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REPORT ON NCI SYMPOSIUM: COMPARISON OF MECHANISMS OF CARCINOGENESIS BY RADIATION AND CHEMICAL AGENTS. II. CELLULAR AND ANIMAL MODELS

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INTRODUCTION

The division of the task of reporting the proceedings of the NCI symposium has been made for practical reasons and not because it is wise to divide a complex process such as cancer on the basis of biological organization. Nevertheless, this segment of the report is devoted to the presentations about studies with in vitro cell systems, in vitro-in vivo systems and whole animals including humans.

The symposium was designed to cover all aspects of carcinogenesis so that the similarities and differences of the manner in which ionizing radiation and chemical carcinogens initiate cancer and complete its expression could be examined. The hope was that the identification of common features and the clearly distinct features would help elucidate mechanisms and indicate areas for new research.

Upton and Miller considered the epidemiological differences in cancer induced by chemicals and ionizing radiation and both held that ionizing radiation induced a broader spectrum of cancers than any given chemical carcinogen. Miller pointed out that very few chemical agents caused cancer in childhood whereas radiation caused leukemia and thyroid tumors. There are tissues that are significantly more susceptible to radiation induction of cancers than others and it was noted that breast and thyroid appeared to be susceptible to radiation but cancers of these organs are not usually associated with the direct action of chemical carcinogens. The question of whether inherited diseases such as ataxia telangectasia (AT) in which the patients' cells show increased radiosensitivity,

have an increased risk of radiation-induced cancer is not settled. It would be an important distinction if such susceptibility was restricted to a particular agent but it is not clear if the type of inherited defect is important in determining whether the increased susceptibility is general or specific. In the case of Xeroderma pigmentosum (XP) the question of excess risks for tumors other than skin will be settled soon. If the incidence of non-dermal tumors is increased it will, presumably indicate a lack of specificity. It is of interest that cells from AT and XP patients that are hypersensitive to ionizing radiation and ultraviolet radiation respectively are not hypersensitive to a number of chemical mutagens and carcinogens.

Miller pointed out that specific cancers were produced in persons who had been exposed in utero to chemicals that were teratogens. In the case of treatment of the mother with diethyl-stilbestrol during pregnancy the probability of the normally rare cancer of the vagina of the daughter is increased considerably. Similarly, diphenylhydantoin treatment may induce neuroblastomas. Radiation which is also a teratogen has not been shown to produce any pathognomonic cancer, although the induction of an excess risk of leukemia and thyroid tumors has been reported.

The great problem of comparing the carcinogenic potency of different agents, or the tissue susceptibility to the individual carcinogens is that the dosimetry for the agents is usually neither adequate or appropriate to compare on the basis of comparable dosimetry. Until it is known what are the salient molecular events in the induction process there can be no unequivocal quantitative comparisons.

There are a number of tumor types that appear to occur much more frequently with radiation than with chemical carcinogens. For example, carcinomas of the mastoid epithelial lining are pathognomic for radium. The tumors probably occur because of high doses from radon gas trapped in the air spaces for protracted periods. Osteogenic sarcomas is an interesting case of a cancer associated with radiation and which, at least in some species, involves a virus whereas bone tumors are not casually associated with chemical carcinogens. Once again it may be a matter of dose level to the target cells.

Tumors in some tissues are more readily induced experimentally with certain chemicals than with ionizing radiation. For example, papillomas of the mouse skin are induced in large numbers by exposure to chemical carcinogens, especially polycyclic hydrocarbons, either alone or followed by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), in contrast ionizing radiation is far less effective. Of equal interest is the marked difference in susceptibility of different tissues in highly inbred strains to whole

body or local irradiation.

Little pointed out that chemical carcinogens and radiation could cause different types of tumors in the lung because of differences in the localization in the lung after intratracheal instillations. Radiation from ^{210}Po induced mainly epidermal carcinomas and adenocarcinomas in the periphery of the lung and benzo(a)pyrene induced tracheal and bronchial epidermal carcinomas. This is a good example of the fact that the cells at risk in an organ may differ depending on the nature of the inducing agent.

It is the ability to both deliver and measure equivalent doses to different tissues of an agent that does not require metabolic agent that makes radiation the carcinogen of choice to investigate the properties of the target cell and systemic factors that determine the susceptibility that are agent independent.

Multistage Carcinogenesis

As Don Borg has said he and I have chosen to highlight the themes, that we thought were threaded through the talks. A recurring theme of the symposium was the multistage nature of carcinogenesis. Features of the multistage characteristics were shown in in vitro systems by M. M. Elkind and A. R. Kennedy in the in vivo-in vitro systems by R. L. Ullrich and M. Terzaghi and in whole animals by F. J. Burns, J. B. Little and H. C. Pitot.

The acceptance that cancer is a multistage process has come because of the evidence from varied sources, in particular the findings in initiation-promotion experiments pioneered in studies of skin carcinogenesis. The epidemiologists consider that a multistage model of carcinogenesis provides a useful framework for understanding the relationship between cancer incidence and time. They distinguish the differences resulting from exposures affecting early stages of carcinogenesis from those affecting the late stages. As yet, it is not clear how radiation and chemicals compare in their action on the early and late stages. The stage dependency may in fact be more a feature of the specific tissue than the specific agent. The epidemiologist deduces at what stage a carcinogen acts from two types of evidence. First, whether risk of excess cancer decreases following cessation of exposure, and second whether the age at exposure influences the risk.

In the case of breast cancer radiation is considered to act at the early stages since exposure at ages greater than about 40-45 years of age does not cause excess risk. The carcinogenic agents in tobacco on the other hand may act on both the early and the late stages.

The concept of early and late stages that the epidemiologist finds useful has not been translated into precise mechanistic terms or models that might be tested. The shapes of the dose-response curves are influenced by the nature of the stages, their number, and in the case of, at least some carcinogens, further exposures to the same or other agents.

The action of radiation or chemical carcinogens cannot be tested on a pristine population, be it cellular or whole animal, because there is always some probability that the cell will transform or the animal will develop a tumor without exposure to the carcinogenic agent. Since, in the case of humans and experimental animals the probability of cancer increases with age it is possible to ask the question what effect does exposure have in a population, some of which must have tumor cells in various stages of development. Despite the example of the breast given above and the claim that the older patients with ankylosing spondylitis, that were irradiated, were at greater risk than the younger patients we know very little about the effects of carcinogens at the different stages. The effect, on what the epidemiologists refer to as the late stage, is assumed to be on a cell population that has already undergone some change.

I have started at the epidemiological level, as did the speakers concerned with humans, because eventually what we learn at the molecular, cellular and tissue level will be melded together with the epidemiological evidence to elucidate cancer in humans. At the epidemiological level it is only possible to distinguish and divide the sequential process of carcinogenesis into two broad categories - early and late. Experimental work on whole animal, tissue and cellular models must supply the finer details of the stages.

The schematic in Figure 1 indicates a possible form of the sequential process of carcinogenesis and the points at which the comparative effects of ionizing radiation and chemical carcinogens might be examined. The simplicity of the schematic conceals among other things, the fact that the mechanisms involved in carcinogenesis are different in different tissues and organs. The problem is that we are attempting to deduce a complex process from very little pertinent information. A further problem is that many of the changes that can be detected may not be relevant. There is a reasonable understanding of what a cancer cell is and is not, and a considerable inventory of the identifying characteristics but many of the important changes are difficult in the development of the cancer cell to detect. Obviously, altered gene expression plays an important role in cancer, and although the understanding of oncogene control is perhaps becoming clearer, we have a very fuzzy picture about gene control or how to distinguish the gene products that reflect a change that is central to induction of cancer.

Returning to the schematic in Figure 1. The delineation of normality is itself not easy. In experimental animals the heritable aspects of susceptibility are clear. Thus, unless it can be shown that the natural incidence of a specific tumor has no influence on the susceptibility for induction by a carcinogenic agent the response to an agent will be determined by the genetic makeup of the specific cells. Such heritable factors that determine the response to carcinogens might range from heritable fragile sites of chromosomes, that in turn correlate with breakpoints involved in chromosomal rearrangements and oncogene expression, to the type of mutational change associated, for example, with retinoblastoma, that reduces the number of further mutations required for transformation. Although the information is scanty in some strains of mice there appears to be a positive correlation between the natural incidence of specific tumors and the susceptibility to the induction by either radiation or chemical carcinogens.

Pitot defined the stages that could be identified in the development of most tumors as initiation, promotion and progression. Initiation appears to have no threshold, obey single hit kinetics, and the resulting change(s) are heritable and irreversible. Promotion tends to be defined as much by what it is not as by what it is - a clear indication of the lack of complete understanding about the process. Slaga considered the major effect of promoters in the skin to be the specific clonal expansion of the initiated cells, that appears to involve both direct and indirect mechanisms. The direct action of promoters on the target cell alters the differentiation capability of the cell. Not surprisingly experimental findings are not consistent with a single stage of promotion. Gene amplification and epidermal cell proliferation are thought to be important in the "second stage" of promotion. Pitot considered that progression resulted from genomic changes that could range from gene amplification to chromosomal translocations.

The carcinogenic process can be discontinuous. A good example described by Miller is radiation-induced breast cancer in the atomic bomb survivors. In humans a single exposure to radiation under 10 years of age induces the changes that eventually result in breast cancer, but after a dormancy of perhaps 30 years. Apparently the young breast is not too impressed by the presumed oncogene changes. In breast cancer the radiation-induced cancers do not appear until the age is reached at which the incidence of breast cancer starts to rise in the unexposed members of the population. It is suspected that an altered hormonal balance that is age dependent is involved. The period of dormancy or latent period decreases with age at exposure. Experimental evidence, that was presented by Ullrich and Terzaghi, shows that expression of initiated mammary cells can be brought about by altering the cell-cell interrelationship. This suggests that expression of initiated cells is controlled at the tissue level as well as the systemic level.

A single high dose rate exposure of radiation can induce the changes in all the targets required to convert a normal cell to a malignantly transformed cell. Recent findings about oncogene activation and the requirement for alteration at two loci is consistent with a two or more target-one step phenomenon. Any further changes in the target cell may come as a consequence of the initial changes in the target cell. The development of variants would be an example.

The identification of the steps in the development of a transformed cell depends on the rate of transition. If the transition takes place very rapidly it may appear that the number of steps is less than if the process is spread out in time.

With chemical carcinogens, especially at relatively low dose levels, the transitions between stages can be relatively slow and so-called premalignant states have been identified and studied by serial sampling, particularly in liver as noted by Pitot. In Figure 2 the possible changes in the pathway of normal cells - to premalignant cell - to malignant cells are shown in more detail than in Figure 1. It is neither clear that the type of carcinogenic agent influences the nature of the stage-to-stage process nor whether different agents act in a qualitatively different manner on the various stages. Agents classified as promoters do act very effectively on the stages between the initiated or latered cell and the appearance of frank malignancy.

Slaga described the evidence that promotion consists of at least two stages and there was some specificity related to the stage affected by various promoters. The demonstration that free radicals play a role in promotion by chemical promoters has raised the question of whether free radical production could be a mechanism that was common to ionizing and ultraviolet radiation and chemical carcinogens. However, certain chemical agents appear to be much more effecti- than ionizing radiations as promoters. The role of ionizing radiation on any stage other than initiation has not been studied systematically. Perhaps, the reason for this is that radiation is effective in single doses and when given in multiple fractions or is protracted over a long time the effectiveness is reduced. In the case of ultraviolet radiation (UVR) it is believed that many of the later exposures in a fractionation regime are "promoting" the lesions induced by earlier exposures. UVR enhances or promotes the expression of cells initiated by other agents including ionizing radiation.

As Little pointed out, promotion or enhancement of tumorigenesis can be due to nonspecific agents. He gave the example of the increase in lung tumors in hamsters exposed to ^{210}Po followed by saline instillations into the trachea.

Much of what is referred to as progression appears to be the

development of variants and proliferation must play a role in this process. Selection due to chromosomal changes and proliferative advantage can change the characteristics of the tumor cell population markedly. Such changes appear to increase the probability that the features we denote as malignancy occur, namely, local invasion and distant metastases. The rapidity of the development of highly malignant variants is probably dose-dependent. Since one assumes the changes in the gene loci that result in initiation are all or none events the difference in rate of the development of variants, and therefore the malignancy, may reflect damage to other DNA sites and particularly chromosome aberrations with subsequent instability of the genome. Many investigators would agree that the degree of malignancy (in itself a rather vague parameter) is dose-dependent but this has not been quantitated satisfactorily.

Weinstein summarized the current state of information about the effects of different carcinogenic agents on the various stages of carcinogenesis (Table I). It can be seen that he believes the gaps in the information lies with ionizing and ultraviolet radiation.

The temporal patterns of cancer incidence have been used to support the thesis that carcinogenesis involved multiple stages. The number of stages has been determined from the exponent of the power function that relates time and cancer yield. Burns outlined the use of both this model and one based on the exponent of the dose-response function which provides an estimate of the number of dose-dependent stages to compare radiation and chemical carcinogenesis. In both models cells are assumed to progress from stage to stage as a result of spontaneous alterations or due to the carcinogen. The altered cells are considered to be viable and capable of clonal growth. Experiments on the induction of skin tumors in the rat by low-LET radiation supported the model that only two events are involved in transition between stages and that one of the events was repairable with a repair lifetime of about 3.5 hours. When the effects of multiple doses of radiation were compared with the effects of single doses the time exponent increased from about 2 to 6 which was similar to the value obtained with multiple doses of chemical carcinogens in the same experimental system. Burns suggested that the increase in the time exponent reflects clonal growth of an early stage and not an increase in the number of stages.

An important difference between multiple exposures to radiation and chemical carcinogens that is suggested by these studies is that repair occurs with radiation but does not in the case of chemical carcinogens. There appears to be additivity of the carcinogenic effects of multiple exposures to certain chemical carcinogens but less than additivity for exposures to gamma or x-rays.

Papillomas induced on mouse skin by single applications of a chemical carcinogen followed by repeated applications of TPA, were

used to study the early stage clones. It is thought that some of the papillomas are clonal expansions of cells in the early stages of the carcinogenic process, perhaps the first carcinogen-dependent stage. When TPA was added to the weekly B(a)P applications the dose exponent dropped from 2 to 1. In terms of the multistage model this reduction can be explained if the inherent amplification in clonal growth has caused the spontaneous transitions to exceed the number of carcinogen-induced transitions. There have not been sufficient experiments on different tissues that have been designed to allow the necessary analyses to know how the stages vary and also whether the nature of the process is dependent on the type of carcinogen.

The investigation of dose-response relationships has been a cornerstone of studies of radiation carcinogenesis. Also, the variations of the conditions of radiation exposure such as fractionation and dose rate have been more extensively studied than with chemical carcinogens both in experimental animals and with in vitro cell systems. Elkind showed that both a reduction in the dose rate and dividing a dose of gamma radiation (low-LET) into five daily fractions resulted in a significantly lower transformation frequency. These results are consistent with repair of sub-transformation lesions. In contrast reducing the dose rate of fission neutron radiation (high-LET) increased the frequency of transformation As Elkind indicated it is of interest that the initial slopes o. the dose-response curves for both low- and high-LET radiation are linear but the effect of reducing the dose-rate of the two types of radiation is different. It is interesting because linear or single track responses are commonly considered to be dose rate independent, but Elkind and his colleagues's findings suggest that the primary absorption events are not completely effective, and that repair can occur and influence the final outcome. In the case of low-LET radiation error free repair reduces the effect but with high-LET radiation for some reason the lower dose rate increases it. Elkind speculated on whether the neutrons induced an error prone repair or whether the protracted exposure acted as a promoter and increased the expression of the initiated cells.

Carcinogenic agents are cytotoxic and cell killing will, of course, reduce the number of cells that can express a transformation. In the case of radiation, Elkind pointed out that tumorigenesis is the net effect of a low probability induction process and a high probability of cell killing. Cell killing may play a role in carcinogenesis in a number of different ways. First, a reduction in carcinogenic effect may be caused by the loss of potential cancer cells. Second, an increase in effect may result in a number of ways: (a) disruption of a tissue with loss of cell-cell communication, (b) loss of cells followed by regenerative cell proliferation which may fix an induced lesion, or add an error, or assist the expansion

of a transformed cell clone, (c) uptake by untransformed and viable cells of DNA from killed cells that theoretically could lead to incorporation and activation of protooncogenes. The probability of the latter occurring especially with low doses must be very small. In a non-renewal or very slowly renewing system such as the liver cell killing and repair of damage appears to be important. The relative effectiveness of cell killing and malignant transformation, in vitro and in vivo is different for different types of agents, chemical, ultraviolet radiation and ionizing radiation and examples were given by Kennedy, Elkind and Ullrich.

The development of in vitro cell systems suitable for quantitative studies of malignant transformation has made it possible to dissect the carcinogenic process at a cellular level and Kennedy and Elkind discussed the results of such studies.

In vitro cell systems consist of cell lines such as C3H 10T1/2 and 3T3 cells as well as primary cultures or cell strains such as Syrian hamster embryo (SHE) cells. If, as has been suggested, the establishment of a cell line involves one of the major changes involved in the development of a malignant cell. Comparative studies on cell lines and cell strains should be extremely informative, since in the cell lines the change to "immortality" has occurred. Furthermore, cell lines such as C3H 10T1/2 are aneuploid, a change that appears to predispose to further changes. In the diploid SHE cell system the susceptibility to transformation by x-rays decreases dramatically with the first few passages. The reasons for this intriguing change in susceptibility is not known.

If the immortal state of cells indicates that one of the targets for transformation has been altered comparison of dose-response relationships, between cell lines and cell strains should be useful. As Kennedy pointed out qualitatively, at least, the dose-responses to radiation appear to be similar in both systems. Experiments designed with target theory in mind must surely have a role in seeking confirmation of the suggestions based on evidence at the molecular level about the number of targets or steps involved in transformation and in discussion Borek referred to such experiments.

The ability to manipulate cells in culture and to expose them to agents at various stages of a sequential process such as in vitro transformation has proven very useful in the dissection of the transformation process and how it may be modified.

The experimental approaches and the interpretation of the results were given by Kennedy who presented an impressive catalogue of experiments that illustrated the range of agents that had been used to "initiate" and to modify the expression of the early events.

Kennedy used Figure 3 to illustrate a working model of the process of in vitro transformation. In this model at least two stages are required for transformation and both enhancement or inhibition of transformation can be carried out with agents applied between the two steps. Kennedy indicated the similarities between induction of malignant transformation by radiation and chemical carcinogens and the apparent similarities of the responses of in vitro and in vivo systems.

Kennedy's presentation made it clear that even in cell strains that malignant transformation is a complex multistage process but that the very fact that it is multistage presented opportunities for intervention, and therefore, prevention of the completion of the process. Kennedy described how protease inhibitors had been used to carry out such prevention. The suppression of transformation by protease inhibitors was found in cells exposed to either radiation or chemical carcinogens.

Kennedy also discussed the evidence that the initial event in the transformation of C3H 10T1/2 cells is a rather common event. There is a great deal of evidence, some of it quite old, that in humans and experimental animals the presence of initiated cells can be demonstrated or inferred, and that they occur much more frequently than do cancers in the same tissues.

The fact that a large number of in vitro experiments had all been carried out by a relatively small number of research groups illustrated a practical, if not scientific, difference between in vitro and in vivo methods, namely, the number of experiments that can be carried out in months rather than years using in vitro systems exceeds greatly the number of animal experiments. However, cancer is not just a cellular disease and the role of the tissue organization and the influencing systemic factors can only be investigated using both in vivo-in vitro systems and whole animals. Ullrich indicated the information that is required, and some that has been obtained using two of a number in vitro in vivo systems now in use. Experiments using tracheal or mammary cells and an epithelial focus assay, plus assays of the emergence of the malignant phenotype have identified stages and the effects of different agents on them. In these experimental systems, that have the advantage of being epithelial, treatments can be carried out in vivo and the effects assayed after manipulation in vitro, and if required the cells can be returned to appropriate sites in animals in order to study host factors.

In these systems assays have been used to identify and quantitate the changes after exposure to chemical carcinogens and ionizing radiation in three phenotypic changes in growth of clonogenic cells: 1) the clonogenic unit gives rise to an epithelial focus, 2) an epithelial focus that escapes senescence and is subculturable, and

3) gives rise to subculturable foci that are tumorigenic when injected into the mammary pad in the case of mammary cells or into the treacheal stripped of its epithelium in the case of tracheal cells. Thus, the stages of development of malignant cells from the time of treatment can be studied sequentially in epithelial cells.

Ullrich showed that mammary tumors in mice can be induced by both radiation and 7,12-dimethylbenz(a)anthracene (DMBA). It was found that comparable incidences of cancer were produced by doses of DMBA that killed few cells but in the case of radiation required doses that resulted in marked cell killing. In the tracheal cell system DMBA appears to be much more effective than radiation in the induction of the initial events. Exposure to x-rays after DMBA treatment was found to reduce the latent period or period required for expression.

CONCLUSION

The point at which the common final pathway for induction of cancer by chemical carcinogens and ionizing radiation has not been identified. Although common molecular targets are suggested by recent findings about the role of oncogenes, the mechanism by which the deposition of radiation energy and the formation of adducts or other DNA lesions induced by chemicals affects the changes in the relevant targets may be quite different. The damage to DNA that plays no part in the transformation events, but that influences the stability of the genome, and therefore, the probability of subsequent changes that influence tumorigenesis may be more readily induced by some agents than others. Similarly, the degree of cytotoxic effects that disrupt tissue integrity and increase the probability of expression of initiated cells may be dependent on the type of carcinogen. Also, evidence was presented that repair of the initial lesions could be demonstrated after exposure to low-LET radiation but not after exposure to chemical carcinogens. In short, there are a number of ways in which radiation and chemical carcinogens may differ qualitatively that influence their carcinogen effectiveness.

There are specific questions about the mechanisms of carcinogenesis that can be answered either more easily or more quantitatively with specific carcinogens. Some of those opportunities emerged from the interchange between workers devoted to one particular class of carcinogen and hopefully the cross fertilization will provide the catalyst for new experimental approaches.

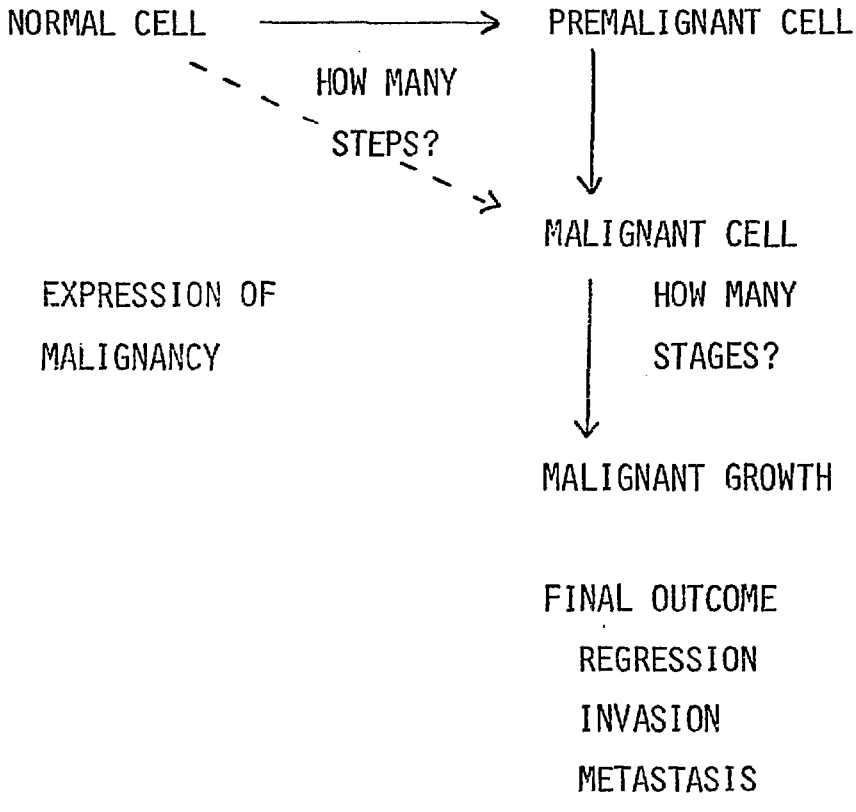
TABLE I

MULTISTAGE CARCINOGENESIS

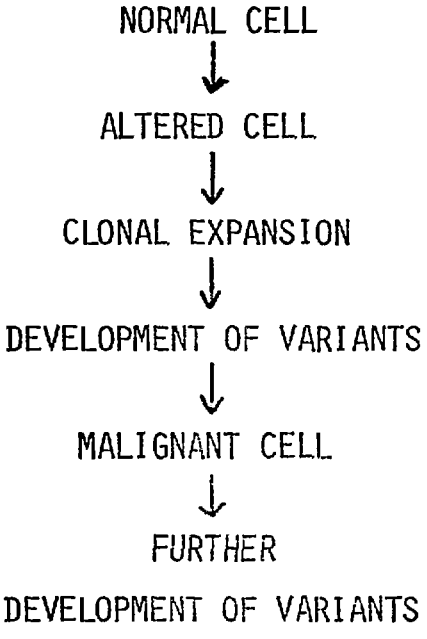
Stage	Carcinogenic Agent		
	Chemicals	UVR	Ionizing Radiation
Initiation	+	+	+
Promoter	+	+	?
Progression	+	?	?
Complete Carcinogen	+	+	+

CANCER INDUCTION

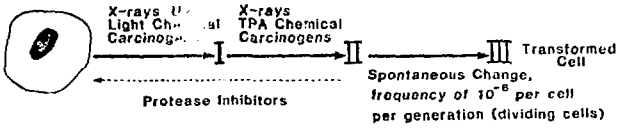
HOW MANY TARGETS?



CARCINOGENESIS



POSTULATED SCHEME FOR THE INDUCTION OF
MALIGNANT TRANSFORMATION IN VITRO



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