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Alternative Hypotheses for the Role of Promotion in Chemical Carcinogenesis

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A new protocol for carcinogenesis in rat liver is described in order that confirmatory experiments might be undertaken concurrently. The basic protocol, designated IPI (initiator + promoter + initiator), is presented in several alternative forms, including the possible use of X-irradiation as the initiator. The rationale is discussed in terms of the two-hit somatic mutation theory of Armitage and Doll, with an initial hit produced by the first dose of initiator and expansion of single cells to sizable clones by promotion thereby increasing the probability of a second hit by the second dose of initiator. The question of relevant mutations was taken up and it was proposed that genes for chalones (C) and for chalone receptors (R) are logical targets for consideration in a two-mutation sequence. Alternative hypotheses pertaining to promoter action were described in terms of possible mechanisms by which nonelectrophilic promoters might simulate a second mutation by increasing or decreasing the levels of a nonchromosomal replicating particle in target cells.

I believe it is worthwhile to discuss rationally derived hypotheses, concepts and beliefs in connection with the cancer problem because I agree with Peyton Rous, who once said, "Beliefs are important, because what men believe determines what men do." I believe that the initiation-promotion model of carcinogenesis is the key to the understanding of the nature of cancer.

When making hypotheses we should endeavor to construct alternative hypotheses and the means to test them (*1*). I offer two alternative hypotheses for the action of the classical promoters, both of which are based on the assumption of a two-hit or multi-hit mutational process.

Promotion as Hyperplasia Due to Blocked Intercellular Communication

Recently it was proposed that the key to the understanding of carcinogenesis in terms of initiation and promotion is the assumption that more than one relevant mutation is required for the production of a promotion-independent cell (*2,3*). It was suggested on the basis of experiments with rat liver that large numbers of stage I single mutant promotion-dependent cells could be produced by a single injection of a low dose of an initiator or a subcarcinogenic dose of a complete carcinogen. These "initiated" stage I cells were assumed to be held in a nonproliferative state by inhibitors (chalones) produced by surrounding adult hepatocytes (*2*).

Administration of a promoter of liver carcinogenesis, e.g., phenobarbital as demonstrated by Peraino et al. (*4*), was assumed to block "intercellular communication" as shown for the phorbol ester promoters by Yotti, Chang and Trosko (*5*) and by Murray and Fitzgerald (*6*) and later confirmed by Newbold and Amos (*7*). Williams (*8*) reported that phenobarbital affected communication between liver cells. Umeda et al. (*9*) found no effect with Chinese hamster cells [but see Trosko et al. (*10*)]. That phenobarbital expands the population of altered cells in rat liver has been established by Pitot et al. (*11*).

Progression through time in the absence of a second dose of initiator was assumed to occur by spontaneous or uncontrolled mutations, with the probability of a second relevant mutation to promoter independence increasing tremendously as the population of single mutant promotion-dependent cells increased under the influence of a promoter (*2,3*).

The term "conversion" was used to represent the experimental production in liver of a second step in the initiation process by a second dose of initiator acting on one or more cells in the expanded popula-

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presence and activity of the transforming growth factors (TGF's) as suggested below.

Promotion as the Dilution or Amplification of a Nonchromosomal Genetic Element

The two-hit hypothesis of carcinogenesis has been widely assumed to imply two mutations in terms of altered DNA structure, and the "simplest hypothesis" (2) for the role of promoters assumes that their effect is achieved by expanding the populations of one-hit cells thereby making a second hit (alteration in DNA structure) more probable, as outlined in Figures 2 and 3. This assumption is fortified by the knowledge that in general the initiators and "complete" carcinogens are electrophiles that alter DNA structure, while the typical tumor promoters are not electrophiles and do not form adducts with DNA moieties (47). In the opening section, promotion as hyperplasia due to blocked intercellular communication (i.e., without alteration in DNA structure) was presented. Without discarding that hypothesis, we must now consider the alternatives by which a nonelectrophilic promoter could act to produce the effect of a second mutation without acting to alter DNA structure.

Two alternative hypotheses must be considered. They are not mutually exclusive nor are they incompatible with the simplest hypothesis. The first is based on recent experiments and proposals by Varshavsky (48,49) in which the powerful skin tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) was shown to dramatically increase the incidence of methotrexate-resistant 3T6 mouse cells in culture (49). It was proposed that the result was due to the production by TPA of "locus-unspecific" extra rounds of DNA replication in many different chromosomal domains, of which the production of greatly increased gene copies for dihydrofolate re-

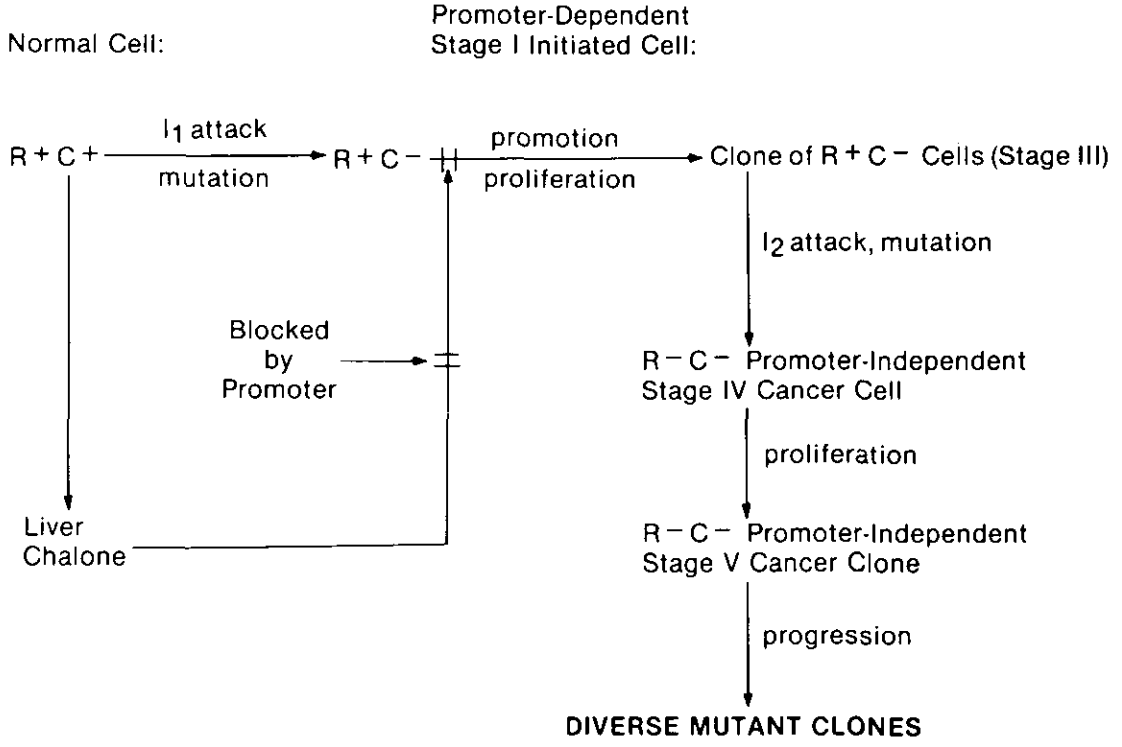

FIGURE 2. The IPI protocol with a hypothesis as to the initiator and promoter functions that would fit the fact of their synergistic action. The symbols $R^{+}C^{+}$, $R^{+}C^{-}$, and $R^{-}C^{-}$ represent the normal genome, the single allelic mutant for the chalone, and the mutant for both the chalone receptor (R) and the liver chalone (C). The liver chalone is defined as a product of adult hepatocytes that inhibits the proliferation of mature or immature hepatocytes. The general hypothesis is that "Cancer results from two or more relevant mutations; promoters enhance proliferation of cells with one relevant mutation, thereby increasing the probability of obtaining a cell with two relevant mutations" (2). The specific hypothesis is that mutations in R and C are relevant, here proposed for the first time. I_1 indicates a single injection of a compound that can be classified as an initiator and that produces structural changes in DNA. I_2 indicates a second treatment by the same or another initiator, also as a single injection. Experiments using radiation in two doses separated by an interval of promotion might be ideal.

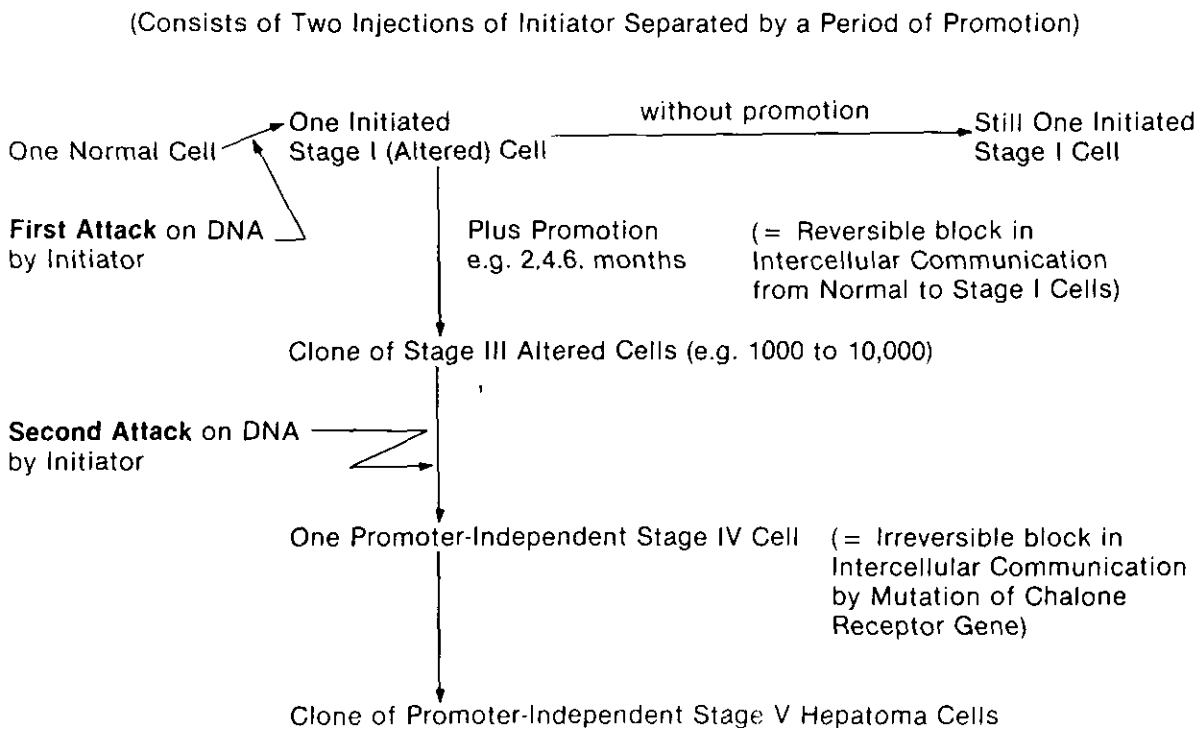

FIGURE 3. A new protocol for chemical carcinogenesis in rat liver. The protocol will be referred to as the IPI since it consists of two injections of an initiator separated by a period of promotion (2). The stages are as described earlier (2). Stage II is omitted here, since operationally stage II is the point at which promoter is discontinued to determine how many clones are at stage III.

ductase (DHFR) was simply a convenient model. The ability to prevent inappropriate DNA replication leading to the generation of "extrachromosomal copies of cellular genes" (*49*) was assumed to be under metabolic control, in other words it might be said to be controlled by intercellular communication. The "constitutive" production of growth factors by tumors (*38,50*) might, according to the Varshavsky model, be brought about by inappropriate gene amplification in the presence of a promoter, or by a "second mutation" needed for "promoter independence" as in Figures 2 and 3.

A second hypothesis for the production of a relevant "second mutation" by a nonelectrophile tumor promoter resembles the Varshavsky model insofar as it assumes the existence of extrachromosomal copies of genetic particles. It was pointed out in 1950 (*51*) that if such elements exist, their total loss (a "second mutation") could be brought about by a speeding up of cell proliferation relative to particle proliferation or by a slowing of particle proliferation relative to cell proliferation. The latter could be the extra property of promoters in addition to the production of hyperplasia. The 1950 proposal was based on a model system studied by Sonneborn and by his student Preer. I have used tables by Preer (*52*) to construct a chart (Fig. 4) showing the total loss of a nonchromosomal replicative particle under conditions that permit cell replication to exceed particle replication assuming a random (Poisson) distribution of particles between two daughter cells. If a lost particle were the source of control for growth factor production, the latter would become constitutive (*38,50*), and the effect would be that of a second mutation.

The Blocked Ontogeny Hypothesis and the Morris Hepatomas

In an earlier report (*53*) it was suggested that the TGFs are fundamentally the product of embryonic cells or stem cells, in any case cells that actively proliferate, while chalones are the products of nonproliferative cells that are either terminally differentiated or near that stage. It was further proposed on the basis of numerous proteins present as so-called fetal and adult forms of homologous proteins such as γ-globin (fetal) and β-globin (adult) that the production of chalones might somehow not only suppress proliferation but suppress the production of TGF's as suggested above.

Thus a concept of hepatocyte maturation can be presented as in Figure 5 in which the hepatocyte genotype is R^+C^+ but the two genes are unex-

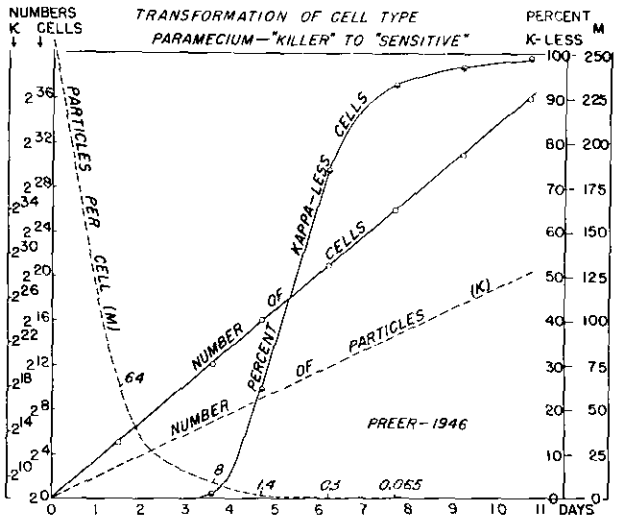

FIGURE 4. An experimental model of the absolute loss of a *replicating particle from cells that can divide relatively* faster than the particles are able to divide. The model is an *alternative hypothesis for a possible "second mutation"* by a nonelectrophilic tumor promoter that could slow the replication of a nonchromosomal replicative particle. The experiments were carried out by Preer (*52*), whose data were used to prepare the above chart. See also Potter et al. (*51*). The organism was *Paramecium aurelia* (Variety 2). Preer estimated the starting number of "killer" particles (K. kappa) at 256 (M) and the growth rate as logarithmic at 1.9 times per day for particles and 3.3 times per day for the cells. Distribution of particles in dividing cells was assumed to be random and was calculated according to the Poisson equation $P_0 = e^{-m}$, where P_0 is the number of cells with no particles, and m is the mean number of particles per cell. In the chart, the dashed lines were calculated. The identity and function of the Kappa particles is not relevant to the model as such.

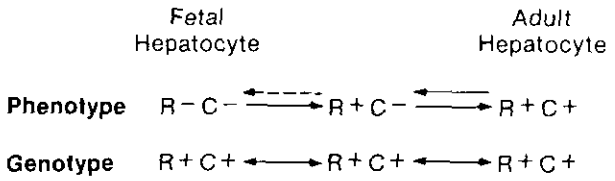

FIGURE 5. The developmental stages of normal hepatocytes. Symbols as in Fig. 2. There are many differences between fetal hepatocytes and adult hepatocytes and it is here assumed that the former do not express either the R or the C gene, while the latter express both. It is also assumed that at least one intermediate stage occurs and that the R^+C^+ phenotype can retrodifferentiate to the earlier forms during liver regeneration, and then undergo "reontogeny."

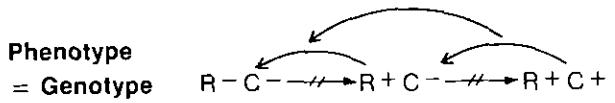

FIGURE 6. *Blocked ontogeny in Morris hepatomas.* Symbols as in Fig. 2. The curved arrows represent mutational *alterations in either or both the R and C genes. Depending* on the mutations there could be blocked or partially *blocked ontogeny (53) at either the* R^-C^- *or the* R^+C^- *stage.*

pressed in the fetal hepatocyte, which is accordingly R^-C^- in phenotype. Unstated in Figure 5 is the strong possibility that the fetal hepatocyte is GF^+ in phenotype as suggested earlier (*53*). It is further proposed that the phenotype changes to R^+C^- and finally to R^+C^+ as maturation proceeds, and that in the case of liver these phenotypic changes are reversible in order to facilitate liver regeneration after partial hepatectomy.

Finally, in Figure 6 it is proposed that the spectrum of Morris hepatomas includes some that are R^+C^- and some that are R^-C^-. The former would require a single mutation and the latter would require two mutations. The R^+C^- type would be highly differentiated, subject to some degree of control by the host, and have receptors responsive to chalones (*34*) and as another example, to glucagon, which we have shown (*54*). The R^-C^- type would be poorly differentiated, rapidly growing, and unresponsive to the liver chalone (*29,30*).

The designations R^+ and R^- in Figures 1-5 represent in the simplest case cells that are either positive or negative but it must be anticipated that there will be a spectrum of affinity constants that will include receptors that are not R^- but that have a weak affinity for the normal chalone. Moreover, the production of chalone will not be a constant but may even vary on a diurnal basis as is the case for the hepatic stimulatory substance (*18*).

Conclusion

As stated earlier, the advocacy of the IPI protocol is not dependent upon the concepts described. What needs to be emphasized is the benefits that will accrue from the cultivation of normal and transformed hepatocytes in serum-free culture in the presence of known concentrations of pure growth factors and chalones. Today the amino acid sequence is known only for EGF (*55*), but the need is for the sequences of all the peptide growth and transforming factors and for the peptide chalones in order to detect homologies, which must form the basis of interaction and competition (*53*). With serum-free cultures (*39*) and pure GFs and chalones (*27*), the receptors and their mechanism of action (*37,46*) can be studied. These studies at the molecular level are needed to finally understand the role of initiation and promotion in carcinogenesis.

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