studied systems (the skin and the liver) are also true in other systems. Panelists proposed and discussed several other systems to study. The bladder would be a good system to study, although it may be complicated by the fact that urine itself acts as a promoting agent. Saccharin was proposed as a chemical to study in this system. It has a strong promoting effect and there are several good initiators. The colon is another system to study.

Dr. Pitot thought it important to develop a good system with a hormonal background. The thyroid is one possibility. It has the advantage that it is cellularly homogeneous. The breast may be more complicated. The kidney may not be a good system to develop because it is cellularly heterogeneous so

tumors may derive from many different cell types, which may have different responses to promoters. The lung is a difficult system to study but important because it is a major route of exposure. Unlike the skin and the liver, the respiratory system of animal models produces lesions that are histologically identical to human lung lesions. In addition, two alternative systems that may hold promise for study are: the Klein-Szanto and Nettesheim tracheal system (which involves a denuded trachea that can be repopulated with human cells to study human tissue) and Craighead and Mossman's system of hamster tracheal explants (Mossman and Craighead, 1978).

Panelists also discussed the possibility of testing promoters in several systems or organs in the whole animal. This would be possible if a universal agent were identified that initiated in a number of organ or tissue systems. Alternatively, a cocktail of chemicals could be used to initiate every organ. This approach would be difficult if promoters are shown to be initiator-specific.

Chemicals

The panelists discussed which agents should be studied further. Several agents were mentioned. Panelists differed in their opinions about which should receive priority. Some panelists thought that studies should focus on chemicals that pose the greatest potential human health hazards. Others felt it was important to enhance the data base for substances that have already been well studied in order to understand how they act in the model systems. The chemicals proposed for study were:

- TPA. This is a good promoter but its promoting ability is limited to skin of various strains of mice. This may be because the ester groups are removed, eliminating its promoting ability.

- Teleocidin may be a better chemical than TPA for promotion studies because it is not metabolized.

- TCDD is a good candidate for study. One area for study is the receptor-TCDD interaction. TCDD actions are believed to be mediated through a receptor, but the affinity of TCDD for the receptor is not related to its toxicology. One explanation may be that TCDD actions are mediated by the affinity of the receptor-TCDD complex for DNA. This needs to be studied.

- Chrysarobin is probably also a good chemical to study, since it promotes tumors at low doses in the skin.

- Sodium phenobarbital.

- PBB.

- Alcohol acts as a promoter in epidemiological studies. However, it generally has not been shown to act as a promoter in the liver in experimental studies. Alcohol changes the total number of altered cells and thus the volume of foci that are present after initiation. It does not increase the number of foci (S. Hendrich, T. Glauer and H.C. Pitot, unpublished observations). This is an interesting mechanism of action if ethanol is a promoter.

- Pesticides.

- Solvents.

Pure Promoters and Nonpromoters

The lack of negative chemicals, i.e., agents that have no promoting ability, was discussed. The lack of negative chemicals has been a problem in testing tumor promoters in in vitro assays. Thus, one needed area of research is the identification of chemicals that act primarily as initiators or promoters.

The panelists agreed that there are ways of determining experimentally whether something is acting as a initiator or promoter; however, the lack of chemicals that have been

identified as pure promoters and initiators makes this difficult.

Human Data

The panelists agreed that obtaining data on promotion in humans and identifying human promoters is a high priority. Several specific suggestions were made for obtaining data on promotion in humans. These are described in Section 11, Species Difference/Human Studies.

Animal Models

The panelists agreed that short-term and long-term animal models should be developed. A variety of animal models were mentioned: (1) a fish model that produces a pathological lesion that looks like a retinoblastoma when exposed to chlorinated aliphatics; (2) a fish model that involves crossing a molly and a swordtail that produces melanoma; and (3) a model for Mendelian-inherited kidney adenoma in rats (Eker and Mossige, 1961). Another model mentioned was Balmain's system in mice whose epidermis has been infected with the Harvey sarcoma virus. Treatment with a promoter induces papillomas, a certain portion of which progress to carcinomas. However, in this system, the presence of the whole virus may complicate the results.

Species and Strain Differences

The panelists agreed that species and strain differences were an important area for both in vitro and in vivo research. They discussed possible ways to investigate species

differences. One panelist proposed that, since TCDD is a good promoter in the rat liver, a model for TCDD using the human liver should be developed so that TCDD action could be compared in both systems.

In Vitro Systems

The panelists discussed the need to improve in vitro screening models to detect promoters, and to develop in vitro models for studying the mechanism of promotion. They agreed this would be a difficult undertaking, not only to select the most appropriate in vitro tests but also setting up parallel studies in animals. The ability of in vitro data to predict in vivo phenomena should be studied, and cell culture systems that are best able to reproduce in vivo conditions should be given priority for study. Serum may be a problem in in vitro testing for promoters; the action of many promoters depends on which lot of serum is used, so serum lots must be carefully screened.

Dr. Huberman stressed the importance of testing promoters in in vitro cell transformation systems, especially in human cell transformation systems. He recommended that the human myeloid leukemia cell differentiation test be studied because it is a relatively simple assay that can detect changes in the expression of various genes including a series of known oncogenes. Dr. Slaga mentioned that no one has yet shown a requirement for a promoter to get cell transformation in an in vitro epithelial system.

Mechanisms

Reversibility

Because of its implications for risk assessment, the question of reversibility of promotion is an important area for study. If promoters have thresholds, then presumably exposure to pure promoters at levels below the threshold will not induce tumors. One panelist raised the following question: If it can be shown that promoters have thresholds, is it worthwhile continuing to model IPI phenomena, since greater knowledge in this area may not lead to different regulatory actions for promoters. The sentiment was that modelling would be worthwhile and efforts should continue in this direction. A validated theory of initiation/promotion/progression would enable quantification of potency at each stage.

One suggested area of research was for statisticians to determine at what level of uncertainty experimental data can be used to describe the behavior of the receptor as a basis for estimating the threshold model. The EPA Carcinogen Assessment Group is currently investigating this.

Receptor Binding

Two approaches to studying receptor mechanisms were suggested: (1) in vitro or in vivo mutation studies and (2) competitive binding studies. Dr. Trosko suggested looking for a cell line that has an EGF-receptor mutation, in which the receptor would bind, but the signal would not be transduced. If the parent and mutant lines gave different responses in the presence of a promoter, that would imply a receptor mechanism. He mentioned a TPA-resistant strain used by Yamasaki et al. (1985) to investigate mechanisms and the role of cell-cell

communication in transformation. This mutant strain is not promoted by TPA, whereas TPA does promote transformation in the parental line. This is one example where a mutant for a receptor-mediated response of TPA in in vitro promotion could be used as a model.

Dr. Pitot suggested locking the receptor up with an irreversible inhibitor. He said that data suggest that TCDD acts as a promoter through a genetic receptor mechanism (although the fact that removal of the thyroid decreases TCDD toxicity may cast some doubt on this theory).

Cell Differentiation

Modulation of cell differentiation studies are important. Certain promoters like phorbol esters and TCDD are extremely effective in modulating differentiation in some cell systems.

Oncogenes

The correlation of oncogenes with tumor promotion should be researched. Cells in which oncogenes are activated may be more sensitive to tumor promoters, e.g., c-Ha-ras can initiate in mouse skin (Balmain).

Phorbol Esters

Studies should be done to find out why phorbol esters promote in some mouse strains but not others. If this mechanism can be elucidated then much of the background data on TPA may be applicable to other systems.

Models

The Two-Stage Birth-Death-Mutation Model

There was support for conducting studies to validate this model. Several research suggestions were made. These are described in Section 7.

Intercellular Communication

The role of intercellular communication in linking tumor promoters to a mechanism of action in vivo should be investigated. Many technologies are available to measure cell-cell communication and its role in growth control and differentiation. Mutants are becoming available for gap junctions, antibodies exist for gap junctions, and the gene for the gap junction has been cloned. Genetic, molecular and cellular experiments with normal cells are needed, both in vitro and in vivo.

Gap junction technology may offer an opportunity to investigate mechanisms of promotion at the cellular level. The ability to apply gap junction technology to liver and skin could be investigated. The scrape loading assay could be adapted to use cells (such as primary human keratinocytes) that metabolize agents in order to study the role of metabolites in promotion.

Areas for future research include the question of how big a clone must be before it is free of inhibition by surrounding normal cells via cell-cell communication. This may depend on the type of cell since not all cells have the same number or size of gap junctions. Identification of the diffusion-suppressing molecule(s) is another possible research

area. Another research area is to perform the scrape technique using whole tissue.

Other Models

The prooxidative model (Cerutti, 1985) should be tested versus the PKC model (Nishizuka, 1986). Data suggest that these two models may not be mutually exclusive, i.e., one may affect the other. These two models should be correlated with the cellular and genetic models that have been proposed, e.g., the cell-cell communication model and the recombination model. A method of testing the cell-cell communication model was proposed: Correlate (1) sustained hyperplasia after TPA treatment with the total absence of gap junctions, and (2) nonsustained hyperplasia in the Syrian hamster with the presence of gap junctions.

Quantification

It appears that the liver can be used to quantitate initiation, promotion and progression. The ability to quantitate these stages in other organs should be developed. Promotion could probably be quantitated in the bladder, skin, colon. It may be difficult to quantitate initiation in the skin unless ways to detect initiated cells can be developed.

Expansion of the NTP Bioassay

One panelist proposed the idea of expanding the NTP bioassay to study the ability of agents to promote. In addition to the standard protocol for carcinogenicity, each agent would be administered to a group of animals that had been

previously exposed to a universal initiator. The expanded protocol could also include studies in which treatments with the agent would be stopped to determine the ability of tumors to regress.

Additional Research Recommendations

The panel made no specific research recommendations but mentioned several other potential areas for research. These include:

- Distribution, metabolism and pharmacokinetics of tumor promoters in various systems.
- The influence of the sequence of administration of agents on the action of promoters.
- Synergism among promoters.
- Spontaneous initiation and promotion.
- The relationship between cytotoxicity and promotion.
- Conversion of benign tumors to malignant tumors.
- Progressors and progression.

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APPENDIX A

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Research Planning for the Development of Risk Assessment Methodologies for Tumor Promotors

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Research Planning for the Development of Risk Assessment Methodologies for Tumor Promotors

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APPENDIX B

AGENDA

FIRST EPA WORKSHOP ON RESEARCH PLANNING FOR RISK ASSESSMENT

Research Planning for the Development of Risk Assessment Methodologies for Tumor Promotors

AGENDA

Tuesday, February 3, 1987

8:00-8:30 a.m.	Registration
Chairmen:	Roy Albert University of Cincinnati Medical Center, Cincinnati, OH Hugh Spitzer Office of Research and Development, U.S. EPA, Washington, DC
8:30 a.m.	Announcements
8:40 a.m.	Welcoming Remarks Vaun Newill, Assistant Administrator for Research and Development, U.S. EPA, Washington, DC
8:55 a.m.	Introduction and Overview Roy Albert and Hugh Spitzer
9:15 a.m.	Panelists' Presentations of Pre-meeting Comment Summaries
10:30 a.m.	COFFEE BREAK
10:45 a.m.	Panelists' Presentations of Pre-meeting Comment Summaries (cont.)
12:15 p.m.	LUNCH BREAK
1:45 p.m.	Panelists' Discussion of Areas of Consensus or Lack Thereof
2:30 p.m.	1. Testable Hypotheses - Biological a. Mechanisms of Action i. What we know now ii. What we need to know
3:30 p.m.	COFFEE BREAK

Tuesday, February 3, 1987 (cont.)

3:45 p.m. 2. Testable Hypotheses - Modeling

a. Possible approaches for integrating promotor activity into risk assessment
i. Mechanisms of action
ii. Biological half-life
iii. Considerations of species differences

5:15 p.m. Closing Discussion

5:30 p.m. Adjourn

6:00 p.m. Workshop Dinner

* * * * *

Wednesday, February 4, 1987

Chairman: Robert Langenbach
NIEHS, Cellular and Genetic Toxicology Division, Research Triangle Park, NC

8:30 a.m. Announcements and Overview of Preceding Day's Issues

9:00 a.m. The Biology of Tumor Promotion

1. Discussion of possible appropriate chemicals that can serve as surrogates for classes of chemicals

2. Need for developing new methodologies

3. Short- and long-term research proposals

10:30 a.m. COFFEE BREAK

10:45 a.m. The Biology of Tumor Promotion (cont.)

12:00 p.m. LUNCH BREAK

1:30 p.m. Modeling

1. Can we integrate promotional activity into current risk assessment methodologies

a. What assumptions are required

b. Identify uncertainties encountered

3:30 p.m. COFFEE BREAK

Wednesday, February 4, 1987 (cont.)

3:45 p.m. 2. Alternative models/approaches

a. Data base required for testing

b. Biological considerations

c. What assumptions are required

d. Identify uncertainties encountered

5:30 p.m. Adjourn

* * * * *

Thursday, February 5, 1987

Chairman: William Farland
Director, Carcinogen Assessment Group, U.S. EPA, Washington, DC

8:30 a.m. Summary of Discussions
Roy Albert and Robert Langenbach

9:30 a.m. Setting Research Priorities for Planning Risk Assessment Methodologies

1. Biological research

a. Long-term

b. Short-term

2. Model development

a. Long-term

b. Short-term

10:30 a.m. COFFEE BREAK

10:45 a.m. Setting Research Priorites (cont.)

12:15 p.m. Closing Remarks

12:30 p.m. Adjourn

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APPENDIX C

PANELIST PREMEETING COMMENTS

United States Environmental Protection Agency
Office of Regulatory Support
Office of Research and Development

First EPA Workshop on Research
Planning for Risk Assessment

PRE-MEETING COMMENTS FOR THE WORKSHOP ON THE DEVELOPMENT OF RISK ASSESSMENT METHODOLOGIES FOR TUMOR PROMOTORS

February 3-5, 1987

Bethesda Hyatt Regency
Bethesda, MD

PRE-MEETING COMMENTS FOR WORKSHOP ON THE DEVELOPMENT OF RISK ASSESSMENT METHODOLOGIES FOR TUMOR PROMOTORS

February 3-5, 1987

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PANELISTS' PRE-MEETING ASSIGNMENT

Topics for Pre-meeting Comments

In an effort to stimulate discussion and to develop consensus or a spectrum of views on key topics, panelists are requested to bring to the workshop written statements on each of the following topics:

1. How do you define tumor promotion? What is the basis for your definition? What are the limitations of the definition?

2. Is it possible to quantitatively separate the promotional activity from the initiating activity of a chemical in assessing the carcinogen risk using the available data set?

3. How would you approach incorporating the qualitative characterization of promotional activity into a risk assessment?

4. What data would you require before accepting the conclusion that a chemical is only a promotor?

5. What chemicals, do you think, would be good candidates for use in developing risk assessment methodologies for tumor promotors?

   What additional research, do you think, is required on these chemicals to better understand their interaction with biological systems?

6. What generic considerations should be given to interspecies extrapolation of the risk associated with exposure to promotors (body wt. vs. surface area; liquid soluble vs. water soluble)?

   What chemical specific considerations should be given in interspecies extrapolation of the risk associated with exposure to promotors?

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Question 1.

Promotion is a term that has been defined by the design of carcinogenesis experiments. Originally the term co-carcinogenesis was used and later the specific experimental regimen delinated the term promoter. Tumor promotion is one of a series of steps whereby normal somatic cells become neoplasic lesions. The carcinogenic process begins with the initiation step in which an agent (initiator) damages a critical cellular target, presumably DNA. Although necessary, the initiation process is not sufficient for neoplastic transformation of a cell. A promotion step is required in which exposure to a second agent (a promoter) results in cellular changes that commit initiated cells to a progressive process that ultimately results in full neoplasitic expression [1-4].

The definitions of initiation and promotion are based on studies of sequential exposures to agents that produce tumors. Early experiments involved a single dermal application of agents such as 7,12-dimethylbenz[a]anthrecene (DMBA) that did not produce tumors in the population of exposed mice or only an occasional tumor. It was observed that repeated topical application of a second agent such as croton oil, which does not produce tumors alone *, caused a high incidence of malignant tumors. Initiation and promotion are defined by a temporal sequence of events that is necessary for tumor production: exposure to an initiator followed by exposure to a promoter. At high doses or repeated exposures, some initiating agents such DMBA act both as initiators and promoters and are therefore called complete carcinogens.

* Promoters may induce a <u>very</u> low incidence of tumors but this is usually attributed mechanistically to previous undetected initiation (background).

In addition to the temporal relationship of exposures necessary to produce tumors, initiators and promoters are characterized by the temporal nature of their effects. Initiating events are believed to occur in a relative short period of time (minutes to hours?). The effects of initiators are persistant, perhaps irreversible, and cumulative at all doses and frequencies of exposure. The promotion phase of carcinogenesis occurs over a longer period of time (weeks to years?). At low doses or low frequencies of exposure the effects of certain promoters appear to be reversible and non-cumulative. Sustained exposures to sufficient doses of promoters are necessary for initiated cells to "evolve" into neoplastic tissue.

A major limitation of the above definition of tumor promotion is that it is an operational definition based on experimental protocols used to produce tumors in amnimals. The biochemical mechanisms underlying tumor promotion are far from clear and different promoters may have vastly different modes of action.

Question 2.*

It is difficult to separate quantitatively promotional and initiating activities of chemicals because most of the available data set does not specifically deal with initiation and promotion as defined in answer one. It has been generally assumed that initiation is a direct result of DNA damage. Most data come from mutation assays or estimating adduct formation that are supposed to reflect underlying DNA damage/binding. Agents that test positive in these assays are assumed to be at least initiators. Agents that do not test positive in these assays but induce tumors are often assumed to be promoters [5]. The relationship of these assays to the exact nature of underlying DNA damage/binding and the relationship of that damage to carcinogenesis are far from clear (see review by Perera [6] and references therein). Until a much clearer picture of the biochemical/physical mechanisms of initiation and promotion are defined, we will have to rely on classical bioassays to differentiate initiators from promoters. In addition, classical bioassays still give us the most relevant data to use in making our best guesses about the relative risks of carcinogenic agents.

* What available data set?

Question 3.

Because of the necessity to repeatedly apply a promoter in early experiments and the fact that stopping the exposures causes a less than 100% incidence of tumors, a kind of reversibility is assumed and or a threshold event (total accumulated dose) is considered to be operating. For this reason there has been some discussion about regulating promoters differently from initiators [5]. There may be some justification for this but it must be emphasized that, as pointed out above, the original definition of promoters was based on the production of tumors in long-term bioassays under a specific set of circumstances. However carcinogens are commonly classified as promoters based upon their mechanisms of action and assumptions abou the biological significance of *in vitro* assays. The present state of scientific knowledge does not allow clear categorization of carcinogens based on their mechanisms of action. In addition, the activity of a promoter in producing tumors depends on the type and degree of initiation involved [7]. Setting a no-effect level for a promoter would require knowledge about qualitative and quantitative relationships between initiators and promoters, and the normal level of background initiation and ambient initiators. Even in the absence of obvious initiators, promoters increase the background incidence of tumors in experimental animals.

Question 4.

To be just a promoter, a substance must have been tested experimentally and fit the definition of a promoter given in answer one. Operationally a chemical may be a promoter but are the mechanisms of action all the same? Data still need to be developed.

Question 5.

Good candidates for studying promotion are chemicals such as arsenic which has been demonstrated to be a carcinogen in human populations but is difficult to demonstrate to be a carcinogen in animal studies. Particular attention should be paid to substances where the potential human exposure is high. Phorbol esters are excellent experimental promoters but how relevant are they to the human situation? Another very intriguing compound that has been repoorted to be a promoter is TCDD. It should be evaluated further as a promoter to determine whether or not the experimental animal data and the human epidimiological data fit.

What about asbestos and/or cigarette smoke?

Question 6.

It is difficult to make generalizations about interspecies differences in dose-response relationships of promoters because very little research has been done in this area [6]. Not only is there a void in terms of species differences but there is a paucity of experimental data for all except skin and perhaps liver. The lung seems to be such an important area for experimental work based upon clues from epidemiology but alas who supports such experimental research.

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5. Weisburger, J.H. and Williams, G.H. (1983): The distinct health risk analyses required for genotoxic carcinogens and promoting agents. Environmental Health Perspectives, 50:233-245.

6. Perara, F.P. (1984): The genotoxic/epigenetic distinction: relevance to cancer policy. Environmental Research, 34:175-191.

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Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promoters

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1.) Tumor promotion is defined based on the initiation-promotion protocol developed in the mouse skin model system. After a single application of an initiator (or an initiating dose of a complete carcinogen), which by itself does not produce skin tumors, repeated applications of a promoting agent are required to produce skin tumors. Most of these tumors are benign papillomas, a small percentage of which may progress to carcinomas. Promoter treatment by itself induces very few tumors.

The end point of promotion is the benign papilloma. Papillomas are heterogeneous in their potential for progression to malignancy, which I believe reflects the heterogeneity of initiated cells. Progression from the papilloma stage proceeds in 2 further stages, which are distinct from promotion: malignant conversion is the progression to malignancy (end point is a squamous cell carcinoma); metastatic conversion is the progression of the malignant tumor to metastasize (end point is a metastasizing squamous cell carcinoma).

This definition is a starting point from which modifications can be made for other tissues or for man. For a process to be called "promotion" (so that mechanistic studies from skin, liver or other animal models are relevant), the process must 1)follow initiation, 2)require repeated exposure and 3)by itself produce few (or no) tumors. Initiation could be the result of repeated, as well as single, exposures, but by itself should produce few tumors. A distinction should be made between promoters and co-carcinogens.

2.) The relative initiating and promoting abilities of a chemical can be assessed (Cancer Res. 43, 2034-2041, 1983) by testing at various dose levels in a 2-stage model utilizing optimal doses of known initiator and promoter. Few chemicals have been tested in this way.

3.) If a chemical is a pure promoter in an in vivo animal model (producing benign tumors with little potential to progress to malignancy), the likely risk to man would be small. The risk would be much greater for an agent active in the malignant conversion stage or co-carcinogenic (by simultaneous treatment) to produce malignant tumors.

4.) For a chemical to be only a promoter, it must be tested for activity as an initiator, a promoter, a malignant converting agent, and a co-carcinogen and be found negative for all stages other than promotion.

5.) Based on the mouse skin model, comparisons should be made between 1)phorbol esters and other promoters which apparently act through protein kinase C, 2)benzoyl peroxide and related compounds such as hydrogen peroxide, 3)hydrocarbon derivatives such as bromomethylbenzanthracene, and 4)anthrones. There are apparently differences between the mechanisms by which these agents act. They also have different potencies when tested for malignant conversion. Shouldn't radiation be considered, or are we only interested in chemicals? The biological effects of phorbol esters on various aspects of control of epidermal proliferation and differentiation are being well-characterized. Similar efforts should be undertaken on the other classes of promoters. What is the role of free radicals in promotion? Do promoters all necessarily act via a similar mechanism?

6.) Hasn't EPA already dealt with these questions from considerations of carcinogen risk?

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promotors

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1. Definition of Tumor Promotion:

The classical definition of "promotor" is as follows: "The enhancement of the carcinogenicity of an agent by a second agent not carcinogenic by itself under the test conditions acting after exposure to the first has ended." This classical definition, as conceived by Rous and Berenblum, is quoted from Williams and Weisburger in A Guide to General Toxicology (Homburger, Hayes, and Pelikan, eds.), published by S. Karger, A.G. Basel/New York, 1983, p. 220.

The two-stage concept of carcinogenicity, postulating a first phase of "initiation" followed by a second stage of "promotion," assumes the existence of an "initiated" or "dormant" cancer cell which can be stimulated into

neoplastic growth by promotors.

The concept of "promotor" must be clearly separated from that of "co-carcinogen," which is any substance enhancing the carcinogenicity of a chemical simultaneously administered and not itself carcinogenic under the test conditions.

Clearly separate from the two above is the concept of "synergism" between carcinogens, i.e., the mixture of two carcinogens may be more carcinogenic than each of the carcinogens alone (J.P. Greenstein, The Biochemistry of Cancer, Academic Press, 1954, p. 79). To the best of my knowledge, these concepts and definitions are still valid.

2. Separation of Promoting Activity from Initiating Activity:

By definition, a promotor never has initiating activity. The few tumors caused by some promotors in mouse skin are assumed to be due to spontaneously occurring latent tumor cells activated by the promotor (Berenblum, Carcinogenesis as a Biological Problem, North-Holland Publishing Co., Amsterdam/Oxford, 1974).

3. Incorporation of Qualitative Characterization of Promotional Activity into a Risk Assessment:

If this question means "how will the presence of a promotor affect a risk assessment?" my answer would be "I do not know." Each experimental data set would have to be judged on its own merits.

4. Data Required to Decide that a Chemical Is only a Promotor:

By definition, a chemical is either a promotor or an initiator. It cannot be both.

5. Candidate Chemicals for Study of Risk Assessment Methodology for Promotors:

Most studies of the mechanism of promotion have been done with croton oil, and later, with its active ingredients, the phorbol esters, and particularly, with Hecker's compound A_1 (Cancer Res. 28:2338, 1968), or TPA (12-o-teradecanoyl-phorbol-13-acetate). Most recently, a number of diverse compounds have been

described as promotors in different experimental settings (Proceedings of the American Association for Cancer Research, March 27, 1986), for example, orotic acid, promoting liver tumors and intestinal carcinogenesis in rats (Rao, et al, abs 561), polychlorinated biphenyls promoting lung and liver tumors induced in infant mice by N-nitrosodimethylamine (Anderson, et al, abs 560), benzodiazepine tranquilizers promoting hepatocellular neoplasms in mice (Diwan, et al, abs 559), long-acting barbiturates promoting tumors in rat liver (Diwan, et al, abs 558), cyclosporine promoting induction of thymic lymphoma in mice by N-methyl-N-nitrosourea (Shinozuka, et al, abs 546), aspirin promoting urinary bladder cancer induced in rats (Sakata, et al, abs 490), peroxides as promotors in the 2-stage mouse skin model (Rotstein, et al, abs 567). These are merely cited as examples of the most recent vintage. There are many other substances claimed to be promotors, some of them of practical importance, such as saccharine and cigarette smoke suggested as promoting bladder cancer (in rats), and asbestos inducing lung cancer (in humans), respectively. Which of these many possible chemicals to use as models for further study of promotion is debatable.

The type of additional research required to better understand the interaction of promotors with biological systems requires a great deal of thought. I doubt that the scientific basis exists for a strictly rational formulation of hypotheses that could be verified by experiments. In some cases, it is found that what may have been considered a case of promotion turns out to be the result of entirely different mechanisms, for example, the case of the interaction of asbestos inhalation and smoking resulting in lung cancer. It appears that cigarette smoking impedes asbestos clearance from the lungs by increasing retention of short fibers (in Hartley strain guinea pigs), the same mechanism which might contribute to the rate of disease seen in asbestos workers who smoke (McFadden, et al, Am. Rev. Resp. Dis. 133(3):372-374, 1986).

In light of recent advances in immunology, some alleged promotors must be re-investigated to determine whether their apparent promoting effect might not be mediated through immunosuppression, which would release dormant cancer cells from immunosurveillance. Cyclosporin might be a paradigm for such situations.

6. Generalizations on Extrapolation from One Species to Another:

Extrapolation from one species to another is always a risky task. It would be a worthwhile exercise in risk assessment to quantify the risk involved in any such procedure.

While it is reasonably safe to extrapolate from acute toxicity tests in animals to humans (with some notable exceptions, such as atropine, which is perfectly safe for rabbits, but fatal to infants of comparable weight), the problem is far more complex when it comes to the transfer of information from animal carcinogenesis tests to the human epidemiological situation. A listing of human carcinogens detected by animal tests (Homburger, in A Guide to Toxicology, S. Karger, A.G. Basel/New York, 1983, pp. 205-208) shows that with proper safeguards, such tests have validity and are usable. However, this is only true in a qualitative way. Expression of numerical risks extrapolated from animal assays are, in most cases, useless, and may be misleading.

In the case of promotors (as opposed to most known initiators), the scientific basis for the planning of

protocols for animal testing is still weak, and much more fundamental research is needed before experiments can be designed that are sure to provide information useful in regulatory decisions. For lack of a thorough knowledge of their mechanism of action we may see ourselves compelled to adopt an arbitrary regulatory stance somewhat akin to the Delaney clause, and regulate that any substance active as a promotor of carcinogenesis in animals must be assumed to be a potential promotor of carcinogenesis for humans.

Conclusion

I conclude with my statement at the end of the chapter "Carcinogenesis - Concepts," in the above-quoted book (p. 211):

"Whatever is known about chemical carcinogens suggests that there are not only the generally recognized classes of initiators, which may induce latent or overt cancer cells, but a multitude of promoters of widely varying chemical structures which may activate latent tumor cells into an overtly cancerous state. Intensive efforts to better understand the carcinogenic process are therefore needed if regulation of carcinogens is ever to be placed on a sound scientific basis."

Premeeting comments
on risk assessment methodologies
for tumor formation

by E. Huberman

1. Tumor promotion is an operational term that characterizes one component of the multistage and multifactorial process of cancer causation. The term can be defined as the process by which (usually) protracted exposure of animals (humans) to noncarcinogenic or weakly carcinogenic agents (promoters) following and separated by time from the administration of a nontumorigenic dose of a carcinogen (initiator) results in a tumor(s) (individual) or in an increased tumor incidence (population).

   This definition is limited because it is not based on a mechanistic understanding of the process of tumor promotion. Furthermore, all currently known tumor initiators are potent carcinogens, and a major fraction of the promoters are capable of eliciting a tumorigenic response by themselves.

2. Optimal experimental protocols that can discriminate unequivocally between phenomena associated with initiation and promotion have not yet been fully developed. Reliable quantitative estimations of relative initiating and promoting activities of a given chemical are therefore yet not possible.

3.

4. To characterize a chemical as being a promoter only, it should have tumor-promoting activity and, be unable itself to initiate tumorigenic activity.

5. The chemicals to be used in developing risk assessment methodologies for tumor promoters should be these that may alter tumor incidence in humans. Until such agents are identified, one should use representatives of currently known classes of tumor promoters. Additional research should therefore be directed toward the identification of tumor promoters in the human environment. Other studies should involve the development of simple but relevant *in vitro* models. In these cell systems, tumor- promoter-induced alterations in gene expression should be studied in the context of malignant cell transformation.

6. In principle, the generic consideration for interspecies extrapolation of the risk associated with exposure to promoters should be the same as for complete carcinogens. However, since we do not yet have clear insights into the importance of such parameters in tumor promtion, a conservative approach should be taken, e.g., test species should be exposed to putative promoters at levels just below the ones producing a toxic effect. Evidence for promoting activity under such conditions should be interpreted as signifying a potential human hazard. Another approach to identifying potential human hazard may involve a comparison of the test agents with other similar agents whose body distribution and metabolism in man are known and whose cellular action *in vitro*, especially in human cells, resembles that of known tumor promoters.

COMMENTS FOR EPA WORKSHOP ON RISK ASSESSMENT METHODOLOGIES FOR TUMOR PROMOTERS

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1. How do you define tumor promotion? What is the basis for your definition? What are the limitations of the definition?

At this point, there are no generally agreed upon definitions of tumor promotion, as many of the classical concepts have been challenged recently and are still quite controversial. In a recent debate on this issue which was published (1), the Chemical Pathology NIH study section could only agree that initiation starts off the carcinogenic process, while promotion finishes the process. Even this definition is at present controversial, as data have now been presented to indicate that a single dose of a promoting agent before initiation can be partially effective as a "promoter".

I define a compound as a promoter if it causes no, or a low level of, tumor formation (or transformation foci in vitro etc.) by itself, but synergistically enhances the yield of tumors or foci when given as repeated exposures after initiation has occurred. My definition would include, for example, saline instillations to the lung as a promotional stimulus. In our studies on lung carcinogenesis, we observed that small amounts of saline (given as seven weekly intratracheal instillations beginning 5 months after the animals were exposed to a single dose of ^{210}Po) could markedly enhance the incidence of lung cancer in hamsters exposed to low doses of alpha radiation from ^{210}Po (^{210}Po is a radionuclide found in cigarette smoke and cigarette smokers' lungs: ^{210}Po alpha radiation is similar to that which arises from Radon gas, plutonium (a byproduct of nuclear power), etc.(2,3). While saline is not a classical promoting agent such as TPA, its effect appears to result in tumor promotion. Thus, many of the classical definitions derived for promoting agents such as TPA are inappropriate for agents such as saline and yet, tumor promotion can clearly be achieved with saline.

As my definition is for the process of tumor formation, it does not cover many of the classical characteristics attributed to promoters, as summarized recently by Weinstein et al.(4) as follows:

A Comparison of Biologic Properties of Initiating Agents & Promoting Agents

Initiating Agents	Promoting Agents
1. Carcinogenic by themselves - "solitary carcinogens"	1. Not carcinogenic alone
2. Must be given before promoting agent	2. Must be given after the initiating agent
3. Single exposure is sufficient	3. Required prolonged exposure
4. Action is irreversible and additive	4. Action is reversible (at early stage) and not additive
5. No apparent threshold	5. Probable threshold
6. Yield electrophiles that bind covalently to cell macromolecules	6. No evidence of covalent binding
7. Mutagenic	7. Not mutagenic

Personally, I still believe that many of these characteristics for promoters are still reasonable ones-such as the reversible nature of changes induced (at early times), the necessity of repeated applications, etc. The most questionable of these characteristics is the lack of mutagenicity of promoting agents, as even classical promoting agents such as TPA have recently been shown to cause chromosome-type mutations (reviewed by Marx (5)).

2. Is it possible to quantitatively separate the promotional activity from the initiating activity of a chemical in assessing the carcinogen risk using the available data set?

If the available data set include two-stage in vivo carcinogenesis experiments in animals, then initiation and promotion can be distinquished relatively easily-as they have been in the extensive literature in this field. If the "data set" refers to human exposures to chemicals, I do not believe that it will be possible to separate the initiating and promoting activity of the agents being studied. I do believe that these activities could be separated with radiation as the initiating agent, however, as described below.

3. How would you approach incorporating the qualitative characterization of promotional activity into a risk assessment?

One approach to determining whether in fact a chemical is a promoter in human populations is to study whether specific agents have the characteristics of promoters in people. From many different types of in vivo, in vitro and human epidemiologic studies, it is now clear that radiation can serve as an initiating agent. Irradiated human populations would be very appropriate as "initiated" groups for detailed studies, as there are now many people who have been exposed to radiation in discrete (and known) doses which we have much information about. (Many of these populations are discussed in detail in the "BEIR" report (6) as well as several other documents). The individuals in such irradiated populations could be questioned about exposures to agents we suspect as being promoting agents.

From previous studies of irradiated populations, the thyroid and the female breast have emerged as the most sensitive tissues for the induction of radiation induced cancer (6). Approximately 20,000 cases of thyroid cancer (in the $>$ 200,000 people irradiated) are expected to occur in this country in people whose thyroid happened to be in the irradiated field when they were irradiated for the treatment of an existing disease/problem- (for examples, thymus enlargement, acne, eczema, etc.- were treated with relatively high doses of x-irradiation in the 1940's and 1950's). As the form of thyroid cancer that is induced by radiation has a very low mortality rate ($\sim$3%, although mortality estimates have varied from 1-10%), most patients who have or have had thyroid carcinoma will be alive to discuss the effects of possible promoting agents in their lives. In fact, there are already "risk" groups that have been defined in this irradiated population. In human populations, it has been observed that the risk of developing radiation-induced thyroid cancer is considerably higher in: 1) females (6), 2) those having a Jewish ethnic background (6), and 3) those who have emigrated from Morocco or Tunisia (7); these data suggest that factors other than the radiation exposure play a very large role in the genesis of this disease. It is thought that the sexual difference is "related to the flunctuating hormonal status in females, with significantly greater variations in the pituitary-thyroid axis and in secretion of thyroid-stimulating hormone than in males" (6). The greater relative risk among the Jewish population could be due to genetic susceptibility; however, all of the increased risk factors considered together suggest that promotional factors, some of which may be present in the diet of "emigrating" populations, may be the most important determinants of whether cancer will result from the random distribution of energy by

ionizing radiation. Clearly, diet is one of the major determinants of the cancer incidence in human populations (8), and many promotional factors are known to be present in the human diet. Many of these irradiated patients in this population have already been "recalled" to determine whether they have a thyroid cancer; thus, they could be an already assembled population available for questioning about their exposures to potential promoting agents.

There are several other populations which have been irradiated which could be studied for possible promoting effects. For example, people treated with I^{131} (5 mCi by mouth) for thyrotoxicosis receive the following doses:

> "A patient with a 30 g gland with 60% uptake and who is treated with 5mCi of I^{131} by mouth will receive the following average tissue doses: 5 rads - whole body, 9000-10,000 rads -thyroid gland, 2-5 rads - hemopoietic tissue, approximately 1 rad to the testes and 2 rads to the ovary" (9).

(In vitro transformation studies suggest that even very low doses (10 rads) of x-radiation are capable of initiating cells (10); thus even the organs receiving the relatively low doses of radiation indicated above are like to have initiated cells following the ^{131}I treatment). A study of these irradiated patients could have "controls" built into the study, as some of the patients will have had part of their thyroids removed surgically, some will be taking a thyroid replacement hormone (and some will not), etc.

Certain hormones are likely to be potent promoting agents for some types of human cancer (for example, thyroid hormones have already been shown to have such promoting activity in vivo (11-13)). In the many populations of irradiated individuals, it is likely that many of the female patients will have been exposed to sex hormones (other likely promoting agents (11, 14-16)) for birth control, to treat symptoms of menopause, etc. Exposure to these hormones could easily affect cancer development in irradiated organs such as the breast, uterus, ovaries etc. Specific hormone use has already been associated with a promotion- like response in animals (17) and humans (18). Like the reversible actions of tumor promoting agents in vivo and in vitro, many tumors in animals, such as mouse mammary tumors, are under hormonal controls in that removal of a necessary hormone can cause tumor regression (17). For human cancer, it has been reported that women who take estrogen as a therapy for postmenopausal problems have a greatly increased risk of developing cancer and that there is a rapid decline in risk following the discontinuation of estrogen use (18). Again, such a reversible effect is what would be expected of a promoting agent operating in human carcinogenesis. Endogenous hormonal promotion in the breast could easily account for the fact that the female breast is the most sensitive human organ to the induction of cancer by ionizing radiation (6, 19).

Many of the solid cancers expected to develop in the atom bomb survivors should be detected over the next decade (there are approximately 30,000 people remaining in the currently ongoing study (begun by the Atomic Bomb Casualty Commission) to determine the cancer incidence occurring in individuals irradiated when the bombs were dropped in Hiroshima and Nagasaki). These studies could be extended to search for promoting factors present in the environments/diets etc. of the Hiroshima and Nagasaki survivors. To determine whether a promotional response has been observed in these studies, the shapes of the dose-response curves, with and without the suspected promoting agent, could be compared.

The presence of a promoting agent in radiation carcinogenesis often results in a linear curve, in both in vivo (20) and in vitro (21) experimental studies, while the curve expected for radiation treatment alone (in the systems cited above) is a quadratic or linear-quadratic curve. The conclusion reached by the most recent report from the Committee on Biological Effects of Ionizing Radiation (6) is that for low linear energy transfer radiation, the dose-response curve for most radiation-induced human

cancer is best represented by a linear quadratic form. Radiation-induced breast cancer in females is a highly notable exception, both for human (6, 19) and animal (22) data, due to its linear dose response; this linear dose response could be due to the endogenous promotion by hormones in breast tissue.

The only other human organ with a radiosensitivity comparable to that of the female breast for the induction of cancer is the thyroid (6, 19). Like the dose response for radiation induced human breast cancer in females, the dose-response curve for radiation-induced human thyroid cancer is also linear (6, 23). As with the breast, the presence of endogenous hormonal promotion could account for the linear relationship. Not only are thyroid hormones known to enhance the cancer incidence in animals (11-13), as discussed above, but it has also been shown that increased levels of thyroid-stimulating hormone result in an increase in the incidence of human thyroid neoplasia (24).

4. What data would you require before accepting the conclusion that a chemical is only a promoter?

The data that it caused no or few tumors in animals by itself but caused an enhancement in tumur formation when given as sequential treatments after an initiating carcinogen.

5. What chemicals, do you think, would be good candidates for use in developing risk assessment methodologies for tumor promotors?

What additional research, do you think, is required on these chemicals to better understand their interaction with biological systems?

Those for which some data exist which suggest that the agent acts as a possible "promoter" and for which human exposure levels can be determined. For examples:

1. drugs such as-

a) valium-inhibits metabolic cooperation (25) as do many other promoters (25) and medical records could give accurate exposure histories, and b) phenobarbital- a promoter for liver carcinogenesis (reviewed in ref. 26); medical records could give accurrate exposure histories.

2. Compounds widely present in the environment, such as pesticides, etc.

a) dieldrin- an example of a potential promoter which, when tested as a carcinogen in animal studies, formed "compound" dependent tumors in vivo (showing the reversibility characteristic of promoter dependent tumors in vivo- in that tumors only remained in the tissue as long as the "promoting agent" was present, and disappeared when the "promoting agent" was removed)

b) DDT, polychlorinated biphenyls, dioxins etc. (levels can be measured in body fat- from mastectomies, etc.) (There is already in vitro data suggesting that such compounds can act as promoting agents, for example see reference 25).

(5b) What additional research do you think is required on these chemicals to better understand their interaction with biological systems?

Further research is needed for those compounds suspected as promoters and for which there is known to be widespread human exposure.

6. a. What generic considerations should be given to interspecies extrapolation of the risk associated with exposure to promotors (body wt. vs. surface area; liquid soluble vs. water soluble)?

a. As promoters are known to be species and organ specific, and we don't know whether any human promoting agents truly exist, there is no clear-cut answer to this question.

b. What chemical specific considerations should be given in interspecies extrapolation of the risk associated with exposure to promotors?

b. As with the question above, we will be able to answer this question only after we have solid human data documenting the fact that there are specific "human" promoters (which have been documented by epidemiologic studies).

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C. ents for the EPA Workshop on Risk Assessment Methodologies for Tumor Promotors

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1. How do you define tumor promotion? What is the basis for your definition? What are the limitations of the definition?

A tumor promotor is a substance which, when administered over an extended period of time, may, through nongenetic toxic mechanisms, appreciably increase the rate of occurrence of neoplastic lesions which have been previously initiated.

This definition is based on the notion that a tumor may be initiated following the occurrence of genetic damage within one or more cells within a specific tissue. Such initiated cells may then undergo malignant transformation to give rise to a histologically or clinically detectable cancerous lesion, with the rate of occurrence of such lesions depending on the potency of the carcinogen. The rate of occurrence of these lesions may be increased by subsequent exposure to a promotor, which serves to increase the pool of initiated cells through nongenetic toxic mechanisms such as cellular proliferation. In order to demonstrate a detectable increase in tumor occurrence rates, it may be necessary to employ moderate to high doses of the promotor for prolonged periods of time in order to induce toxic effects sufficiently great so as to promote the development of the lesions induced by the initiator.

This definition of promotion is based on the concept of expanding the pool of initiated cells within a given tissue. Even without promotion, some initiated cells may undergo malignant transformation as a result of further exposure to the initiator. It is thus difficult to distinguish between a substance which demonstrates promotional activity as defined here and one which enhances the development of fully differentiated cancerous lesions. It is also possible that substances with promotional properties may also possess initiating activity, thereby precluding the classification of some compounds as either pure initiators or pure promotors.

2. Is it possible to quantitatively separate the promotional activity from the initiating activity of a chemical in assessing the carcinogen risk using the available data set?

Separation of initiation and promotional effects within an initiation/promotion system requires special bioassay protocols. At a minimum, treatment groups involving (i) short-term low-level exposure to the initiator, (ii) long-term high-level exposure to the promotor, (iii) a combination of these two regimens (with the promotor being administered following the initiator) and (iv) unexposed controls. This would allow direct measurement of the effects of the initiator and promotor separately as well as the effect of the initiator/promotor pair, and the corresponding calculation of a quantitative measure of carcinogenic potency for these three cases.

Assuming initiation and promotion to be based on genetic and nongenetic mechanisms, it may be possible to separate these two effects through the application of a series of short-term tests designed to measure genotoxicity and nongenetic effects such as changes in cell kinetics and metabolism. Without direct bioassay information on tumor occurrence rates, however, it may be difficult to obtain good quantitative information on initiation and promotional activity for use in carcinogenic risk assessment.

The Moolgavkar-Knudson two-stage model of carcinogenesis suggests another possible approach to quantitatively separating initiation and promotional activity. This stochastic birth-death-mutation model assumes that two mutations, each occurring at the time of cell division, are necessary for a normal cell to become malignant. Initiating activity may be quantified in terms of the rate of occurrence of the first mutation, which transforms a normal cell to an intermediate or initiated cell. The second mutation then transforms intermediate cells to cancerous cells. In this model, promotional activity is quantified in terms of the difference between the birth and death rates of intermediate cells. Application of this approach in practice requires both bioassay data on tumour occurrence and supplementary <u>in vitro</u> data on cell kinetics in order to estimate all of the unknown model parameters required to guage initiation and promotional activity.

3. How would you approach the qualitative characterization of promotional activity into a risk assessment?

In analogy with general toxicity other than carcinogenicity, it may not be unreasonable to postulate the existence of a no-effect level below which nongenotoxic promotional effects may occur. In this case, a suitable safety or uncertainty factor be applied to the experimentally observed no-effect level may represent a viable method of risk assessment. Since the existence of a no-effect level for genotoxic effects is less well accepted, this approach should not be considered with initiators which may be effective even at very low doses.

4. What data would you require before accepting the conclusion that a chemical is only a promotor?

In order to establish that a chemical acts only as a promotor and not as an initiator, it would be necessary to demonstrate negative results in bioassays designed to rule out the possibility that the substance is either a complete carcinogen or an initiator. Assuming that initiators act through interaction with genetic material, evidence against the presence of initiating activity would also be provided by the observation of negative results in a suitable battery of short-term tests for genotoxicity. In practice, this may be the only way to demonstrate a lack of initiating activity, since it would not be feasible to carry out a series of bioassays for initiating activity using a variety of known promotors.

5. What chemicals, do you think, would be good candidates for use in developing risk assessment methodologies for tumour promotors?

Since the phenomenon of initiation/promotion is complex and not fully understood, it seems desirable to select compounds for which a useful body of data on carcinogenic potential (including information on both initiating and promoting activity) and genotoxicity already exists. Saccharin, for example, is known to promote urinary bladder lesions initiated by FANFT and BBN, and has been subjected to extensive toxicological testing.

What additional research, do you think, is required on these chemicals to better understand their interaction with biological systems?

In order to better understand the biological interaction of promotors with biological systems, it is important to improve our understanding of the initiation/promotion mechanism itself. This may be addressed using specially designed in vitro and in vivo studies intended to provide further information in this regard.

6. What generic considerations should be given to interspecies extrapolation of the risk associated with exposure to promotors (body wt. vs. surface area; lipid soluble vs. water soluble)?

There is little empirical evidence to support the use of gross indicators such as body weight or surface area in extrapolating between species with carcinogens generally, including promotors.

In the absence of such evidence, it is difficult to recommend a generic method of species conversion. In the case of body weight vs. surface area, simplicity would suggest the use of body weight for species extrapolation although prudence may dictate to the use of more conservative surface area conversions.

What chemical specific considerations should be given in interspecies extrapolation of the risk associated with exposure to promotors?

Since many substances must undergo some form of metabolic activation in order to exert their toxic effects, it is of interest to determine the dose of the test compound delivered to the target in addition to the dose administered exogeneously. This will be of particular interest when the delivered dose is not directly proportional to the administered dose, as will occur when one or more steps in the metabolic activation process are saturable. Prediction of the delivered dose in the target species may be possible using physiologic pharmacokinetic models, but must be done on a compound specific basis.

Since promotors may act through cellular proliferation, it may also be useful to study their effects on cell kinetics in different species.

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1. Define Tumor Promotion

Tumor promotion is a process manifested by an increase in the number of tumors (relative to controls) in uninitiated or chemically initiated animals due to subsequent repetitive treatment with a promoter. The definition is a generalization covering most model systems used to study the phenomenon and which may also be applicable to the human situation. The limitations of the definition are that it does not describe any model precisely, and assumes spontaneous as well as carcinogen-induced tumor cells are promotable.

2. Quantitative Separation of Initiation and Promotion Activities for Carcinogen Risk

With our present understanding of initiation and promotion, and the model systems available, it is not now possible to quantitatively separate these activites and thus not possible to assess risk due to each individually. For certain model systems (skin and liver) it may be possible to state that a limited number of chemicals (i.e. TCDD, TPA, teliociden) behave primarily as promoters. But evidence that this is not system-specific (i.e. species, organ specific) is lacking. In addition, for TPA, the most thoroughly studied promoter known, there is still a debate as to whether it is a weak carcinogen. However, this does not mean some relative separation of activities is unfeasible.

3. Qualitative incorporation of promotional activity into risk assessment

First, I would obtain ample usable qualitative data. If specific properties of a chemical, such as promotional activity are needed, a more comprehensive testing approach is also needed. However, I am not certain currently available tests for promoters are entirely adequate; but I believe, with system modification and/or improvement greater insight into a chemical's promotional activity can be obtained. In a sense, each chemical would require some basic research based on the nature of the chemical and the information sought.

4. Data needed for a chemical to be only a promoter

It is not now possible to show that a chemical is only a promoter. The reason for this is that promotion of "spontaneously" initiated cells cannot be separated from cells which could be initiated and then promoted by the presumed promoter. Until we can differentiate between spontaneously initiated and chemically initiated cells there will always be some level of uncertainty. Furthermore, the absence of genotoxic activity in short-term tests by a rodent carcinogen may suggest promotional activity; but the limitation(s) of present-day short-term tests makes such conclusions equivocal. In summary more knowledge about the carcinogenic process itself is needed.

5. Chemicals useful for developing risk assessment methodologies and additional research needed.

Chemicals which are believed to act via a nearly entirely promotional mechanism would be good candidates to start. Examples of such chemicals would include TCDD, PBB, teliociden and TPA. Eventually, chemicals with both initiation and promotional activities, and chemicals with only initating activities (if any can be found) should be studied and all three resultant risk assessment models compared.

In addition, I suggest that chemicals be studied for which there is (or will be) human exposure data (phenobarbital, valium, etc.) and which can also be studied in model systems for promotional activity. With this approach, model systems and mathematical extrapolations can be developed and validated relative to effects in humans.

Additional research is needed to understand mechanistic differences between initiation, promotion and progression at the tissue, cellular and genetic level. Understanding the causes of organ and species differences would contribute greatly to practical (risk extrapolation) and mechanistic knowledge. As the mechanisms of promotion for different chemical classes are probably varied, research considering different mechanisms, rather than a unifying mechanism should be conducted.

6. Generic Consideration and interspecies extrapolation

Too few interspecies studies have been done to answer this question.

There are species differences in response to promoters as evidenced by different mouse, rat and hamster skin responses to TPA. However, many more studies are needed before extrapolations could be conducted with confidence.

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promotors

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1. The concept of tumor promotion has been derived from the early experimental work of Rous, Mottram, Berenblum, Shubik and others in which it was found that a single application of coal tar or a polycyclic hydrocarbon to the skin of rabbits or mice in subcarcinogenic amounts could result in the induction of skin tumors if it was followed by wounding the skin or by repeated application of croton oil, a powerful irritant. In these classical experiments the carcinogen was described as the initiator and the croton oil as the promoter. Subsequent work by many investigators confirmed and extended these findings and led to the conclusion that cancer arises in a series of stages and similar conclusions were drawn from studies of human cancer epidemiology. The original criteria for a tumor promoter in the mouse skin model have been recently restated (Hicks, 1983) as follows:

The operational criteria originally defined for a promoter in the mouse skin model were:

(i) that it should not be carcinogenic *per se*;

(ii) that it should not increase tumour yield if administered before the initiating carcinogen;

(iii) that when applied after an initiating, sub-carcinogenic dose of the carcinogen, it should accelerate the rate of development of tumours and thus increase the total, time-related tumour incidence;

(iv) that the total yield of tumours produced should be dose-related to the initiator not to the promoter, providing the promoter is used in excess of the minimum amount required to promote all initiated cells;

(v) that unlike initiation which can take place rapidly during a single exposure to the initiator and which is a permanent event, promotion requires long exposure to the promoter before the changes induced become irreversible.

These criteria have served as guidelines for the application of the concept of promotion to tumor induction in other organs including liver, bladder, colon. Although there is wide acceptance that the concept of promotion can be extended beyond the mouse skin system, in most cases all of the criteria have not been met.

Much recent work has been done on the biochemistry and molecular biology of tumor promotion but a very large part of this has involved the use of only one compound, the diterpene ester 12-O-tetradecanoylphorbol-13-acetate (TPA). It is probably premature to incorporate the findings of these studies into the criteria of a tumor promoter and those listed above, derived from the classical whole-animal experiments, should be used to define tumor promotion.

With these considerations in mind, the definition of a tumor promoter provided by I.B. Weinstein, as follows, is recommended:

Tumor promoters can be defined as compounds that have weak or no carcinogenic activity when tested alone but result in markedly enhanced tumor yield when applied repeatedly following a low or suboptional dose of a carcinogen (initiator). A possible extension of this definition may be considered, again according to Weinstein, as follows:

At the biochemical level, it appears that the major difference between initiators and promotors is that initiators (or their metabolites) bind covalently to cellular DNA; but this is not the case for tumor promoters.

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2. Presumably this question refers to complete carcinogens which induce tumors after single or multiple doses without additional treatment with other agents. If the chemical in question is known to be an initiator and if it is accepted that initiation involves genotoxic activity and interaction with DNA it is difficult to see how any promotional activity it might have, e.g. interaction with membranes, could be separated from additional genotoxic activity. Such a separation would not be helpful from a regulatory standpoint.

3. Important qualitative characteristics of promotional activity for risk assessment are that it is claimed to be reversible and non-additive and that, in contrast to initiation, threshold levels of activity can be demonstrated. If these claims are true, a compound with only promotional activity could be regulated in the same way as one for which there is no evidence of carcinogenic activity.

4. In principle, data would be required to show that the chemical had no carcinogenic activity by itself. However, this criterion has never been met, even with TPA or phenobarbital, the two most widely investigated promoters. In practice, the decision would depend on minimal or apparent total lack of carcinogenic action *in vivo* and *in vitro* and minimal or total lack of mutagenic activity in a variety of tests. Absence of genotoxic action in its wider sense presumably could not be required since TPA, the promoter par excellence, has been shown to induce DNA damage in several ways.

5. The literature on tumor promotion has been dominated by TPA, first in the classical mouse skin model and then in the various *in vitro* cell culture systems in which sequential changes comparable to *in vivo* promotion have been described. More recently phenobarbital has been increasingly used as a promoter in the several initiation-promotion systems involving rat liver. Although other chemicals have been described as tumor promoters in various systems they have not been sufficiently studied to be good candidates for use in developing risk assessment methodologies. Until more information is available on the

mechanisms for action of the other known promoters, TPA and phenobarbital are probably the best examples for assessment methodologies.

A major field of additional research with these agents would be to investigate their biological actions for points of similarity which might throw light on possible common molecular mechanisms underlying their common biological property of acting as a tumor promoter.

6. Interspecies extrapolations of the risk associated with exposure to promoters poses an extremely difficult problem because most of the work on TPA has been with the mouse and that on phenobarbital has been mainly in the rat. There may not be enough data currently available to allow meaningful interspecies extrapolation.

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promoters

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1. The stage of promotion in the natural history of neoplastic development is operationally defined as the reversible expansion of the initiated cell population. Mechanistically this reversible expansion is the result of altered gene expression induced by the presence of a promoting agent.

   The basis for this definition is the fact that the carefully defined instances of tumor promotion, skin and liver, demonstrate reversibility of this stage, and the action of virtually all promoting agents induce reversible effects in cells. Furthermore, all known promoting agents alter gene expression in one way or another.

   The limitations of this definition are to our advantage in the understanding the natural history

of neoplastic development in that previous interpretations of "tumor promotion" involved all changes beyond initiation to include malignancy. Again, in those well-defined systems demonstrating multistage carcinogenesis, the reversible stage of promotion precedes the irreversible stage of progression when malignancy appears.

2. In multistage hepatocarcinogenesis it is possible to quantitate both promotional and initiating activity of a single chemical and to use such values in assessing the carcinogenic risk of a particular agent. In the present chronic bioassay systems utilized it is not possible to quantitate or distinguish initiating, promoting, or complete carcinogenic activities.

3. If an agent demonstrates no DNA-damaging activity but exhibits promotional activity, then the characteristics of tumor promoters (see 4) must be taken into account in risk assessment. These include the reversibility of its effects and its concomitant threshold leading to permissible exposures for intermittent periods.

6. Information on interspecies extrapolation of risk associated with exposure to promoting agents should include the following:

Pharmacokinetics and disposition based on body weight, surface area, body lipid content, etc.

Effect of intermittent exposure and "stop" experiments.

Presence of receptors specific for promoting agent involved.

Chemical considerations in interspecies extrapolation should take the following into consideration:

Metabolism of the promoting agent with respect to metabolites formed and possible DNA damaging intermediates formed in one species and not in another.

Action of the agent in producing an alteration in gene expression, especially with respect to its interaction with specific receptor molecules.

4. The following data would be required in order to characterize a chemical exclusively as a promoting agent:

   a. induces the reversible alteration of genetic expression in cells.

   b. lacks DNA-damaging activity either as a simple mutagen, a clastogen, or an agent altering the structure of DNA by indirect means.

   c. induces the reversible expansion of one or another histogenetic initiated cell population *in vivo*.

5. Estrogens or other steroid or polypeptide hormones, phenobarbital, carbon tetrachloride, butylated hydroxyanisole, ethanol, nicotinamide, and lead acetate.

   Careful determination as to the DNA-damaging action of these chemicals should be carried out including effects *in vitro*, clastogenic activity, and potential DNA damage resulting from indirect effects of these chemicals. The reversibility of their effects in expanding initiating cell populations should be better understood in those tissues where this effect occurs.

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promotors

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This written contribution will be restricted to discussing strategies for developing batteries of tests to identify promoters and to differentiate these from complete carcinogens, non-genotoxic carcinogens, non-carcinogens and to possibly refine the methodologies further to differentiate between potent, moderate and weak promoters and finally, to explore the possibility of differentiating between promoters acting at different stages of the tumor progression process.

This approach is strongly influenced by our experience of the analysis of data bases relating genotoxicants/mutagens to carcinogens. Moreover, it is our hope that this could result in the development of cost-effective as well as highly predictive batteries of tests for promoters while avoiding the costly mistakes that were made in the development of short-term tests for genotoxic carcinogens.

Before we delve into tumor promoters, let us first briefly summarize the situation as it exists with respect to genotoxic carcinogens:

1. Approximately 17,000 chemicals have been tested in one or more short-term tests. This does not include the results generated by industrial and contract laboratories.

2. There are more than 100 different short-term tests. As a matter of fact, a recent IARC study lists in excess of 150 of these.

3. As a result of this unorganized effort, the data base is best characterized as incomplete, i.e., very few chemicals have been tested in a broad spectrum of tests. The few carcinogens that have been widely tested are primarily alkylating agents or other strong electrophilic species.

4. There are only about 1,000 chemicals that have been adequately tested for carcinogenicity. Among these, there is a predominance (about 85%) of carcinogens. This does not reflect the chemical universe and poses problems when devising algorithms to predict carcinogenicity based upon the results of short-term tests.

5. In order to use these incomplete data predictively, carcinogenicity bioassay results had to be pooled with respect to species and organ specificity. Because of the paucity of data on specific tumors, it is almost impossible to analyze the results of short-term tests with respect to their predictivity vis-a-vis organ and species specificity.

6. No plans for systematic testing were developed even after it was realized that the performance of individual tests, or combinations of these, were not predictive.

7. There is no universal concensus as to what constitutes a positive or a negative result in a specific assay. Thus classifications are frequently incompatible. This is further aggravated by the fact that there is no

agreement on how batteries of tests containing mixed results are to be interpreted.

In view of the abovementioned situation, it is but now that systematic approaches are being developed to use the wealth of available information to devise methodologies that would allow one to use the data in a predictive fashion as well as in the design of strategies for the assembly of predictive batteries for genotoxic carcinogens.

It should be pointed out that in developing such strategies we have heretofore paid very little attention to the philosophical goals of the testing program (the goals of a regulatory agency being different from those of a manufacturer of a widely used over-the-counter medicinal agent). Nor have we addressed the societal cost of the misclassification of carcinogens on the basis of the results of short-term tests. Such misclassifications will occur unless we take into consideration the fact that there is no perfectly predictive test, each assay is characterized by a different spectrum of false positive and false negative responses. Accordingly, to devise successful strategies for the deployment of short-term tests to identify tumor promoters, a concerted effort will be required. The model could be of the type that our colleagues at the National Cancer Research Center in Japan, under the leadership of Dr. Sugimura, have undertaken, namely a targeted approach.

Some of the requirements for the development of adequate data bases of tumor promoters should include the following:

1. We need one or several standard animal systems to identify tumor promoters against which other assays will be calibrated. This requires agreement on the choice of one or several systems such as the two-stage mouse skin assay, the two-stage urinary bladder assay, the induction of hepatic foci, the two-stage forestomach assay, etc. Additionally, in each instance a single protocol must be agreed upon as well as the a priori interpretation of the assay

2. The choice of a panel of chemicals is also very important. It is essential that tumor promoters as well as non-tumor promoters be included. Promoters must not predominate in the data base as presumably they do not predominate in the universe either. For future structure activity considerations, a broad spectrum of chemical classes of promoters should be included .

The choice of the short-term assays is very critical. We do not want to repeat the mistakes that were made in the development of genotoxic screening assays, i.e., a large number of tests, but each tested with a restricted number of chemicals. Initially, because tumor promoters may be organ-specific, a battery consisting of ten short-term tests should be selected and tested with all of the test chemicals. If this approach is chosen, the choice of the endpoints is most important. We do not want to include a variety of tests that all measure the same endpoint, i.e., inhibition of cell to cell communication or a variety of cell transformation assays (Table 1). Rather it is essential that initially each of the major endpoints (Table 2) be represented by a single system using an

agreed protocol, e.g., altered foci formation (Pitot), inhibition of cell to cell communication, and enhancement of cell transformation. Moreover, before such studies are undertaken, the protocol must define as to what constitutes positive and negative responses and moreover when a positive response is obtained, what constitutes a weak, moderate or strong response.

During the early developmental phase , genotoxicity tests should be included. This could be accomplished by the parallel testing of the candidate chemicals for their ability to induce gene mutations, chromosomal aberrations, cell transformations. Alternatively, the chosen chemicals can include agents that have already been so tested.

The next step involves the establishment of criteria on how to analyze the results especially with respect to the analysis of mixed results. The goal being to reduce the 10 initial tests to a battery consisting of 3 or 4 highly predictive tests that ideally should be able to predict a variety of tumor promoters differing in species and organ-specificity (however, see below). Our experience indicates that Bayes' theorem is a very powerful tool for making such predictions even when mixed results are obtained as it provides a numbered index of risk (from 0 to 100%). Additionally, it is also very useful in the batteries selection process.

In developing the algorithms for selecting batteries, we have to bear in mind that unlike the unique target (i.e., DNA) involved in genotoxic carcinogenesis, we may find that with promoters each target organ may

be characterized by a different spectrum of predictive tests, i.e., we may have a different battery for liver as compared to bladder promoters.

In the selection of batteries we have to be able to differentiate between tumor promoters, complete carcinogens, non-genotoxic carcinogens and non-carcinogens. This may require the use of so-called mixed batteries, for example, a complete carcinogen might be described as one that is positive in a battery consisting of a subset of genotoxicity tests and a subset of tests designed to identify tumor promoters. We then have to determine by appropriate analysis and modelling, a priori, the level of required response in each subset. Finally we must be able to differentiate between non-genotoxic carcinogens and promoters, this also might be accomplished by a different mixed battery of tests in which for example we may require negativity in a battery of genotoxicity assays and positivity in a battery for tumor assays. Again, the level of response in each subset of tests will have to be predetermined by appropriate analysis and modelling.

If we are fortunate enough to develop a data base and to identify a number of tests, possibly five or six assays which are predictive of tumor promotion, then we can set about to devise batteries which reflect certain philosophical or scientific requirements; such as (1) a battery to differentiate between weak, moderate and potent promoters, (2) risk averse or (3) cost averse batteries. Finally, we might attempt to take into consideration the societal cost of misclassification of tumor promoters and to develop models to estimate the various scenarios and to construct

batteries to satisfy these requirements. Thus, for genotoxic carcinogens it has been suggested that the societal cost of false negatives is ten times that of a false positive and batteries to meet these criteria have been constructed.

With respect to additional methods for predicting tumor promoting activity, structure activity relationships present another target. Two main approaches can be taken. One of these involves the application of the artificial intelligence system (such as the CASE program developed at Case Western Reserve University). Such an approach requires a data base composed of active as well as inactive chemicals as well as a measure of their potency, or lack thereof. CASE requires a learning set of approximately 30 chemicals divided among positive and negative responses for each of the data sets, i.e., endpoints under consideration, e.g., bladder tumor promoters. CASE allows the merging and comparison of data bases to investigate, for example, whether the same mechanism underlies liver and skin tumor promotion. The advantage of such an approach is that it can then be used in a predictive mode to predict not only activity, or lack thereof, but also the expected potency, and it is totally independent of operator biases.

Another approach involves the use of computer graphics to match regions on the various molecules that may have the same configuration. This approach has been taken by Drs. I.B. Weinstein and T. Sugimura. However, in order to be effective and to restrict the number of possible models, x-ray crystallographic patterns of some of the key molecules are

needed. This greatly simplified the analysis by restricting the number of possible solutions.

When undertaking such studies, it is evident that a number of structural determinants have to be taken into consideration. For example, the active phorbol esters all appear to share a single (or family of) receptor site(s) which is (are) structurally determined. However, this is not necessarily a stumbling block in the application of the CASE program or a combination of the CASE program and computer graphics. Thus, in a recent study on the structural basis of the activity of inhibitors of bacterial DNA gyrases, it was found that there were two types of structural determinants that were crucial; (a) a species-dependent determinant controlling entry into the cell (i.e., permeability), and (b) a unique determinant that involved the inhibition of the intracellular DNA gyrase and was independent of species. There is a similarity between DNA gyrase and the tumor promoters with respect to the fact that we already know that there may be specific receptor sites (e.g., for TPA-like promoters) that are promoter/species specific and may affect entry or be related to effects other than tumor promotion. Additionally, however, there may be unique structural determinants which are related to the intrinsic tumor-promoting activity proper. Obviously, there could be a family of such determinants thay may be organ-specific. The data base to be developed should resolve these possibilities.

In summary, it would appear that techniques are available for identifying and predicting the activity of tumor promoters as well as for

providing mechanistic information. However, to assure sucess will require a concerted effort using standard protocols, an agreed upon group of chemicals, an a priori agreement on the interpretation of test results and the application of recently developed computer-based methods.

Table 1

Inhibition of Intercellular Communication

Inhibition of metabolic cooperation - V79
- HPC
- Human teratocarcinoma

H^3-Uridine exchange in C3H/10T½

Citrulline incorporation - V79
- Human fibroblast

Dye transfer - Balb/c 3T3
- V79
- Syrian hamster cells

Permeability of tight junction - MDCK cells

etc.

Transformation and Enhancement of Transformations

C3H/10T½ - focus formation
Syrian hamster embryo cells - Morphological transformation
Balb 3T3
Rat embryo fibroblasts
Mouse epidermal cell line JB-6
Enhancement of SV40 ts - Swiss 3T3

Table 2

Some Tests Used to Identify Tumor Promoters

Activation of EB-virus
Inhibition of TPA-induced activation of EB-virus
Release of lipid metabolism
Stimulation of arachidonic acid metabolism
Reduction of nitroblue tetrazolium
Skin irritation
Ornithone decarboxylase in vitro and in vivo
Modulation of cell differentiation : Human promyelocytic cells +
: Friend erythroleukemia cells -
: Hamster cells -
: Sea urchin - etc.
Aggregation: Lymphocytes
Adhesion of promyelocytic cells

DNA damage
Stimulation of DNA synthesis
Agglutinability by concanavalin A
Aneuploidy in yeast
Co-recombinogenicity in yeast
Inhibition of neurite formation
Gene amplification
Transformation assays
Inhibition of intercellular communication
Altered foci formation
etc.

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promotors

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1. Tumor promotion can be defined both operationally and mechanistically. The operational definition of tumor promotion is the process that leads to the induction of tumors when a weak or noncarcinogenic agent is given repetitively after a subthreshold or approaching a subthreshold dose of a carcinogen (initiation). Tumor promotion must be defined in relationship to tumor initiation. Mechanistically, tumor promotion is defined as the process by which an agent brings about the selective expansion of initiated cells which increases the probability of malignant transformation. Initiation may be defined as a change in a target tissue which induces an essentially irreversible alteration such that subsequent treatment with a tumor promoter expresses this event.

In the absence of a method to measure and identify initiated cells, it is impossible to

rigorously classify compounds as pure initiators or pure promoters.

2. Yes, it is possible to quantitatively separate the promotional activity from the initiating activity of a chemical in assessing the carcinogenic risk. This can be achieved by testing a given chemical at several dose levels as an initiator using a standard promoter and as a promoter using a standard initiator. However, in attempting to classify agents quantitatively as either a promoter or an initiator, it is generally recognized that most agents appear to exhibit both activities to a variable degree.

3. In order to incorporate the qualitative characterization of promotional activity into a risk assessment, it would be necessary for an unknown compound to meet the operational definition of a tumor promoter in a given tissue in several different species. For example, it would be difficult to accept an agent as a human skin promoter if it was a recognized promoter only in mouse skin.

4. This is a very difficult question to answer since to my knowledge, there are no known pure promoters. This is especially notable in extensively studied

compounds. In order to designate a promoter as pure, it would be necessary to secure extensive dose-response data on a given chemical following testing as a complete carcinogen, a tumor initiator, and a tumor promoter in a large number of possible target tissues from several species. If these conditions were met then one could be reasonably certain that the tested compound was only a promoter (pure promoter). This is highly unlikely, however, due to the distinct possibility of spontaneously initiated cells.

5. Since major observable differences exist in tumor promoters from various species and organs, it would be necessary to select a number of chemicals in order that all major target tissues would be represented. The following chemicals would be useful:

   Teleocidin

   TPA

   Chrysarobin

   Benzoyl peroxide

   Phenobarbital

   Saccharin

Although others might be considered, these compounds are especially useful due to the available data base. However, extensive dose-response data is also needed on these compounds in both tumor studies as well as a number of short-term parameters. In addition, the effects of these compounds on human tissue in culture are desperately needed.

6. This is also a very difficult question to answer since a number of the generic considerations for interspecies extrapolation depends to a certain degree on the target tissue of the tumor promoting agent. For example, a skin tumor promoter may have different considerations than the liver in terms of using body weight or surface area. Penetration into the skin is difficult for highly water soluble compounds, whereas lipid soluble compounds can penetrate quite easily. Consequently, the route of exposure is very important. The possible metabolism and pharmacokinetics of a chemical in various species are very important in interspecies extrapolation.

Comments for the EPA Workshop on Risk Assessment Methodologies for Tumor Promoters

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1. Definition of Tumor Promotion

The concepts of initiation and promotion are operational, in that they describe distinct phenomenological observations in experimental animal model systems demonstrating that carcinogenesis in many organ systems of several species consists of multi-steps.

I accept the Berenblum definition, and the Boutwell-type of animal protocols of initiation and promotion. That is, tumors are produced experimentally when an animal is exposed to a single subthreshold dose of a "carcinogen" (1), followed by a repetitive or continuous exposure to a non-carcinogenic stimuli. I also accept the idea that initiation and promotion involve distinctly different molecular/cellular mechanisms. In addition, I feel promotion is the rate-limiting step of carcinogenesis (2).

The basis of my definition is the result of the historical whole animal studies, plus hundreds of molecular, biochemical and cellular studies on agents/conditions, in vivo and in vitro, which can influence either one or both phases. In addition, the definition is, in part, re-inforced by my own research experience studying agents which have been shown to be "carcinogens, initiators and promoters".

The major limitations to the definition are: (a) The concepts do not imply any molecular or cellular mechanisms for the two stages, other than the fact that initiation appears to induce an irreversible alteration in a cell's genome, and that promotion must involve the clonal expansion of the initiated cell; (b) also, physical objects such as implanted solids, or wounding, burns, etc. can also "promote"; and (c)

the practical problem of distingusihing what really constitutes "carcinogens" or non-carcinogens? (3)

2. Since I'm not sure what the question is asking, I'm going to answer on the basis of what I believe are the distinctions between carcinogens and initiators and between initiators and promoters.

By definition, a "carcinogen" is an agent which can accomplish both mechanisms underlying initiation and promotion. When a "carcinogen" is a carcinogen, it can initiate and promote. When a carcinogen is given at "subthreshold doses", it can initiate but can not promote. Therefore the difference between carcinogen and initiator is basically the dose level. In practical terms, this dose level seems to be the threshold, in tissues, between no detectable tissue damage and detected necrosis. (4)

Promotional activity on the other hand, in the case of mouse skin papillomas and rat liver enzyme altered foci, seems to involve, among other things, the clonal expansion of the initiated cell. Therefore, without mitogenic activity, clonal expansion, by definition, can not occur.

Clearly, agents which can mutate cells, via any mechanism (i.e., error-prone replication off of normal DNA; or error-prone repair off of damaged DNA), have been shown to be good initiators. At low doses, rare mutations can occur without much cell death, thus being consistent with the concept of "initiator". At high dose levels, both mutations and cell death would occur. The cell death, caused by a

high dose of an mutagen, would induce regenerative hyperplasia, thus allowing one to refer to this high dose of a mutagen as a "carcinogen".

Promotion can be induced by an agent or condition which would allow a single initiated cell to clonally expand, either by non-cytotoxic, mitogenic stimuli (i.e., growth factors, 2',4',5',2,4,5-HBB) or cytotoxic-induced hyperplasia (wounding, high dose of "carcinogen" or non-genotoxic cytotoxins). (5)

3. First, from my experience, there are promoters and then there are promoters! The literature makes it quite clear that promoters can be classified many ways (i.e., TPA-type which bind to receptors and act as hormones at ngm levels; DDT or phenobarbital-types not needing receptors and working at ugm levels; saccharin or NTA-types, which work at mgm levels). Also, there are promoters which can be metabolized (i.e., TPA) or excreted (i.e., saccharin), and others which are not biodegraded nor excreted (i.e., PBB's).

   Therefore, these factors of mechanism of action and of biodegradation-excretion must be accounted for.

4. Aside from my own personal bias related to my hypothesis on the cellular mechanism of tumor promotion (i.e., inhibition of intercellular communication) (6), I would have to know that the agent can not induce point mutations, using only a few mutation markers. I do not accept any bacterial mutation assay data as relevant to the mammalian situa-

tion. I also do not accept SCE's, UDS, or alkaline elution data as equivalent to "genotoxicity". Moreover, the TK^- or TG^r markers must be accompanied by ouabain-resistance before I would accept any data on a chemical's presumptive mutagenicity (7).

5. Based on my previous comments in (3), I believe there are <u>at least</u> three or four distinct biochemical mechanisms of promotion. TPA is a classic model for what I believe to be a relatively rare type of environmental promoter. To me, it is a good model for endogenous growth factor or hormone-types of promoters (i.e., those needing receptors). I believe, PBB, Dieldrin, and Phenobarbital to be excellent models for those typical environmental promoters which, being lipophilic, need no membrane receptor to trigger their effects. In addition, NTA or saccharin represent another distinct class. Finally, TCDD seems to be in a class, quite distinct from all the rest, which might again represent a rare, but important environmental promoter. In general, since I feel promoters must be, among other things, <u>mitogens</u>, research on the ways these model compounds can be mitogens must be studied (8).

6. In my personal opinion, one major area which has been ignored, except in a few laboratories, is that of synergisms/antagonisms between environmental promoters and endogenous factors (growth factors, such as EGF, or hormones). This might explain sex, developmental stage, and tissue differences.

While I understand the historic role of the whole animal model to test and study promoters and promotion, I believe we are almost at the

stage of in vitro modeling with various normal human cell systems (keratinocytes, kidney epithelium, hepatocytes). Therefore, I do not hold any hope for whole animal to whole human extrapolation, since I believe there are too many intervening factors.

In answering the last question on the nature of chemical specific considerations needed for extrapolation of risks, based on my experience working with different kinds of promoters on different kinds of cells from different organisms, including human fibroblasts and epithelial cells (9), I'm not sure one can generalize for all types of promoters (i.e., TPA versus PBB versus NTA). However, I have found that if a given promoting chemical has a measureable effect, in vitro, on one cell type of one organism, it has the same cellular effect on the similar cell type of another organism. The chemical can have dramatically different effect on a different cell type of the same organism. Any difference between the chemicals effect on the whole animal on one species and the potential risk to the human probably is due to the indeterminable intervening physiological/immunological factors unique to the human individual.

One of the most important considerations, in my view, must be, "Is the chemical metabolized or excreted in the humans." If not, it has the potential to accummulate and reach critical mass levels needed for promotion to occur.

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APPENDIX D

EDITED TRANSCRIPTS OF TUMOR PROMOTERS WORKSHOP
(Feb. 3-5, 1987)

EDITED TRANSCRIPTS OF TUMOR PROMOTERS WORKSHOP[1] (Feb. 3-5, 1987)

FEBRUARY 3

TAPE 1

Vaun Newill: The purpose of this workshop is to seek your advice in identifying research that will help to reduce the uncertainty in one aspect of the risk assessment procedure. In 1982, the EPA Office of Toxic Substances held a workshop on tumor promoters. Scientists were asked to address the issue of how to incorporate information on promoter activity into risk assessment. The participants acknowledged the need to incorporate such information into risk assessment but were not able to offer the Agency guidance on how to address the issue. Recently, both the Science Advisory Board in its review of perchloroethylene and the OPTS panel on dioxin recommended that the EPA consider integrating promotional activity into the traditional risk assessment. We have therefore convened scientists familiar with the issue to help set research goals to improve the scientific bases for addressing promotional activity in risk assessment. We are not seeking guidance for specific chemicals. We are seeking guidance on identifying research to fill knowledge gaps in assessing the potential hazard associated with exposure to promoters as a class of substances, and on how to prioritize this research according to its impact and utility for risk assessment.

Hugh Spitzer: We are seeking guidance for prioritizing research on issues of tumor promotion.

Roy Albert: In the EPA guidelines for carcinogen risk assessment that were adopted in I think September 1985, the whole issue of promoters was waffled by saying that in the absence of any evidence to the contrary, promoters should be considered as complete carcinogens for risk assessment. Everyone felt this was unsatisfactory, but there was not enough consensus to develop an alternative approach in terms of either a qualitative judgement of how likely an agent is to be a promoter, or quantitatively, how great a cancer risk it might pose for given levels of exposure. So for practical purposes, EPA currently regards agents that show signs of being promoters as complete carcinogens. This has been done for dioxin. So I'll present one view of assessing the risk posed by promoters. Some key questions one might ask to identify a promoter include:

> Is there reversibility with respect to dose, i.e., if you interrupt the dose, is the response more markedly

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[1]Complete citations for references in this appendix are provided in Section 14, References, of the main report.

diminished than with the total dose? (This is an accepted pattern of behavior in the skin. I don't know if it's the case in the liver.)

Is the initial response one of benign tumors?

Is there a long interval to cancer (e.g., 3 to 6 months in skin)?

There is some evidence of dose-response for proliferation as related to promoting activity.

Short-term tests, cell-to-cell communication, activated oxygen radical, SCE, etc.

I don't think that the issue of dose with respect to malignancy has been well established (i.e., does the malignancy of tumors diminish as dose decreases?). The assumption with carcinogens is that as dose diminishes the incidence of tumors diminishes but not the severity of malignancy. This is very different for noncarcinogens.

I offer this list as a strawman as an example of a way to evaluate promoters. Not all promoters will show all these characteristics.

Henry Pitot: In systems where the multistage phenomenon has been well characterized, I think that reversibility is almost an absolute characteristic of tumor promotion. In the skin and the liver this is certainly true. In other systems, such as the bladder or the breast, where the stages have not been adequately characterized, I don't think you can yet make a statement such as this. I would argue that, for this discussion, we should stick to systems that are well defined.

Roy Albert: Do you agree that the first lesions of promoters are benign?

Tom Slaga: That's obviously true in the skin and liver. Benign tumors at least come before malignancy.

Henry Hennings: When we promoted for only 5 weeks (DMBA initiation, DBA promotion), we only got one-fourth as many papillomas as we did with 10, 20 or 40 weeks promotion, but we got just as many carcinomas. This suggests that there is a big difference in papillomas with regard to whether they will progress to carcinomas. Every carcinoma apparently arose from a papilloma.

Tom Slaga: I'd like to emphasize that different promoters behave differently. Some substances give few papillomas but many carcinomas. Dr. Saldez, Dr. Conti (sp?) and I have done an extensive study currently in press at the NAS where we

looked at all the benign tumors. Even early after tumor promotion, most benign tumors are diploid. As treatment progresses, around 40 weeks (with treatment twice a week), all benign tumors are aneuploid with sections that could be called carcinomas. So even benign tumors could have a lot of characteristics as carcinomas if they are analyzed in detail.

Roy Albert: I think the point of what you are saying is that the regressible characteristic of promoted lesions is the characteristic of one component of the population. The promoter brings out a spectrum of transformations in terms of graded degrees of malignancy which range from very low transformation to those that are well on their way to cancer. I wonder whether that is not a property of the initiator, i.e., that the initiator is producing a spectrum of transformations and the regressiblity is a reflection of that.

Henry Hennings: I think the initiator gives a spectrum of initiated cells and the first one that are promoted by TPA for example are those that are furthest along. The papillomas that are promoted for only 5 weeks with TPA don't regress. With longer promoter treatment, you hit a peak and then there is regression (in Sencar mice).

TAPE 2

Roy Albert: We could add another characteristic to the list: A promoter is a poor progressor in advancing papillomas to carcinomas.

Henry Hennings: I agree with this but it depends on the agent. In our studies, after treatment with the initiator and promoter we got many papillomas; then we treated with a third stage agent to look for the progression of papillomas to carcinomas. When we continued TPA treatment, we found no increase in the progression from papillomas to carcinomas. With urethane or 4-NQO, we found an increase in the number of carcinomas.

Tom Slaga: We looked at promoters such as benzyl peroxide and chrysarobin and cytotoxic agents such as acetic acid and hydrogen peroxide. Though certain promoters can act as progressors, we find as potent agents that are just as effective. So we are not sure of the mechanism for bringing about what we think is a second selection process through cytotoxicity which selects out more aggressive cells and leads to cancer.

Roy Albert: Is there a strong association between agents that are progressors and genotoxicity?

Tom Slaga: Initial studies by Hennings and Yuspa suggested that. But we're not sure it's all genotoxicity, even though

all the compounds have a cytotoxic base. We did several experiments where we kept raising the dose of TPA which does not act as a progressor. If you raise the dose of TPA to the point where it becomes very cytotoxic, it still does not act as a progressor. So it's not as simple as just cytotoxicity. There is something we don't understand.

We tried several isomers of the diol (sp?) epoxide of benzopyrene as progressors where we applied them once when the benign tumors were present. The minus antidiol epoxide which is a noninitiator in the skin is the most potent of all the agents we have looked at as a progressor to the point where all our mice had multiple carcinomas. So there are agents that are extremely potent. We just don't understand what is going on.

Henry Pitot: In the liver, thus far, there is a clear distinction between a progressor and a promoter. And I think if we look closely in the skin we will find that the same thing is true. With phenobarbital in a carefully defined system you get very few carcinomas that take a long time to appear. You could argue that if the progressor effect is genotoxic in the liver, then the incidence of the conversion of a cell in the stage of promotion to progression is probably as low or lower than the initiation stage itself. That is, you can get thousands of cells initiated, but only one or two that develop carcinoma. The liver has the advantage that you can identify single initiated cells and their early clonal progeny. I would argue that we should separate promotion from progression. I think we can do it in the skin and the liver.

Roy Albert: What are examples of liver progressors?

Henry Pitot: The same as in the skin. One example was reported by Scherer et al. (1984). They initiated with diethylnitrosamine, promoted with phenobarbital, and gave the second dose (in this case an akylating agent - ENU) and got the same effect. In the liver, you can follow the lesions resisting the second dose from very early on. You get the beginning of a microcarcinoma within a focus or nodule. We now think we have methods to quantitate the foci which provides a quantitative measurement of the effectiveness of a progressor agent.

Bob Langenbach: It seems that you are saying that repetitive treatment with an initiator is required to bring about this response. The differences between promotion, initiation and progression are converging.

Henry Pitot: No, I think what is happening is that we have been able to dissect the stages much better. Initiation can be likened to a point mutation which alters a cell but which in no way makes it malignant. A second alteration is probably a much more complicated mutation such as a clastogenic effect. We

have been thus far using classical initiating agents - alkylating agents - all of which are clastogenic. We don't have a system yet in which we have tried other things. It may well be that agents that are purely clastogenic may turn out to be progressors. I think that we are clarifying the picture. I think we must distinguish the reversible stage of promotion from something beyond that which we have recently been calling progression.

Roy Albert: That could be the first landmark of this workshop. Does everyone agree that we are talking about three stages - initiation, promotion and progression - and that there are agents that are associated predominantly with these three steps?

Peter Magee: How does the development of liver tumors after application of DEN, phenobarbital and ENU differ from application of DEN only by repeated dose?

Henry Pitot: By using Scherer's technique you can dissect the stages. If you just give a complete carcinogen continuously you can't distinguish the stages. Scherer's system is a model system that allows you to dissect, quantitate and determine something about each of the stages.

Roy Albert: Is it fair to say that all initiators are progressors although not all progressors are initiators?

Henry Pitot: I think benzoyl peroxide may be an example of a progressor that is nongenotoxic.

Jim Trosko: Anything that damages DNA can be a mutagen. Furthermore, agents that damage DNA not only act as mutagens but can also kill cells at appropriate doses, i.e., they are cytotoxic. However, there are many cytotoxic agents that do not mutate cells. The example of acetic acid as a progressing agent is probably associated with its cytotoxicity rather than any mutagenic action whereas a mutagen as a progressor may also be a progressor by its cytotoxic nature rather than mutagenicity. From what I understand, anything that can induce cytotoxicity which then would force compensatory hyperplasia can act as a promoter. So I am saying that it is important for us to acknowledge cytotoxicity regardless of the mechanism.

Roy Albert: Cytotoxicity is a mushy term.

Bob Langenbach: Henry and Tom, in the systems you have described, if you omit the promoter from this approach and apply the initiator and then the progressor, do you get malignant tumors?

Henry Hennings: It depends on the agent. Urethane gave no malignant tumors. 4-NQO gave a few malignant tumors.

Bob Langenbach: How about if you substitute a tissue-damaging agent such as turpentine for a promoter in an initiation/promotion/progression study?

Tom Slaga: This experiment has not been done. If you initiate, then wait and give a progressor, the majority of the agents that have been used (MNNG, EMU) will give tumors because they are complete carcinogens. If you do those studies with acetic acid (initiate, wait and give acetone, then acetic acid), you do not get any significant level of tumors. If you initiate with TPA to get benign tumors followed by promotion, you don't express any additional benign tumors.

Bob Langenbach: Are promoters really of hazard relative to DNA-damaging agents if such agents are responsible for both initiation and promotion?

Henry Hennings: In our model system, the promoter is just expanding the population of initiated cells so you have a much bigger target.

Tom Slaga: The risk from a promoter is a function of the background of spontaneous initiation, i.e., how many initiated cells you have when you start. How can we define a pure promoter when there is this background?

Henry Pitot: There is also spontaneous promotion. We have many promoters built into our own organism and we eat many promoters. So I would argue that in humans we should be more worried about promotion than initiation.

Eliezer Huberman: Isn't the problem that we don't have pure promoters, initiators or progressors? Are there any agents that act primarily in one of these three stages. Such agents would be extremely helpful.

Henry Pitot: In the liver, we have at least two promoters: phenobarbital and dioxin, which have little if any effect on the other stages of carcinogenesis. But dioxin has peculiar pharmacokinetics because it has a very long half-life, so a single dose is actually a continuous dose. For these chemicals there is no evidence in the liver of initiation.

Eliezer Huberman: Shouldn't we concentrate on these agents?

Tom Slaga: In the skin, stage-specific agents can be identified. Urethane is an initiator in the skin, but can cause cancer in other tissues. Chrysarobin and benzoyl peroxide are fairly pure promoters in the skin. Likewise the diolepoxide of benzo(a)pyrene can be considered a pure initiator.

[BRIEF DISCUSSION OF EXPERIENCE WITH INDIVIDUAL CHEMICALS]

Roy Albert: But my understanding is that agents may have different properties in different tissues...

TAPE 3

Roy Albert: ....so it is difficult to extrapolate from animals to humans. How do you know if the agent will behave the same way? I hear that nongenotoxic agents can be progressors.

Henry Pitot: My definitions are initiation of a cell produces some irreversible change in that cell which is inherited by its progeny which means the cell could potentially become malignant. Promotion is the reversible expansion of the initiated cell population. Progression is an irreversible change in a cell that likely involves a major structural change in the DNA - either a clastogenic event, a major deletion, or a translocation - so the cell is malignant.

Roy Albert: This workshop has been the most vigorous exposition of progression that I have heard. So I think that a consideration of both promotion and progression are within the scope of this workshop - and even initiation as a stage in carcinogenesis.

Eliezer Huberman: I consider initiation to be a genotypic change that does not necessarily result in malignancy; promotion, on the other hand, I consider to be the stage that involves the conversion of an initiated cell to a tumor cell and the clonal expansion of these cells.

Henry Pitot: In the liver, we can demonstrate that in the presence of a promoter, the initiated cell population expands. Once the population achieves independence of the presence of the promoter, we would argue that it is in the stages of progression. If you remove the promoter, the majority of cells in the altered foci disappear. We have isolated these cells and put them back into an animal. They only survive as long as the promoter is there.

Eliezer Huberman: The expanded cell population in tumor promotion represents simply a quantitative difference between tumor initiation and promotion.

Henry Pitot: Yes.

Roy Albert: But in the skin a promoter can produce both reversible and irreversible lesions.

Henry Pitot: The same thing can happen in the liver. All I am saying is that once you have gone to irreversibility, you are likely in the stage of progression.

Peter Magee: How do you distinguish an initiated cell from one that simply reacted with the initiator?

Henry Pitot: Japanese investigators have recently claimed that they can demonstrate 1 in 1,000 cells initiated in the liver using a different marker. I am not ready to accept that. If you apply a promoter, only about 1 in 10 or 1 in 100 of these cells expand to a colony. So many of the marked cells don't act like they have been initiated. So I would say that the initiated cell population is only that population that can expand in the presence of promoter. Only a few cells will expand in the absence of promotion, but very slowly. One could argue that this is spontaneous promotion.

Henry Hennings: We found that if we initiate and then wait for 5 to 20 weeks before promotion, we get papillomas earlier. The simplest way to explain this is that the clone of initiated cells has increased in that time. We typically get papillomas 2 to 3 weeks earlier.

Roy Albert: Do we have a consensus on these definitions: that progression is an irreversible change in DNA towards malignancy and promotion is the reversible expansion of initiated cells? NO COMMENT ON AGREEMENT OR DISAGREEMENT FROM THE PANELISTS.

Dan Krewski: I was wondering if we should look at the two-stage birth-death-mutation model that Moolgavkar developed because that leads to very precise notions for initiation, promotion and progression. If those precise definitions correspond to what you have just indicated, then I would agree.

Roy Albert: I think the striking thing here is the notion that initiated cells are so common.

Anne Kennedy: Work in Wisconsin by Clifton et al. (1984) has shown that if you carcinogen-treat cells and then put them into an animal, you only need a few cells to give rise to a cancer. If you can go down to 20 cells treated with the carcinogen and it causes cancer in animals, it is not a single base mutation that is the initiating event. Single base mutations are not going to be in that high a proportion of cells. So if the initiating event is common, we need to look at things quite differently.

Henry Pitot: If you treat with a DNA-damaging agent at sufficient levels, you can skip promotion. So we are dealing

with a cell that is both initiated and progressed simultaneously. There is a lot of evidence in liver that high doses almost immediately create cells that are malignant from the start. Even Williams has claimed that when you feed AAF continuously one can identify carcinomas that arise de novo. So there may be doses of chemicals where you skip promotion and go right to progression.

Anne Kennedy: Similar experiments in other labs have shown that even low doses of carcinogens can lead to foci formation.

Henry Pitot: I wouldn't agree with your conclusions for some of those experiments. But I agree that mutation is quite common. But I question the Japanese assumption that just a change in a marker indicates an initiated cell.

Anne Kennedy: Stenback, Peto and Shubik (1981) gave different doses to mouse skin over orders of magnitude and got the same final tumor incidence when all the animals were promoted. Their experiments indicated that initiation was a common event even at low doses.

Jim Trosko: When a mutagen is applied, millions of cells are exposed, but not all DNA lesions will lead to a mutation. Not all cells are in the same cell stage, some are stem, committed or differentiated. Another question is: Where does the mutation occur? Not all mutations will affect the potential for promotion. When we refer to initiated cells, we are referring to a select number of cells in which a few events occur in a select number of genes, even though all the cells were exposed to the initiator.

Roy Albert: From a risk assessment standpoint, it might be useful to try to characterize an agent in terms of the individual actions and its potency with respect to these individual actions. For example, if an agent were purely a promoter, then it would probably be less dangerous at low doses than another agent that had an irreversible component to its action. Perhaps ultimately we may be able to dissect an agent according to the balance between initiation, promotion and progression.

COFFEE BREAK

Eliezer Huberman: The concept that promotion is simply an expansion of initiated cells that are already transformed to tumor cells does not agree with studies in which the hamster embryo colony cell assay was used. In this cell transformation system, we treat <u>single cells</u> seeded for colony formation with a low dose of a carcinogen (i.e., an initiator) and then a couple of days later incubate the cultures with a tumor promoter (e.g., a phorbol diester). Thus, the frequency of

intiated cells in the control and tumor-promoter treated dishes is the same. Yet, the frequency of transformed colonies in cultures treated with this tumor promoter is higher than in cultures of cells that had been treated only with the low dose of the carcinogen. If it was simply a question of expansion, you wouldn't be able to see the effect of tumor promoters in the hamster colony assay. Furthermore, removal of the phorbol ester results in the reversion of most transformed colonies to a normal phenotype.

Roy Albert: No, it is not a question of expanding the number of initiated cells, but rather the clonal outgrowth of initiated cells. So the number of initiated cells doesn't change but it makes the initiated cells proliferate rapidly to form a clone.

Eliezer Huberman: But even that is a problem, because if you remove the promoter from the dishes, more than 80% of the transformed colonies will revert to the normal phenotype.

Jim Trosko: In a recent paper in Science (Herschman and Brankow, 1986), UV was used as an initiator in a cell system, and only after TPA did they find transformed colonies. When they plucked these colonies out and grew them in the absence of TPA, they looked normal, but when they cocultivated those pure initiated clones with normal cells they didn't see any foci except in the presence of TPA. So I would suggest that TPA removed a suppression.

Eliezer Huberman: I'm arguing that a tumor promoter can directly alter the phenotype of the cell, but this alteration requires continuous presence of the tumor promotor. I would, therefore, suggest that the definition of tumor promotion be modified.

Henry Pitot: If you remove the TPA and then reintroduce it, are the cells phenotypically different, or are you dealing with a phenotype that is set within the initiated cell and you are getting selective growth of those cells in the presence of promoting agents?

Eliezer Huberman: In our case, we are dealing with a colony from a single cell.

TAPE 4

Eliezer Huberman: ....We must inject into the definition of tumor promotion that the promoter induces the initiated cells to convert from a normal phenotype to a tumor phenotype.

[SOME MORE DISCUSSION OF HUBERMAN'S EXPT. HENRY PITOT HAS SEEN SIMILAR RESULTS IN THE LIVER]

Henry Pitot: Suppose we alter the definition to say "the reversible expansion and the reversible alteration of gene expression in the initiated cell population"?

Eliezer Huberman: Yes, and I would alter the order.

Roy Albert: So does everyone agree that "promotion is the reversible alteration of gene expression and the reversible expansion of initiated cells." [NO ONE DISAGREES]

Freddy Homburger: What you are talking about works in the Sencar mouse but may not work in another strain or species. We should make an effort to study these things in different species to see if there are significant species differences. Then we could conclude with more certainty whether we could extrapolate to humans.

[ROY ALBERT AND TOM SLAGA AGREE]

Anne Kennedy: I don't think even an exhaustive study of species will help us know what might affect humans. I think we must do human studies to get data on human risks. We already know that promoters are extraordinarily species and organ specific.

[HENRY PITOT TALKS ABOUT PHENOBARBITAL EFFECTS IN HUMANS VS. ANIMALS]

PRESENTATION OF PANELIST PREMEETING COMMENTS (the name of the panelist presenting the comments is underlined):

Eula Bingham: I have trouble translating animal data to humans. I would like to see us talk about human experience with promoters and then back up and see how that fits the experimental animal data. Cigarette smoke is one possible candidate for this. Arsenic and dioxin are another.

Henry Pitot: DDT is clearly a promoting agent for the liver in rodents. There have been at least 30 epidemiological studies for DDT, none of which indicate any carcinogenic effect in humans. So you have a relatively high exposure of a compound which, by our definition, is producing reversible effects yet you see nothing in humans to indicate its (DDT) carcinogenicity.

[DISCUSSION OF DDT]

Eula Bingham: I was suggesting starting with a chemical that does have an effect in humans.

Henry Pitot: I would argue the other way. It is important that we find out what a known promoter does in the human.

Bob Langenbach: I agree. If we are going to do animal experiments, we might as well do them on chemicals for which we have or will someday have human data.

[MORE DISCUSSION OF DDT AND HOW VALID THE DATA ARE]

Bob Langenbach: Maybe the dose was not high enough in humans to see promotion.

Jim Trosko: Two papers are coming out (Aylsworth et al., submitted) that show synergism between two promoters (TPA and DDT). Although these two substances seem to act very differently, there is a tremendous synergism. This may pose another element of complexity. There will be other confounding factors that we won't measure, especially in human studies. Synergism is another potential area for study.

Henry Hennings: I think that progression can be divided into two or more stages. We have called the first stage "malignant conversion," which is the conversion of a papilloma to a squamous cell carcinoma. The next stage is metastasis. Promotion must come after initiation. I think we need to be careful about our definitions. In terms of human exposure, I would be much more concerned about progressors than promoters. John Scribner in a 1983 paper in Cancer Research looked at bromomethylbenzanthracene.

TAPE 5

....He used the approach of testing in stages to determine whether something is a promoter, initiator or progressor. Just take a defined model system, and test the chemical separately with a known initiator and a known promoter. Few chemicals have been tested in this way....Some agents are better than others at giving carcinomas versus papillomas.

[TOM SLAGA TALKS ABOUT B(a)P MECHANISM OF ACTION]

Freddy Homburger: [Presents his premeeting comments (see Appendix C).] Knowledge of the mechanism of cancer is too fragmentary to permit any conclusive risk assessment, except from human epidemiology data. Risk assessment for promoters is even more complex.

Eula Bingham: What is there about TPA or anything else that takes a papilloma to a carcinoma? What is the significant biochemical event?

Henry Hennings: In our experiments, TPA is not good at converting papillomas to carcinomas. I think it is something present in the initiated cell. I think you have a spectrum of initiated cells, some of which are predestined to be carcinomas

from the beginning and you can promote those cells more easily than the others.

Tom Slaga: I believe like Henry that you have a whole spectrum of initiated cells with different degrees of initiation, and that is what makes the whole process so complicated.

Roy Albert: Does anyone know the mechanism of regression of papillomas? This is an area of research that we could recommend.

Eliezer Huberman: At the present time we don't know the mechanisms of any of the stages in tumorigenesis, namely, tumor initiation, promotion and progression. We have discussed the possibility of clonal expansion and inductive processes, and there is also the possibility that immune surveillance is involved. I think we have to analyze not only the mechanism of tumor promotion but also that of tumor intiation and progression since each step may involve a different mechanism. I also have a problem with the fact that most tumor promoters that we use were originally isolated as irritants. So *a priori* we have selected a group of tumor promoters that are irritants. We may be missing other classes of tumor promoters.

Roy Albert: But irritation is not a characteristic of liver promoters. Does phenobarbital in the liver induce cell proliferation?

Henry Pitot: It depends on what reference one cites. [He mentions specific studies.] I think that in the liver, the evidence argues more that the known liver promoters do tend to increase DNA synthesis, at least transiently.

[PANELISTS DISCUSS NANCY COLBERT'S STUDIES WITH GENES]

Anne Kennedy: We do not yet have one human promoter. I think a top priority is to establish what data could establish promotion in humans. My comments concern what data we could look at to establish promotion in humans. Many years ago we promoted with saline instillations to the lung. As an initiator, we used polonium 210, which is an alpha-emitting radionuclide that is in cigarette smoke and is like the radiation we are exposed to in radon gas and its daughter products, plutonium from the nuclear power industry. It is a relatively widespread environmental contaminant. A relatively low dose of polonium 210 to hamster lungs produced few lung tumors. However, promotion with 7 installations of saline 5 months later results in lung tumors in 22-44% of exposed animals. Saline instillations themselves do not cause cancer. At present, some uranium mine workers who have been exposed to alpha radiation from the radon daughter products are having

their lungs washed with saline to remove the material. This is also being done for people exposed to asbestos. This has happened in Boston, Texas and Colorado. So there are some two populations that could be studied for promotional effects from bronchial lavage. .....We get peripheral lung adenocarcinomas. The uranium miners get primarily bronchogenic carcinomas....

TAPE 6

.....I want to challenge the concept of irreversibility of the initiating lesion. Our studies indicate that the first event is potentially, though not usually, reversible. If protease inhibitors are given sometime after the radiation exposure, there is no transformation. We think that some agents such as protease inhibitors can completely revert cells to a noninitiated state whereas other agents cannot.

Several agents are highly antipromotional in in vitro systems. When we do look at humans we could look at the shape of the curve to determine if promotion is occurring. In in vitro transformation systems the curve is the same (essentially a quadratic or linear quadratic) no matter what the initiator is. The presence of a promoter makes the curve linear. This is a dramatic effect.

....TPA and other agents can promote transformation in cells 13 generations after initiation.

There are many human populations that could be studied for promotion with radiation as the initiating agent. These are the radium dial painters that were exposed to alpha-emitting radionuclides that led to bone cancer. Also, people who were given radium treatments in the '40s and '50s for various medical problems.

The studies that I think would be most appropriate are thyroid cancers. From irradiations of people carried out in the '40s and '50s, we are expecting about 20,000 cases of thyroid cancer in this country. These are people who were irradiated for various benign disorders - eczema, acne, thymus enlargement, etc. They have found that in these groups it is higher in women, Jewish people, people who have emigrated from Tunisia or Morocco, people who have immigrated to Hawaii. They have defined many risk groups. The people are alive because mortality is extremely low (1-3%) for thyroid cancer, so they are around for purposes of interviewing. The way they tell if you have thyroid cancer is to give you a scan with an isotope like iodine 131 that gives a dose of 200 rads to the adult thyroid. That is the optimal dose for the induction of cancer in many different systems. This would be an excellent progressor - a yearly dose of radiation.

There is a dose-response relationship for many of the several different kinds of cancers that have developed from radiation. The thyroid and the female breasts have the highest sensitivity. These have linear curves. The breast is a tissue that is under strict hormonal controls and has perhaps a built-in promotional agent and maybe this is why the curve is linear. The same is true for the thyroid. We know that thyroid hormones act as promoters in several in vivo and in vitro systems. The linear response suggests that a promoting agent may be important in the genesis of those kinds of cancers.

I think the uranium miner studies are the best potential data for human promotion. Many houses in Massachusetts have exposure to levels of radiation like those in uranium mines. Radiation is an environmental agent that is capable of initiating cells. The curve for induction of lung cancer in white uranium miners who smoke is linear. When the first study of the Colorado plateau uranium miners was performed, the nonwhite miners (American Indians) who didn't smoke had a nonsignificant incidence of cancer. But recently Indians have been getting cancer at an elevated rate, and the cancer incidence curve is now significantly above control levels.

Here are in vivo/in vitro data from three labs through 1984. These data allow investigators to know how many cells have been exposed to carcinogens and give rise to tumors. Clifton et al. (1984) used fairly high doses of radiation to get the effects. They found that at 20 cells per graft site, approximately half the animals got tumors. It is hard to envision a single base mutation in a large proportion of those cells which could lead to the tumors. Dr. Pitot, don't you assume that the initiating event is the same, whether the dose of carcinogen is high or low?

Henry Pitot: The dose does matter, because with a high dose, you are getting initiation and progression almost simultaneously. I would argue that if you want to look at initiation you should isolate the event. If you telescope it, you are looking at complete carcinogenesis.

Anne Kennedy: If the initiating event is common and there are many initiated cells around, then the rate-limiting steps in carcinogenesis are the later ones. If the first event is common, then it is not a single base mutation in DNA which occurs with a frequency of about one in a million. The frequency of the initiation event makes a difference in how you go about studying it. If it is a common event, adduct removal studies, for example, to my thinking are meaningless. If we think it is common, then perhaps we should be focussing on promotion and progression.

Henry Pitot: You cannot say that saline is a promoting agent. It starts a chain of events in the animal that alters the hormonal environment which alters gene expression. In the whole animal, you can give many things which we would never think of as promoters, but they change the internal environment in such a way that the end result of many factors in tumor promotion.

Freddy Homburger: The instillation of saline or anything else into the hamster lung cannot be compared with human response. The hamster takes it without any general response - no adrenal enlargement, hormonal change, or struggle. I think we are a little too quick to label something as a promoter just because we see an increased tumor incidence when it is administered following exposure to a carcinogen. In recent experiments with guinea pigs (McFadden et al., 1986), it was shown that the retention of small particles of asbestos is increased by the inhalation of cigarette smoke. Therefore their effect could be enhanced, but this would not be promotion. With saline, you may just be changing the dynamics of the disposal of polonium particles.

Roy Albert: Freddy Byrnes (sp?) did an experiment with radiation on the back of the rat using doses that were marginally tumorigenic. We did skin stripping which produces a brisk proliferative response and we plucked hairs once a month, and nothing happened. So maybe cell proliferation works in some places but it doesn't in others.

Henry Pitot: That points out that Eliezer Huberman's addendum to the definition may be very important.

LUNCH

Dan Krewski: [Reads his definition of promoter from his premeeting comments. See Appendix C.]

TAPE 7

The multistage model assumes that a cancerous lesion occurs following the completion of k distinct stages which are usually thought of as being some kind of mutational event. This model does a good job of describing the age-incidence curves for human cancer which are generally related to some power of age. One problem is that you may require a model with five or six stages in order to adequately describe some human cancer data. This may not be biologically reasonable.

An alternative model is the Moolgavkar, Venzon and Knudsen (M-V-K) model which is a stochastic birth-death-mutation model that involves only two stages. The advantage of this model is that it incorporates explicitly information on the kinetics of

tissue growth and differentiation. It involves only two stages relating to mutational events and it seems to be consistent with much of the experimental and epidemiological data that we have in the area of carcinogenesis. The model assumes that you begin with normal stem cells that can either divide into normal progeny or be killed or you can have mutation resulting in one normal daughter cell and an intermediate or initiated cell. The same things can happen with the population of initiated cells. They can divide, die, or undergo a second mutation to produce a fully transformed malignant tumor cell along with another intermediate cell. [SEE FIGURE 1 IN REPORT] It follows that the age-specific incidence for cancerous lesions under this model at time t is:

$$I(t) = u_1 u_2 \int_0^t x(s) e^{(a_2 - b_2)(t-s)} ds$$

This involves the two mutation rates u_1 and u_2 and the number of normal cells x(s) in the tissue at time s and another term that takes into account the birth (a_2) and death (b_2) rates for intermediate cells. The mutation rates determine what the overall level of tumor incidence is going to be under the model, and the second term, which involves normal tissue growth and the birth and death rate of initiated cells, determines the shape of the curve. If we define initiator, promoter and progressor in terms of this model we get the following definitions:

An initiator is a substance that increases the rate at which the first mutation occurs, i.e., it increases u_1. If it is a genetic lesion, it may be reasonable to assume that anything that increases u_1 may also increase u_2 although the magnitude of the two changes may differ. If this is so, then prolonged application of an initiator may result in the induction of a neoplastic lesion.

A promotor is substance that increases the pool of intermediate cells available for subsequent malignant transformation. This can happen either by increasing the birth rate of the intermediate cells - a_2 - or decreasing the death rate - b_2 - or both. So it is the difference ($a_2 - b_2$) that is important.

I would like to define a progressor as something that increases u_2. [THE GROUP AGREES WITH THIS]

Do we want to consider an intermediate or initiated cell as a neoplastic lesion? [THE GROUP RESPONDS NO]

Do we want to consider an expanded colony of such intermediate cells as a neoplastic change? [THE GROUP AGREES THAT IT IS NOT NORMAL]

A complete carcinogen is something that would increase the rates of occurrence of both the first and second mutations. Is it also necessary to have promotion occurring in order for a complete carcinogen to exist? I would say no. So a complete carcinogen could involve either initiation and progression alone or all three stages. [THE PANELISTS AGREE]

What would we call an agent that increases the rate at which a malignant lesion develops? [THE PANELISTS AGREE THIS WOULD BE CALLED A GROWTH FACTOR]

Roy Albert: This model is a simplification of reality. There is probably a spectrum of lesions of graded malignancy all the way across. For instance, there are papillomas that regress and those that don't.

Dan Krewski: Promotion was suggested as being largely a reversible phenomenon, but how would you reverse the expansion of a pool of cells? Why would cells suddenly start to die when you remove the promoter?

Jim Trosko: I think it is because all these studies have ignored intercellular phenomena. Traditionally we have thought that cancer lies within a single cell, but people such as Potter (1981) argue that cancer involves relationships between cells. This kind of a model does not capture that interaction. So the interaction between cells could be responsible for the reversibility of the hyperplastic effects. Normal cells can suppress the phenotype of this premalignant lesion if there is normal interaction, but if promoters block that suppressing effect, then not only does it cause clonal expansion but it prevents the phenotypic alteration which I and others believe is prevented by some intercellular signal. We must develop models with interactive features.

Dan Krewski: That sounds very reasonable.

Tom Slaga: The way it stands, the initiated cell would continue to gain number all the way through until you promote. It may change slightly, but it would not be double or triple the tumor response.

Jim Trosko: Bell (1976) in 1973 or so presented a mathematical model where he suggested that the premalignant cells could become autonomous when they reached a critical mass. So that implies that once the pool of initiated cells gets large enough, it negates the interactive effect.

Henry Hennings: We know that there are papillomas that are TPA-dependent and there are also autonomous ones.

Roy Albert: It is not clear why papillomas regress.

Dan Krewski: I don't think anyone has mentioned the initiation/promotion/initiation (IPI) type of study which is relevant for separating promotional and progression effects. If we agree with the model that we are increasing the pool of initiated cells, if we a second initiator to enhance the second mutation rate, u_2, we should get a dramatic increase in the crop of malignant lesions that you observe at the end of the study. There are several studies where that is the case.

Substances may possess initiating, promoting and progressing activity. In this two-stage model, if the mutation rate per intermediate cell division ($u_2/[u_2+a_2]$) is considered to be a constant, then an agent that increases the proliferation of the intermediate cells must also increase the mutation rate for the second stage. So if it is a constant, then substances that possess promotional activity within the context of this model may also demonstrate some potential for progression as well.

Roy Albert: That relates to the question of whether simply increasing cell turnover would accomplish promotion and progression. That would tend to support your formulation. I think the model fits what we have been talking about. What does this model do for us in terms of risk assessment?

Dan Krewski: That is a difficult question. To apply the model you will need data on normal cell growth, on cell kinetics at the proliferation stage, and on tumor occurrence as a function of dose. So more data will be needed before the model can be applied. We need data on how the mutation rates vary with dose. The model has been applied to both experimental and epidemiological data and it seems to fit well.

Curtis Travis: I think the model can be used because it provides a theoretical framework with which to interpret experiments or to propose experiments. We can't use it for risk assessment until we know more about mechanisms. One research suggestion is to start with background cancer rates in mice, rats and humans, and measure regular mitotic rates as a function of age. Then the only parameter that you are missing for this model is the mutation rate. That is supposed to be a constant. So there is only one unknown constant, and you want to see if you can reproduce the age-specific cancer rates in mice or rat livers. We have done this, where we found the background mitotic rates for liver as a function of age, assumed a mutation rate of 10^{-8} and were able to reproduce the age-specific cancer rates in rats just from the model. We are trying to do it for mice now. It could also be done for humans.

Another suggestion involves tetrachloroethylene, for which there are long-term cancer bioassay data for mice. Increased

cell turnover rates as a function of dose in mice have been measured. If we can determine the background mutation rates in mice from the background cancer rates in mice, you can combine this with the actual measured increased cell turnover rates in liver from TCE application, use the model to predict the increase in tumor rates and compare it with the actual data. Thus, the model provides a conceptual framework with which to propose and interpret experiments. You could take a look at the increased cell turnover rates in the mouse liver, apply the model and make predictions as to the enhancement you should have seen in the background tumor rates. Moolgavkar did it for breast tumors, although this work could be improved. Almost every parameter in this model is obtainable. The model provides a strong theoretical framework and driving force for experimental work in this area.

Jim Trosko: I agree. Potter's IPI paper (Potter, 1981) outlines this model. One prediction of this framework is that after initiating groups of animals with the same dose of initiator, but promoting for different periods of time, the pool of initiated cells would vary depending on the duration of promotion. Then, depending on the period of promotion, you could expose the cells to the same level of a new initiator. This would be a way to test the model. I don't think this experiment has been done: (1) initiate, (2) promote for various lengths of time, and (3) then initiate at those different promoting times.

Henry Hennings: We did this, but the data are not published yet. We promoted for 5, 10 or 20 weeks after initiation by DMBA giving urethane or 4-NQO in the third stage. We found that the response to urethane or 4-NQO was best with the shorter promotion. This study will be published in the Abstracts of the American Association for Cancer Research in 1987.

Tom Slaga: We have done similar experiments. I think once you get papillomas on the backs, the chemicals have difficulty penetrating all the cells.

Bob Langenbach: Henry Pitot, in your rat studies, you see a levelling off of the number of what you believe to be spontaneous tumors in the animals that you promote, which means that most of the spontaneous tumors probably occurred during embryogenesis or neonatal life. Do you think that spontaneous initiation continues to occur during the animal's lifetime?

TAPE 8

Henry Pitot: Our data suggest that spontaneous initiation in the rat liver reaches a maximum somewhere between 6 weeks and 3 months of age. After that time up to almost a year of age there is no significant change in the total number of initiated

cells in the liver. We have interpreted that to mean that the process of fixation of whatever the requirement for cell liver replication in the initiation process is lost just because the liver cell doesn't replicate significantly after that time.

Dan Krewski: Why don't those initiated cells revert?

Henry Pitot: We think that the promotion of these spontaneously initiated cells is due to endogenous and dietary factors. We have found that the normal chow diet is an effective promoting agent for the liver. We think it might be the plant estrogens that are present which vary with the time of year. Semisynthetic diets eliminate much of this effect. I don't think the skin people have that problem.

Roy Albert: Can you compare the response to phenobarbital in rats with different background occurrences of liver tumors so that you can see whether or not the logic of the system holds up - i.e., if you have a higher incidence of spontaneous tumors, it might suggest a higher amount of initiation and you would expect a larger yield with a given dose of phenobarbital?

Henry Pitot: The number of spontaneous foci is 3 or 4 orders of magnitude lower than what you would get when you initiate with an agent. So it doesn't really contribute anything when you do the experiment. It looks as if the Fischer rat has a higher incidence of spontaneous initiation than the Sprague Dawley rat.

Roy Albert: The whole tenor of what we are saying is that promoters act on spontaneous initiation when you don't give an initiator and that is why you get tumors. So it ought to follow that the more spontaneous initiation you have, the more tumors you have.

Henry Pitot: Can you use the model for single doses of initiators?

Dan Krewski: Yes. One thing we are looking at is what are the effects of changes in the dosing pattern over time on carcinogenic risks.

Bob Langenbach: At a recent meeting at NIEHS, Kinzel reported that pretreatment with TPA followed by an initiator some weeks later and then a second-stage promoter increased the number of tumors (Furstenberger et al., 1985). This may mean that there is a memory for the TPA treatment.

Tom Slaga: We have repeated these experiments up to 10 weeks. The memory has nothing to do with cell proliferation. By all indications, the skin is back to normal.

Bob Langenbach: This memory for promoter treatment could be an exception to our definition of promotion. Secondly, our definition assumes that carcinogen-induced initiated cells and spontaneously induced initiated cells are both promotable. Are all initiated cells the same and do they all respond in the same way to a given tumor promoter? Even among very potent tumor promoters, there are possibly very different mechanisms. For example, TPA interacts with protein kinase C and also inhibits metabolic cooperation, but TCDD does not do well for either one. ....So assays for tumor promoters (in addition to the skin and liver systems), which take into account all possible mechanisms, are needed. Within the NTP bioassay, it may be possible to use a universal initiator where the test chemical would also be used with a group of animals that were previously initiated with a universal initiator. Alternatively, stop studies may be useful to see if tumors in test-chemical-treated animals can regress. ....[Presents his premeeting comments. See Appendix C.] However, the identification of nonpromoting chemicals is difficult, which may be a reflection of our level of understanding of promotion. In recent studies with NIOSH, we attempted to identify chemicals that did not have promoting activity to be used for validating Trasko's V79 metabolic cooperation assay. When we took into account results from the skin, liver, mammary, lung, and colon systems, we were unable to come up with a chemical that was not a tumor promoter in at least one of those systems. For example, phorbol - the standard nonpromoter in the skin system - is a promoter in a mammary system and a leukemic system. So, I don't think looking at just one system is sufficient to determine whether a chemical is active or inactive as a promoter.

Tom Slaga: I think there are ways of determining experimentally whether something is acting as a initiator or promoter, at least theoretically. But if we don't have known pure initiators or promoters, this would be difficult to do.

Bob Langenbach: It seems to me that, if nothing else, the spontaneous level of tumors, which TPA will promote, will always interfere with a final statement of unequivocal as an initiator. As I understand risk assessment, there is a need to quantitatively say that there is no initiating activity and this may not be possible at present.

Tom Slaga: Together, all the studies suggest that in general TPA will give you some papillomas and even fewer carcinomas.

[DISCUSSION OF IVERSON/HUBERSON DATA]

Roy Albert: Do you find initiated foci in human liver?

Henry Pitot: In patients that have either hemosiderosis or a hemachromatosis, you can see focal areas where the hepatocytes have very little iron pigment, but these foci don't look like what we find in rats.

Roy Albert: Is this good enough to use from an epidemiological standpoint?

Henry Pitot: You'd have to take multiple sections of the liver which might be difficult to impossible.

Peter Magee: [Presents his premeeting comments. See Appendix C.]

TAPE 9

Roy Albert: Has anyone tried treating mice with antipane (sp?) to see if you can eliminate the TPA tumors.

Tom Slaga: Other protease inhibitors have very little effect on this initiation if given at the time of initiation. But whether they reverse initiation later in time has not been looked at.

Roy Albert: Is the nature of the lesions produced after PB application the same for initiated and noninitiated cells?

Henry Pitot: The foci look the same.

Henry Pitot: I will talk about how one might quantitate the three stages in the liver system. You can in the liver quantitate the potency of a single chemical for the different stages. The system we use is analogous to the skin with one exception: initiation in the liver must take place during cell proliferation. We stimulate cell proliferation using a partial hepatectomy. Then we administer diethylnitrosamine (you can use many other agents), followed by the promoter usually continuously in the diet or drinking water or gavage. We look at lesions by using histochemical markers and in situ hybridization looking at oncogene expression.

Using computers, you can quantitate the foci. We use three different markers. We have found foci within foci (very occasionally). If you do the IPI experiment, the foci within foci (where one marker clearly only involves a small part of the population of the focus) increases by at least an order of magnitude. We interpret this as a transition from promotion to progression because these are morphologically carcinomas, whereas the focus is quite different. In the future we hope to quantitate the foci within foci, which should allow one to determine in a quantitative manner the transition from promotion to progression.

In the liver system you can develop an initiating index and a promoting index which is a way of quantitating the potency of these two factors. The initiating index is the log of the number of foci (corrected for the background level) per liver per millimole of the compound given in a single dose. The initiation index for TCDD and phenobarbital is zero. [He discusses the I index for other chemicals.] The promoting index is the volume occupied by the foci in the liver in the presence of the promoter divided by the volume of the foci in the absence of the promoter per millimole of the agent per week. We are measuring the ability of the promoter to expand the population. It is relatively independent of time. [He discusses the P index for various agents and answers questions about these indices.] You have to take the threshold into account. If the rate of promoter administration does not exceed the threshold level, one cannot calculate a promotion index.

Freddy Homburger: How are the foci counted?

Henry Pitot: This is a computer plot of three serial sections stained for three different markers. You can overlay this and determine the phenotype of each focus and the number and the volume. We have shown that it is invalid to simply count the number of foci when, as in most cases in our studies, they have unequal diameters. You must look at the foci/volume. Area and volume occupied by foci are equal, so you can get the index from the area. The number of foci depends somewhat on shape. Most are spherical. A few are ellipsoid. ....Different phenotypes grow at different rates.

TAPE 10

...the problem with the skin is that you cannot identify the initial initiated cells. In the skin, you could get a promotion index based on the papillomas.

Steve Nesnow: Have you looked at the effect of the promoter on the I index and vice versa - the nature of the initiator on the promotion index?

Henry Pitot: Not very extensively. [He speculates on mechanisms of growth.]

COFFEE BREAK

Herb Rosenkranz: I will describe how you would go about setting up a test battery to identify promoters. [He presents his premeeting comments. See Appendix C.]

Roy Albert: Are any of the noncarcinogenic mutagens pure initiators?

Herb Rosenkranz: This was looked at initially but it was said that they hadn't been tested adequately....We systematically went through all mutagenic and genotoxic potencies using the TD50 values accomplished by Gold et al. (1984; A carcinogenic potency data base of standardized results of animal bioassays; Environ. Health Persp. 58:9-319) about a year ago on 800 chemicals. We found no correlation between carcinogenic potency and short-term test potency. There is a correlation between carcinogenic potency as expressed by the TD50 and the +/- response in some of the short-term tests. For example, UDS is only responsive to potent or moderate carcinogens. Some tests respond to weak and moderate carcinogens and also noncarcinogens (Ennever and Rosenkranz, 1987; Mutagenesis 2:39-44).

Roy Albert: Has there been any attempt to relate carcinogenic potency to initiation?

Herb Rosenkranz: Not as far as I know..

TAPE 11

Bob Langenbach: As I said earlier, one problem we have had in selecting chemicals to put in in vitro assays for tumor promoters is identifying chemicals that have been adequately studied to show that they are not tumor promoters.

Herb Rosenkranz: ...We combined the data base of PAHs and some standard tumor promoters and we did find some structure among the PAHs (and it is not the bay (sp?) region) which appeared to be correlated with promoting ability. So there are structural determinants which contribute to promoting ability.

Jim Trosko: There are many different mechanisms for mutations (e.g., aneuploidy vs. point mutations).

Roy Albert: How many chemicals does it take to build up an understanding of structure and promoting activity relationship?

Herb Rosenkranz: If you use the computered automated structure evaluation (CASE) system and if you are dealing with a single endpoint that is measuring a finite biological relationship, then a data base of about 15 active and 15 inactive chemicals is sufficient to give you high reliability. If you are dealing with a multifunctional event such as a mutagen or carcinogen, you need 50 to 60 chemicals and even then the probability of being correct is not as high. ...We have the positive chemicals, we don't have the negative ones.

Jim Trosko: I have said that everything will end up being a tumor promoter under the right conditions. [He talks about two similar chemicals that behave differently: 2,4,5-$\bar{2}$,$\bar{4}$,$\bar{5}$

hexabromobiphenyl vs. 3,4,5-$\bar{3}$,$\bar{4}$,$\bar{5}$-hex.] If you test in high enough doses in a cytotoxic protocol, you find that chemicals that do not otherwise show activity then show promoting activity. ....I have no reason to doubt that anything that is cytotoxic in the liver is a promoter. That is my gut feeling.

FEBRUARY 4
MORNING

Tom Slaga: I don't feel that there are enough data available to perform risk assessment for promoters. We don't have that many tumor promoters (probably less than 30 or 40 have been reasonably studied). Most tumor promotion studies are mechanistic. Few are designed to consider risk assessment. We need extensive dose-response data for specific promoters as well as frequency of application. We need short-term studies for morphological and biochemical data. We need species and tissue comparisons. We need data on effects in human cells in culture (since you can't test humans) and we need to know how these compare to the various rodent species that the majority of people use.

My definition of promotion: The modulation of the expression of growth in differentiated related genes in initiated cells resulting in the selection and clonal expansion.

I think the definition of promotion that we developed here is realistic. We must be sure that our definition does not just pertain to the skin. We can't use just a subthreshold dose since in most systems the carcinogen given as initiator does give some tumors.

I don't know what the value would be of giving a promoter before initiation. I feel that the interval between initiation and promotion is important in an operational definition. I think that the promoter must be still effective after a reasonable time (at least several months) after initiator application and I think this should be incorporated into our definition of tumor promotion.

I think we should also consider the bladder. I think it is a good two-stage system. Saccharin as a promoter would be a nice model to add overall to the system. The colon may also be worth adding even though the data are not yet that extensive.

TPA is a good promoter but it is limited in its promoting ability in various strains of mice and in other species. The problem may be that the ester groups are removed in many different tissues which may eliminate its promoting ability. Teleocidin may be a much better chemical to pick over TPA for promotion studies because you don't have to worry about removal of the ester groups. I think TCDD is a good candidate for

study. Chrysarobin is probably also good, since you can induce tumors with low doses in the skin. Sodium phenobarbital would also be good. We need to look at these compounds and other promoters in different tissues, as well as distribution, metabolism and pharmacokinetics.

We don't have any pure promoters. The greater the potency of tumor promoter, the greater the possibility that you will get some tumors.

Curtis Travis: The question is what data do you need to do a risk assessment.

[DISCUSSION OF TCDD]

Roy Albert: For risk assessment purposes, if something is a promoter, then there may be threshold below which it may be safe. The implications of what we are talking about are that for compounds that have more than one type of activity, we can take apart the various components and consider them separately.

Curtis Travis: In risk assessment, we are interested in prevalent environmental chemicals. Those are the chemicals that we should be looking at. We now think that every chemical has some initiation and some promotion properties. We must find a way of separating those properties for risk assessment. I think a chemical like TCE is acting mostly through promotion, so that at low doses most of its activity would disappear. If it's an initiator, then there is no threshold.

Tom Slaga: Unless you have a real data base and extensive dose-response then you can't make judgments about other chemicals.

Curtis Travis: Let us study environmental chemicals and get a data base for them.

Eliezer Huberman: Is it true that promoters have thresholds?

Tom Slaga: This assumption is based on a limited amount of data. We need more dose-response data.

Roy Albert: The argument is made on the basis of reversibility. Presumably reversibility is due to the fact that the effects of the agent are countered at some rate. As the dose gets low, the rate of producing the effect is lower than the rate of counteraction, so you get no effect.

TAPE 13

Henry Pitot: The basis for the no-threshold in the DNA damaging effects is also purely theoretical.

Dan Krewski: Could we look at Dr. Slaga's definition of promotion?

Tom Slaga: We have added "growth in differentiated related genes."

Henry Pitot: It leaves out the point of reversibility.

Jim Trosko: Modulation implies an epigenetic mechanism that occurs all the time in normal cells, e.g., during differentiation of the cell cycle. It's a fact documented in the literature that those chemicals identified in promotion systems do have that property to turn on and off genes through nongenetic mechanisms. It is modulatable and reversible.

Dan Krewski: If we are going to try to associate the notion of threshold with promotion, we don't want to have a definition that implies that promotion can occur through genetic mechanisms. I got a sense from yesterday's discussion that there may well be a genetic component associated with promotion.

Anne Kennedy: Six of the tests in Dr. Rosenkranz's Table 2 (in his premeeting comments) that are used to identify promoters are based on a genetic endpoint.

Roy Albert: This give promoters a little initiating ability. Does the issue of cytotoxicity fit in with your definition?

Tom Slaga: Yes it does. You can bring about selection in different ways, e.g., direct effect on the initiated cell, increase or decrease in differentiation, and selective cytotoxicity.

Roy Albert: Selective toxicity allows the outgrowth of cells that are resistant, but that doesn't fit the definition of reversible gene expression.

Tom Slaga: The modulation of growth can be by selective toxicity.

Jim Trosko: When cells die, they release compounds that trigger the surviving cells to go into wound healing.

Tom Slaga: We can still modify my definition to take into account reversibility.

Bob Langenbach: I think we are trying to put some of these things into categories for which we don't really have the information. There are also cytotoxic chemicals that aren't tumor promoters so cytotoxicity alone is not sufficient.

Henry Hennings: "Selective" is the important factor.

Bob Langenbach: I don't think we can rule out unequivocally that some promoters may cause genetic damage.

Eliezer Huberman: Bob raised an important point. Peter Cerutti and others assume that genetic damage is a critical component of tumor promotion.

Henry Pitot: The oxygen radical effects are most likely working at the interface between promotion and progression. Progression involves DNA damage/alterations in a major way. Oxygen radical effects are clearly indirect. Whenever you have an indirect effect, you will also have a threshold. So one could argue that the oxygen radical effect will show the same sort of thing. I think this effect is most likely working to take a cell in the reversible stage of promotion and place it into progression. Benzoyl peroxide and hydrogen peroxide are probably the best current examples of progressors....The concept of stages of promotion is unique to the skin. In multistage carcinogenesis in rat liver no such stages have been identified.

Roy Albert: It's my impression that reversibility has not been tested on a scale that would permit it to be evaluated adequately. For instance, when you stop promoting with TPA in the skin, you get regression of the papillomas, but there is something left there, and when you start TPA again, the lesions pop right out. In the liver, has the reversibility been tested thoroughly?

Henry Pitot: Not with numerous compounds. Yes with phenobarbital, and the choline-deficient diet, and AAF as a selecting agent. In the liver, as I think in the skin, the stage of promotion in all models of the two-stage phenomenon can be shown to be reversible.

Eula Bingham: What are the data on reversibility in the skin?

Tom Slaga: This is difficult to answer because so many different mouse models have been used. Only CD1 has been extensively studied for reversibility. Initially you get complete reversibility, but if you stop promotion after about 4 to 8 weeks, there is a little bit that is irreversible because you have set some of the initiated cells to the point where they will go all the way through. A few studies have been done in which promotion was stopped after say 4 to 6 applications and then restarted 6 months later to see if you can recoup the effect. Those tumors appear much more quickly so there appears to be a residual effect.

Henry Hennings: I'm not sure we would expect a papilloma to regress all the way back to a single initiated cell. It probably regresses back to a micropapilloma.

Eula Bingham: We don't have much data on regression.

Tom Slaga: The skin is at a disadvantage over the liver in that we can't detect initiated cells which is the only way to really quantitate promotion and its reversibility.

Eliezer Huberman: In the second step of promotion, do you see the same foci as those that appeared the first time?

Tom Slaga: We can't tell this in the skin.

Bob Langenbach: Could you explain how regression occurs from a full-blown papilloma?

Tom Slaga: I don't know any study that has done the extensive histology necessary to determine this. ...When you do a progression experiment, where we induce a fixed number of benign tumors, the chances are that about 30% might regress. When you give a progressor, in every case, all tumors that become squamous cell carcinomas from the papillomas decrease in size before they become _____, and that is why we think that cytotoxicity is involved there. You decrease the tumor size by greater than 50%. When it reaches the smaller size, then it looms up into a carcinoma. (The promoter is not still being given when you give the progressor.)

Roy Albert: Isn't there a major difference in reversibility in the liver and the skin? In the liver, you get hyperplastic nodules at the expense of the normal liver and when you stop promotion there is remodelling and the loss of cells.

Henry Pitot: That's the picture in the Solt-Farber liver model. In our system and Shulte-Herrmann's system and probably Peraino's system, it appears that the cells in the promoted foci die in the absence of the promoter.

Jim Trosko: I think we should discuss some of the three to five current models to explain the mechanism of promotion. I have identified at least 12 areas that I consider gaps that we should discuss to deal with promotion. I would like to introduce three brand new technologies that I think will give insight into testing the three to five models.

One hypothesis is the activation of the protein kinase C molecule by a variety of tumor promoters. Another is the Troll-Cerutti model - the prooxidative oxygen radical model. These are biochemical and molecular models. A third model, a cellular model, that we have presented...is the inhibition of

gap junctional intercellular communication by chemical tumor promoters. It is possible that the molecular/biochemical models can be integrated with the cellular model, since there is evidence linking PKC with cell-cell communication. There are also genetic models, e.g., the genetic recombination model. I would like to present the cell-cell communication model.

TAPE 14

The crux of that model is that once the initiated cell is formed, it is surrounded by and communicating with its normal neighbors. This introduces a higher order of biology into the Moolgavkar two-stage model because the phenotype and future of the initiated cell will depend totally on the communication properties of the normal neighbors. In the literature, there are a variety of mechanisms by which one can clonally expand the initiated cell, simply by removing the suppressive, contact-inhibiting effects of the normal neighbors, by wounding, surgery, physical irritation, etc.

We now have direct evidence that many known growth factors work by blocking contact inhibition. A few of the growth factors have been shown to have promoting properties. We have tested over 100 chemicals. We tested several chemicals in vitro and later in vivo and we were able to predict that these would be promoters.

You can also clonally expand an initiated stem cell by putting a solid (e.g., plastic or metal) next to an initiated cell. That solid is not communicating so expansion is not inhibited.

Promoters may also cause normal cells to proliferate. The difference is that the normal cell after proliferation goes into terminal differentiation. Initiated cells can't do this. Once the critical mass of the initiated cells gets large enough, the suppressing effects of the normal neighbors will be diluted out. This is the essence of the cell-cell communication model. We have developed a short-term test to test this model. This assay has more applications than just tumor promotion.

The process of intercellular communication is mediated by a structure called the gap junction found in virtually all normal cells in every organ. Small molecules below 1,000 daltons are transferred between these gap junctions. Cells in tissue that are coupled by gap junction have all their critical molecules and ions below 1,000 daltons in equilibration. Gap junctions are modulated by drugs, food additives, nutrients, endogenous growth factors, biological toxins, pollutants, neurotransmitters, hormones, heavy metals, etc.

It appears to us using in vitro models to test cell-cell communication that there are at least three classes of promoters: those that have receptors and work at nanogram levels (hormones, TPA, TCDD, etc); those that don't seem to need receptors (DDT, PBB, etc.) but just melt into the membrane because they are lipophilic - they usually work at microgram levels; and those that don't need receptors but are not lipophilic (saccharin) - they usually work at milligram levels.

How does this link to gap junctions? Four intercellular "second" messengers seem to be responsible for modulating gap junctions. One is PKC. One is calcium. One is pH. One is cyclic AMP. The first three close gap junctions. Cyclic AMP increases gap junction function in certain cells.

There is a brand new technology which I think can be used to test thresholds, reversibility, synergisms, antagonisms that have been speculated in the animal promotion model. This technology enables us to bypass rodent cells. We can use human cells - any human cell. We call this technology the scrape loading/dye transfer technique. It is extremely simple. You grow cells to confluence, which we feel mimics the normal situation in solid tissues. We take two dyes - lucifer yellow and rhodamine red dextran - and put these on living cells. These won't go in the membranes of living cells. The yellow dye is a small molecular weight dye so that when it does get into the cell it can easily go through the gap junction. The red dye is too large. Now we scrape the cells, which is just like wounding. The dye will go into the cells along the wound line where the membranes are temporarily disrupted. The membrane heals within milliseconds, trapping the dye in the cells along the edge. You dump the dye out, wash the cells, and immediately put it under a fluorescent microscope with two filters, take a picture and you will see both dyes at the edge. You then put the cells back in the incubator with and without a presumptive modulator of cell-cell communication. Five minutes later you take another picture of the cells. If the cells have good gap junction function, the yellow dye will diffuse away from the edge but the red will not.

We can use this to study dose-response. You can see a clear no-effect level.

Drs. Lowenstein and Borek pointed out about 20 years ago that most cancer cells seem to have defects in their gap junction communication. This technique corroborates this. Tumor cells don't seem to communicate at all. Some oncogenes seem to block cell-cell communication when they are expressed in the appropriate cell.

There aren't many synergism studies in the cancer field. There are even fewer studies of synergism in tumor promotion. There are studies on antagonism trying to block TPA action. We believe that some promoters have their action mediated by PKC (a protein kinase enzyme, a phospholipid, calcium-dependent enzyme). Therefore, if you have two chemicals - one of which will stimulate the phospholipid component of the PKC and the other which modulates calcium - you will have a much more effective activator of PKC than either one alone. We conducted an experiment to see whether synergisms could occur in modulating gap junctions. We used phospholipid-activating chemicals and calcium-modulating chemicals. DDT blocks the efflux of calcium through the membrane. We then added TPA alone and got a nice dose-response curve. Same with DDT. We then held TPA constant and added DDT over the same dose range. They showed synergistic rather than additive effects. We also found synergism between unsaturated fatty acids and DDT. I suggest that a study of synergism of promotion in the liver and the skin be done. PKC is present in both. I would say, do DDT and TPA in the skin, and I would bet you would get an effect level. ....The other two assays are photobleaching and a biological assay to measure communication. We have tested the metabolic cooperation assay for V79 in over 100 chemicals and we have found that it takes at least 3 days in V79 before the cooperative donor cell can die or be rescued. We have found that, in the scrape loading/dye transfer assay, TPA will block communication in liver cells, but only for an hour or two. In V79, it takes days. So these assays measure two different responses: a transient response and a more long-term response. Also, the V79 system uses serum which contains growth factors so we think that the assay may not reflect in vivo conditions. We think the scrape assay is a better mimic of in vivo conditions because we can use serum-free media.

Roy Albert: How big does a clone have to be before it's free of cell-cell communication?

Jim Trosko: That is an important question but I don't think anyone can answer it at this time. Not all cells have the same number or size of gap junctions. We don't know what the diffusion-suppressing molecule(s) is or are. In cells that communicate well, we can determine the rate of communication (i.e., dye diffusion) which varies between different types of cells. We can quantitate all of this with a laser machine.

Henry Pitot: In the initiation/promotion system in the liver, the lab chow diet appeared to have a synergistic effect with promoters. It is possible that these diets may be synergistic with many compounds tested in chronic assays. ....We always test compounds in their crude state because that is the way they are in nature. The diet may be "natural" but it is not like the human diet. Secondly, you are not answering

the question of whether a compound is truly a promoter or has to act with something in the diet. This is a question that the regulatory agencies will have to deal with.

Jim Trosko: We have tested synergism with DDT and aldrin and found that they were additive. We also added quercetin, which is an inhibitor of PKC and we completely blocked the TPA effect on cell communication.

Tom Slaga: How long do you wait after you scrape with the toothpick? Do all cells take an equal amount of time to repair this damage?

Jim Trosko: We haven't studied the latter. The loading time is extremely rapid because cell membrane healing is a very fast process. Not all toothpicks will work the same. Some cells lay down collagen and fibernectin. So if you use the blunt end of the toothpick you may lift up the collagen but you won't affect the membrane. ....All of these studies are done at noncytotoxic doses. You know if you have reached the cytotoxic when dye goes into all the cells.

Bob Langenbach: Have you done this with mouse dermal cells?

Jim Trosko: To my knowledge at least 75 different cell strains and lines have been used. But we are just using human cells.

Bob Langenbach: I think it would be a good opportunity to test whether the system is doing what you think to test mouse, rat and hamster dermal cells and see if the blockage is related to promotion because you have sensitive and resistant species there. It would be interesting to test mouse, rat, and hamster dermal cells.

Jim Trosko: We have done that. ....I challenge whole animal people to do it in whole tissue.

Bob Langenbach: How do you limit your doses?

Jim Trosko: There is a built-in visual demonstration of the cytotoxic level.

TAPE 15

[DISCUSSION OF c-Ha-ras ONCOGENE)

Henry Pitot: We have not seen any transcriptional activation of the protooncogenes - c-myc, c-Ha-ras or Ki-ras - in foci or nodules. We have seen it in carcinomas. With one exception, mutational activation of protooncogenes in rat hepatocarcinogenesis has been either nonexistent or only

occasionally seen. But you can get mutational activation of the c-Ha-ras gene in carcinomas of the mouse liver. It has also been shown in mouse adenomas. Recently, we have found fairly consistent transcriptional activation of the c-raf gene, both in nodules and in carcinomas in the rat liver. One may demonstrate that some but not all foci exhibit transcriptional activation of the c-raf protooncogene by in situ hybridization. We are interested in what the phenotype of these foci is. Some foci show a lowering or absence of the gap junction protein by the immunohistochemical technology, although some show normal levels. The question is, if we do the overlays, do those that express c-raf also have a low level of the gap junction protein?

Tom Slaga: We have looked at the expression of several different oncogenes by promoters in mouse skin in vivo and do not find any change except from benign papillomas. There are studies that activated c-Ha-ras will lead to papillomas if you get it in by skin scraping and then give a tumor promoter, but I don't know of any studies that suggest that the oncogenes are involved in vivo in terms of promotion....There has not been increased expression of c-Ha-ras in epidermal cells before tumor formation.

Henry Hennings: Our studies indicate that c-Ha-ras can be the initiating step or the malignant conversion (progression).

[MORE DISCUSSION OF c-Ha-ras]

Henry Pitot: I think the evidence in the oncogene work is preponderantly that activation of oncogenes by mutation or transcription does not occur during promotion. However, the work in the skin by Balmain leaves it open as to exactly when you get mutational activation of at least the c-Ha-ras gene during multistage carcinogenesis in the skin. There also may be some question in the mouse liver. But by and large, the data indicate that promotion does not involve activation of protooncogenes. ....Studies suggest that most become activated during progression. Balmain's and the Miller's work suggest that there is a mutation of c-Ha-ras that probably occurs during initiation. But is that the direct cause of the whole process? I don't think that has been shown.

COFFEE BREAK

ROY ALBERT SUMMARIZES

1. The concepts of progression and progressors were introduced. There was general agreement on them. This is the first time that I am aware of that there has been such general agreement on the use of the term and its consideration for risk assessment.

2. There was consensus on the definition of promoter - that it is a reversible alteration of gene expresion and the reversible expansion of initiated cells. We didn't define initiation or progression. Problems with the concept of mechanisms of initiation may make that definition difficult.

3. Another important point that was brought out was that one can quantitate initiation and promotion and possibly progression. Possible every agent could have properties of each of the three functions. Different agents would have different balances of these activities. This could be an important consideration in risk assessment in terms of the characterization of agents, particularly if their activities could be quantitated, so one might express potency or relative potency of the three types of agents.

4. We discussed whether reversibility is inherent in the definition of promotion. This implies a threshold. There was some concern that reversibility may not be complete in all systems. There was a thought that reversibility may be complete in the liver but not in the skin based on the recurrence of promoted lesions.

5. We discussed synergism of promoters. This has not been considered in risk assessment before. It may be an important factor.

6. There was considerable discussion of the mechanisms of promotion. We concluded that promotion doesn't involve the activation of oncogenes although the evidence in the skin is incomplete since you see activation of oncogenes in promoted lesions but not in the skin itself. The presumption is that the initiated cells had activation of oncogenes which are demonstrable by clonal expansion but that is not really testable unless you can get to the cellular level. Oncogene activation does play a role in initiation and progression, at least in the sense that a double dose of c-Ha-ras has been demonstrated to push the cells towards malignancy. Cell-cell communication as a mechanism of promotion is also being linked to oncogene activation.

7. The mechanism of initiation is not clear. Initiation has been described here as a sudden irreversible change which is irreversible over a long period of time. However, there is evidence that the initiated state is so common that the original concept of an initiated cell as one that is mutated doesn't really fit in terms of the expected frequency of mutations. The alternative that has been suggested is that it is some irreversible differentiation change in the cells. How this can be brought about and what it really means is not clear. Conventionally, initiation is thought to be linked to genotoxic agents and yet the evidence doesn't support the

notion that it is a mutation. If it is a genotoxic change that is linked with a irreversible change in differentiation, what sort of genotoxic effect are we talking about?

8. There is now a mathematical model - Moolgavkar's - that incorporates the concept of both initiation and promotion and progression. It might be useful to test some of our biological notions to see if the model expresses the biology.

BOB LANGENBACH SOLICITS COMMENTS FROM THE PANEL ON RESEARCH NEEDS AND RECOMMENDATIONS. BASED ON THEIR COMMENTS, HE DRAWS UP THE FOLLOWING LIST:

1. Species and strain differences
2. Other organs, colon, bladder, etc.
3. In vitro systems - human
4. Chemicals to use?
5. Differentiation, organ, chemical variation
6. Mechanisms, O_2, PKC, etc.
7. Animal models, short- and long-term compare to data from in vitro
8. Oncogenes - role? antagonisms?
9. Chemicals for human hazard - promoters
10. Potencies: initiators, promoters and progressors
11. Benign: malignant, reversibility
12. Sequence of administration
13. Progressors

Freddy Homburger: We need more data on species and strain differences in in vivo and in vitro....using inbred animals as well as first generation hybrids.

Eliezer Huberman: (1) We need more work in I/P protocols for organs other than the skin and the liver, e.g., the colon and breast and other organs that are relevant to cancer induction in man. (2) We also need to expand the number of chemicals that have been shown to act as tumor promoters. (3) I suggest that more emphasis be given to testing promoters in in vitro cell transformation systems, especially those using human cells. (4) Studies on modulation of cell differentiation should also be included because there is evidence that certain tumor promoters like phorbol esters and TCDD are effective in modulating differentiation processes.

Tom Slaga: Since a lot of the data are based on phorbol esters, I think studies should be done to find out why these compounds don't work in other species or mouse strains. It may be related to removing the ester groups. This is worth knowing because then maybe much of the background data on TPA could spill over into other systems.

Jim Trosko: (1) We need to test the available hypotheses that we have. The prooxidative model should be tested versus the PKC model. There is evidence now that these two models may not be mutually exclusive, i.e., one may affect the other. And we should try to correlate those two models with the cellular and genetic models that have been proposed, namely the cell-cell communication model versus the recombination model for example. (2) The role of intercellular in linking tumor promoters to a mechanism of action in vivo should be investigated, i.e., comparing the well-demonstrated in vitro phenomenon with in vivo. (3) We should research the correlation of oncogenes with tumor promotion.

Bob Langenbach: Certain oncogene-infected cells may be more sensitive to tumor promoters. It is a good suggestion. Maybe it is a kind of testing approach that we could utilize to see if a chemical is really a promoter? Henry, in your earlier papers you propose utilizing several systems in addition to the animal bioassay for identifying tumor promoters and how much of the chemical's activity may be due to promotion compared to initiation.

Henry Pitot: I showed a slide of recent work based primarily on the liver system. I think you potentially could get these quantitative relationships from that system. You could get systems, particularly in solid organs to do the same thing. Certainly for promotion, you could do it in the bladder, skin, colon. For surface organs it may be more difficult to quantitate the initiation unless you develop spreads. Many years ago, Roy Albert showed some beautiful pictures of spreads with microlesions in them. These were animals that were irradiated as I recall. I have often wondered if something like that couldn't be used for bladder and colon where you could see the early lesions and really quantitate initiation. I think however that the chronic bioassay is essential to see which tissues are involved, so I think the two have to be done in concert.

Jim Trosko: If you could correlate, for example in the Sencar mouse, sustained hyperplasia after TPA treatment with the total absence of gap junctions as opposed to the nonsustained hyperplasia in the Syrian hamster with the presence of gap junctions, that is a test of a model. No one has done that.

Peter Magee: Maybe we should focus our research on chemicals that are of greater concern as human hazards because of their higher expcsure.

Dan Krewski: Maybe we should expand on the data base that Dr. Pitot presented yesterday where we had a measure of potency for the initiating and promoting activity of about a half dozen agents. ...Get these values for more chemicals.

Eula Bingham: We need to research the mechanism of reversibility and the threshold question. Is there a threshold?

Bob Langenbach: We should also look at benign to malignant conversion.

Jim Trosko: Some of the best studied promoters - PCBs, PBBs, phenobarbital, BHT and TPA - depending on the circumstances may act as anti-initiators also. If given before the carcinogen, they protect the animal. If given after, they promote.

Roy Albert: The issue of what is spontaneous initiation is a good one. The relationship of cytotoxicity and promotion is important from a risk assessment standpoint. Synergism in promoters is another area for study.

Bob Langenbach: Let's begin discussion of item #1. What kind of studies are needed to understand the differences between the rat and the mouse?

Roy Albert: TCDD is a good promoter in the rat liver. Could we develop a model using the human liver so we could compare TCDD action in both systems?

Henry Pitot: We need to learn more about it. The most reasonable way to do this would be to look at receptor-TCDD interaction. The current argument is that all TCDD actions are mediated through the receptor. But the affinity of TCDD is in no way related to its toxicology. One can argue that maybe it is the affinity of the receptor-TCDD complex for DNA. So a lot of work is needed in this area.

TAPE 16

Tom Slaga: We need to look at why you don't get sustained hyperplasia after repetitive treatment with TPA in rat, hamster, etc. Differences from mouse.

Jim Trosko: I challenge whole animal people to take the gap junction technology and see if it can be adapted to liver and skin.

Tom Slaga: You have to be careful with cell culture systems because they don't maintain what is going on in vivo. You can put the liver cells into the spleen, the breast cells, etc. Maybe there are systems like that where you maintain more of the tissue relationship. Those systems should be studied more.

Jim Trosko: Another line of investigation is the epidemiological data on chemicals known to be promoters in animals, such as the linkage of known saturated fatty acids

with breast tumors in rats and the change in the American diet in the last few years. In the comparative models, we should be studying chemicals that could be studied epidemiologically.

Henry Pitot: Alcohol is an interesting compound. Epidemiologically it acts as a promoter. But only one or two experimental studies have shown it to act as a promoter in the rodent liver. It does not change the number of foci after initiation. It changes their volume. It increases the number of cells in the foci that are there. So this is an interesting mechanism of action if ethanol is a promoter. This may be a compound to study.

LUNCH

Hugh Spitzer: Are the dose-response and frequency issues that were brought up yesterday the issues that we need to address to begin to get the data we need for extrapolation between species and to humans?

[DR. PITOT EXPLAINS TO DR. KREWSKI WHAT IS MEANT BY REVERSIBILITY. THERE IS FURTHER DISCUSSION OF TCDD. DR. MAGEE ASKS WHAT IS MEANT BY GENOTOXIC. A DISCUSSION OF THIS FOLLOWS.]

TAPE 17

Bob Langenbach: What other systems are available for study besides the skin and liver? Is the bladder ready as a screening system?

Henry Pitot: The bladder is complicated because the urine itself is a promoting agent.

Bob Langenbach: Another problem is that the initiator also induces tumors sometimes.

Tom Slaga: I think the bladder or maybe the colon is the best third system to study after the skin and liver.

Henry Pitot: The thyroid may be a good system. There hasn't been a good system developed that has a hormonal background. Since many hormones may be promoters, an endocrine system might be important to develop. The breast is possible but may be complicated. The thyroid is nice because it is cellularly homogeneous. The kidney is a possibility but it is cellularly heterogeneous so the kidney tumors can be derived from many different cell types. If each cell type has a different response to a promoting agent, it could get complicated.

Roy Albert: The lung is notable for being bad, but it is an important system.

Tom Slaga: The Nettesheim tracheal system is a nice one because they have a denuded trachea which they can repopulate with human cells and get human tissue growing there.

Herb Rosenkranz: I think studies should be conducted in the lung because this is an important route of exposure for humans.

Freddy Homburger: I would suggest Craighead and Mossman's system of hamster trachea explants.

Hugh Spitzer: How will these systems help us for risk assessment?

Henry Pitot: We need to find out if promoting characteristics in the two systems are true in others. If not, then we must rethink the whole thing. You can't base risk assessment on two systems.

Herb Rosenkranz: The respiratory systems produce lesions that are histologically identical to human lung lesions. This is not the case in the skin and the liver. So there is a much closer morphological association.

Peter Magee: Methylnitrosourea might be considered as a universal initiator. I can think of about five organs where it produces tumors.

Bob Langenbach: If we had a universal initiator that worked in several systems, we could test suspect promoters in these systems or organs in the whole animal using the same initiator.

Roy Albert: You could also use a cocktail of nitrosamines that was designed to initiate every organ.

Dan Krewski: Aren't promoters initiator-specific?

Bob Langenbach: I think that is a fundamental question and we don't have enough data to answer it.

Tom Slaga: I think the only place I know of whether they are initiator-specific is the lung adenoma model. In the liver and skin, you can change the initiator and the promoter still works.

Bob Langenbach: I think the consensus is that there is merit in looking at the various organ systems to look at the universality of the phenomenon in testing. ..I would like to ask Drs. Huberman and Kennedy what they think the in vitro transformation systems might offer for identifying and studying promoters.

Anne Kennedy: I think the problem with testing for promoters using in vitro systems is that promotion in vitro is highly serum dependent. The action of many promoters depends on which lot of serum is used. So lots must be carefully screened.

Tom Slaga: No one has shown a requirement for a promoter to get cell transformation in an in vitro epithelial system, especially the skin. This is puzzling.

Jim Trosko: You may be promoting just by the way you set up the experiment in an in vitro system.

Bob Langenbach: Let's talk about which chemicals should be studied.

Roy Albert: Chlorinated solvents and pesticides.

TAPE 18

Tom Slaga: We need to test chemicals to develop the model systems first, and then look at the ones of concern to humans. For model development we would want to focus on chemicals for which there is a data base and which are known to be promoters.

Herb Rosenkranz: We should look at the list of 50 or so chemicals drawn up by Upton et al. (1984).

Bob Langenbach: From that discussion, I ended up with TPA, PB, PBB and TCDD.

Tom Slaga: I would add teleocidin and maybe chrysarobin.

Eliezer Huberman: I believe that one way in which some chemical promote tumors is by altering cellular differentiation processes. I would recommend that more work be done in this area. The human myeloid leukemia cell systems are useful for such studies because they provide simple assays for testing the ability of tumor promoters to induce differentiation processes.

Bob Langenbach: I am concerned about the possible role or need for metabolism to manifest promotion.

Jim Trosko: Russ Malcolm's study showed that there are several compounds that don't block cell-cell communication, but their metabolites do. My question is how relevant are the metabolites. Under normal circumstances (where there isn't cytotoxicity), I think the metabolites have as their target the membrane or some cytosolic fraction. We could adapt the scrape loading assay to cells that can metabolize agents like the primary human keratinocytes.

Bob Langenbach: Do we have any promoter that has to be activated?

Tom Slaga: I don't know of any. Unless you have a complete carcinogen, that may have both an initiator and a promoter among its metabolites. It could be instructive to study this.

Dan Krewski: A model that can lead to quantitative predictions of risk at various doses would be a useful tool for risk assessment for initiators and promoters. We talked about normal cells that change to become initiated cells. The initiated cells could replicate to form an expanded pool of such cells. An initiated cell could undergo further transformation to lead to a malignant tumor cell.

I was happy until the end of yesterday with thinking of the first change as involving some sort of genetic lesion in which there was mutation or DNA damage. I wanted to make that same assumption for the second transformation. I wanted to think of the process of expanding the population of intermediate cells as being a nongenetic mechanism which would involve for example recurrent cytotoxicity to stimulate cell proliferation or perhaps the preclusion of terminal differentiation in order to reduce the death rate of the intermediate cells.

First, I would like to ask whether the assumptions of which are genetic and nongenetic are correct.

Roy Albert: Experimentally there is evidence that initiation is linear.

Dan Krewski: We have to model the dose dependency of this first and second transition rates. Most people who model carcinogenesis would probably like to assume that they are linear functions of dose. We also have to model the dose dependency of the birth and death rates of the initiated cells. I don't think they should be linear functions of dose because they may have thresholds since we are talking about nongenetic mechanisms.

How would we get at all the parameters in this model? First, the age-incidence curve does not involve the birth and death rates of unaltered cells (a_1 and b_1) simply because we can think of the original tissue mass as being sufficiently large that it can be described as a deterministic rather than a stochastic process. All that enters in is the number of cells in that tissue as a function of time. So what we do need in the way of data is information on the growth of the tissue of interest as a function of age.

Henry Pitot: If you have a fixed number of initiated cells, does u_1 drop out?

Dan Krewski: These are just constants that scale the position of the age-incidence curve and they will be dose-dependent. The higher the dose of the initiator, the greater the incidence of lesions at the end of the process. So you would leave this constant in.

Jim Trosko: You are trying to set this up for the IPI?

Dan Krewski: That is a good question. I wanted to mention another version of this model developed by Leon Ellwein which essentially does the same thing but in much more detail. In his version, the various transformation rates can be time-dependent.

TAPE 19

Dan Krewski: In the standard IP protocol, the first data requirement is the growth rate of the normal cells within your tissue. This is something you could get fairly readily. Second, you need information on the birth and death rate of the intermediate or initiated cells. For that, you would have to go the laboratory assays for promotion that we have been discussing in detail. Third, you would need information on the transformation rates for initiation and progression (u_1 and u_2). Here you would have to go to a 2-year, long-term rodent bioassay. I haven't looked at whether you could get these rates from a single standard bioassay or whether you would have to use some kind of IPI protocol to factor out the progression step.

I(t) is the time rate of appearance of lesions in the bioassay. You could (1) use skin and count them; (2) use serial sacrifices; (3) assume that the lesion of interest is rapidly fatal, in which case the survival time of the animal would serve as the proxy for the actual time to tumor induction; or (4) assume that death as a result of tumor occurrence in a bioassay is independent of death from competing causes in which case, we could statistically separate out time to tumor. These are fairly classical problems and we do have approaches for them.

The idea of this model is that you get data on tissue growth and cell proliferation from studies separate from the bioassay, and then you factor in bioassay data. Knowing these parameters, you try to estimate transformation rates and possibly use an IPI to separate u_1 and u_2.

In the bioassay, unexposed controls would allow you to measure spontaneous initiation. The spontaneous rate of tumor

formation in the controls is not a measure of the spontaneous rate of initiation, but we would get a measure of this transformation rate from the tumor occurrence rate in the control animals if we knew all of the other parameters in the model from these separate experiments.

Little s is a dummy variable that indexes time going from zero to t.

Henry Hennings: How do you take care of spontaneous progression?

Roy Albert: In the presence of constant internal level of promotion, the number of benign lesions that one might get would be a measure of u_1, but the rate of progression of these benign lesions to malignant lesions would be a measure of u_2.

Dan Krewski: That is correct.

Roy Albert: For instance in the lung adenoma system, you get a lot of benign tumors and few carcinomas. From that you could get an independent measure of u_1 and u_2.

Dan Krewski: I think the statistical community needs to figure out exactly what kind of data we need to estimate the specific parameters in the model - to separate u_1 and u_2.

Herb Rosenkranz: Would the partial hepatectomy in the liver system be a problem? Can you correct for that?

Dan Krewski: Yes, we would have to have information on a_2 and b_2 that would be relevant to the conditions under which the bioassay would run. We don't do partial hepatectomy in rodent bioassays.

Henry Pitot: Partial hepatectomy will change only u_1 in our system. You can get around that by initiating during the neonatal period for u_1 and if you do an IPI experiment, then do a partial hepatectomy for u_2. We administer a single dose of the initiator 24 hours after hepatectomy. Then you can wait a year to give the promoter. In Farber's selection protocol, you are forcing cell replication in the presence of the selecting agent so you are allowing the altered cells to grow in the presence of an inhibitor of end cells.

Curtis Travis: I don't think the partial hepatectomy would affect u_1 at all because that is the mutation rate which should be constant per cell division. All the hepatectomy does is increase the cell division which affects the birth rate and x(s)...As I said yesterday, we took background cancer rates [I(t)] in rats as a function of age. We found x(s) - the

growth rate of the liver as a function of age - from the literature. We also assumed that the initiated cells would be growing at the same rate ($a_2 - b_2$) as the background cells, estimates of which were obtained from the literature. We then had all the parameters except u_1 and u_2. We assumed that these were the same and that they were 10^{-8}. We ran the model and exactly reproduced the age-specific incidence of cancer rates in rats. We used the NTP data so we knew the background rate down to about one-tenth of a percent. We did this for the liver in rats. We will be doing it in mice and humans.

Dan Krewski: To model dose dependency, we would then have to have control and exposed animals.

Curtis Travis: For TCE, which was shown to be carcinogenic in mice, we already have a dose-dependence for increase in cell turnover rate. It has been measured at the levels used in the animal bioassay. Dow Chemical Company measured the increase in mitotic rate as a function of dose. It is a threshold phenomenon. As effective dose to the liver, it is zero for a while, then you reach the threshold and it starts increasing linearly with effective dose to the liver. I want to put these data into this model. I will assume that TCE has no genotoxic effects so u_1 and u_2 would be the same as background. We know the x(s) and I will put in ($a_2 - b_2$) from the data. We should be able to predict the age-specific incidence of cancer from the TCE bioassay. If we can, then that will prove in my mind that TCE is working solely through a promotional mechanism. I feel that we need just this kind of research in this area - studies on particular chemicals, determine increased cell turnover rates as a function of dose, plug them into these models and see if we can predict the cancer bioassay rates that have been observed.

Dan Krewski: If we wanted to do a full examination of initiation/promotion we would have to do a bioassay involving various levels of exposure to the initiator in order to establish how these mutation rates vary with dose.

Curtis Travis: Several refinements need to be made. When you are measuring increased cell turnover rates you are really measuring increased turnover rates of the normal cells and not the foci. I have assumed the turnover rates are the same. Also, this model assumes that these rates are time-independent. I think that the rate of foci growth will increase with time. We need to do experiments where we look at the livers at different times to see if those volumes are increasing at a different rate.

Roy Albert: Does it make any difference that you get a spectrum of initiation at least in the skin? Small doses or

short-term application of promoters gives a papilloma that shows a lower rate of regression than with larger or longer or stronger doses. Does it matter that on the malignant side, there is no objective measure of malignancy such as growth rate? There is also a spectrum of malignant response.

Henry Pitot: If you use a sufficiently small dose of initiating agent, you won't get the spectrum. The same is true in the liver. At high doses, you telescope the whole thing and get a spectrum. If you want to model initiation and promotion, you must make sure that when you initiate, that is all you do - that you do not get any progression or promotion.

Bob Langenbach: In the models, do you really measure b_1 and b_2 and if so how? I think u_2 is really progression and not promotion. I'm not sure that $(a_2 - b_2)$ is an adequate representation of the promotion.

Jim Trosko: [Draws diagram.] This is what I would refer to as promotion (a_2): the nonmutagenic event that clonally expands all the initiated cells. On further promotion these will give more and more initiated cells. Depending on where you put mutagen 2 you should increase the target size of the number of initiated cells. Now, presumably this occurs for spontaneous tumors also. This model is a test of whether you need two hits.

Dan Krewski: I agree completely with Jim. His expanded model is the same as mine. ... By doing IPI you will estimate u_1 and u_2. The two mutagens don't have to be the same.

Jim Trosko: The point Tom made yesterday is that the same mutagen at u_1 may be very effective as an initiator but may not be as effective as u_2 because of the pharmacodynamics after the clone gets large. That is why I suggested using X-rays because you don't have to worry about metabolism or selective mutagenicity of the cells.

Dan Krewski: If the same agent affects u_1 and u_2 and we knew this for sure, I think that we could estimate the product of those two from the IP protocol.

What kind of studies would I do? We would use doses of an initiator of 0, 0.5 and 1 (think of 1 as the MTD) in a single application. You would have chronic exposure of a promoter at three doses - say 0, 0.5 and 1. This allows you to study dose-response for the promoter and initiator and you could add a second initiator which may or may not be the same as the first and this would be single administration in IPI protocol at various times after promotion. You could have various time lags. You could administer the initiator at various points in the animal's lifetime. Basically, you would need several doses

of the initiator and of the promoter in order to model dose dependency within the model. If you wanted to separate out the rates at which the two mutations occur you would have to add in the second initiating application.

You can do smaller experiments. If spontaneous initiation is occurring at a sufficient rate for your promoter to be effective, then you don't have to administer an initiator...

TAPE 20

Freddy Homburger: What is the purpose of this model?

Dan Krewski: To predict effects at low exposures. But the model must be tested experimentally before it can be used for risk assessment.

Roy Albert: IPI experiments are expensive. Could they be done in tissue culture? Are the clones produced in the tissue culture promotion assay malignant?

Eliezer Huberman: It depends on the assay. In the hamster embryo cell system, only a small fraction of cells became malignant.

Roy Albert: If you further treat these colonies with a carcinogen, can you make them malignant?

Eliezer Huberman: We never tried an API protocol. We should do this experiment.

Peter Magee: If the IPI were done, what agents would we use?

Roy Albert: I would use a direct-acting agent in both cases because you cannot be sure of the metabolism.

Bob Langenbach: I think it should be done systemically as an appendage to the normal NTP bioassay.

Tom Slaga: In the skin, you could use MNNG as the initiator and TPA as the promoter and urethane as the second initiator.

[DISCUSSION TO CLARIFY THE MEANING OF THE TERMS IN THE MODEL AND WHAT WOULD HAPPEN TO THOSE TERMS AND WHAT YOU MIGHT SEE IF DIFFERENT TYPES OF AGENTS ARE USED ON THE THREE STAGES OF AN EXPERIMENT.]

Dan Krewski: We could call the IPI study IPP or IPC for progressor or converter at the third stage.

END FEBRUARY 4

FEBRUARY 5
MORNING - CONCLUSION

TAPE 20

ROY ALBERT SUMMARIZES

We talked mostly about long-term research that dealt with mechanisms of action. In the area of initiation, we talked about the issue of uncertainties in mechanisms with respect to irreversible differentiation versus the induction of mutation. In the area of promotion, we discussed approaches in terms of receptor binding with reversibility and the role of promoters in terms of gap junctions and cell-to-cell communication.

We didn't talk much about the mechanisms of action of progressors, except to say that progression is probably related to DNA damage. The issue of whether there are agents that don't damage DNA that can cause progression was left open. Such agents would be particularly sinister because they wouldn't necessarily show up on bioassays.

We alluded to the importance of species differences in response to initiation, promotion and progression. We need to understand these differences from a mechanistic standpoint.

TAPE 21

...be able to develop some basis for predicting whether humans are going to respond to promoters. It seems that promoters have more extreme differences in species and strain responses than carcinogens, because strong carcinogens are notable for attacking multiple strains and species. This is not necessarily the case with some of the promoters that have been studied. This makes risk assessment even more difficult because of the difficulty of extrapolating animal data to humans.

The issue of synergism of promoters was discussed, with some evidence for DDT and TPA. This opens up a new area, both in terms of mechanisms and identification of the kinds of promoters that are likely to interact.

The need for more extensive models capable of demonstrating promotion was expressed, particularly models that are more relevant to the major human cancers, i.e., colon and lung. to enhance extrapolation of animal data to human data.

We discussed mathematical modelling of a two-stage promotion model and ways and means of validating the model. Also, we talked about whether tissue culture could be used for this purpose, primarily by initiation/promotion and second initiation studies.

We discussed the need to improve in vitro screening models. We agreed that this would be a formidable undertaking, not only to select the most appropriate in vitro tests but to set up parallel studies in animals. However, many people think that promoters can be as great or greater environmental hazard than carcinogens. So there was a feeling that this enterprise is needed. It would probably be undertaken by the NTP rather than EPA.

We discussed how to quantitate initiation, promotion and progression. This has been done in the liver. There was sentiment that this should be extended at least to the skin and perhaps to other tissues. There is a fair amount of agreement that agents can be promoters as well as initiators. Eventually, we may reach a point where we assess these characteristics separately for each chemical.

We have essentially been talking about an NIH long-term research program. If these areas were well funded, one could expect results in 5 to 10 years that may or may not be applicable. It leaves open the issue of what kinds of short-term studies might be useful to EPA for risk assessment. One key issue is low-dose extrapolation. There is evidence and opinion that promoters are reversible with thresholds. The question is how do you demonstrate this. The other issue is whether an agent that shows behavior as a promoter in one tissue also demonstrates that behavior in another tissue, even in the same animal, and then whether there are differences among species and strains. These are the gut issues of the controversy of agents of current regulatory concern.

Bob Langenbach: We need more studies in rodents and human epidemiology to help to better understand species differences. Henry Pitot's explanation of thresholds was very informative - how a threshold for a promoter may differ from a threshold for an initiator. We could use further discussion on how to accurately determine such a threshold, and how such a threshold would be used in risk assessment. We discussed mechanisms of tumor promoters, especially receptor-mediated mechanisms. We didn't discuss other types of promoters. Research is needed in this areas.

With regard to in vitro screening, I personally believe that the current genotoxic systems we have today are not doing the job they were designed and promised to do. If the field of short-term tests is to contribute to carcinogenesis screening, we will need in vitro and short-term models to identify chemicals acting by a promotional mechanism that are not picked up in the genetic toxicology systems.

We developed a list of chemicals to study: teleocidin, phenobarbital, PBB, TCD, chrysarobin and, with some

qualifications, TPA. We should also consider weaker promoters such as saccharin. Chemicals to which humans are exposed and for which there will eventually be epidemiology data should also be studied.

Oncogene involvement and the relative potencies of promoters versus initiators, either within the same chemical or in different chemicals were not well addressed.

Another point to consider is how useful are models that only give papillomas or foci in risk assessment? Do we need carcinogenesis as a final endpoint in these systems?

I'm beginning to think that progression may be as important as promotion. We need to further consider the promotion/progression interaction and overlap in doing risk assessment.

Bill Farland: I'd like the panel's responses to two questions:

1) What data would allow us to determine that a chemical has the ability to promote?

2) What data would allow us to determine that a chemical has the ability only to promote?

The first information we need is that a chemical has the ability to produce tumors in an animal system.

Henry Pitot: Promoters are carcinogens, i.e., they cause an age-specific increase in neoplasms. The only question is the mechanism by which carcinogenesis occurs. In answer to the first question, anything that results in a neoplasm may have promoting action.

Roy Albert: I disagree that promoters are carcinogens. Promoters serve to expand the cell population at any early stage of transformation before they are malignant, and it's in that expanded cell population that you get progression toward malignancy, so they heighten the likelihood of developing cancer. If you have an agent that has been clearly demonstrated to be a promoter with initiation/promotion studies, and you think it's working by receptor binding, which implies reversibility and the existence of a threshold, how do you demonstrate this in a persuasive way? Since this is reversible and doesn't include the tumorigenic process, except by inference, if we had a decisive way to demonstrate this, it would have an enormous impact on the risk assessment approach to the evaluation of these agents, because it would imply the use of a completely different extrapolation model than low-dose linear extrapolation.

Jim Trosko: If you could find a cell line that has an EGF-receptive mutation, where it may bind but the signal is not transduced, and you have the parent line where the receptor is there and the EGF binds to it, in one case you should get a response and not in the other. You have a specific molecule needing a specific receptor for a biological effect, and if you remove the promoting activity you would be proving the case. That would be better than using a drug that would interfere with signal transduction because drugs usually have multiple effects.

Herb Rosenkranz: Just looking for a binding site may mislead you, since the site may have nothing to do with the activity.

Henry Pitot: What Roy was saying is how do we demonstrate that that receptor binding is related to the response we are talking about. I think TCDD has been demonstrated to work through a receptor, with the possible exception of the thyroid effect (if you remove the thyroid you decrease the effect). If you eliminate the receptor it doesn't work. The receptor is genetic. Some animals have it, others don't.

Roy Albert: Can you take the animal with the receptor and inactivate the receptor?

Henry Pitot: You can lock the receptor up with an irreversible inhibitor.

Roy Albert: I think this is an important area for study.

TAPE 22

Tom Slaga: All promoters that have been looked at in detail do have some carcinogenic activity. But I think that in most cases where they have been extensively studied, they do not show a dose response. If it does not show a dose-response, then if you have spontaneously initiated cells, you express those relatively easily so you don't have a dose-response by the so-called carcinogen as a promoter.

Henry Pitot: TCDD, phenobarbital and saccharin have dose-response.

Tom Slaga: If you have a finite number of spontaneously initiated cells, those are going to saturate fairly easily.

Henry Pitot: At the maximally tolerated dose, I agree.

Peter Magee: Weinstein defines tumor promoters as compounds that have weak or no carcinogenic activity when tested alone, but result in markedly enhanced tumor yield when applied repeatedly following a low or suboptimal dose of a carcinogen

initiator. Is everyone happy say that promoters are carcinogens?

Jim Trosko: If we all agree that carcinogenesis is due to initiation, promotion and progression, then we must decide whether the mechanism for initiation is discrete from the mechanisms underlying promotion and progression. But we don't know the mechanisms yet so we can't say if they are discrete.

Henry Pitot: I don't see any problem with promoters as carcinogens because I can't think of any known promoter that is not carcinogenic by the definition that it increases the age-specific incidence of neoplasms in a set strain of animals. Let's not destroy the original definition but rather dissect it into it's original components.

Bob Langenbach: At an NIEHS meeting 3 or 4 months ago, the consensus was that promoters are a class of carcinogens. Implicit in the defintion we agreed to earlier at this workshop was that promoters were carcinogens because it was an increase in the number of tumors in an animal which is very nearly the definition of a carcinogen.

Roy Albert: The more important question is how do promoters behave, in terms of whether you can extrapolate from animals to humans, and whether or not one should use a threshold model. Can an agent that acts as a promoter in one organ act as a whole carcinogen in another?

Eliezer Huberman: We need to focus on what are the mechanisms underly the promotional event.

Bill Farland: We have said that one of the mechanisms is a receptor mechanism. There were two suggestions that one could use mutation studies in vitro or in vivo and competition studies - competitive binding studies - to look at mechanisms. Are there any other suggestions?

Henry Pitot: To say that something is a promoter, these characteristics should be present: 1) There is no direct DNA damaging or altering effect. 2) In many cases a receptor mechanism mediates the effect of the agent. 3) There is a maximal effect of the promoting agent in producing tumors following initiation in the absence of toxicity (this is very questionable but has been shown in the two major systems). 4) There is an experimentally measurable threshold. 5) The effects at both the cell and gene level are reversible. 6) The effects of the promoting agent are modulated by environmental means (e.g., aging, alteration of the hormonal environment of the animal).

Roy Albert: I agree with this list.

Bill Farland: This type of list provides a weight of evidence for saying that something is a promoter.

Henry Pitot: From a practical standpoint, we must first determine whether a substance is carcinogenic in a long-term bioassay. Then you ask whether it is an initiator or promoter, etc. And, if you want to be pure, you do it for each organ system.

Bill Farland: In different tissues, you may be looking at different mechanisms of promotion.

Roy Albert: Radiation is an example of an agent that acts as a complete carcinogen in some tissues and as a promoter in others.

Eliezer Huberman: Hormones are examples of substances that have receptors in various parts of the body yet may affect these different parts in different ways.

Roy Albert: How do we show the existence of threshold?

Henry Pitot: In the liver you have a baseline level of foci. You can go to a level of no change in phenobarbital and, possibly, dioxin.

Jim Trosko: Cell-cell communication may play a role in tumor promotion with regard to thresholds. Cells in a 3-D tissue are surrounded by other cells and communicating with them by hundreds of gap junctions. If we assume that knocking out a gap junction by one molecule - a promoter - is a one-hit event, then we have an explanation for thresholds.

Dan Krewski: Shouldn't we use the term NOEL instead of threshold?

Bill Farland: Are we able to say that threshold in the liver is not a pharmacokinetic situation - that you are not dealing with dose to the liver - in terms of demonstrating the threshold? Are we talking about the dose to the liver or to the animal as being a threshold?

Henry Pitot: Eighty to ninety percent of ingested material gets to the liver.

Tom Slaga: Regarding the promoter-only list, I would rank the "maximum effect follows initiation in the absence of toxicity" as one of the most important and put the concept of threshold and "effects are reversible" as the key things. Receptors are less important. 1, 2 and 3 are the key things.

Peter Magee: What is the criterion of direct DNA damage?

Henry Pitot: That the compound itself chemically reacts with DNA, intercolates with DNA or directly causes a cision of the chain. You are dealing with one chemical reaction versus two or more.

Bill Farland: If one wants to build characteristics for the weight of evidence of something being only a promoter, the evidence of no direct DNA binding is the strongest piece of evidence you can have. But this is not sufficient to demonstrate that an agent is a promoter.

Jim Trosko: What about chemicals that don't initiate but are very cytotoxic and because of cytotoxicity also act as promoters, e.g., 3,4,5-$\bar{3}$,$\bar{4}$,$\bar{5}$-hexabromobiphenyl?

Henry Pitot: You probably can't use highly cytotoxic compounds because cytotoxic effects will occur before you express all the initiated cells. If you get into that hedge, you have to eliminate maximal effect and go to the others.

Roy Albert: Why do you call it maximal? Isn't it because you get a bigger response with an initiator than without?

Henry Pitot: No, you get a dose-effect with dioxin and phenobarbital, but that dose goes up to a point and then quits. I don't think that's true in the absence of toxicity of a complete carcinogen. You keep initiating and getting tumors until you get toxicity.

Dan Krewski: Shouldn't there be something in this list to rule out the possibility of progression, perhaps a criterion that would suggest the occurence of benign lesions rather than malignant?

Henry Pitot: That's why I said no direct DNA damage. I argue that indirect DNA damage produced by agents that have no initiating activity is more closely related to progression. So you will have a problem if you let indirect DNA damage be part of the definition since the agent may have progressor activity.

Bob Langenbach: I'm not sure our definition for promoters eliminates other types of epigenetic carcinogens as defined by Gary Williams - chemicals that may cause gene amplification or gene rearrangement and such events that also cause cancer but aren't really initiators.

Henry Pitot: Maybe we should say no direct DNA structural alteration. I would argue that gene amplification is involved in progression.

Herb Rosenkranz: Hydroxyurea does cause direct DNA damage but it also induces free radicals. So I think it fits in.

TAPE 23A

Peter Magee: How would you measure direct DNA damage?

Henry Pitot: Types of direct DNA structural alteration are: alkylation (adduct formation), intercalation (demonstrate by spectral techniques), scission of the chain.

Bill Farland: The chemistry is the information that you have. Do we have experiments that allow us to identify the threshold phenomenon? The classic animal experiment is not the one to pursue to demonstrate a threshold.

Henry Pitot: The demonstration of a threshold is always open to question. You can demonstrate an experimental threshold with many complete carcinogens, but nobody believes it's there, for good reasons. With promoters, you can demonstrate there is a threshold and argue that it is there. The liver system allows you do this, and the skin and, I think, the lung (at least with BHA). Certainly, in the bladder you get a measurable threshold.

Bill Farland: How about the demonstration of maximal effect in the absence of toxicity?

Henry Pitot: It has been demonstrated in the liver and skin. I don't know if it has been demonstrated in other tissues.

Roy Albert: I think that another criterion for promoters is the induction of benign tumors which take a long time to go to carcinomas.

Henry Pitot: As a pathologist, I object to this. What is meant by benign tumor - a callus in the bone, a neoplastic nodule in the liver, a papilloma in the skin, or an adenoma in the liver?

Roy Albert: I am talking about a benign precursor lesion that goes to a malignancy.

Henry Pitot: I would not buy that, because once you have an adenoma in the liver, for practical purposes it is a carcinoma. Once you have a papilloma that has dysplasia in the skin, you will also get carcinoma. You need to distinctly define benign tumor, both with respect to morphology and to natural history. A reversible benign mass of promoted cells would be fine as a definition, but I don't think you can do that on a morphologic basis. Morphology cannot distinguish between a lesion which is still reversible and one which is permanent.

Bill Farland: What are some other characteristics that one would look for to describe reversibility?

Henry Pitot: One characteristic implied in that definition is that the biochemical effect on gene expression of that compound is reversible. That can be easily measured.

Bill Farland: What about in vitro systems? How could they contribute to this type of definition?

Anne Kennedy: I think they could be very useful....Isn't it true that some promoters will be working by a nonreceptor mechanism but you just need more of them. How will that distinguish a promoting agent? We have already demonstrated most of the other things in vitro for the classical promoters and we could do it for other agents whose mechanism is unknown, so I think it would be easy to study these things in vitro.

Eliezer Huberman: I agree with Anne. We do not, however, see in vitro a threshold of promotion of cell transformation. From my experience with tumor promoters like teleocidin and TPA, one can establish a reasonable dose response. We also can see reversibility of the transformed phenotype in hamster embryo colony assay. I would say that 80 to 90% of the phenotypic changes are reversible.

Henry Pitot: I think the question of the presence or absence of a threshold cannot be ascertained experimentally for complete carcinogens or promoters. But for promoters, one should be able to demonstrate them experimentally.

Eliezer Huberman: I agree, but where is the threshold?

Henry Pitot: That depends on the strength of the chemical. It can vary dramatically. If you are dealing with a receptor mechanism and you know the equilibrium constant of the ligand, you should be able to predict the actual concentration of the threshold level. You can do this with dioxin. It turns out that the threshold for promotion may be as much as an order of magnitude higher than what the receptor data predict in practical terms.

Anne Kennedy: How could you tell if something was directly causing DNA structural alterations or not? For example, with estrogen, we know that the estrogen receptor complex is in the nucleus and doing things. When you measure aneuploidy, which strikes me as a structural alteration, how do you know whether that is indirect or direct for an agent?

Henry Pitot: If you don't get an alkyl group and you don't get direct intercalation in a cell-free system, you could think of all sorts of ideas. But the weight of evidence for the hormones is that you are dealing with indirect effects.

Bill Farland: We are trying to get too fine.

Henry Hennings: Should we include anything about cytotoxicity on the list? In the skin at least, with promotion you have a balance between cytotoxicity and promotional effects. All skin promoters that I know of give you hyperplasia in the skin that may be regenerative as a result of cytotoxicity. I think that some promoters may act by a selective cytotoxicity.

Henry Pitot: If we include it, we would have to have a hedge because there is no cytotoxicity in the liver with phenobarbital and dioxin. I don't think there is cytotoxicity in the mammary gland with prolactin as the promoter. In the thyroid and the kidney there is no cytotoxicity.

Henry Hennings: So we might want to say that cytotoxicity is tissue- and compound-specific. Not all the things on the list are necessarily criteria since not all promoters act in that way. I think the three that Tom Slaga mentioned are the most important. I would limit the list to these three and also include no direct DNA structural alteration.

Bill Farland: So DNA interaction becomes the fourth criterion.

Henry Pitot: I would still argue that the receptor is important, certainly in the skin and the liver the major known promoters act through a receptor mechanism.

Roy Albert: I agree for a different reason, which is that the force of calling something a promoter based on these criteria markedly affects the characterization of the low-level dose-response. If you limit it to those that have demonstrable receptor mechanisms, you have something to talk about, otherwise you are just waving your hands. So the inclusion puts you in a much better position to make statements about dose-response.

Bob Langenbach: But I don't think we can assume that all promoters act by receptor mechanisms.

Henry Pitot: One thing about receptors that is important is that it is very clear that some of the best known promoters are tissue-specific. I don't understand how that is possible unless there is some other mediating mechanism; receptors seem the best candidate that we have at the moment.

Roy Albert: I am concerned that the list does not contain the essence of promotion which is clonal expansion of transformed cells. We can quibble about whether benign is benign, but I think the list should contain this property.

Eliezer Huberman: I think that the critical point is selective clonal expansion.

Bill Farland: The list would be titled "Criteria for Chemicals that Can Only Promote." These elements would constitute the weight of evidence for one to make a finding that a chemical is essentially only promoting.

Anne Kennedy: It seems that me that most people still think of progression as an outgrowth of a more aggressive variant from a malignant tumor. I like this definition better. But we should recognize that it is not the classical definition.

Bill Farland: At a meeting this fall at NIEHS on promoters, there was much discussion that the definition of progression was changing to represent the types of things we have been talking about.

Eliezer Huberman: We are back to the original definition of progression. The previous definition dealt with conversion of the tumor cell to its final stage of metastasis, invasiveness, etc. In a way we didn't change much, but we are now defining where it starts.

Bill Farland: Let's get back to the question of: What are the types of information that allow us to identify or characterize promotional activity? What types of experiments would we want to do? We looked at mechanisms of promotion. The first thing we discussed was receptors. We talked about how we could examine receptors using mutants and competitive studies. What are other mechanisms and data that we should examine. What about cytoxicity?

Eliezer Huberman: I propose a working hypothesis of carcinogenesis, especially of its promotion step. I suggest that tumor formation may, in principle, result from continuous expression of growth facilitating genes which, as a result of some types of genetic changes during tumor initiation, were placed under the control of genes that are expressed during normal cell differentiation. Therefore, some chemicals may promote tumor formation by inducing cell differentiation processes in initiated cells.

Peter Magee: Wouldn't DMSO be a promoter on mouse skin?

Henry Hennings: It isn't a promoter on mouse skin.

Anne Kennedy: DMSO is active at very low concentrations as an inhibitor of transformation.

[MORE DISCUSSION OF DMSO AND ITS SUITABILITY FOR STUDY]

Jim Trosko: In a recent study, Rivedal et al. (1985) used TPA-resistant cells to investigate mechanisms and the role of cell-cell communication in transformation. Here is a mutant that doesn't respond to TPA, is not promoted by TPA, and for which communication is not blocked by TPA, whereas in the parental line TPA does promote transformation. So here is one example where a mutant for a receptor-mediated response of TPA in in vitro promotion could be used as a model.

Bill Farland: So that would be another suggestion for the use of mutants in the receptor category.

TAPE 23B

Jim Trosko: There are all kinds of technologies to measure cell-cell communication and its role in growth control and differentiation. Mutants are becoming available for gap junctions. Antibodies exist for gap junctions. They have cloned the gene for the gap junction. I see both genetic molecular and cellular experiments with normal cells not only in vitro using cell-cell communication as an endpoint but cell-cell communication in transformation assays and in vivo such as the kind of studies that Henry is doing in the liver. There is a hypothesis to be tested and technologies to measure this endpoint of cell-cell communication. There are mutants and antibodies available. The biochemistry to link the receptor PKC to that endpoint is out there. We now need to formulate good experiments to test the hypothesis.

Roy Albert: We may want to consider adding this to the laundry list for promoter-only chemicals - namely that they show decreased gap junction characteristics.

Henry Hennings: With regard to the induction of differentiation, we have shown that with normal epidermal cells in culture, treatment with TPA induces terminal differentiation in about half the cells. The other half appear to be unaffected. They can then proliferate. If the initiated cells were among that population that is not induced to terminally differentiate, then this would give a selective mechanism for how TPA works. We now have "initiated" cell lines which give papillomas when put on an animal. These cell lines do not respond to TPA by a terminal differentiation as normal cells do. So this is a reasonably good possibility for explaining how TPA works.

Bill Farland: This could enable us to sequester particular cell groups that respond differently from other cells.

Dan Krewski: I would like to suggest moving the last two lines on the list - cytotoxicity and selective clonal expansion - to the top half of the slide since those are properties of

promoters that work in the direction of cell proliferation and effects on cell kinetics.

Bill Farland: I think the suggestion is that if one has information on cell kinetics that allows one to start to describe promotional activity. If one can go as far as to say that the cell kinetics point toward a clonal expansion of initiated cells, then you can go further and talk about promotional activity only. As long as you include mechanistic studies in general, but in terms of your specific definition you focus on clonal expansion of initiated cells, then I think we have captured that.

Roy Albert: The advantage of this type of laundry list is that it permits you to set up criteria for each item in terms of the kind of evidence needed.

Bill Farland: What types of data do we want to develop with regard to the models that we will be exploring, e.g., the Moolgavkar model which is a leading candidate for modelling multiple activities and responses at low doses? Roy has indicated that some of these studies may be very expensive. If we are going to collect those data, we need to think carefully up front what those data are, what they will tell us and how they will fit into the model. One suggestion I heard was that we should start to get a handle on rates of cell division in particular organs. Let's discuss the feasibility of this. Do the data exist in the literature? What studies are needed? Can we use in vitro systems to collect those data?

Jim Trosko: The Moolgavkar-Knudsen model was developed based on patients with retinoblastoma. This human model may be an excellent candidate for study. We are finding that tumors are appearing in many other sites than the eyes. This is a case of an inherited gene mutation, where all the somatic cells in the embryo will be initiated. Presumably promotion has occured in the eye because of the differentiation of the eye tissue. Chemotherapy is inducing very high cancers in other tissues in the survivors. Closer examination of the survivors of therapy for retinoblastoma may give risk assessors some assessment of the progressor step. If retinoblastoma can be used as a model of IPI, then we should be able to look at animals that are genetically susceptible to organ site cancers and determine whether they have inherited an I state or a P state.

Bill Farland: I heard of a fish model that produces a pathological lesion that looks like a retinoblastoma when exposed to chlorinated aliphatics. The questions is whether this is a promotional event. So there are animal systems that could be characterized as models for some specific issues.

Roy Albert: There is an old fish model that involves crossing a molly and a swordtail that produces melanoma.

Bill Farland: That is a system that could be used to evaluate that carcinogenic response.

Peter Magee: There is a model for kidney tumor in rats, first described by Eker and Mossige in 1961. I know someone who has these rats. This is a Mendelian inherited tumor.

Dan Krewski: The two most important areas that I think are relevant to risk assessment are the question of thresholds for promoters and the modelling of initiation/promotion phenomena using something like the two-stage birth-death mutation model. If we look at thresholds, then we are back into the arena of of general toxicology where we establish a no-effect level by a suitable uncertainty factor in order to arrive at an acceptable level of exposure. If that is satisfactory, then maybe that is as far as we need to go, and we should focus our efforts into elucidating that (1) we do have initiation/promotion phenomena and (2) the criteria for "only promotion" are satisfied. Then the rest of the story from the regulatory point of view would be fairly straightforward. If you wanted to go beyond that and do modelling, the advantages I see would be that it would perhaps allow you to validate a theory of initiation/promotion in quantitative terms. It would be nice to see the concepts embodied in the two-stage birth-death mutation model supported by experimental data. It would also allow one to calculate a measure of potency for initiation, promotion and progression from one large unified data set. But it may not lead to any different regulatory actions if we accept a hypothesis that promotion is a threshold phenomenon. So we need to ask: What are we going to gain in going beyond identifying something as a promoter to developing a mathematical model to describe the process?

Bill Farland: If we are going to fully characterize the data, then we may find ourselves at a point where it doesn't matter how we treat the data mathematically. We may get to the same point, but we have done a service by characterizing that information carefully.

Dan Krewski: My experience has been that the margin of safety approach will often lead to a substantially different result than the mathematical modelling approach to setting safe levels of exposure.

Bill Farland: I think we still want to pursue data to model initiation/promotion phenomena. The Moolgavkar model is the best model we now have explain the current thinking about the multistage model for carcinogenicity. The question is can we get reasonable data to flesh out this model and validate it.

Dan Krewski: If we want to start working with the model, I think we could take the example of retinoblastoma and follow up

the cases that have been treated for that lesion. That would be a worthwhile data base against which to fit the model. Animal species - fish or rodents - that would allow you to induce retinoblastoma in the laboratory would be another fruitful approach to testing the model. In general, if we could get a good initiation/promotion system, where we knew a lot about the mechanism of promotion, e.g., we knew that cytoxicity was responsible for proliferation of the initiated cells, and we could quantitate the rate of cell proliferation using various in vitro or in vivo studies in the laboratory, and then couple that with bioassay data to fit the whole model, this would be very worthwhile. We need a large bioassay with multiple doses of initiator and multiple doses of promoter. Maybe we could even factor in the progressor. I think that would be the ultimate data base that we could generate for purposes of model validation.

Peter Magee: Could we use the kidney model that I mentioned?

Dan Krewski: It doesn't have to be retinoblastoma, but I like it because we know quite a bit about the mechanism and it seems to fit nicely with the two-stage birth-death-mutation model. The idea of doing it in several species is very attractive. Other tumors that are genetically determined would be good candidates for study.

Jim Trosko: The classic that led us into this mechanistic era of cancer is xeroderma pigmentosum. This is a recessive disease that predisposes the individual to the initiation action of UV light in the skin, where most of the tumors are formed in the skin, but not all. I think that Ken Kraemer (1980) at NCI has been using this model to look for nonskin tumors as evidence of the progressor. Since UV can't penetrate internally, these tumors must be the result of exposure to chemical initiators. This could be used to test IPI.

Bob Langenbach: Another possible system to look into is Balmain's system where he has infected the skin of the backs of mice with a virus containing the V or C c-Ha-ras. With treatment with a promoter, he then gets papillomas, a certain fraction of which progress to carcinomas. In this model, you may have eliminated the need to calculate the u_1 term.

Anne Kennedy: In that system, the whole virus is put in, which brings in other functions. Many of the studies that have concluded that the activation of c-Ha-ras is the initiating event have been done with Richard Mulligan's shuttle vector which brings in functions attributable to myc. So you can't conclude that c-Ha-ras activated by a single base mutation is the initiating event.

Dan Krewski: The question of which initiation/promotion system is relevant for applications of the birth-death-mutation model is quite important because ...

TAPE 24

...I understand that the skin and liver work best. For purposes of modeling, I am not attracted to the skin because many of the lesions that we are concerned with from the regulatory point of view are not skin lesions. The liver would be much more advantageous if a partial hepatectomy is not required as part of the protocol because then the bioassay and in vitro cell proliferation could be done under comparable circumstances. I think that the bladder with saccharin might be a good thing to study because we do have several good initiators, and there is a strong promoting effect. Saccharin has some initiating activity on its own, albeit it very weak. Maybe an elaborate study of that compound with varying doses of the initiator and saccharin as the promoter would provide good information on how well the model works. It may even provide indices of potency for saccharin as both a promoter and an initiator.

Bill Farland: Cohen and Ellwein at Nebraska have been doing that work with the saccharin data base. Hopefully a critical look at their work might lead us to make some suggestions about what additional data might help to tie the loose ends together.

Roy Albert: Maybe statisticians should look at receptor behavior and how one would characterize it in terms of deciding what is a threshold level.

Dan Krewski: Statisticians can determine a level at which there is no statistically significant increase in the response over background, but that is not necessarily a good estimate of a true threshold level corresponding to no elevation in risk.

Roy Albert: That is not what I was driving at. There is a theoretical description of receptor behavior. Then you look at a specific receptor and from that you attempt to describe the theoretical behavior but this has uncertainty in terms of the experimental data. If you want to use the observed behavior to describe the behavior of the receptor as a basis for estimating what could be threshold model, this involves statistical considerations.

Bill Farland: We are doing some of that right now. Steve Bayard is working on it in our group. They are looking at TCDD.

Bob Langenbach: Maybe the skin would be a good system to look at because of the tremendous data base.

Roy Albert: Would Peter describe the genetically based kidney tumor system?

Peter Magee: They are adenomas. Half of the progeny get them, half don't. The data were published by R. Eker (1961) in Nature. The strain is delicate.

Herb Rosenkranz: There is the human model of PUV-A therapy. Those that have received it and then get X-ray therapy rapidly get tumors. Would that fit into the model?

Bill Farland: You must also remember that these are individuals who have psoriasis which might be a means for proliferation of cells.

END WORKSHOP

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