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THE QUANTITATION OF CELLULAR KINETICS FOR THE MODELLING OF CHEMICALLY-INDUCED CARCINOGENESIS

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FOR THE COMMANDER

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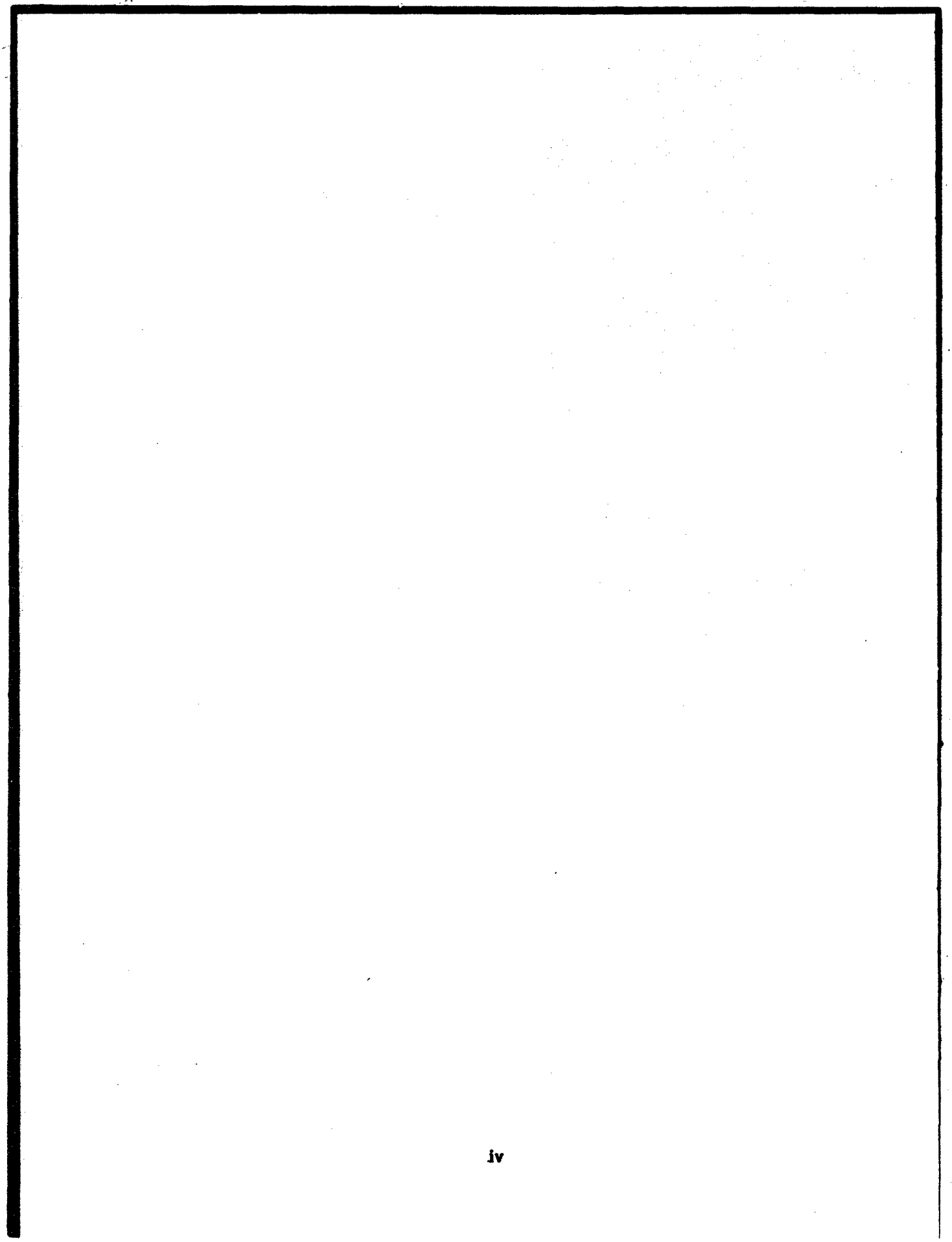
ABSTRACT

This report, which was prepared for the Armstrong Aerospace Medical Research Laboratory, presents the results of an independent evaluation of selected relevant scientific literature. It identifies and discusses experimental methods that are potentially useful for the quantitation of parameters that are necessary for the modelling of chemically-induced cellular changes that occur in carcinogenesis. The report presents an overview of the cell dynamics of carcinogenesis in terms of a conceptual two-stage model and briefly evaluates experimental approaches which may be applicable, focusing especially on the liver and lung of rodent species.



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EXECUTIVE SUMMARY

INTRODUCTION

The Air Force Installation Restoration Program (IRP) is involved in assessing and managing health risks from exposure to toxic chemicals at hazardous waste sites on Air Force installations. In human health risk assessment, data from epidemiological studies are valuable when available. However, data from animal studies are usually extrapolated to humans because the human data are often insufficient. Thus, uncertainties arise concerning the relevance of the animal data to humans.

The pioneering efforts of the Armstrong Aerospace Medical Research Laboratory (AAMRL) in the development of physiologically-based pharmacokinetic modelling has the potential to provide a biological basis for extrapolating pharmacokinetic data from experimental animals to humans, and thereby enhance the accuracy of risk assessments at hazardous waste sites and facilitate the effective use of resources in the restoration of IRP sites. However, physiologically-based pharmacokinetics alone cannot account for interspecies differences in cellular activity in response to a given dose of a carcinogen. Interspecies differences in cellular dynamics must be defined to accurately and reliably estimate cancer risk in humans from animal studies.

The cellular dynamics of carcinogenesis can be described by a conceptual two-stage model with model parameters that, in theory, can be represented by measurable cellular parameters. The AAMRL has obtained the assistance of The MITRE Corporation to provide an independent assessment of the available information pertinent to defining these parameters and to identify potentially useful experimental approaches to quantifying the relevant parameters.

THE CELL DYNAMICS OF CARCINOGENESIS

The two-stage model of carcinogenesis describes carcinogenesis mathematically in terms of the occurrence of specific mutations which define the two stages of the model and in terms of the rates of cell division and cell death of both normal and mutated cells.

The first mutation occurs with a frequency of μ_1 per normal cell division to produce an intermediate cell from a normal cell. The intermediate cells are subject to the second mutation of the two-stage model. The second mutation occurs with a frequency of μ_2 per intermediate cell division to produce a tumor cell, which has the ability to grow and proliferate to form a tumor. The two-stage model also describes tumor incidence as a function of the rate at which normal cells divide (α_1 divisions/cell/unit time), the rate at which normal cells die (β_1 deaths/cell/unit time), the rate at which intermediate cells divide (α_2 divisions/cell/unit time), and the rate at which intermediate cells die (β_2 deaths/cell/unit time).

In principle, these six parameters (α_1, β_1, μ_1, α_2, β_2, μ_2), together with estimates of the total number of normal cells in the tissue and the growth fraction, allow the two-stage model to describe the appearance of tumor cells over a given time interval. According to the two-stage model, chemical carcinogenesis is the result of the perturbation of one or more of these parameters. The two-stage model is capable of producing excellent curve-fitting of various age-specific cancer incidence data from epidemiological studies, suggesting the potential predictive value of this model for human cancer risk assessment. Accurate and reliable measurements of the relevant cellular parameters (birth rates, death rates, and mutation frequencies) in experimental animals and humans remain to be determined.

In the context of the two-stage model, initiation is the result of a specific mutation in a single normal cell to produce an intermediate cell either spontaneously or by the interaction between a chemical carcinogen and the nuclear DNA. Initiation corresponds to the first mutation in the two-stage model. Promotion is the stimulation of initiated (intermediate) cell proliferation which can result in the clonal expansion of solitary initiated cells to produce discrete foci or nodules that are phenotypically distinguishable from normal tissue. The second mutation of the two-stage model converts an intermediate cell to a tumor cell which possesses the ability to grow and proliferate to produce a tumor.

In the liver, distinct subpopulations of hepatocytes can be identified during hepatocarcinogenesis which appear to correspond to the two stages of the two-stage model. Promotion results in the appearance of altered hepatocyte foci and nodules. Each altered hepatocyte focus and nodule is probably the product of the clonal expansion of a single initiated hepatocyte. While most altered hepatocyte foci and nodules that develop during promotion "remodel" or disappear following removal of the exogenous promoter, some persist. Under certain experimental conditions, the cells of some persistent nodules may exhibit properties characteristic of tumor cells.

In the lung, the development of adenomas appears to be consistent with the two-stage model. Pulmonary adenomas, which are benign lesions that appear prior to malignant adenocarcinomas in mice, may be composed of the intermediate cells from which adenocarcinomas arise.

EXPERIMENTAL APPROACHES TO QUANTIFYING THE PARAMETERS OF THE TWO-STAGE MODEL

Cell birth is the production of a pair of daughter cells from the mitotic division of a parent cell. The interval between consecutive mitotic divisions of a cell is called a cell cycle. Since cell division occurs once per cell cycle, the rate of cell birth is the inverse of the time required for the completion of the cell cycle. Accordingly, the cell birth rate (α) can be estimated by elucidating the cell-cycle time.

The cell-cycle time can be determined in populations of asynchronously proliferating cells by a "fraction labelled mitoses" approach in which cells in the DNA-synthesis (S) phase are pulse-labeled

with a single type of DNA precursor (e.g., ^{3}H-thymidine or bromodeoxyuridine). Alternatively, double-labelling approaches have been described which may be especially useful for estimating the cell-cycle time of the proliferating cells of human tissues *in vivo*. In addition, multilabelling approaches can be used in combination with flow cytometry, image cytometry, or fluorescence microscopy to study the cell-cycle kinetics of even small subsets of mixed cell populations exhibiting heterogenous properties. In principle, these approaches may permit the determination of the cell-cycle time for specific types of normal cells, initiated cells, and tumor cells present in a tissue or organ.

The very low mitotic activity characteristic of unstimulated normal hepatocytes and many types of lung cells in adults will probably preclude obtaining reliable division rate estimates for these cells solely by means of the fraction labelled mitoses method or the double-labelling approach. In addition, the pulmonary cells may be especially problematic because the cell-types of the lung are typically very difficult to identify and distinguish from each other. These problems may best be addressed by developing multiparameter analytical approaches that simultaneously or sequentially identify cells according to cell type and ploidy, whether or not they are in a proliferating mode, and their position in the cell cycle. These approaches may be combined with modifications of the fraction labelled mitoses or the double-labelling methods to provide the reasonable estimates of the cell-cycle times and birth rates.

The cell death rate (β) is the number of viable cells lost from a cell population during an interval of time. The cells in most tissues, especially in adult animals, usually do not die with the temporal regularity required for mathematical modelling. The possible indices of cell death based on directly measurable parameters will provide no temporal information from which the rate of cell death (β) might be estimated. However, the rate of cell death may be evaluated indirectly from the rate of change in total cell number and the rate of cell birth.

The kinds of mutations to be incorporated into the two-stage model represent a small fraction of the total number of mutations possible. An assumption of the two-stage model is that the first mutation confers both a growth advantage and phenotypic alteration to the initiated cell which are both expressed on exposure to a promoter. The second mutation confers to the cell the potential to progress to tumor.

Quantitating the frequency of mutation from normal cells to intermediate cells (μ_1) may be facilitated by the clonal expansion of each mutated cell to a detectable population of altered foci and nodules. In the liver, for example, initiated cells can be selectively and intensely induced to proliferate to produce altered hepatocyte foci and nodules by means of modifications of experimental protocols described in the scientific literature. Quantitative stereology and related methods can be used to determine the number of altered hepatocyte foci and nodules per cubic centimeter or per total liver. The number of altered foci and nodules may be used to estimate the number of mutations that have occurred from which, in conjunction with normal cell birth rates (α_1), the mutation frequency (μ_1) can be calculated.

The mutation frequency of intermediate cells to tumor cells (μ_2) can be inferred from the number of tumors or persistent nodules that arise in a tissue, assuming that each tumor and persistent nodule is a clonal expansion of a single cell in which the second mutation of the two-stage model has occurred. The number of tumors can be counted in bioassays and this number can be used, in conjunction with intermediate cell birth rates (α_2), to calculate the mutation frequency (μ_2). Alternatively, the number of persistent nodules containing cells which retain the ability to grow and progress to tumor, as assessed for example by means of splenic transplantation, could be used in conjunction with intermediate cell birth rates (α_2) to calculate a more reliable and accurate estimate of the mutation frequency (μ_2).

The mutation frequency (μ_1) of pulmonary epithelial cells may possibly be estimated by counting adenomas if the stem cell-type from which adenomas arise can be definitively identified and characterized. The estimation of the mutation frequencies of other types of lung cells is limited by the characteristically low rate of cell mitotic activity, and by the difficulty in identifying and distinguishing specific cell types from the other types of lung cells. More study is needed to provide experimental approaches amenable to estimating mutation frequencies in lung cells.

In summary, this report provides an independent review of selected, relevant literature and identifies cellular parameters that are experimentally accessible in the liver of laboratory rodents. Although no reliable measurements of the relevant cellular parameters are presently available, the technologies and experimental approaches currently in use to study the liver in the laboratories listed in appendix B can be modified and combined to provide reasonable values for the parameters of the two-stage model. In principle, similar approaches may be developed to estimate cell birth rates, death rates, and mutation frequencies of these tissues. The predictive value of the current two-stage model for the extrapolation of bioassay data in risk assessment can be evaluated by comparing empirically derived tumor incidence data with tumor incidences predicted by the model using experimentally derived parameters.

SECTION 1

INTRODUCTION

The Air Force is involved, through the Air Force Installation Restoration Program (IRP), in a large task of assessing and managing human health risks that may arise from toxic chemicals present at hazardous waste sites on Air Force installations. A complete human health risk assessment includes hazard identification, dose-response assessment, exposure assessment, and risk characterization (National Research Council, 1983). Hazard identification is the determination of whether or not a causal relationship exists between exposure to a chemical and the occurrence of cancer or other health problems in humans. Dose-response assessment is a quantitative description of the relationship between the degree of exposure and the probability that the adverse health effect will occur. Exposure assessment is the evaluation of the extent of human contact with the chemical. Risk characterization is the estimation of the situation-specific probability that the adverse health effect will occur in humans, on the basis of both the dose-response assessment and the exposure assessment, and the definition of the uncertainties associated with the estimation.

Data from epidemiological studies are valuable for hazard identification and dose-response assessment. However, both hazard identification and dose-response assessments for humans are usually extrapolated from animal studies because the human data are often not sufficient. Thus, uncertainties arise concerning the relevance of the animal data to humans, and from considerations such as possible differences between animals and humans in the metabolism and physiological distribution of the chemicals of concern.

The Armstrong Aerospace Medical Research Laboratory (AAMRL) provides technical support for the IRP. The AAMRL is a leader in the field of pharmacokinetics, especially as it relates to the quantitative description of the absorption, distribution, metabolism, and excretion of toxic chemicals. Classical pharmacokinetics depicts pharmacokinetic data, such as blood concentrations or exhalation of a volatile chemical over time, by means of differential equations with terms that usually have no physiological correlates (O'Flaherty, 1987). In contrast, physiologically-based pharmacokinetic modelling depicts pharmacokinetic data by means of differential equations with terms that represent specific biochemical and physiological processes involved in the metabolism and fate of an absorbed chemical (Conolly, et al., 1988a). The physiologically-based pharmacokinetic modelling (also referred to as extrapolation-based pharmacokinetic modelling) developed by the AAMRL and others has the potential to provide a biological basis for extrapolating pharmacokinetic data from experimental animals to humans, and thereby enhance the accuracy of risk assessments at hazardous waste sites and facilitate the effective use of resources in the restoration of the sites.

The time-dependent changes in the concentration of a chemical of concern in a specific tissue can be elucidated from the equations of the physiologically-based pharmacokinetic model. The tissue-specific concentrations estimated from the model can be related to the occurrence of an adverse effect in the laboratory animals and used, together with the physiologically-based parameter values

characteristic of humans, to predict the doses likely to produce a corresponding effect in humans (e.g., Reitz et al., 1990).

A number of chemicals of concern in the IRP are carcinogens. Interspecies extrapolation of the data from animal bioassays is one of the most important and difficult tasks in carcinogen risk assessment. Physiologically-based pharmacokinetics facilitates the extrapolation by accounting for differences between laboratory animals and humans in relevant biochemical and physiological processes to enable the estimation of target-tissue-specific concentrations.

However, physiologically-based pharmacokinetics alone cannot account for interspecies differences in cellular activity in response to a given tissue-specific concentration of a carcinogen. Interspecies differences in cellular dynamics must also be defined to accurately and reliably estimate cancer risk in humans from animal studies (Conolly, 1990). The quantitative description and modelling of the cellular processes involved in carcinogenesis, such as the rates of cell division and cell death, and frequency of cell mutation, in humans and laboratory animals, may reduce the uncertainties associated with risk characterizations that are based on the extrapolation of animal data to humans.

The cellular dynamics of carcinogenesis can be described by a conceptual two-stage model with parameters that, in principle, can be represented by measurable cellular parameters (Moolgavkar and Luebeck, 1990; Conolly, et al., 1988a; Conolly, et al., 1988b; Conolly, 1990). The cellular parameters (birth rate, death rate, and mutation frequency) that may be measured to describe the incidence of cancer in terms of the two-stage model are not well defined. Reliable measurements of the cellular parameters remain to be determined in both experimental animals and humans.

The AAMRL has obtained the assistance of MITRE to provide an independent assessment of the available information pertinent to defining these parameters in biological terms and to identify experimental approaches that are potentially capable of yielding data from which the parameters could be quantified. This report describes experimental approaches which appear to be applicable, based on an independent evaluation of selected relevant articles from the scientific literature, focusing especially on the liver and lung of rodent species.

Section 2 discusses the cell dynamics of carcinogenesis in terms of the two-stage model and defines the relevant parameters. Section 3 identifies and describes experimental approaches from which reliable estimates of the relevant cellular parameters may be derived. Section 4 is a brief evaluation of the approaches identified, which are discussed in terms of the liver and the lung. Section 5 summarizes the major conclusions of this report.

SECTION 2

THE CELL DYNAMICS OF CARCINOGENESIS

2.1 DEVELOPMENT AND APPLICATION OF THE TWO-STAGE MODEL

A conceptual two-event model of carcinogenesis was derived empirically by curve-fitting epidemiological data to relate the incidence of deaths due to cancers to the age of the victims at the time of death (Armitage and Doll, 1954; 1957). This model mathematically conceptualized cancer development stochastically (probabilistically) in terms of the occurrence of one or more cellular events, termed "hits," that lead to the development of tumors. The occurrence of these events is increasingly probable with increasing age of the individuals at risk.

The kinetic, conceptual two-stage model of carcinogenesis proposed by Hethcote and Knudson (1978), and refined by Moolgavkar and associates (1978, 1980, 1981, 1990), combines the stochastic elements of the two-event model of Armitage and Doll with the deterministic elements represented by the birth and death rates of cells in the target tissue.

The two-stage model of Moolgavkar and associates is more widely applicable to the incidence rate patterns of cancer in human populations, as compared to the purely stochastic model of Armitage and Doll. Cancer incidence rates in human populations generally exhibit one of three major patterns: (1) a steady rise in the incidence rate with increasing age; (2) a steady rise in the incidence rate with age up to early adulthood, followed by a slower rise in the incidence rate with increasing age; and (3) a rise in the incidence rate to a peak at some time during life, followed by a decline in the incidence rate with increasing age. The two-stage model of of Moolgavkar and coworkers is capable of predicting all of these patterns, while the earlier model of Armitage and Doll can describe only the continuing increase in cancer incidence rate with age (Cohen and Ellwein, 1990; Cohen and Ellwein, 1990b; Moolgavkar, et al., 1980; Moolgavkar and Luebeck, 1990).

2.1.1 Description of the Two-Stage Model and Identification of Parameters

The two-stage model developed by Moolgavkar and associates (Moolgavkar, 1986; Moolgavkar and Luebeck, 1990) describes carcinogenesis mathematically in terms of the occurrence of specific mutations which define the two stages of the model and the rates of cell division and cell death of both the normal and the mutated cells. A schematic depiction of this model is presented in figure 2-1.

One underlying assumption of the two-stage model is that two rare events, occurring sequentially and often separated by a considerable time interval, are required for a tumor to arise from a population of normal cells. These rare events are likely to be specific mutations that most probably occur during cell division. The first mutation occurs with a frequency of μ_1 mutations/normal cell

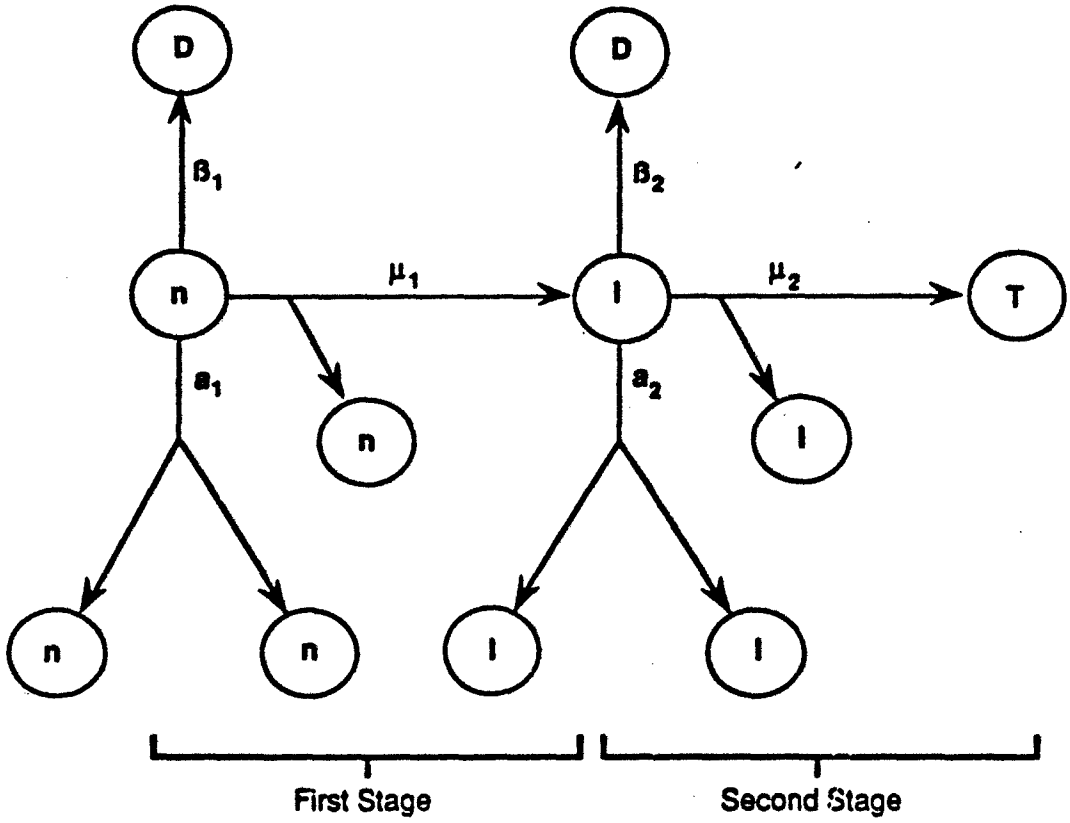

Figure 2-1. The Two-Stage Model of Carcinogenesis

division to produce an intermediate cell. The intermediate cells are subject to the second mutation. The second mutation occurs with a frequency of μ_2 mutations/intermediate cell division to produce a tumor cell which has the ability to grow and proliferate to form a tumor.

The two-stage model describes tumor incidence as a function of the rate at which normal cells divide (α_1 divisions/cell/unit time) and the rate at which normal cells die (β_1 deaths/cell/unit time) which, together with the frequency of the first mutation (μ_1 mutations/normal cell division), determine the number of intermediate cells produced during a given interval of time. In addition, tumor incidence is described as a function of the rate at which intermediate cells divide (α_2 divisions/cell/unit time) and the rate at which intermediate cells die (β_2 deaths/cell/unit time) which, together with the frequency of the second mutation (μ_2 mutations/intermediate cell division), determine the number of tumor cells produced during the time interval.

Thus, the parameters that must be defined in order to describe tumor incidence by means of the two-stage model are the following, assuming that both cell birth and cell death are exponential functions of time (Conolly, et al., 1988b):

1. α_1, the birth rate of proliferating normal cells in divisions x cells^{-1} x time^{-1}

2. β_1, the death rate of normal cells in deaths x cells^{-1} x time^{-1}

3. μ_1, the frequency of mutations that produce intermediate cells during division of normal cells in mutations x divisions^{-1}

4. α_2, the birth rate of proliferating intermediate cells in divisions x cells^{-1} x time^{-1}

5. β_2, the death rate of intermediate cells in deaths x cells^{-1} x time^{-1}

6. μ_2, the frequency of mutations that produce tumor cells during division of intermediate cells in mutations x divisions^{-1}

In principle, these six parameters, together with estimates of the total number of normal cells in the tissue and the fraction of the normal cells which are actively proliferating (growth fraction), allow the two-stage model to describe the incidence of tumor cells over an interval of time. The background values of α_1, β_1, μ_1, α_2, β_2, μ_2, together with estimates of the total number of cells in the tissue and the growth fraction, will allow the two-stage model to describe the spontaneous incidence of tumors in unexposed populations. The model assumes that chemical carcinogenesis is the result of chemically-induced perturbation of one or more of the model parameters (Moolgavka and Luebeck, 1990). For example, a chemical that induces an increase in the birth rate of proliferating intermediate cells (α_2) will increase the probability of producing a tumor cell from intermediate cells over a given time interval. Similarly, a chemical may increase the probability of producing an intermediate cell from normal cells by stimulating some of the non-proliferating normal cells to divide, increasing the

growth fraction. Alternatively, a chemical could increase the number of intermediate cells produced from normal cells by directly increasing the frequency of mutations (μ_1).

The following describe the relationships between the parameters enumerated above for the time interval t between time = t_0 and time = t_1 (see appendix A for derivations).

If the proliferating cells of a tissue exhibit a doubling time (cell-cycle time) of T_c, then

$$\alpha = 1/T_c = \ln(n_{t1}/n_{t0})/0.693t$$

where,

α = divisions/proliferating cell/unit time
T_c = doubling time
n_{t1} = number of proliferating cells at t_1
n_{t0} = number of proliferating cells at t_0
t = $t_1 - t_0$

If the total number of cells in the tissue and the growth fraction remain constant with time, as may be expected in most adult tissues, then

$$\beta = \alpha GF$$

where,

β = deaths/total number of cells/unit time
GF = growth fraction

The mutation frequency of normal cells (μ_1) to produce intermediate cells is related to the birth rate of normal cells (α_1) by the following (Moolgavkar and Knudson, 1981):

$$\mu_1 = i/[\alpha_1 \int_{t_0}^{t_1} X_n(t)dt]$$

where,

μ_1 = number of mutations/normal cell division
i = number of intermediate cells arising from normal cells between t_0 and t_1
α_1 = number of normal cell divisions/proliferating normal cell/unit time
$X_n(t)$ = number of proliferating normal cells at time = t

Similarly, μ_2 is related to α_2 by the following,

$$\mu_2 = Tu/[\alpha_2 \int_{t_0}^{t_1} X_i(t)dt]$$

where,

μ_2 = number of mutations/intermediate cell division
Tu = number of tumor cells arising from intermediate cells between t_0 and t_1
α_2 = number of intermediate cell divisions/proliferating intermediate cell/unit time
$X_i(t)$ = number of proliferating intermediate cells at time = t

2.1.2 Human Cancer Epidemiology as Described by the Two-Stage Model

The incidence rates of many common carcinomas steadily increase with age. The two stage model of Moolgavkar and colleagues generates age-specific incidence curves that closely fit the epidemiological data for these types of cancers when the growth of the normal tissue is represented by a Gompertz curve or by a logistic birth-death process (Moolgavkar, et al., 1990; Karlin and Taylor, 1975; Tan, 1986). For example, Moolgavkar and Knudson (1981) showed that the two-stage model can generate an excellent description of the age-specific incidence of lung cancer among nonsmokers and can quantify the impact of different levels of smoking on lung cancer risks. In addition, the model reveals the significant influence of smoking-induced increases in cellular proliferation on lung tumor development.

Breast cancer in women is the best known example of a cancer which displays a biphasic pattern (rapid and slow phases) of increasing age-specific incidence rate. Moolgavkar, et al. (1980) fitted the breast cancer incidence data from each of six populations by means of the two-stage model. The populations studied were from the United States, Denmark, Finland, Slovenia, Iceland, and Japan. Excellent agreement was obtained between the curves generated by the model and the epidemiological data for each of these diverse populations because the model can account for the changes in the cellular proliferation of mammary epithelium in response to menarche and menopause. Other epidemiologic features of breast cancer, such as the apparent protective effect of an early full-term pregnancy, can also be explained within the framework of the two-stage model.

Finally, the two-stage model is capable of describing the incidence rates of retinoblastoma, a rare cancer exhibiting an incidence rate that peaks in childhood and then declines with increasing age (Moolgavkar and Venzon, 1979; Moolgavkar and Knudson, 1981). The model of Moolgavkar and associates can describe the incidence rate patterns of both forms of retinoblastoma, the autosomaldominant form and the sporadic form. Likewise, the two-stage model can generate an excellent description of the pattern of the incidence rates of testicular carcinoma, which peaks at around the age of 30-35 years.

Thus, the two-stage model developed by Moolgavkar and colleagues is capable of producing excellent curve-fitting of various age-specific cancer incidence data from epidemiological studies, suggesting the potential predictive value of this model for human cancer risk assessment. As discussed above, the two-stage model depicts carcinogenesis by means of equations with terms (α, β, μ) that represent specific cellular parameters (birth rate, death rate, and mutation frequency). Accurate and reliable measurements of the relevant cellular parameters remain to be determined.

2.2 INITIATION AND PROMOTION IN THE CONTEXT OF THE TWO-STAGE MODEL

There is no consensus for a single unified theory of the mechanism of carcinogenesis. However, at least three separate classes of cellular processes are generally believed to participate in carcinogenesis: initiation, promotion, and progression (Pitot, et al., 1990).

Initiation is the result of a genetic change or mutation in a single cell arising either spontaneously or caused by the interaction between a chemical carcinogen and the nuclear DNA. Thus, the initiated cells possess heritable alterations. Initiation corresponds to the first mutation in the two-stage model, and initiated cells are the intermediate cells representing the first stage of carcinogenesis (Pitot, et al., 1987).

Initiation is a rare event, and the number of initiated cells arising directly from the interaction between a carcinogen or a carcinogenic metabolite and the genetic material is minuscule compared to the number of normal cells remaining in the tissue. Furthermore, initiated cells often must be stimulated to express phenotypic alterations which allow them to be distinguished from normal cells. Consequently, the detection of initiated cells is generally not possible without the second class of cellular processes associated with carcinogenesis, namely promotion (Pitot, 1990).

Promotion is the selective stimulation of DNA synthesis, mitosis, and cell division in initiated (intermediate) cells resulting in an increase in the rate of initiated-cell proliferation. Promotion can stimulate the clonal expansion of solitary initiated cells to produce discrete foci or nodules which are phenotypically distinguishable from normal tissue (Moolgavkar, et al., 1990; Rabes, et al., 1982).

Promotion can result from either the direct stimulation of cell proliferation by chemical mitogens or the indirect stimulation of cell proliferation by mechanisms which induce cell division in cells that survive exposure to cytotoxic chemicals (Goldsworthy, et al., 1990). Direct mitogenicity typically yields additive hyperplasia, characterized by increased weight of the tissue with no signs of necrosis. In contrast, cytotoxicity yields regenerative cell growth, characterized by normal or reduced tissue weight accompanied by signs of necrosis.

The genes of proliferating cells appear to be much more likely to undergo mutations than those of non-proliferating cells. One reason for this enhanced susceptibility is that cell division may be necessary prior to completion of DNA repair in a mutated cell for the permanent "fixation" of the

mutation (Cayama, et al., 1978; Columbano, 1982; Farber, 1984; Farber and Cameron, 1980; Pitot, et al., 1987). Further, the single-stranded DNA of proliferating cells is more exposed and unprotected from mutagenic attack (Goldsworthy, et al., 1990; Maguire and Rabes, 1989). Thus, promotion enhances the probability that mutations will occur in initiated cells both spontaneously and as the result of carcinogen exposure.

Accordingly, carcinogenic activity has been classified as either genotoxic or nongenotoxic. Genotoxic carcinogens react directly with the genetic material to increase the frequency of mutations (μ_1 and μ_2) that may potentially result in a tumor. Promoters (nongenotoxic carcinogens) are cytotoxic or mitogenic chemicals that selectively enhance the probability of mutation by increasing the rate of intermediate cell birth (α_2) and/or decrease the rate of intermediate cell death (β_2). Some carcinogens may exhibit both genotoxic and nongenotoxic properties.

In addition to initiation and promotion, at least one more rare event, commonly assumed to be a second mutation, must occur for intermediate cells to progress to tumor development (Farber and Cameron, 1980; Moolgavkar, 1983; Pitot, et al., 1987; Shulte-Hermann, 1985). In the context of the two-stage model, this event corresponds to the occurrence of the second mutation that converts an intermediate cell to a tumor cell, and tumor cells represent the second stage of carcinogenesis. The term progression, which has historically been used inconsistently in the cancer literature, has recently been suggested as a descriptor of this event (Pitot, et al., 1987; Moolgavkar, et al., 1990; Pitot, 1986).

2.3 LIVER CARCINOGENESIS

Carcinogenesis in the rodent liver has been extensively studied. Distinct subpopulations of hepatocytes can be identified during hepatocarcinogenesis which appear to correspond to the two stages of the two-stage model.

Promotion of initiated hepatocytes, by means of partial hepatectomy or exposure to mitogenic or cytotoxic agents, produces discrete foci and nodules of initiated hepatocytes which can be recognized as phenotypically distinct from normal hepatocytes (Cayama, et al., 1978; Peraino, et al., 1986; Rushmore, et al., 1987; Solt and Farber, 1976; Solt, et al., 1977). The foci of cells produced are sometimes referred to as enzyme-altered foci because the phenotypic alterations that characterize the initiated hepatocytes can generally be recognized as increases or decreases in the activities of certain enzymes. Nodules are the products of the focal proliferation of altered hepatocytes which are visible to the naked eye (2 to 3 mm in diameter) and exhibit clear-cut boundaries with respect to the surrounding normal liver on histological examination (Farber, 1984; Farber and Cameron, 1980). Both altered hepatocyte foci and nodules are regarded as putative tumor cell precursors by many authors (Cayama, et al., 1978; Emmelot and Scherer, 1980; Enomoto and Farber, 1982; Farber, 1984; Farber and Cameron, 1980; Popper, et al., 1960; Pugh and Goldfarb, 1978; Solt, et al., 1977).

Each altered hepatocyte focus and nodule is probably the product of the clonal expansion of a single initiated hepatocyte (Emmelot and Scherer, 1980; Kerler and Rabes, 1988; Pitot and Sirica,

1980; Pugh and Goldfarb, 1978; Scherer and Hoffmann, 1971; Tsuji, et al., 1988). Thus, the number of altered hepatocyte foci and nodules reflects the number of intermediate cells arising directly from normal cells to enter the first stage of the two-stage model.

Most altered hepatocyte foci and nodules that develop during promotion "remodel" or disappear following removal of the exogenous promoter (Enomoto and Farber, 1982; Bursch, et al., 1984). The initiated cells of remodelled altered hepatocyte foci and nodules are indistinguishable from normal hepatocytes (Enomoto and Farber, 1982; Pitot, et al., 1987). However, some altered hepatocyte foci and nodules persist following removal of the promoter. A subset of the persistent altered hepatocyte foci and nodules contain cells that exhibit higher proliferative rates relative to normal and intermediate cells, both in the presence and absence of the promoter. Unlike normal and intermediate hepatocytes, these cells retain the ability to grow rapidly and autonomously to produce cells with the appearance of tumor cells, either *in vivo* on transplantation into normal animals or *in vitro* (Miyazaki, et al., 1989; Kerler and Rabes, 1988).

Thus, under some experimental conditions, the cells of some persistent nodules may exhibit properties characteristic of tumor cells (Pitot, 1990). Further, each persistent altered hepatocyte focus or nodule containing cells possessing an enhanced potential to grow and proliferate may be the product of a single tumor cell. If so, then the number of persistent altered hepatocyte foci and nodules containing these cells reflects the number of tumor cells arising directly from intermediate cells to enter the second stage of the two-stage model.

2.4 PULMONARY CARCINOGENESIS

The lung consists of several different cell types, as well as several anatomically distinct regions. Thus, there are a number of different types of cancers found in the lung corresponding to the different possible cellular origins of lung cancer.

The most studied animal model for lung cancer is pulmonary adenoma in mice. Although the susceptibility to the induction of pulmonary adenomas varies greatly with the mouse strain tested (Malkinson, 1989), the relatively high incidence of adenomas in mice generally facilitates the screening of lung carcinogens (Busby, et al., 1989). On the other hand, the relatively high susceptibility of mice compared to other species raises the question of the relevance of the mouse adenoma model to the study of human pulmonary carcinogenesis.

Pulmonary adenomas in mice are benign tumors which, like malignant bronchiolo-alveolar carcinomas in humans, can develop either from alveolar type II cells or from the bronchiolar nonciliated Clara cells. Human bronchiolo-alveolar carcinomas account for only 1 to 5 percent of human lung cancer cases, which suggests that the mouse model is relevant for only a small percentage of human pulmonary carcinogenesis.

The development of adenomas appears to be consistent with the two-stage model. Pulmonary adenomas are benign lesions that appear prior to malignant adenocarcinomas in mice. Thus, the temporal relationship between adenomas and adenocarcinomas is consistent with the idea that adenoma cells are intermediate cells from which adenocarcinomas arise. In addition, adenomas that appear spontaneously in some mouse strains are increased in size and number by chemicals that stimulate cell proliferation, lending support to the proposal that adenomas may be composed of intermediate cells.

However, cell proliferation and tumor development are not consistently correlated in the lung. For example, Witschi (1986) reported that butylated hydroxytoluene, which possesses the ability to stimulate cell proliferation under some circumstances, can enhance pulmonary tumorigenesis even in the absence of detectable cell proliferation. In addition, oxidant gases which induce cell proliferation do not produce a clear effect on pulmonary tumorigenesis (Witschi, 1988). Thus, the possibility that adenocarcinomas can arise directly from normal cells rather than from proliferating adenoma cells cannot be discounted based on the available information.

An animal model for small cell carcinoma would be especially useful because this form of lung cancer is highly malignant and is associated with cigarette smoking in humans. Small cell carcinoma probably develops from lung neuroendocrine cells (Gould, et al., 1983). The development of an animal model for small cell carcinoma and related bronchopulmonary carcinomas has proven to be difficult because the stimulation of the proliferation of neuroendocrine cells in laboratory animals is often not associated with increased neuroendocrine tumor incidence.

Neuroendocrine cells exist in animal and human lungs both as solitary cells and as clusters of cells known as neuroepithelial bodies. Pulmonary carcinogenesis in humans is associated with an increase in the number of neuroendocrine cells in the lung. Further, the cells of small cell carcinomas and related carcinomas typically exhibit the secretory characteristics of neuroendocrine cells (Gould, et al., 1983). Neuroendocrine cells in both humans and laboratory animals proliferate in response to a number of toxic agents, including high or low oxygen concentrations, oxidant gases, asbestos, and carcinogenic amines. However, the stimulation of the proliferation of lung neuroendocrine cells by many of these agents fails to produce an increased incidence of neuroendocrine tumors in experimental animals. This demonstrates the difficulty associated with developing an animal model for the study of neuroendocrine cell-derived lung tumors in humans.

However, an animal model has recently become available. Schuller, et al. (1988) demonstrated that pulmonary neuroendocrine cancer develops in hamsters exposed simultaneously to diethylnitrosamine (DEN) and hyperoxia. Either hyperoxia alone or DEN alone produces neuroendocrine cell proliferation, but not neuroendocrine tumors in the hamsters. On the other hand, the carcinogenicity of DEN alone, at doses that stimulate neuroendocrine cell proliferation, is evidenced by the production of adenomas from Clara cells followed by the appearance of adenocarcinomas. Thus, DEN is a complete carcinogen for Clara cells, capable of promoting as well as initiating these cells. In contrast, promotion induced by hyperoxia must be combined with DEN-induced initiation to produce tumors from the neuroendocrine cells in the lungs of hamsters.

SECTION 3

EXPERIMENTAL APPROACHES TO QUANTIFYING THE PARAMETERS OF THE TWO-STAGE MODEL

3.1 THE CELL CYCLE

Proliferating somatic cells progress through a series of morphologically distinguishable phases as they divide. This sequence of phases, which is repeated for each generation of cell divisions, constitutes a cell cycle.

At the light microscopic level, dividing cells exhibit characteristic changes in the cell nucleus, including dissolution of the nuclear envelope, manifestation of chromosomes, and emergence of the mitotic apparatus. These traits define cells in the mitosis (M) phase of the cell cycle, which is followed by the division of the parent cell into two daughter cells (cytokinesis). After cell division, the cells appear to be "resting" and exhibit nuclear and other features which define interphase of the cell cycle. The cells remain in interphase until the next mitosis.

The average amount of time that elapses between consecutive mitoses in a population of proliferating cells is the mean cell-cycle time. Figure 3-1 is a schematic diagram of the cell cycle as determined histologically.

The number of cells in mitosis per total number of cells examined histologically is termed the mitotic index (MI). Thus, the MI is a measure of the percentage of cells that are dividing at a given time. The MI alone provides no information about the average time interval required for proliferating cells to traverse the cell cycle, nor does it indicate the magnitude of the growth fraction.

A cell in interphase progresses through a series of processes in preparation for the next mitosis, although the cell appears to be quiescent under the microscope. One period during interphase is characterized by especially high biochemical activity, corresponding to the replication of nuclear DNA. This period is termed the synthesis (S) phase. S phase begins with the start of DNA replication and ends with the complete duplication of the nuclear DNA.

The period between the end of M phase and the beginning of S phase, during which DNA is not replicated and biosynthetic activity is relatively low, is termed gap 1 (G_1). Similarly, the period between the end of S phase and the beginning of M phase is referred to as gap 2 (G_2). Thus, interphase can be subdivided into three phases (G_1, S, G_2) on the basis of biochemical observations. G_1, S, and G_2, together with M phase, constitute the four phases of the cell cycle, as depicted schematically in figure 3-2.

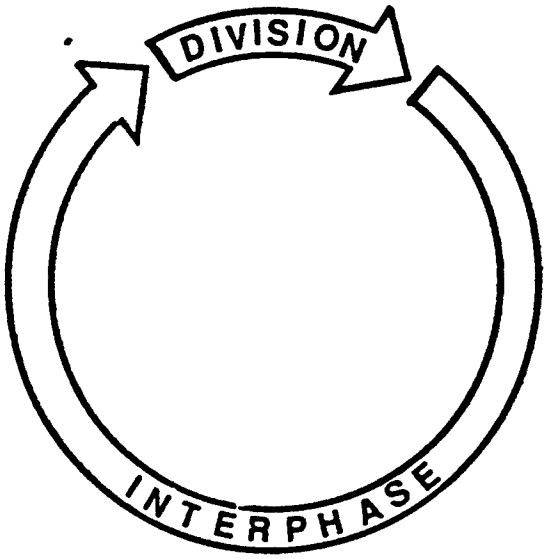

Figure 3-1. Cell Cycle as Determined Histologically

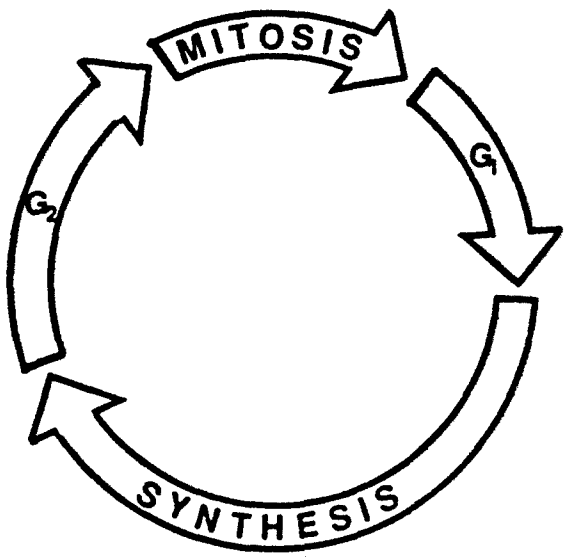

Figure 3-2. Cell Cycle as Determined Biochemically

The duration of a complete cell cycle can be subdivided into the time intervals required for proliferating cells to progress through each of the four phases of the cell cycle. Mathematically, this can be expressed as follows:

$$T_c = t_{G1} + t_S + t_{G2} + t_M$$

where,

T_c = cell-cycle time
t_{G1} = time required to progress through G_1
t_M = time required to progress through M
t_{G2} = time required to progress through G_2
t_S = time required to progress through S

or,

$$T_c = \Sigma t_{ph}$$

where t_{ph} = time required to progress through a given phase of the cell cycle

If the cell-cycle time (T_c) is constant, and each of the four phases of the cell cycle can be represented as a compartment of cells through which proliferating cells traverse, then the ratio of the number of cells in a given compartment (n_{ph}) to the time required for each cell to progress through that compartment (t_{ph}) will be constant for a steadily growing cell population. This concept can be expressed mathematically as follows:

$$\frac{n_{ph}}{t_{ph}} = K_{ph}$$

where,

n_{ph} = number of cells in a compartment
t_{ph} = time required for proliferating cells to progress through that compartment
K_{ph} = flux parameter for the compartment

In the steady state condition in which there is no net growth and no net reduction in the number of cells in the tissue, the flux parameters for individual compartments (K_{ph}) are equal to each other and also equal to the flux parameter for the entire four compartment system (K). Therefore,

$$\frac{n_{ph}}{t_{ph}} = \frac{N}{T_c} = K$$

where,

n_{ph} = number of cells in a compartment
t_{ph} = time required for proliferating cells to progress through that compartment
N = total number of cells in the entire proliferating cell population
T_c = cell-cycle time
K = the flux parameter for the entire proliferating cell population

Rearranging,

$$T_c = t_{ph} \times N/n_{ph}$$

Normally, the average time required for S, G_2, and M phases of the cell cycle is relatively constant and is a species-specific and cell-type-specific characteristic of the proliferating cells in a tissue.

However, even under controlled conditions, some variation exists in the cell-cycle time in "homogeneous" populations of proliferating cells. These cells can pause for a variable time interval (G_1) after mitosis, which accounts for much of the nonuniformity in the cell-cycle time. It appears that the cells must proceed past a specific point in G_1, termed the restriction point, before they can commit to the continuation of the cell cycle. Each cell at the restriction point has an equal probability of proceeding past the restriction point per unit of time. The probabilistic nature of this event contributes greatly to the variation in cell-cycle time.

The cells of tissues in adult animals generally do not proliferate at a steady rate. Adult tissues have usually completed their growth phase, and the cells of these tissues either divide occasionally to replace lost cells (e.g., skin or urothelium of the bladder) or lose the ability to divide (e.g., skeletal muscle or nervous tissue). In addition, the time interval per total number of cells in the tissue (proliferating and nonproliferating) from one cell division to another varies from as little as < 12 hours for epithelial cells lining the small intestine to as long as 2 years or more for hepatocytes (Alberts, et al., 1989).

In comparison, the time required for any dividing cell to progress through M phase is brief (usually about 30 minutes, but not longer than 2 hours). Thus, the time required to progress through M phase usually represents a very small fraction of the time interval per total number of cells in the tissue (proliferating and nonproliferating) from one cell division to another. Consequently, the MI is below the experimental limit of detection in many "static" adult tissues.

Similarly, the DNA-precursor pulse-labelling techniques described below often fail to yield a labelling index (LI), defined as the percentage of cells that incorporate the precursor into nuclear DNA during a given time interval. However, long-term continuous DNA-precursor labelling studies can distinguish proliferating cells from nonproliferating cells. The latter cells, which do not

incorporate DNA precursors but can re-enter the cell cycle near the restriction point (R) in G_1, are said to be in G_0 (figure 3-3). The growth fraction is related to the number of G_1 and G_0 cells by the following:

$$GF = (N - n_{G0})/N = (n_{G1} + n_M + n_{G2} + n_S)/N$$

where,

N	=	total number of cells
n_{G0}	=	number of cells in G_0 phase
n_{G1}	=	number of cells in G_1 phase
n_M	=	number of cells in M phase
n_{G2}	=	number of cells in G_2 phase
n_S	=	number of cells in S phase

Populations of cells with large G_0 cell subpopulations relative to the proliferating cell subpopulation, such as is found in many normal adult tissues, will be characterized by relatively small growth fractions.

Where GF<1, the relationship $T_c = t_{ph} \times N/n_{ph}$ becomes,

$$T_c = t_{ph} \times GF(N/n_{ph}) = GF(t_S/LI)$$

where,

$GF \times N$	=	number of proliferating cells in a population with N total number of cells
t_S	=	time required to progress through S phase
LI	=	labelling index

The above relationships show that the cell-cycle time (T_c) can be determined from the proportion of proliferating cells in a given phase of the cell cycle (n_{ph}/N), the time required for the proliferating cells to progress through that phase (t_{ph}) and the growth fraction (GF).

Two of these measurements, namely the proportion of cells in S phase of the cell cycle (n_S/N = LI) and the growth fraction (GF), can be determined by the specific labelling methods described below. The time of S phase (t_S) can be estimated by the approaches described in section 3.2.

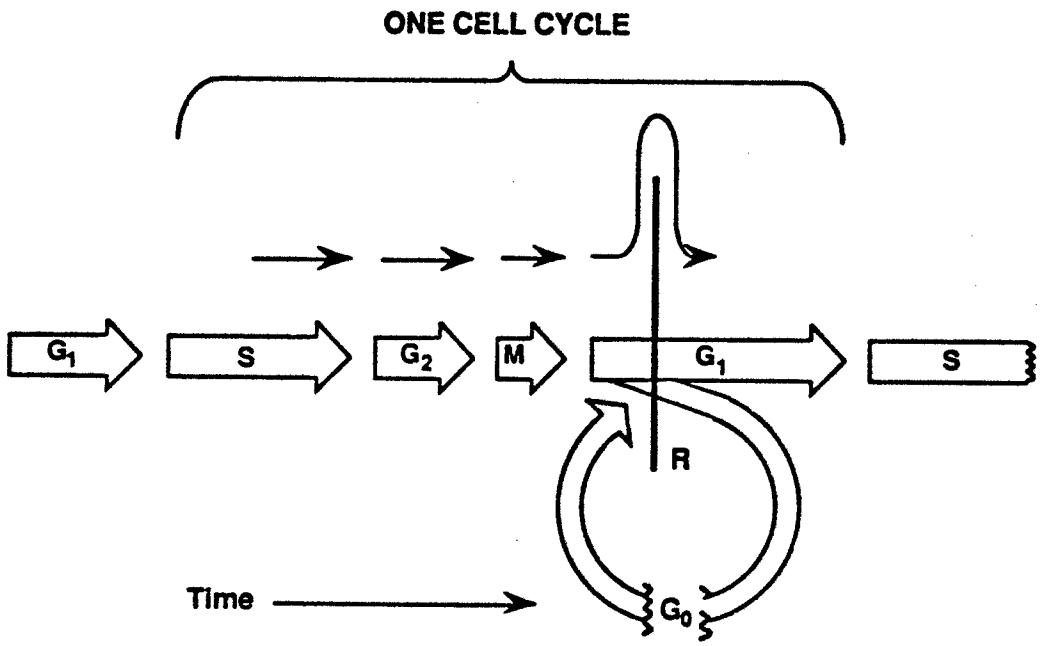

Figure 3-3. Cell Cycle in Tissues Containing Both Proliferating and Non-Proliferating Cells

3.1.1 ^{3}H-Thymidine Labelling

Exogenous thymidine, a DNA-precursor nucleotide that can be radiolabelled with tritium (^{3}H), is commonly used to label DNA. DNA labelled *in vivo* with ^{3}H-thymidine can be extracted from a tissue or organ and monitored by means of liquid scintillation counting to provide a rapid means for estimating the degree of replicative DNA synthesis. However, this approach requires a rigorous DNA extraction procedure and does not allow histopathological evaluation of the tissue.

Alternatively, cells undergoing replicative DNA synthesis (S phase) can be identified and quantified by *in vivo* ^{3}H-thymidine labelling followed by fixation of the tissue or organ and autoradiography. This approach allows the tissue or organ to be evaluated histopathologically and readily distinguishes S phase from unscheduled DNA synthesis based on the degree of labelling (Doolittle, et al., 1987; Mirsalis, et al., 1982). Also, this approach often provides a reliable means for measuring the LI which is the percentage of cells that incorporate a specific DNA precursor into DNA during a given time interval. Thus, the LI reflects the percentage of cells entering S phase during the labelling interval. Cells labelled in S phase are more readily recognized than those in M phase because grains in an autoradiograph are much easier to recognize and characterize than mitotic figures in histological sections. Thus, the LI can provide a more definitive identification of the labelled cell type than the MI (Goldsworthy, et al., 1990). In addition, the LI is typically more sensitive to increases in cell replication than the MI because, among other reasons, the duration of S phase is usually substantially greater than that of M phase.

However, an increase in the LI after a single pulse-label injection may be due to an increased number of proliferating cells, to an increased duration of S phase, or to a combination of both (Wachsmuth, 1987). The magnitude and extent of replication can be evaluated in longer-term studies by continuous *in vivo* labelling over several days, using an implanted osmotic pump (Goldsworthy,, et al., 1990), to provide a LI that is less dependent on changes in S phase duration or diurnal variations in cell proliferation.

Like the MI, the LI alone provides no information about the time interval required for the completion of the S phase (t_S).

3.1.2 Bromodeoxyuridine Labelling

S-phase cells can also be identified and quantified by labelling with bromodeoxyuridine (BrdUrd) *in vivo* followed by immunohistochemical staining with monoclonal antibodies directed against BrdUrd (Wynford-Thomas and Williams, 1986).

Like ^{3}H-thymidine labelling, BrdUrd labelling can provide the means to determine a LI. Flow cytometric analysis of BrdUrd labelling provides little basis for establishing definitive or quantitative lower end cut-offs for determining the degree of labelling to be scored as positive. However, S-phase cells labeled with BrdUrd can be examined directly by fluorescence microscopy (Gunduz, 1985). This approach allows the determination of the location of fluorochrome binding within a single cell

(e.g., cytoplasm, nucleus, or nucleolus), and the identification of artifacts (e.g., nonspecific binding of the fluorochrome), so that cells can be more reliably scored as positive or negative for the BrdUrd label. In addition, fluorescence microscopy allows the differentiation of cell types, such as tumor cells, lymphocytes, macrophages, and fibroblasts, and fragmented or broken cells in mixed cell populations.

Alternatively, the proportion of S-phase cells in tissue samples can be rapidly determined by means of flow cytometry to measure the indirect immunofluorescence of BrdUrd-labelled cells treated with anti-BrdUrd and fluorescein-labelled goat anti-mouse γ-globulin (Sugihara, et al., 1986). The antigenicity of BrdUrd in formalin-fixed tissues can be unmasked by enzymatic digestion (proteinase) combined with acid hydrolysis, allowing the possibility of flow cytometric study of paraffin-embedded tissue.

3.1.3 Immunostaining With the 19A2 Anti-Cyclin/PCNA Antibody

Cyclin/proliferating cell nuclear antigen (PCNA) is a highly conserved acidic nuclear protein with an apparent molecular weight of about 36,000 which is synthesized in late G_1 and S phase (Galand and Degraef, 1989). Cyclin/PCNA is the auxiliary protein of DNA polymerase δ. The 19A2 autoantibody found in the serum of patients with systemic lupus erythematosus recognizes cyclin/PCNA and can be used to identify cells containing cyclin/PCNA.

S-phase cells in methanol-fixed, paraffin-embedded animal tissues can be specifically identified by immunostaining for cyclin/PCNA with the commercialized monoclonal antibody 19A2 followed by a fluorochrome-conjugated antibody specific for the 19A2 antibody (Galand and Degraef, 1989). Further, the percentage of cells in S phase may be determined rapidly and objectively in a large population of cells by means of immunostaining for cyclin/PCNA combined with flow cytometric monitoring.

Like the MI and the LI, the percentage of cells in S phase determined solely by means of 19A2 anti-cyclin/PCNA immunostaining would provide no information about the time interval required for the completion of S phase from which the total cell-cycle time (T_c) might be estimated.

3.1.4 Other Labels for Studying the Cell Cycle

Antibodies have been identified which may facilitate the discrimination between proliferating cells and G_0 cells, namely the Si87 anti-nucleolar antibody and the mouse monoclonal antibody Ki67.

Immunostaining with the Si87 anti-nucleolar antibody, found in the serum of a scleroderma patient, results in specific fluorescence of the nucleoli in the tissues and organs of mice, rats, and humans (Dubben, 1990). The Si87 antibody can be used to discriminate between proliferating cells and nonproliferating (G_0) cells because the morphological features (size, shape, and number) of the nucleoli depend on cell cycle and growth state. Cells immunostained with Si87 and counterstained with fluorochrome-conjugated antibodies can be studied for total immunofluorescence by means of

flow cytometry, permitting rapid and objective measurement of nucleolar immunofluorescence intensity (Dubben, 1990). In addition, the interpretation of the flow cytometric data can be facilitated and confirmed by parallel morphological detection of Si87 in tissue sections using fluorescence microscopy.

Gerdes, et al. (1984) showed that mouse monoclonal antibody Ki67 recognizes a human nuclear antigen that is present in proliferating cells (G_1, S, G_2, and M phases) but is absent in nonproliferating cells (G_0). Thus, flow cytometric analysis of cells immunostained with monoclonal antibody Ki67 may allow a simple and rapid estimation of the growth fraction of human cell populations (Baisch and Gerdes, 1987). However, Silvestrini, et al. (1988) have raised some questions about the capacity of Ki67 to distinguish between G_0 and G_1 cells.

3.2 BIRTH RATE

Cell birth is the production of a pair of new cells from the mitotic division of one cell. As discussed above, the interval between consecutive mitotic divisions of a cell is termed a cell cycle. Since cell division occurs once per cell cycle, the rate of cell birth (number of cell divisions/proliferating cell/unit time) is the inverse of the time required for the completion of the cell cycle (time/cell division/proliferating cell). Accordingly, the cell birth rate (α) can be estimated by elucidating the cell-cycle time.

3.2.1 Mean Cell-Cycle Time as Determined from Fraction Labelled Mitoses Curves

The durations of the various phases of the cell cycle and the total cell-cycle time can be determined in nonsynchronized populations of cells by a "fraction labelled mitoses" method developed by Quastler and Sherman (1959) and modified by Shimada and Langman (1970). The assumptions that underlie the fraction labelled mitoses approach are that (1) the population of cells under study is dividing at a steady rate, (2) the cell-cycle time (T_c) is constant, and (3) each phase of the cell cycle is of constant duration. In actuality, cell-cycle time is not constant. The cell-cycle time can be affected by external factors such as changes in the availability of critical nutrients and alterations in the concentration of stimulatory (mitogenic) or inhibitory factors. *In vitro*, the cells' environment can be controlled to mitigate the effects of most of these external factors.

The fraction labelled mitoses procedure is carried out in the following manner. Populations of proliferating cells are exposed to a pulse of a labelled DNA precursor (usually ^{3}H-thymidine) at time = 0. Cell or tissue samples are then obtained at various times (time = t) during a post-pulse interval that can range from 30 minutes to 40 hours and prepared for histological analysis and DNA-labelling determinations.

When ^{3}H-thymidine is used, the sampled tissue is subjected to autoradiographic analysis. Silver grains developing in the autoradiograph over the nucleus of a cell indicate that the nuclear DNA in the

cell is labelled as a consequence of DNA synthesis occurring during the ^{3}H-thymidine pulse exposure.

Labelled nuclear DNA will remain in the nucleus as long as the DNA molecule remains intact. Labelled nuclei represent cells in S, G_2, and M phases of the cell cycle at the sampling time, because the label that is incorporated into the DNA during S phase remains in the DNA through G_2 and M phases. However, the labelled nuclei of cells in M phase can be distinguished from S and G_2 phase nuclei by means of histological examination. Thus, the sampled tissue can be scored for number of mitoses that exhibit labelling and total number of mitoses (labelled and unlabelled). The ratio of labelled mitoses to the total number of mitoses is termed the "fraction labelled mitoses."

A fraction labelled mitoses curve is then generated by plotting fraction labelled mitoses as a function of time, as exemplified in the hypothetical curve presented in figure 3-4. The duration of S phase (t_S) is estimated from the portion of the curve representing the time interval during which 50 percent or more of the mitoses are labelled. This value can be obtained directly from the graph. In figure 3-4, t_S is approximately 7.5 hours.

The proportion of cells in S phase (n_S/N) can be estimated from the ratio of the number of labelled, non-mitotic nuclei (S and G_2 phases) to the total number of non-mitotic nuclei (G_1, S, and G_2 phases). For example, a hypothetical labelling index for a rapidly proliferating tissue, such as intestinal epithelium, could be 0.4. Thus, the fraction labelled mitoses approach provides numerical estimates for both n_S/N and t_S.

If $T_c = t_S \times N/n_S$, as shown in section 3.1 ($T_c = t_{ph} \times N/n_{ph}$), then:

$$T_c = t_S/LI$$

where,

T_c = cell-cycle time
t_S = S phase duration as estimated from the fraction labelled mitoses curve
LI = labelling index

Substituting the hypothetical values in the example above,

$$T_c = \frac{7.5}{0.4} \text{ hours} = 18.75 \text{ hours}$$

The birth rates of proliferating cells even in tissues with small growth fractions (GF) can be determined by the fraction labelled mitoses approach, where $T_c = GF(t_S/LI)$ as discussed above, if the proliferating cells can be distinguished from the cells in G_0. The growth fraction may be estimated, for instance, by immunostaining the tissues with the Si87 antinuclear antibody (Dubben, 1990) or similar antibodies. T_c could then be calculated from GF, t_S, and LI.

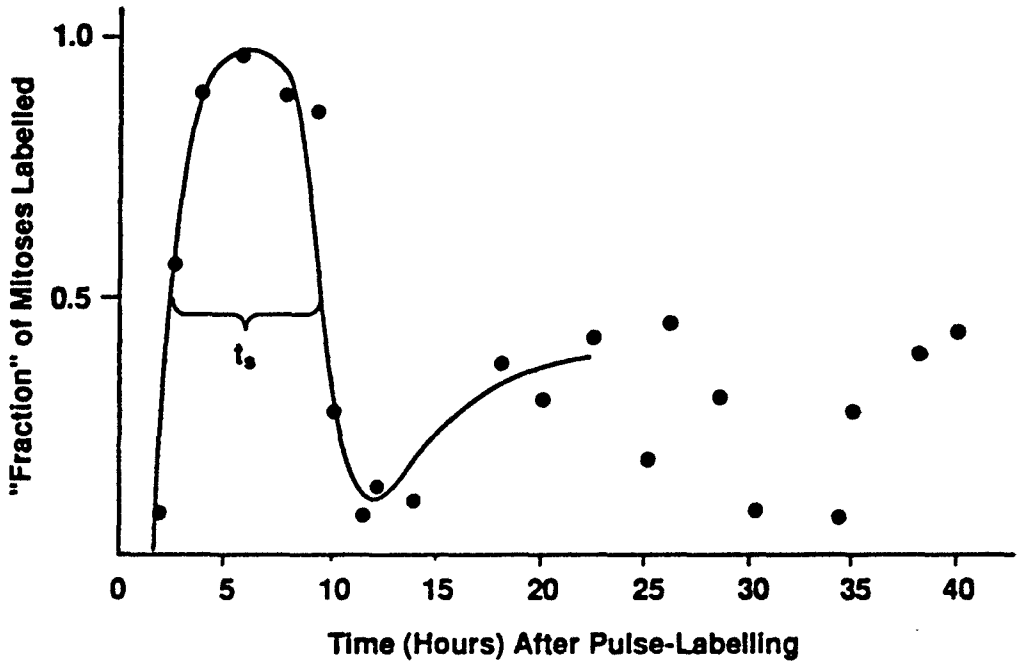

Figure 3-4. Hypothetical Fraction Labelled Mitoses Curve

3.2.2 Mean Cell-Cycle Time as Determined by Multi-Labelling Approaches

3.2.2.1 ^{3}H-Thymidine Autoradiography Combined with BrdUrd Immunohistochemistry

In combination with ^{3}H-thymidine autoradiography, BrdUrd immunohistochemistry allows reliable double labelling, and may be useful for measuring the duration of S phase and the doubling time (total cell-cycle time) *in vivo*.

For example, Raza, et al. (1987) studied the kinetics of leukemic cells, using the double-label method, by first infusing BrdUrd intravenously for one hour to label S-phase cells in nonlymphocytic leukemia patients. The percentage of S-phase cells (LI) was estimated in post-infusion cells collected from bone marrow aspirates and biopsy samples. The post-infusion bone marrow aspirates were incubated with ^{3}H-thymidine *in vitro* for one hour. The average duration of S phase (t_S) was calculated using the following formula:

$$t_S = \frac{DL + {}^3HTdr}{BrdUrd} \times t$$

where,

t_S	=	average duration of S phase
DL	=	number of double-labelled cells
^{3}HTdr	=	number of cells labelled solely with ^{3}H-thymidine
BrdUrd	=	number of cells labelled solely with BrdUrd
t	=	time interval between the two labels, taken as 1-hour for patients receiving a 1-hour BrdUrd infusion

The logic behind this double-labelling approach is as follows (Wimber and Quastler, 1963): three groups of labelled cells will be observed when an asynchronous cell population in a steady state is pulse-labelled with BrdUrd and a short time later (t) labelled with ^{3}H-thymidine, namely, cells singly labelled with BrdUrd, cells singly labelled with ^{3}H-thymidine, and doubly labelled cells. A fraction of the cells, proportional to t_S, will be labelled during the ^{3}H-thymidine treatment, and some of these cells will be double-labelled. During the interval between treatments (t) a number of cells, proportional to t, will progress past S phase and will be labelled only with BrdUrd. If none of the labelled cells divide, then, because t_S is proportional to the total number of ^{3}H-thymidine-labelled cells, including those that are double-labelled, and t is proportional to the number of cells single-labelled with BrdUrd,

$$t_S \times BrdUrd = t \times ({}^3HTdr + DL)$$

and rearranging,

$$t_S = (DL + {}^3HTdr)t/BrdUrd$$

Accurate assessment of the cell-cycle time (T_c) from the t_S depends on the following assumptions (Wimber and Quastler, 1963): (1) the cell population is in steady state so that the number of cells lost equals the number of cells born; (2) cell division in the population is asynchronous so that each proliferating cell has an equal chance of being at any point in the cell cycle as any other proliferating cell; (3) maturation, death, or loss of cells from the population occurs after division, and prior to S phase; and (4) the population exhibits only minor variations in generation time. Under these circumstances, the average total cell-cycle time (T_c) can be calculated using the following formula, which follows from the previous discussion:

$$T_c = \frac{t_S \times GF}{LI}$$

where,

T_c	=	average total cell-cycle time
t_S	=	duration of S phase
GF	=	growth fraction
LI	=	labelling index (n_S/N)

Similar double-labelling approaches may be developed for determining average cell-cycle times in the liver, lung, and other tissues and organs of humans and experimental animals.

Antigen-expression approaches to cell-cycle investigation may also be amenable to multi-labelling protocols followed by flow cytometric or fluorescence microscopic analysis. For example, human tissues labelled *in vivo* with a DNA precursor, such as BrdUrd or ^{3}H-thymidine, can be methanol-fixed and paraffin-embedded for histological examination and then labelled with anti-cyclin/PCNA or similar antibody for estimating S-phase duration (t_S) by the double-labelling approach. In addition, the growth fraction may be estimated by immunostaining the tissues with Si87 (Dubben, 1990) or similar antibody. The total cell-cycle time (T_c) could then be calculated from the GF, t_S, and LI as discussed above.

3.2.2.2 Two-Parameter Flow Cytometry of Cells Counterstained with Propidium Iodide

Propidium iodide stains DNA and can be used to quantitate the total DNA content in a cell. Flow cytometric analysis of BrdUrd/propidium iodide double-labelled cells allows rapid characterization and sorting of a population of cells based simultaneously on BrdUrd incorporation and total DNA

content (Crippen and Jones, 1989; Dolbeare, et al., 1983). The analysis of cells labelled with BrdUrd, *in vivo* or *in vitro*, and counterstained with propidium iodide can be used to identify proliferating S-phase cells (anti-BrdUrd-fluorescence) and discriminate among diploid (2X) and tetraploid (4X) cells, as well as aneuploid, octaploid (8X), and other multiploid cells (propidium iodide-fluorescence intensity).

Further, BrdUrd/propidium iodide double-labelling may allow discrimination between S-phase cells (BrdUrd-labelled; >2X to 4X propidium iodide) and the non-S phase diploid progeny of proliferating cells (BrdUrd-labelled; 2X propidium iodide). Thus, evaluation of the bivariate (BrdUrd/propidium iodide) DNA distributions may be a rapid means to measure a relatively accurate and reliable LI, reflecting the percentage of cells entering S phase during the BrdUrd-labelling period, and can account for non-S phase BrdUrd-labelled diploid progeny of the proliferating cells, BrdUrd-unlabelled cells with S phase DNA content, and DNA-synthesizing tetraploid and higher-ploid subpopulations of cells that may be found, for example, in the normal liver of humans and experimental rodents (Mahon, et al., 1979).

A possible alternative to BrdUrd/propidium iodide double-labelling is antibody Ki67/propidium iodide double-labelling, which may be used to distinguish proliferating from nonproliferating human cells and simultaneously measure DNA-fluorescence intensity in a cell sorter (Baisch and Gerdes, 1987).

3.2.2.3 Cell-Cycle Measurements in Specific Subsets of Mixed Cell Populations

Multi-labelling approaches may be used to recognize and determine the labelling indices and cell-cycle times of even small subsets of mixed cell populations exhibiting heterogenous properties. In particular, the BrdUrd-staining approach may allow the simultaneous flow cytometric analysis of BrdUrd-labelling and specific membranous, cytoplasmic, and nuclear antigens (Crippen and Jones, 1989).

In multiparameter flow cytometric analysis, cells are stained with mixtures of fluorescent dyes and then passed through several illuminating beams of different wavelengths, separated in space (Shapiro, 1983). Correlated measurements of as many as eight parameters can be made. In addition to antigen expression, parameters which may be used to distinguish cell types include cell size, cell shape, redox state (measurements of NADH fluorescence), lectin binding (surface sugars), total protein, basic protein, sulfhydryl groups, DNA content, chromatin structure, surface/cytoplasmic receptors, enzyme activities, and many others.

In principle, these parameters may permit the identification of the various types of normal cells present in a specific tissue or organ, and the discrimination between normal cells, initiated cells, and tumor cells. However, the automated flow cytometric approach requires the identification of specific markers for the cell populations of interest.

In addition, flow cytometry requires the use of single-cell suspensions. Fresh or frozen tissue must be mechanically or enzymatically disaggregated. Del Bino, et al. (1989) developed a method to mechanically release nuclei from frozen tumor samples to distinguish diploid from aneuploid cells by means of flow cytometry. It may also be possible to disaggregate cells from paraffin blocks for flow cytometrical analysis (Del Bino, et al., 1989; Silvestrini, et al., 1988). However, the results obtained from tissues in paraffin blocks may not be comparable to those obtained from fresh or frozen tissue because (1) the high temperatures associated with paraffin treatment destroys many cellular products and proteins in the tissue, and (2) the dehydration associated with the treatment results in cellular shrinkage and loss of saccharides and other cellular components.

Alternatively, a DNA-image cytometry approach has been developed which permits the objective analysis of multi-labelled cells based on the "Leiden TV Analysis System" (Cornelisse and Van Driel-Kulker, 1985). The cells are selected by an image segmentation algorithm instead of by visual examination. Visual inspection is performed after automated selection to identify artifacts that may not have been automatically rejected.

DNA-image cytometry allows the evaluation of multiple parameters in single cells either by use of different light wavelengths or by sequential staining procedures (Ailison, et al., 1984). In addition, DNA-image cytometry permits relatively rapid evaluation of large aggregates of cells in tissue slices and, presumably, ready identification of cells as normal, initiated, or tumor cells by means of histochemical examination. Also, the cells analyzed by this approach can be visually inspected to validate the methodology and verify the results.

3.3 DEATH RATE

The cell death rate (β) is the number of viable cells lost from a defined cell population over a specified interval of time (number of deaths/cell/unit time). Ideally, β should be estimated for the two-stage model from an objective, directly measurable parameter that quantifies cell death. Even with such a parameter, the estimation of the rate of cell death in a tissue would require that cell deaths could be expected to occur with temporal regularity based on a predictable schedule or a characterizable cycle.

However, the cells in most tissues, especially in adult animals, usually do not die with temporal regularity. Although dying cells progress through characteristic sequences of identifiable morphological and biochemical changes, the manifestations of cell death provide no evidence that the occurrence of cell deaths in adult tissues follows a temporal program. Further, no cell cycle can temporally characterize cell death, since the death of a cell ends with its irreversible removal from the cell population. In contrast, as discussed above, cell births in proliferating cell populations follow a temporally regular cycle. Thus, any directly measurable parameter that quantifies with cell death can provide, at most, only a measure of the number of dead and dying cells in the cell population at the time of examination.

3.3.1 Manifestations of Cell Death

Cell death can be the result of either extracellular factors, such as injury caused by cytotoxic agents, or intracellularly-programmed termination of cell viability (Wyllie, 1981; Kerr, et al., 1972). Extracellularly-caused death is known as necrosis, and programmed single cell death is referred to as apoptosis.

In general, necrosis and apoptosis can be distinguished readily, although recourse to ultrastructural examination may be necessary. Necrosis is characterized by cellular edema (cytoplasmic swelling) and mitochondrial damage. In contrast, apoptosis is characterized by progressive contraction of cellular volume (cytoplasmic condensation), preservation of the integrity of the mitochondria and other cytoplasmic organelles, and dense peripheral aggregation of chromatin or aggregation of chromatin into hemilunar caps in the nucleus. Electron microscopy shows that apoptotic bodies, which are small roughly spherical or ovoid cytoplasmic fragments that can be detected with the light microscope, represent a small fraction of the total number of cell remnants present (Kerr, et al., 1972).

Apoptosis in the liver is normally a short-lived phenomenon lasting less than four hours (Schulte-Hermann, et al., 1987). Although apoptosis may be difficult to detect and measure (Goldsworthy, et al., 1990), small numbers of apoptotic bodies have been observed in histological sections of many healthy tissues and organs (Kerr, et al., 1972). Apoptosis preferentially eliminates old cells that did not replicate during normal tissue or organ growth (Schulte-Hermann, et al., 1987).

The incidence of apoptotic bodies is severalfold higher in altered hepatocyte foci than in normal resting liver (Schulte-Hermann, et al., 1987). In addition, numerous apoptotic bodies are sometimes observed in rapidly growing malignant tumors (Sarraf and Bowen, 1988). The rate of tumor enlargement may depend primarily on the balance between apoptosis and mitosis (Kerr, et al., 1972).

3.3.2 Potential Indices of Cell Death

Several approaches may be developed to provide indices for apoptosis and necrosis based on the manifestations of the cell death processes. However, as discussed below, each of the potential approaches falls short of providing a useful index of cell death or a death rate (β) which can be applied to the two-stage model.

Necrotic cells break down and fragment to produce particles of cellular debris which appear both in the extracellular space and within the nearby healthy phagocytizing cells of the tissue. Thus, the degree of necrosis that has recently occurred in the tissue is reflected by the amount of cellular debris. However, necrotic cells cannot be quantified from cellular debris because necrotic cells fragment into particles which vary in size and number and can be displaced by vascular activity or phagocytosis by mobile macrophages. Further, an examination of cellular debris provides no information about the time interval during which necrosis occurred.

Supravital dyes such as trypan blue can be used to identify necrotic cells, prior to their fragmentation, and to distinguish necrotic cells from both viable cells and apoptotic cells. Supravital dyes passively enter swelling necrotic cells and are excluded by the undamaged cell membranes of viable and apoptotic cells. However, the amount of supravital dye taken up by the tissue is not directly proportional to the number of necrotic cells present, and the method requires the microscopic examination of individual cells to score them as viable dye-excluding cells or necrotic cells. In addition, supravital dyes can provide no temporal information with which to characterize necrosis.

Nile blue sulfate can be used to identify cells undergoing apoptosis. Nile blue sulfate is an alkaline dye that stains apoptotic cells because these cells typically have acidic cytoplasm. However, nile blue sulfate also stains macrophages and phagosomes (membrane-bound vesicles containing phagocytized material). Further, like the supravital dyes, the amount of nile blue sulfate taken up by the tissue is not directly proportional to the number of apoptotic cells present and provides no temporal information from which apoptosis might be modelled.

Bursch, et al. (1990) described a method to estimate the duration of apoptosis both in altered liver foci and in mitogen-induced hyperplastic liver in rats following termination of mitogen treatment. The number of apoptotic bodies in liver stained with hematoxylin and eosin were counted under the light microscope and expressed as a percentage of intact hepatocytes. A semilogarithmic plot of the apoptotic body incidences was used to determine the half-life of the histologically visible stages of apoptosis. Bursch, et al. (1990) suggested that the cell loss rate by apoptosis can then be calculated from the following formula:

$$\text{cell loss/unit time} = (i \times f)/d$$

where,

- i = incidence of apoptotic bodies
- f = factor to correct for the formation of more than one apoptotic body from an apoptotic cell
- d = duration of the visible parts of apoptosis

Thus, the method depends on reliable estimates of the average number of apoptotic bodies formed from each apoptotic cell (f). Further, as mentioned above, apoptotic bodies that can be detected with the light microscope represent only a small fraction of the total number of cell remnants present. In addition, the method does not allow d, the visible part of apoptosis, to be estimated directly from tissues in which the apoptotic body incidence might be expected to be constant with time, such as in the normal untreated adult liver.

Necrosis releases cell contents into the blood stream. Thus, serum glutamate-pyruvate transaminase activity or other tissue-specific enzyme activities may provide indices of cellular damage and necrosis. However, the correlations necessary to provide a useful index of necrosis by means of this approach have not been adequately determined.

Differences in fluorescence between viable cells and dead cells may provide useful approaches to separating and quantifying dead cells by means of flow cytometry. For example, the refractive index between intact cells and cells with damaged membranes is commonly exploited in immunofluorescence analysis to discriminate dead cells, which tend to stain non-specifically with fluorescent antibodies, from intact cells bearing the surface antigen under study. Presently, dead cells cannot be sufficiently discriminated from viable cells to provide a useful index of cell death by means of measuring differences in the refractive index. However, this or similar approaches may be developed to provide a more reliable index of cell death.

3.3.3 Cell Death Rate as Estimated from the Cellularity of the Tissue

Indices of apoptosis and necrosis may be among the basic parameters of tumor development because, as represented in the two-stage model, cell death may be as critical as cell proliferation for the selective growth of precancerous cells (Goldsworthy, et al., 1990; Sarraf and Bowen, 1988). However, as discussed above, the possible indices of cell death based on directly measurable parameters will provide no temporal information from which the rate of cell death (β) might be estimated.

On the other hand, the rate of change of the total number of cells in a tissue or organ is a function of the difference between the rate of cell birth and the rate of cell death. Thus, the rate of cell death can be estimated indirectly from the rate of change in total cell number and the rate of cell birth without further experimental input.

3.4 MUTATION FREQUENCY

Each gene typically contains numerous sites at which mutations can occur spontaneously or as the result of exposure to mutagens, including genotoxic carcinogens. However, most mutations do not lead to phenotypic alterations and, therefore, are not detectable. Furthermore, the kinds of mutations to be incorporated into the two-stage model, which lead to the production of tumor cells from normal cells, represent a very small fraction of the total number of mutations that might occur spontaneously or as the result of exposure to a genotoxic carcinogen. An assumption of the two-stage model is that the first mutation confers both a growth advantage and phenotypic alteration to the initiated cell; both of these are expressed on exposure to a promoter. The second mutation of the two-stage model further abrogates growth control, conferring the potential for the cell to progress to tumor.

3.4.1 Frequency of Mutations of Normal Cells to Intermediate Cells

3.4.1.1 Frequency of Mutation Estimated from Number of Altered Foci and Nodules

As discussed above, the mutation of a normal cell to an intermediate cell is a rare event that is not directly detectable. However, foci of altered cells (e.g., altered hepatocyte foci and nodules) probably

arise as the result of the clonal expansion of intermediate cells following exposure to a promoting stimulus. These lesions are often designated enzyme-altered foci, because they typically contain abnormal amounts of various enzymes.

Quantitating the frequency of mutation from normal cells to intermediate cells may be facilitated by the clonal expansion of each mutated cell to a detectable population of enzyme-altered foci and nodules. The number of clonally-derived altered foci and nodules may be used to estimate the number of mutations that have occurred from which, in conjunction with normal cell birth rates (α_1), the mutation frequency (μ_1) can be calculated.

3.4.1.2 Characteristics of Altered Foci and Nodules

Histochemical and other approaches can be developed based on differences such as those enumerated below. In principle, these could be used to differentiate intermediate cells from normal cells and estimate the number of clonally-derived altered foci (Farber, 1984; Fiala, et al., 1972; Kalengayi, et al., 1975; Pitot, 1990).

Altered hepatocyte foci and nodules may exhibit one or more of the following enzymic alterations: (1) increased glutathione S-transferases (placental), DT-diaphorase, alkaline phosphatase, epoxide hydrolase, UDP-glucuronyltransferase 1, or γ-glutamyl transpeptidase, and (2) decreased ATPase, glucose-6-phosphatase, β-glucuronidase, serine dehydratase, acid phosphatase, total microsomal cytochromes P-450, cytochrome b5, or in one of several mixed-function oxidase activities.

Altered hepatocyte foci and nodules may exhibit a number of other phenotypic alterations, in addition to altered enzyme activities, including the following: (1) increased levels of glutathione, (2) increased levels of glycogen, and (3) resistance to iron accumulation (also referred to as deficiency to store iron or iron exclusion).

In addition, altered foci and nodules may exhibit the following: (1) enhanced basal rate of proliferative activity, manifested, for example, by elevated DNA synthesis and mitosis, and nuclear changes suggestive of a "more open" or "more available" genome for transcription, (2) aberrant methylation of nucleic acid bases, (3) damaged DNA (e.g., Wahba, et al., 1988), and (4) aneuploidy.

Finally, altered foci and nodules may also exhibit the following: (1) expression of a specific cytosolic antigen with a molecular weight of 21,000 (not lost in remodeled altered foci and nodules), (2) incidence of apoptotic bodies increased severalfold higher than normal resting liver, (3) growth advantage *in vitro* (e.g., Kerler and Rabes, 1988), and (4) resistance to cytotoxicity (hepatotoxicants and carcinogens) *in vivo* and *in vitro*.

3.4.1.3 Experimental Protocols for the Identification and Quantitation of Altered Foci and Nodules

Experimentally, promotion of initiated hepatocytes, by means of partial hepatectomy or exposure to mitogenic or cytotoxic agents, is required to produce discrete altered foci and nodules of initiated hepatocytes which can be recognized and counted by gross or microscopic examination (Cayama, et al., 1978). Most altered foci and nodules which develop during promotion "remodel" or disappear after the promoter is removed (Enomoto and Farber, 1982). Further, the re-expression of the carcinogen-induced altered phenotype in remodelled foci and nodules requires a strong growth stimulus. Thus, many foci may be missed in the absence of promotion or in tissue examined some time after a promoting agent has been removed by metabolic and excretory mechanisms (Farber and Cameron, 1980). Missed foci will result in an underestimation of μ_1.

To facilitate the detection of altered foci, initiated cells can be selectively and intensively induced to proliferate *in vivo* to produce nodules by means of one of several well-known treatment protocols (Emmelot and Scherer, 1980; Farber and Cameron, 1980). For example, nodules 1-2 mm in diameter develop in the livers of Fischer rats five to ten days after the following sequence of treatments ("Solt-Farber" protocol): (1) a single dose of up to 200 mg diethylnitrosamine/kg body weight; (2) two weeks later, dietary exposure to 0.02 percent 2-acetylaminofluorene, an hepatocarcinogen and potent inhibitor of normal cell proliferation, for one week; and (3) partial hepatectomy or treatment with a liver mitogen combined with continued oral exposure to 2-acetylaminofluorene to selectively stimulate the proliferation of initiated cells (Cayama, et al., 1978; Solt and Farber, 1976; Solt, et al., 1977).

The basis for the selection of the altered hepatocytes in this and similar experimental protocols is that initiated cells exhibit resistance to the cytotoxic action of a continuously administered carcinogen (Columbano, et al., 1982; Emmelot and Scherer, 1980; Farber, 1973). In particular, the Solt-Farber protocol relies on the carcinogen 2-acetylaminofluorene to create the selection pressure for initiated hepatocytes. Evidence exists to support the idea that, at the doses used, 2-acetylaminofluorene does not induce initiation in the liver of adult rats until after four to five weeks of treatment. Also, the 2-acetylaminofluorene treatment appears to have little effect on the probability of the second mutation (Cohen and Ellwein, 1990). Ideally, noncarcinogenic cytotoxic agents may be found which can serve as suitable replacements for 2-acetylaminofluorene and the other carcinogens used in these selection protocols (Solt, et al., 1977).

In any case, the strong selection drive provided by the Solt-Farber and similar experimental protocols greatly accelerates the appearance of altered hepatocyte foci and nodules from initiated cells against a background of essentially nonproliferating normal hepatocytes. The number of altered hepatocyte foci and nodules can be easily counted and, assuming that each altered hepatocyte focus and nodule is the product of the clonal expansion of a single initiated cell, used to estimate the frequency of mutation from normal cells to intermediate cells.

The number of altered hepatocyte foci and nodules can be counted in individual tissue sections and the number can be expressed as altered hepatocyte foci and nodules per square centimeter (Ghosal, et al., 1987; Ito, et al., 1988). However, this method can be inaccurate and misleading if used to estimate changes in the number of altered hepatocyte foci and nodules in the entire liver (Pitot, 1990). Alternatively, quantitative stereology and related technologies can be used to determine the number of altered hepatocyte foci and nodules per cubic centimeter or per total liver (Campbell, et al., 1982; Campbell, et al., 1986; Enzmann, et al., 1987; Pugh, et al., 1983). The stereologic methods, while considerably more labor intensive, typically provide much more reliable estimates of changes in the number of altered hepatocyte foci and nodules in the whole liver.

3.4.2 Frequency of Mutation of Intermediate Cells to Tumor Cells

The mutation frequency of intermediate cells to tumor cells is a rare event that is not directly detectable. However, this mutation frequency can be inferred from the number of tumors or persistent nodules that arise in a tissue. The number of tumors or persistent nodules reflects the number of mutations of intermediate cells to tumor cells, assuming that each tumor or persistent nodule is produced by clonal expansion of a single cell in which the second mutation of the two-stage model has taken place.

3.4.2.1 Frequency of Mutation Estimated from Number of Tumors

The number of tumors can be counted in bioassays to estimate the number of mutations that have occurred. This number can be used, in conjunction with intermediate cell birth rates (α_2), to calculate the mutation frequency (μ_2).

3.4.2.2 Frequency of Mutation Estimated from Number of Persistent Altered Foci and Nodules

While most foci and nodules developing during promotion "remodel" or disappear following removal of the exogenous promoter, some nodules persist (Emmelot and Scherer, 1980; Enomoto and Farber, 1982). It is reasonable to expect that a tumor cell produced within a population of intermediate cells, as the result of the second mutation of the two-stage model, will give rise to a persistent nodule which, in turn, can progress to a tumor (Emmelot and Scherer, 1980; Farber, 1984; Farber and Cameron, 1980; Pitot, 1990; Solt, et al., 1977). Thus, the number of persistent nodules could be used, in conjunction with intermediate cell birth rates (α_2), to calculate the mutation frequency (μ_2). Experimental protocols such as those discussed in section 3.4.1.3 may facilitate the detection of persistent nodules.

Splenic Transplantation of Cells from Altered Foci and Nodules to Assess Growth Ability. The cells of persistent nodules which retain the ability to grow and progress to tumor can be distinguished from the intermediate cells by observing cellular growth on splenic transplantation (Finkelstein, et al., 1983; Lee, et al., 1983; Miyazaki, et al., 1989). Cells isolated from each altered hepatocyte foci or nodules can be transplanted into the spleens of syngeneic animals to assess the

ability of the cells to grow and produce hepatocellular tumors. Generally, the cells of early "premodeled" remodelling nodules, like normal hepatocytes, typically grow slowly and diffusely in the spleens, and gradually replace the splenic "pulp" over many months (Farber, 1984; Finkelstein, et al., 1983; Lee, et al., 1983). In contrast, some cells of the persistent nodules grow relatively rapidly and grow as nodules in the spleen. In addition, many of the cells derived from the persistent nodules exhibit the appearance of cancerous hepatocytes after development in the spleen. The splenic-transplantation assay has proven to be sensitive to the enhanced growth ability of the persistent nodular cells examined. Presumably, the enhanced growth ability observed is conferred to these cells by the second mutation of the two-stage model.

Histochemical Approaches to Distinguishing Between Persistent and Remodelling Altered Foci and Nodules. Persistent nodules may also be distinguished from remodeling nodules by means of histochemical staining procedures under certain experimental conditions. For example, after carcinogen treatment and a recovery period, Enomoto and Farber (1982) conducted a selection procedure consisting of 2-acetylaminofluorene exposure combined with two-thirds partial hepatectomy. After removing the selection pressure, the glutamyl transpeptidase-positive hepatocyte nodules observed could be divided into those exhibiting completely uniform staining and those exhibiting irregular, patchy staining indicative of the partial disappearance of glutamyl transpeptidase.

In addition, Enomoto and Farber (1982) found that the difference in the gross appearance of the two types of nodules was obvious. Remodelling nodules, unlike persistent nodules, showed a progressive merging with the surrounding liver. Further, a clear-cut difference in proliferative activity was reported. The LI of the hepatocytes in the uniformly stained nodules was substantially larger than the LI of the hepatocytes in the nonuniformly stained nodules and in the surrounding liver. These observations taken together suggest that persistent altered hepatocyte foci and nodules may be readily distinguished from remodeling nodules by histochemical approaches.

However, as discussed by Enomoto and Farber (1982), it remains to be determined whether the failure to remodel, as manifested by uniform staining, was the result of a loss in growth control in the more or less autonomous hepatocytes of persistent nodules or the result of an unknown mitogenic stimulus in the liver. This question might be resolved by means of parallel histochemical and splenic-transplantation studies (Farber and Cameron, 1980).

It is also important to note that heterogeneity in histochemical markers is characteristic of altered hepatocyte foci, nodules, and tumors (Peraino, et al., 1986; Peraino, et al., 1984). No single marker invariably appears in all of these persistent proliferative lesions and tumors, and no single marker can be used to distinguish all of the small number of persistent proliferative lesions from the large number of remodelling lesions. In particular, glutamyl transpeptidase, the most prevalent marker in altered hepatocyte foci, is probably not the most prevalent marker in tumors. Thus, the variability of the altered phenotypes that may be encountered should be taken into account in the development of histochemical approaches to discriminate persistent altered hepatocyte foci and nodules from remodelling altered hepatocyte foci and nodules.

Further, many persistent nodules identified histochemically might not retain the ability to grow and progress to tumor (Emmelot and Scherer, 1980; Farber and Cameron, 1980). Until this question is resolved, the conservative tack for the purpose of risk assessment would be to assume that every persistent nodule will eventually produce a tumor.

Other Approaches for the Early Identification of Tumor Cells. It may also be possible to distinguish remodelling altered hepatocyte foci and nodules from persistent altered hepatocyte foci and nodules by studying alterations in the expression of oncogenes, proto-oncogenes, and tumor suppressor genes (Farber, 1984). For example, the p53 gene appears to be a tumor suppressor gene that loses its suppressing activity when mutated to the forms that have been found in numerous cancers. Accordingly, altered p53 may be characteristic of some types of persistent altered hepatocyte foci and nodules.

SECTION 4

EVALUATION: APPLICATION OF EXPERIMENTAL TECHNIQUES TO LIVER AND LUNG

Rotstein, et al. (1984) evaluated the cell cycle kinetics in regenerating rat liver and in carcinogen-induced rat liver nodules by the method of Quastler and Sherman (1959) as well as by means of a computer analysis of the fraction labelled mitoses curves. However, the very low mitotic activity characteristic of unstimulated normal hepatocytes (e.g., deFazio, et al., 1987) and the many types of lung cells in adults will probably preclude obtaining reliable division rate estimates for these cells solely by means of the fraction labelled mitoses method or the double-labelling approach described by Raza, et al. (1987). In addition, the pulmonary cells may be especially problematic because the cell-types of the lung are typically very difficult to identify and distinguish from each other.

Pulmonary epithelial cells proliferate at a relatively rapid rate that can be stimulated further by toxic injury. The proliferation of several cell types in the lung in response to oxidant insult has been measured in a number of studies (Evans and Shami, 1989). The LI has been used to estimate S, G_2, and M phase durations for alveolar macrophages and epithelial cells in pulmonary airways and alveoli.

In contrast, pulmonary endothelial and interstitial cells and other types of lung cells are refractory to quantitative kinetic study because these cell-types cannot be consistently and conclusively identified and discriminated from each other (Evans and Shami, 1989). Unperturbed neuroendothelial cells appear to divide at a very low rate (Sorokin and Hoyt, 1989). Mitotic neuroendothelial cells and ^{3}H-thymidine-labelled neuroendocrine cells are rarely observed in labelling studies. Thus, the background rates of cell birth have not proven to be measurable by current techniques.

Technical problems such as these may best be addressed by developing multiparameter analytical approaches that simultaneously or sequentially identify, and possibly sort, cells according to cell type and ploidy, whether or not they are in a proliferating mode, and their position in the cell cycle. These techniques may be combined with modifications of the fraction labelled mitoses or the double-labelling approaches to provide the data needed for the application of the two-stage model.

Estimating the frequencies of mutation (μ_1) of normal hepatocytes to intermediate cells and from intermediate cells to tumor cells (μ_2) may be facilitated in experimental animals by means of modified experimental protocols which selectively and intensively induce the mutated hepatocytes to proliferate and produce recognizable altered hepatocyte foci and nodules.

The mutation frequency (μ_1) of pulmonary epithelial cells may possibly be estimated by counting adenomas, if the stem cell-type from which adenomas arise can be definitively identified and characterized in terms of birth rate (α_1). Estimating the mutation frequency (μ_1) of neuroendocrine

cells would require the ability to identify neuroendocrine-derived intermediate cells and distinguish them from malignant cells. The estimation of the mutation frequencies of other types of lung cells is limited by the low level of cell mitotic activity and by the difnculty in identifying and distinguishing specific cell types from the other types of lung cells. Much more study is needed to provide the information required and the experimental approaches amenable to estimating mutation frequencies in lung cells.

SECTION 5

CONCLUSIONS

Interspecies differences in cellular dynamics must be defined to accurately and reliably estimate cancer risk in humans from animal studies. The cellular dynamics of carcinogenesis can be described by a conceptual two-stage model with model parameters that, in theory, can be represented by measurable cellular parameters. The six parameters of the model (birth rate of normal cells α_1 death rate of normal cells β_1 mutation frequency of normal cells to produce intermediate cells μ_1, birth rate of intermediate cells α_2, death rate of intermediate cells β_2, and mutation frequency of intermediate cells to produce tumor cells μ_2), together with estimates of the total number of normal cells in the tissue and the growth fraction, allow the two-stage model to describe the incidence of tumor cells over a given time interval. Accurate and reliable measurements of the relevant cellular parameters in experimental animals and humans remain to be determined.

The cell birth rates of normal and intermediate cells (α_1 and α_2, respectively) can be estimated by determining the cell-cycle times characteristic of normal and intermediate cells. In principle, multi-labelling approaches can be used in combination with flow cytometry, image cytometry, or fluorescence microscopy to discriminate specific types of normal cells and intermediate cells in a tissue or organ. The cell-cycle times could then be determined in populations of asynchronously proliferating cells by the fraction labelled mitoses or double-labelling approaches. The available information suggests that multilabelling approaches, combined with the fraction labelled mitoses or other methods described in the scientific literature, may provide the means to estimate cell-cycle times for normal and initiated cells of specific cell types even in mixed cell populations exhibiting heterogenous properties and characteristically very low mitotic activity. In particular, many markers (e.g., γ-glutamyl transpeptidase) have been described for distinguishing initiated hepatocytes from normal hepatocytes. Much less is known about initiated pulmonary cells.

The possible indices of cell death based on directly measurable parameters will not provide the temporal information required for the estimation of the rate of cell death. However, the rate of cell death may be determined indirectly from the rate of change in total cell number and the rate of cell birth. For example, $\beta = \alpha GF$ in tissue that is not growing and for which the growth fraction (GF) remains constant with time, as may be expected in many adult tissues. The relationships between β, α, GF, and the total number of cells become much more complex in growing tissue.

An assumption of the two-stage model is that the first mutation confers both a growth advantage and phenotypic alteration to the initiated cell, which are both expressed on exposure to a promoter, and the second mutation confers to the cell the potential to progress to tumor.

The frequency of the first mutation can be calculated from the number of intermediate cells that arise directly from normal cells over a given time interval together with the normal cell birth rate (α_1). The available information indicates that intermediate cells in the liver can be selectively induced to

proliferate to yield altered hepatocyte foci and nodules by means of experimental protocols described in the scientific literature. Since each hepatocyte foci and nodule is probably the product of the clonal expansion of a solitary intermediate cell that has arisen by mutation directly from normal hepatocytes, the number of altered foci and nodules may be a reasonable estimate of the number of times the first mutation of the two-stage model has occurred in the liver. The number of altered hepatocyte foci and nodules per cubic centimeter or per total liver can most accurately be determined by quantitative stereology and related methods. Pulmonary adenomas may be the products of the clonal expansion of intermediate cells and may, therefore, be analogous to altered hepatocyte foci and nodules in the liver. However, the stem cell-type from which adenomas arise must be adequately identified and characterized, and the relationship between adenomas and adenocarcinomas must be more definitively established before the number of adenomas can be assumed to represent the number of intermediate cells that have arisen from normal cells.

The mutation frequency of intermediate cells to tumor cells (μ_2) can be estimated from the intermediate cell birth rates (α_2) and number of tumors that arise in a tissue during a bioassay, assuming that each tumor is a clonal expansion of a single tumor cell in which the second mutation of the two-stage model has occurred. In the liver, the number of persistent altered hepatocyte foci and nodules containing cells exhibiting the growth ability characteristic of tumor cells may provide a more reliable and accurate estimate of the mutation frequency (μ_2).

In summary, this report identifies biological parameters that correspond to the mathematical parameters of the two-stage model of carcinogenesis. Experimental methods exist for the quantitation of these biological parameters. The quantitation of the parameters for liver is possible using the approaches described here. The application of the approaches to the quantitation of the parameters for other tissues and organs will most probably require further technological developments and biological advances. Experiments that quantitate these parameters and compare the tumor incidence predicted by the model with experimentally observed tumor incidence will test the value of the two-stage model for the extrapolation of bioassay data to humans in health risk assessment.

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APPENDIX A

RELATIONSHIPS BETWEEN THE MODEL PARAMETERS OF THE TWO-STAGE MODEL

The following derivations describe some of the relationships between the parameters of the two-stage model, given some simplifying assumptions.

If the cell population of a tissue is growing exponentially with a doubling time (cell-cycle time) of T_c, and all of the cells in the tissue are proliferating (GF = 1), then

$$n_{t1} = n_{t0}e^{0.693t/T_c}$$

where,

n_{t1} = number of proliferating cells at t_1
n_{t0} = number of proliferating cells at t_0
t = $t_1 - t_0$
T_c = doubling time

Since, on average, each cell will divide once every T_c,

$$1/T_c = \alpha$$

where α = number of cell divisions/proliferating cell/unit time.

Substituting,

$$n_t = n_{t0}e^{0.693t\alpha}$$

and solving for α,

$$\alpha = \ln(n_{t1}/n_{t0}) / 0.693t$$

In an adult tissue that is composed of one kind of cell, in which there is no net growth, and for which the growth fraction (GF) is constant over time, n, the number of proliferating cells in the tissue, remains constant and, αn = number of divisions/unit time.

If β = total number of deaths/total number of cells/unit time, then, where N = total number of cells (proliferating and nonproliferating), βN = total number of deaths/unit time. Since there is no net growth,

$$\alpha n = \beta N$$

and,

$\beta = \alpha n/N.$

Since GF = n/N,

$\beta = \alpha GF.$

Assuming that the number of intermediate cells that arise from normal cells between t_0 and t_1 is a random variable that has a Poisson distribution with expectation (Moolgavkar and Knudson, 1981):

$$\int_{t_0}^{t_1} m_1 X_n(t)dt$$

where,

m_1 = number of mutations/proliferating normal cell/unit time
$X_n(t)$ = number of proliferating normal cells at time = t

Then,

$$i = m_1 \int_{t_0}^{t_1} X_n(t)dt$$

where i = number of intermediate cells arising from normal cells between t_0 and t_1.

Solving for m_1,

$$m_1 = i/[\int_{t_0}^{t_1} X_n(t)dt]$$

Since,

$\mu_1 = m_1/\alpha_1$

where,

μ_1 = number of mutations/normal cell division
m_1 = number of mutations/proliferating normal cell/unit time
α_1 = number of divisions/proliferating normal cell/unit time

then,

$$\mu_1 = 1/[\alpha_1 \int_{t_0}^{t_1} X_n(t)dt]$$

Likewise,

$$\mu_2 = T_u/[\alpha_2 \int_{t_0}^{t_1} X_i(t)dt]$$

where,

μ_2 = number of mutations/intermediate cell division
T_u = number of tumor cells arising from intermediate cells between t_0 and t_1
α_2 = number of cell divisions/proliferating intermediate cell/unit time
$X_i(t)$ = number of proliferating intermediate cells at time = t

APPENDIX B

LEADING INVESTIGATORS/LABORATORIES

Duration of S Phase (t_S)

Fraction Labelled Mitoses

Rotstein, J., et al., 1984, "Cell Cycle Kinetics of Rat Hepatocytes in Early Putative Preneoplastic Lesions in Hepatocarcinogenesis," *Cancer Research*, Vol. 44, pp. 2913-2917. (Department of Pathology, University of Toronto, Toronto, Ontario, Canada M5S 1A8; Department of Mathematical Sciences, McMaster University, Hamilton, Ontario, Canada L8S 4J9; Institute of Pathology, University of Munich, Munich, Federal Republic of Germany.)

Double Labelling

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Labelling Index (LI)

Short-Term DNA-Precursor Labelling

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