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STOCHASTIC DESCRIPTION OF INITIATION AND PROMOTION IN EXPERIMENTAL CARCINOGENESIS

E.G. LUEBECK and S.H. MOOLGAVKAR

Fre ' Hutchinson Cancer Research Center, Public Health Sciences Division. Seattle, WA, USA

Summary. - *Hepatocarcinogens are known to give rise to enzyme altered foci (EAF) in the liver. Since EAF are regarded as intermediate on the pathway to cancer, the promoting effect of chemical compounds on their growth is of critical importance in the assessment of the cancer risk. Using a stochastic description based on the two-mutation model we outline a quantitative analysis of EAF in the rat liver.*

KEY WORDS: carcinogenesis, enzyme altered foci (EAF), initiation-promotion, two-mutation model.

Riassunto (Descrizione stocastica dei processi di iniziazione e promozione nella cancerogenesi sperimentale). - *E' noto che i cancerogeni epatici danno luogo nel fegato alla formazione di "foci" con enzimi alterati (EAF). L'effetto di promozione da parte di agenti chimici sulla crescita degli EAF è di importanza critica nella stima del rischio cancerogeno, in quanto si ritiene che gli EAF agiscano come intermediari nel processo che dà luogo al cancro. Utilizzando una descrizione stocastica basata sul modello "a due mutazioni", viene qui delineata un' analisi quantitativa degli EAF nel fegato del ratto.*

PAROLE CHIAVE: cancerogenesi, foci con enzimi alterati (EAF), iniziazione-promozione, modello "a due mutazioni".

Introduction

Among the many causal elements of carcinogenesis much attention is now given to answer questions related to the interaction of agents with the target tissue and its cellular dynamics. This interest builds mainly on the existence of histologically distinct cell clones which appear in early carcinogenesis in certain tissues (colon, liver, pancreas etc. [1-3]). It is now widely accepted that malignant tumors evolve from such intermediate (premalignant) clones [4-6]. Consequently, it is also of interest to quantitate the properties of initiation and cellular proliferation of intermediate cells since the outcome of malignant tumors will clearly depend on this.

The classical example for initiation and promotion (I-P protocol) is the mouse skin painting experiment. The mouse epidermis is first initiated with a single application of a chemical agent and thereafter treated with a promoter substance which causes benign tumors (papillomas) to appear while the reverse procedure does not yield the tumors. The tumors are believed to be made out of intermediate cells and subsequent promotion is believed to enhance their growth into visible benign lesions. The protocol suggests that carcinogens be categorized as either initiators or promoters or both depending on their ability to influence each one of these steps. The analysis of such observations is, so far however, lacking a quantitative measure of initiating and promoting potencies of the agent. We shall make an attempt toward defining such a measure.

Another experimental system, the one we shall be concerned with here, is the rat liver and the observation of hepatocarcinogenesis under various experimental conditions. After the animal is exposed to a carcinogen a number of enzyme-altered focal lesions appear in its liver. The animals are serially sacrificed at various time points after treatment has begun and sections are taken from their liver lobes. These sections are prepared into histologic slides to mark the specific phenotype of the primed (initiated) cells in these lesions. Common markers include those for ATPase (adenosine triphosphatase), G6Pase (glucose-6-phosphatase), GGTase (γ-glutamyl transpeptidase) activity in the cell. Often, serial sections are marked for multiple phenotypes. Observed, then, are the number and size of transections on a histologic slide per unit area at a given time. The quantitative analysis of all these data is therefore intrinsically statistical and best performed by maximizing the total likelihood as constructed from a realistic carcinogenesis model. For a detailed description of this procedure see ref. [7].

At present it is not known which phenotypes are necessary "doorway" states for the development of cancer and which ones are not. It is clear, however, that such alterations stand at the beginning of the hepatocarcinogenesis process defining a rate limiting genomic event which, in this context, is also identified with the notion of cellular initiation.

To be specific we will be looking at ATPase deficient focal lesions in rats which were exposed to various levels of NNM (N-nitrosomorpholine) in their drinking water. All animals either died of natural causes or were sacrificed at different time points determined by the experimental design (Table 1). Serial sections from their livers were taken and examined for enzyme-altered foci. We are grateful to Dr. Michael Schwarz from the German Cancer Center in Heidelberg, West Germany, for making these data available to us.

The model

In order to understand the implications of our conclusions a brief review of the basic model assumption is in order here. The two basic features of the model (Fig. 1) are:

Initiation and conversion. - Transition of normal target cells into cancer cells via an intermediate stage in two rate limiting, irreversible and hereditary (at the level of the cell) steps. Intermediate cells are assumed to be generated from normal cells as a non-homogeneous Poisson process with intensity $\nu(s)X(s)$, where $\nu(s)$ can be thought of as the rate of initiation per cell per unit time and $X(s)$ in the number of normal cells at risk at time s. The second step is the conversion of intermediate cells into malignant cells with rate $\mu(s)$ per cell and unit-time. Precisely, an intermediate cell divides into one intermediate cell and one malignant cell with rate $\mu(s)$.

Table 1. - *Details of NNM experimental*

Dose (ppm)	Number of animals	Number of transections	Range of treatment times (in days)
0.0	36	84	112-686
0.1	30	114	225-686
1.0	23	123	217-616
5.0	17	217	210-441
10.0	18	217	204-364
20.0	19	345	196-399
40.0	19	554	84-217
Total	162	1654	

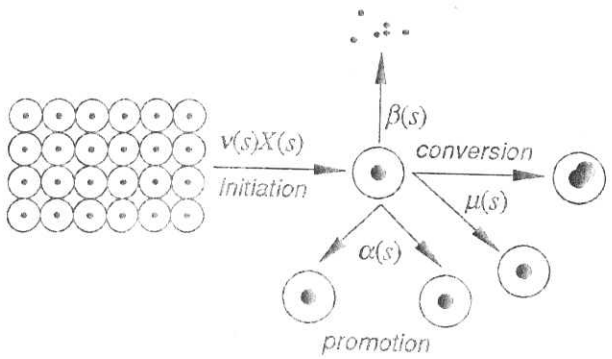

Fig. 1. - Graphical representation of two-mutation model.

Promotion. - Growth (clonal expansion) of intermediate cells as a stochastic birth-death process with cell division rate $\alpha(s)$ and death (differentiation) rate $\beta(s)$. An agent is a *promoter* if it increases the net growth rate (α-β) and an *anti-promoter* if it decreases it.

This is basically a generalization of the recessive oncogenesis model by Knudson [8] and has been shown to be consistent with a large body of experimental and epidemiologic data [9].

Under the assumption of constant proliferative (birth-death) parameters α and β, like in situations of chronic exposure to carcinogens, the quantitative analysis of hepatocarcinogenesis experiments can actually be based on a few simple formulas. More complex patterns of dosing, including the use of a partial hepatectomy to give a mitotic stimulus for enhancing the effect of an initiator in acute exposures (Pitot protocol) can also be expressed properly in this framework by straightforward extensions [10-12]. Following mathematical expressions ($\nu' = \nu X$) are at the core of our analysis:

Mean number of intermediate cells per cm³

$$I(t) \simeq \int_0^t \nu' \exp[(\alpha - \beta)(t - s)]ds$$

and volume fraction $f_v = I/X$

Number of nonextinct foci per cm³

$$\Lambda(t) = \int_0^t \nu' [1 - p_0(t, s)]ds$$

where $p_0(t,s)$ is the probability of extinction of a clone by time t which was generated at time s and is given by

$$p_0(t, s) = \frac{\beta - \beta \exp[-(\alpha - \beta)(t - s)]}{\alpha - \beta \exp[-(\alpha - \beta)(t - s)]}$$

Asymptotically, $p_0(t,) \rightarrow \beta/\alpha$ as $t \rightarrow \infty$.
Note, neither $I(t)$ nor $\Lambda(t)$ are directly observable. However, Wicksell [13] showed that $f_v = f_A$ with f_A being the ratio of all transectional areas to the total area of the slide. Also, it follows from Wicksell's transformation that the number of observed transections per unit area, say n_A, can actually be expressed by Λ and mean foci diameter r.

$$n_A = 2r\Lambda$$

The probability of finding a non-extinct clone consisting of m cells at time t

$$p_m(t) = \frac{\nu'}{\alpha\Lambda}\frac{\left(\frac{\alpha}{\beta}p_0(t,0)\right)^m}{m}$$

Note, if ν' is assumed to be constant, ν' pulls out from under the integral in the expression for $\Lambda(t)$ so that p_m no longer depends on ν'.

To go from a discrete to a continous (3-D radial) size distribution g_3:

$$p_m = \overset{m=(r/r_c)^3}{\longrightarrow} g_3(r)$$

where m is the ratio of focus to cell volume.

Further, to obtain a 2-D radial size distribution from the 3-D one, we use the Wicksell transformation [13]:

$$g_3(r) \overset{\text{Wicksell transf.}}{\longrightarrow} g_2(x) = \frac{x}{r}\int_x^\infty \frac{g_3(r)}{\sqrt{r^2-x^2}}\,dr$$

It is worthwhile to pause and reflect on these expressions as they bear some simple features and consequences for the analysis of data on the growth of such intermediate clones. These considerations should be viewed in conjunction with previous attempts to analyse such data using only summary information on the volume fraction f_V, mean foci size [14] and categorized size distributions which were laboriously reconstructed using stereological methods [15, 16]. These complications are essentialy avoided here because of our 2-D likelihood construction using Wicksell's transformation. In the framework of the two-mutation model the following points can now be made:

- from the volume fraction alone one cannot determine whether an agent is an initiator (acting through ν) or a promoter (or anti-promoter) increasing (or decreasing) $(\alpha\text{-}\beta)$. Recall, $(\alpha\text{-}\beta)^{-1}$ is roughly the doubling time for the intermediate cell number.

- the number of *observed* intermediate foci depends upon both the rate of initiation as well as the growth kinetics of the initiated cells. Not only are there small unobservable foci in the tissue of interest but many of them may simply not survive if cell death is prevalent. As a matter of fact, in rapidly dividing cell populations cell death (apoptosis) or differentiation must almost balance the number of newly created cells to otherwise avoid an explosive growth of focal tissue ($\beta/\alpha \sim 1$). So even when $(\alpha\text{-}\beta)$ does not change with dose a large death rate β (and therefore a larger birth rate α will lead to a small number of large foci while for lower β the converse will happen: one will find a large number of small foci (see the 4-DAB/NDEOL experiment by Schwarz *et al.* [17]). Consequently, one cannot infer from a large number of observed foci alone that an agent is an initiator;

- when an agent affects the size distribution of intermediate foci then it must have promoting (or anti-promoting) activity.

It is sometimes argued that only non-extinct foci need to be considered in assessing the cancer risk since malignant transformation can apparently only proceed from those alive and one need not to worry about foci lost along the path toward malignancy. However, at any given time, especially when the initiation and death rates of intermediate cells are high, one may have a large number of small clones many of which, of course, would become extinct in the future, but some of which could undergo malignant conversion prior to extinction. It is therefore important to consider the complete "stochastic history" of all the generated intermediate clones.

The example

We briefly review the data obtained from the NNM experiment of M. Schwarz *et al.* [18, 19, 7] and their analysis. In short: a total of 173 female Lewis rats,14 weeks of age, were subjected to different concentrations of NNM in their drinking water (0, 0.1, 1, 5, 10, 20, 40, 80 ppm). The animals were sacrificed at various ages and their livers examined for ATPase deficient foci. In particular, measurements on the number of observed two dimensional transections per square cm and their radii as functions of treatment time (in days) and dose (in ppm) were obtained. Since the smallest transections are more likely to be missed we have restricted our analysis to transections larger than 60 μm in radius. Also a few very large transections (> 500 μm) were dropped from the analysis. The (altered) cell radius, r_c, is approximately 12 μm [20] and the number of normal cells per cm^3, X, is assumed to be constant. According to the experimenters animals in the highest dose group (80 ppm) showed signs of toxicity and were not included in the analysis. Further, a preliminary dose by dose analysis showed an almost linear dose response in $(\alpha\text{-}\beta)$ while the parameters $\beta/\alpha \sim 0.99$ for most dose groups. This suggests an analysis with one constant parameter $\gamma = \beta/\alpha$, common to all dose groups. This choice of analysis is further motivated by the fact that individual estimates of α and β are not very robust. For γ near 1 small differences in γ are translated into large changes in α and β. It is therefore useful to estimate the parameter gamma on the basis of all available data. As a result the parameter estimates for this analysis are generally tighter than corresponding ones from the preliminary (dose by dose) analysis. The numerical results of our analysis are shown in Table 2 and graphically depicted in Figs 2-4. More details on the data analysis, likelihood construction and maximization can be found in ref. [7].

Fig. 2 shows the rate of initiation per cubic centimeter of liver and the net growth rate of ATPase deficient foci $(\alpha\text{-}\beta)$ as functions of the dose of NNM. The best fitting regression lines through the points are also shown. Based on the linearity of the dose response a simple definition of initiation and promotion potencies can immediately be given by the ratio of the slope and intercept of the corresponding regression lines. Using the maximum likelihood lines we find that

$$\nu X(d) = 5.9 + 10.93\ d$$
$$\rightarrow \text{initiation potency} = 1.85\ \textit{per ppm}$$

and similarly for the "replicative" parameter $(\alpha\text{-}\beta)$

$$(\alpha\text{-}\beta)\ (\text{d}) = 0.009 + 0.00031\ \text{d}$$
$$\rightarrow \text{promotion potency} = 0.034\ \textit{per ppm}$$

From this result we would conclude that NNM is rather a strong initiator but a week promoter. This does not mean, however, that the promotional effect of NNM is unimportant since intermediate cell clones grow exponentially under the model while initiation occurs only linearly (see the expression for $I(t)$. So, sooner or later, the promotional effects will dominate the tumor risk.

The value of $(\alpha\text{-}\beta)$ ranges from approximately 0.0054 per cell per day in the control group to 0.021 per cell per day in the 40 ppm dose group. These values correspond to doubling times of approximately 128 and 33 days, respectively. Of particular biological interest is the rate of cellular division α. Our estimates range from 0.5 per day per cell, corresponding to an average cell cycle time of about 2 days, in the lowest dose group, to about 2 per cell per day, corresponding to an average cell cycle time of about 12 h. It seems that only fetal cells in mammals could cycle this fast, indicating that the data here cannot be used to reliably estimate α. Furthermore, the assumption of an exponential waiting time distribution for rapidly cycling cells is only a

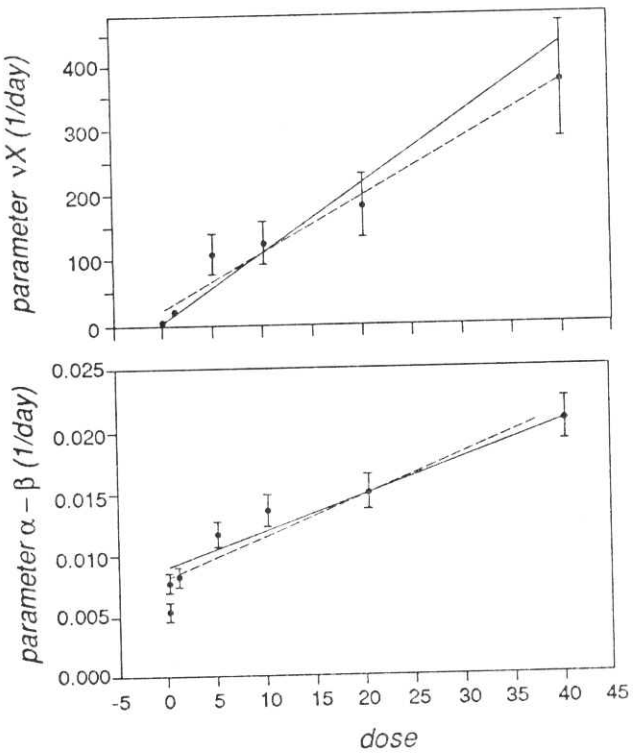

Fig. 2. - Parameter estimates (and 95% confidence intervals) plotted against dose of NNM in parts per million. The parameter νX estimates the number of altered cells generated per day per cm³ of liver. The parameter $(\alpha\text{-}\beta)$ is the net rate of growth of altered cells. The solid lines are maximum likelihood lines, the dashed lines are least square regression lines through the estimates.

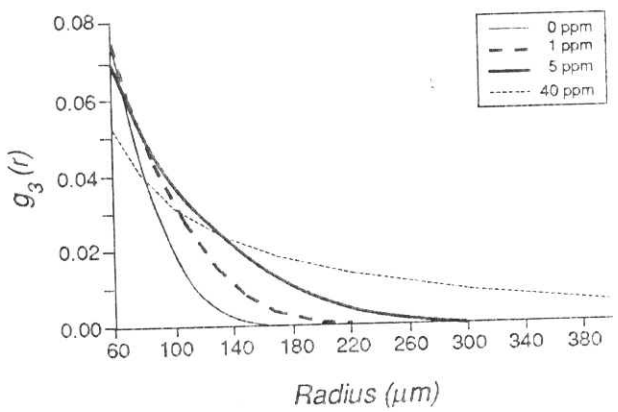

Fig. 3. - The 3-D density function (truncated at 60 μm) of the distribution of radii of foci after 300 days of treatment.

Table 2. - *Parameter estimates and their standard errors (SE). Standard error are computed from the observed information matrix*

Dose group	Parameter	Estimate	SE
0.0	$\alpha\text{-}\beta$	0.0054	0.367×10^{-3}
	ν	10.41	1.57
0.1	$\alpha\text{-}\beta$	0.0077	0.384×10^{-3}
	ν	7.88	1.25
1.0	$\alpha\text{-}\beta$	0.0082	0.399×10^{-3}
	ν	20.91	3.19
5.0	$\alpha\text{-}\beta$	0.0115	0.550×10^{-3}
	ν	110.6	15.3
10.0	$\alpha\text{-}\beta$	0.0136	0.656×10^{-3}
	ν	127.6	17.1
20.0	$\alpha\text{-}\beta$	0.0153	0.730×10^{-3}
	ν	188.0	25.3
40.0	$\alpha\text{-}\beta$	0.0213	0.893×10^{-3}
	ν	384.2	45.9
All	α/β	0.99	0.128×10^{-3}

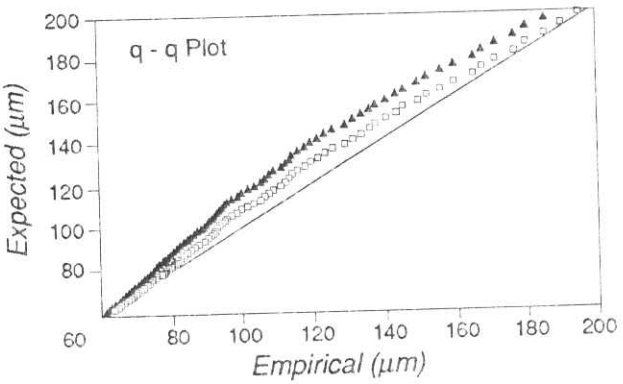

Fig. 4. - Q-Q plot of expected 2-D transectional distributions of radii versus the observed distribution for transection radii 60 μm < r < 500 μm and doses 0 to 20 ppm (▲ esponential model, □ Gompertz model).

crude approximation. It would be helpful to have additional experimental information on cell cycle times, death or differentiation rates as well as physiological constraints derived from the various phases of the cell cycle for the tissue(s) of interest.

Fig. 3 shows the density function for the distribution of sizes of foci in 3-D with radii larger 60 μm at time 300 days after the start of the experiment. Note the shift to larger sizes with increasing dose (proliferative effect).

Finally, we undertook a first step in assessing the possibility of non-exponential time dependent growth of intermediate cell clones. The assumption of clones of independently cycling cells is most likely too idealistic. By allowing for an age (age of the clone) dependent stochastic expansion of initiated cells we hope to take into account collective effects which possibly stem from unorderly gap junction activities [21] or other cell to cell communication failures. This may either lead to a slow-down of the clonal expansion, possibly due to effects of crowding and/or cytotoxic responses, or lead to accelerated growth, e.g. through selective processes and environmental adaption. Our approach was to let $(\alpha-\beta)$ become a simple exponential function of time (to be precise: a function of the age of the clone)

$$(\alpha-\beta) = b \exp(- at) \text{ where } -\infty < a < +\infty$$

and to keep $\gamma = \beta/\alpha$ constant. This introduces one additional parameter into our analysis, say the "shape" parameter α. It can be shown [22] that this will actually lead to Gompertz growth of the mean clone size. In other words, if $N(t-t_0)$ is a random variable for the number of intermediate cells in a given clone which was first initiated at time t_0 then the mean size of this clone becomes

$$E[N(t-t_0)] = \exp\left[\frac{b}{a}(1 - \exp(-a(t-t_0)))\right]$$

Using the same maximum likelihood procedure as before (excluding dose group 40 ppm which did show a slight slowing of clonal growth, possibly due to increasing toxicity), we find that the intermediate cell clones prefer to grow super-exponentially (log-likelihood ratio test is 25 on 1 degree of freedom). We estimated $a = -(0.32 \pm 0.09) \times 10^{-2}$.

To visualize this improvement percentiles of the expected 2-D size distribution of transection radii, one from the exponential model, the other from the Gompertz growth model, are plotted versus the empirical distribution of all transections from dose groups 0 through 20 ppm in the "window" 60 μm < r < 500 μm. This, so called Q-Q plot [23], is shown in Fig. 4 and is considered a fairly sensitive test for the equality of two given distibutions. Indeed, were the two distributions identical the Q-Q plot would have to lie on the 45 degree line. From Fig. 4, we also see that both models systematically underestimate the number of smaller transections, and consequently, the number of smaller foci. However, it is also clear that the more general model is a substantial improvement over the exponential mean growth model.

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