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ALTERED HEPATIC FOCI: Their Role in Murine Hepatocarcinogenesis

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INTRODUCTION

The recognition that the appearance of malignant disease in an organism is a reflection of a number of convergent processes, usually described as stages, has become one of the foremost areas of knowledge in the field of oncology. Notwithstanding the substantial epidemiologic and experimental evidence of extended latent periods between exposure to carcinogenic agents and the appearance of cancer, it was from the early studies of Rous and Kidd (1), Mottram (2), and Berenblum & Shubik (3) on the development of the two-stage process of epidermal carcinogenesis in the mouse that such descriptive information was placed on a firm experimental basis. However, because epidermal neoplasms were seldom induced by dietary or parenteral administration of putative carcinogenic agents, it was not until the multistage nature of carcinogenesis in other tissues was described that the impact of the natural history of carcinogenesis became a significant factor in both basic and applied cancer research. Of the extra-epidermal tissues studied, multistage carcinogenesis of the liver in both the mouse and the rat has become the best known.

Prominent among early investigations of experimental carcinogenesis of the liver were those of Farber (4), Goldfarb & Zak (5), and Gössner &

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Friedrich-Freksa (6). In the former two laboratories (4, 5), the importance of lesions known as "regenerating nodules" in the pathogenesis of hepatocellular carcinoma was proposed. The latter investigators (6), on the other hand, described focal cellular alterations in hepatocytes characterized by a deficiency in the histochemical staining of the enzyme glucose-6-phosphatase. These investigators, particularly in a later study (7), suggested that these focal lesions or "islands" played a role in the development of carcinoma or in some instances were themselves the direct precursors of malignant hepatic lesions. Similar focal lesions identified by hematoxylin and eosin (H & E) stains were later described both in rats (8) and in mice (9), although in the latter instance there was no distinction between benign neoplasia, preneoplasia, and hyperplasia.

After these earlier studies, other histologic and histochemical characteristics of such islands, or altered hepatic foci (AHF) as referred to in this text, were described. Hori (10) and later Kitagawa (11) described several histochemical alterations in such focal lesions in livers of rats administered 3'-methyl-4-dimethylaminoazobenzene (3'-MDAB) or 2-acetylaminofluorene (AAF), respectively, in the diet. Later, Bannasch and his associates (12, 13) described the accumulation of glycogen or "glycogenosis" in focal lesions of livers of rats administered several hepatocarcinogens, including thioacetamide and nitrosomorpholine. Prominent among these earlier studies were developments resulting from the observation by Fiala et al (14) of an increased expression of the enzyme γ-glutamyl transpeptidase in both mouse and rat hepatomas. Subsequently, Kalengayi et al (15) demonstrated the presence of γ-glutamyl transpeptidase (GGT) activity in focal lesions of the livers of rats administered aflatoxin B_1, and other studies (16–18) confirmed such investigations in both early and late lesions of hepatocarcinogenesis in the rat. Together, these investigations set the stage for the extensive studies that have followed, emphasizing the importance of histologic, histochemical, and immunohistochemical studies of early focal lesions in the development of neoplasia in murine liver.

Morphology, Classification, and Phenotypes of Altered Hepatic Foci

As indicated above, some of the histologic and histochemical characteristics of AHF were described more than two decades ago, but since then many studies have characterized AHF both with respect to their morphology and their phenotypes as designated by histochemistry and immunohistochemistry. The reader is referred to several reviews for a more extensive discussion of this subject (19–21).

MORPHOLOGY Morphologic investigations of AHF with the light microscope and standard histologic stains (e.g. H & E) have been extensive during

the past two decades and especially in the last few years. The morphology of AHF under the light microscope in rat liver has been studied somewhat more extensively than comparable lesions in other rodent species. The first consensus on the nomenclature of such lesions was reported in a 1975 workshop (22), when the terms "foci of cellular alteration" and "neoplastic nodule" were used to denote lesions designated as AHF in this review and as hepatocellular hyperplasia or hepatocellular adenoma in other publications (23). In 1980 (24), a panel of pathologists published a monograph depicting in color the morphology of such lesions. A workshop held in Japan in 1985 (25) came to similar conclusions and specified the nomenclature for these lesions.

The detailed histomorphology of AHF has been studied extensively by Bannasch and his coworkers (13, 26). His classification of AHF, which extends that of earlier proposals (22, 25), is descriptive and includes clear-cell, acidophilic-cell, intermediate-cell, tigroid-cell, basophilic-cell, and mixed-cell AHF (26). Although most of these lesions were induced by carcinogenic nitrosamines, several studies have also investigated the morphology of spontaneous, or background, AHF. Ward (27) found AHF in livers of 2-year-old Fischer 344 rats used as untreated controls in carcinogen bioassays. The morphology of these lesions was quite similar to those of earlier reports. Recently Harada et al (28) have undertaken a careful quantitative evaluation of such spontaneous AHF in the same strain of animals. In their quantitative investigations they demonstrated that most of these AHF were of the basophilic- and clear-cell variety, the former predominantly in females and the latter predominant as spontaneous lesions in males. In a related study (29), these investigators demonstrated that the morphologic characteristics of chemically induced AHF were significantly different from those seen in spontaneous AHF.

Ultrastructural studies of cells within AHF have been relatively infrequent. Hirota & Williams (30), using the characteristic of iron exclusion to localize cells within AHF under the electron microscope, revealed some cells with few morphologic abnormalities and others with increases in glycogen, smooth endoplasmic reticulum, altered Golgi complexes, and abnormal bile canaliculi. Basophilic foci showed numerous free polyribosomes and parallel arrays of rough endoplasmic reticulum. Similar findings had been reported earlier by Merkow et al (31) in studies of hyperplastic (neoplastic) liver nodules that developed in rats fed 2-AAF.

The histomorphology of comparable lesions in mouse liver has evolved over the last two decades from the original study by Walker et al (9) that classified liver tumors in mice as to types (*a*) and (*b*), the latter being more aggressive and anaplastic. Frith & Ward (32) have used these terms to designate hepatocellular adenomas and carcinomas, respectively, as the preferred terminology. A later publication (33) proposed a classification of AHF and neoplasms of the liver in mice somewhat similar to that in the rat. Becker

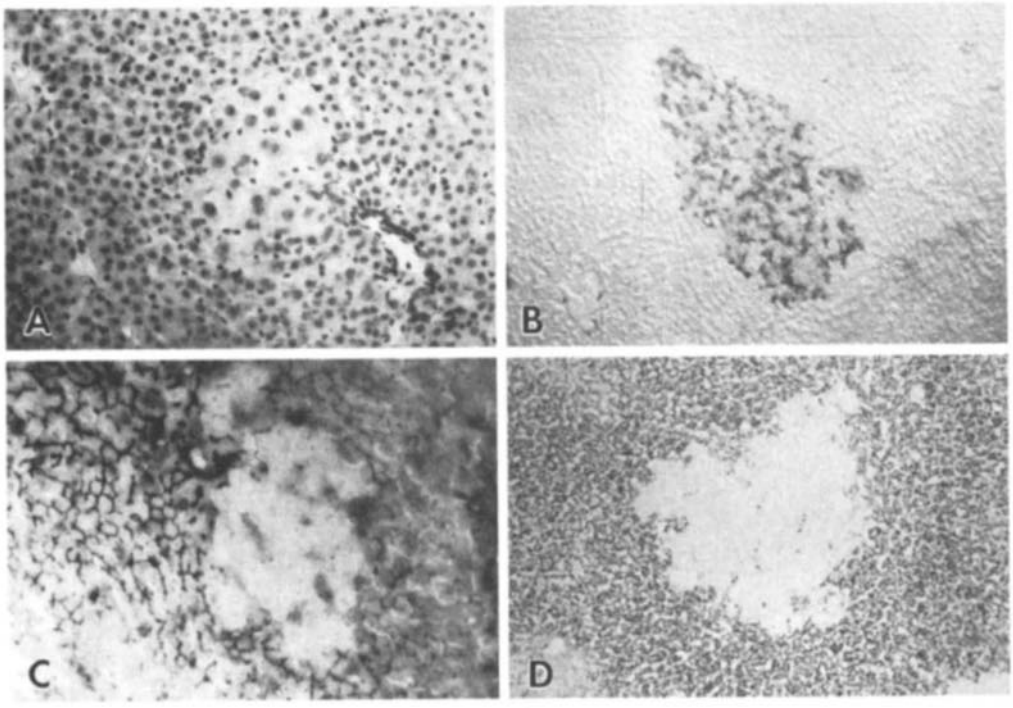

Figure 1 Photomicrographs (×400) of altered hepatic foci exhibiting various phenotypic changes. A. Hematoxylin and eosin-stained frozen section exhibiting an altered hepatic focus noted by the large pale cytoplasm and large nuclei in the group of cells in the center of the micrograph. B. AHF stained for γ-glutamyltranspeptidase. The surrounding hepatocytes do not stain with the substrate whereas the enzyme activity can be noted on the cellular membranes and the bile canaliculi of the cells in the AHF. C. Deficiency of canalicular ATPase. The bile canalicular staining of normal hepatocytes and its absence in the AHF can be easily noted in the photograph. D. Altered hepatic focus exhibiting a loss of staining for the gap junction protein, connexin 32, as seen in human liver. Staining of individual gap junctions can be noted within the normal hepatocytes.

(34) also proposed a more detailed classification of mouse liver tumors, which divided the (*a*) and (*b*) categories into four types representing different patterns of cellular growth. Lipsky et al (35) studied the hepatocellular adenomas and carcinomas in BALB/c mice and demonstrated significant similarities between these two morphologic lesions. They, and others too (9, 33), suggested that the adenoma was a precursor of the hepatocellular carcinoma in mouse liver.

HISTOCHEMISTRY The morphologic variations seen in cells of AHF are relatively limited compared with the wide variety of expression of various genes in cells of AHF as monitored by enzyme, immunohistochemistry and other histochemical markers (Table 1). Whereas earlier studies on the phenotype of AHF used a variety of enzyme histochemical techniques, more modern methods have employed immunohistochemistry and in situ hybridization. Of the other histochemical markers used, the most common is the deficiency-of-iron staining following administration of colloidal iron to the animal (36).

Although the listing seen in Table 1 refers to altered expression of genes in AHF in the rat, some studies have been concerned with AHF in other rodent species. Moore et al (66) demonstrated that the placental form of glutathione S-transferase (GST-P) is expressed at high levels in focal lesions that occur both during pancreatic carcinogenesis and hepatocarcinogenesis in the hamster. Lipsky et al (67) reported that hepatocellular adenomas in the mouse exhibited a deficiency of glucose 6-phosphatase (G6Pase) and succinate dehydrogenase, as well as iron staining following iron loading. In addition, these authors reported an increase in GGT activity in such lesions, although others (68, 69) demonstrated an increase in GGT in spontaneous hepatomas in mice only after chronic administration of phenobarbital. Later studies by Vesselinovitch et al (70) demonstrated that AHF in mouse liver exhibited a deficiency of G6Pase and an increase in glucose-6-phosphate dehydrogenase (G6PD). Variable changes were seen with ATPase and glyceraldehyde-3-phosphate dehydrogenase. Koen et al (71) reported that some AHF in mice expressed α-fetoprotein. In a recent study using the polymerase chain reaction, Buchmann et al (72) demonstrated specific mutations in the c-H-*ras* gene in the mouse in approximately one third of AHF.

Quantitation of Altered Hepatic Foci

Although the morphology and histochemical staining characteristics of AHF are of considerable interest, the specific quantitative parameters of these lesions are most important in understanding their natural history and the development of malignant neoplasia in the liver. The critical parameters in the analysis of whole animal studies related to the development of neoplasia are

the number of AHF, the volume of the liver occupied by the total number of AHF (percentage of liver mass), and the phenotype of the AHF as determined by the use of multiple markers for their enumeration and volume quantitation. Each parameter makes a specific and critical contribution to the understanding of the action of the carcinogenic agent during the multistage process of carcinogenesis.

QUANTITATIVE ENUMERATION OF AHF There are two principal methods to determine the ***number*** of AHF induced in the liver by a variety of agents or appearing spontaneously in animals not subject to any specific treatment. The first, which is relatively simple but can also be inaccurate and misleading, counts AHF on individual tissue sectors and determines the number of AHF per square centimeter or some similar two-dimensional parameter (73, 74). Such values have been used as endpoints in experiments to reflect changes in the numbers of AHF occurring in the entire organ. The other method determines the number of AHF per cubic centimeter or per total liver by use of

Table 1 Histochemical reactions of cells of altered hepatic foci in the rat[1]

Histochemical reactions	Change	References
Enzyme histochemistry		
Enzyme		
Acid phosphatase	−	20
Aldehyde dehydrogenase	+	20, 37
Alkaline phosphatase	±	11, 20
α-Naphthylbutyrate esterase	−	20
β-Glucuronidase	−	11
Canalicular ATPase	+	11
D-T Diaphorase	+	20, 38
Deoxyribonuclease	−	39
5'-Nucleotidase	+	20
γ-Glutamyltranspeptidase	+	16, 17, 18
Glucose 6-phosphatase	-	7, 11
Glucose 6-phosphate dehydrogenase	+	20
Glyceraldehyde-3-phosphate dehydrogenase	+	40
Glycogen accumulation	+	12, 20
Nonspecific esterase	+	41
Phosphorylase	−	11, 41
Ribonuclease	−	42
Succinic dehydrogenase	±	11
UDP-glucuronyltransferase	+	43
Immunohistochemistry		
α-Fetoprotein	±	11, 44
Albumin (in analbuminemic rats)	+	45
Albumin (in normal rats)	−	46
c-*fos* gene product	-	47

Table 1 (*Continued*)

Histochemical reactions	Change	References
Enzyme histochemistry		
c-Ha-*ras* gene product	+	48
Connexin 32 (27 kD gap junction protein)	−	47
Cytochrome P450 (methylcholanthrene-inducible)	±	49
Cytochrome P450 (phenobarbital-inducible)	±	49, 50
Epoxide hydrolase (PN antigen)	+	51
γ-Glutamyltranspeptidase	+	52
Glucose-6-phosphate dehydrogenase	+	53
Glutathione S-transferase (placental form)	+	54, 55
Glutathione S-transferases (A & B forms)	+	56
Glutathione S-transferases (B & C forms)	+	57
NADPH cytochrome P450 reductase	±	57
Plasma membrane antigens	±	58
Pyruvate kinase (Liver form)	+	59
Serine dehydratase	−	60
Tryptophan oxygenase	−	61
Other Histochemical Markers		
Marker (Function)		
Fibronectin	±	62
Glutathione content	+	63
Iron deficiency	−	36
Lipid peroxidation	−	64
In situ Hybridization		
Albumin	−	46
γ-Glutamyltranspeptidase	+	65

[1] + = Increased expression of the marker in a significant number or majority of AHF; − = decreased expression of the marker compared with expression in non-AHF hepatocytes; ± = slight or variable increased and/or decreased expression of the marker relative to non-AHF hepatocytes.

quantitative stereology or related technologies (75–77). Although the accuracy of this latter stereologic method is in part a function of the total number of AHF analyzed (the greater the number, the more accurate the analysis), it gives a much better representation of changes in the entire liver than do two-dimensional analyses. An experimental and mathematical demonstration of this analysis was reported several years ago from this laboratory (78), in which it was pointed out that only when all AHF have the same mean diameter are the two-dimensional and three-dimensional analyses proportional. Where AHF are heterogeneous in size, as occurs in most experimental and in all natural circumstances, the stereologic or three-dimensional analysis is necessary in order to determine with a reasonable degree of accuracy the total number of AHF in the organ. Several studies have

now demonstrated (79–82) that individual AHF reflect the clonal development of an altered, presumably initiated, hepatocyte and thus the accurate determination of the number of AHF can reflect the effectiveness of an agent as an initiator of these lesions.

VOLUME PERCENTAGE OF AHF IN LIVER Of equal importance but with different implications is the determination of the *volume* of the AHF occupied in the entire liver. With this parameter, as pointed out elsewhere (75–77), the area percentage of the AHF as determined by direct computation from the two-dimensional tissue section is equal to the volume percentage as determined by one of several methods. The Delesse method is preferred because it does not depend on the size or shape of the transections of the AHF (75). Alterations in the volume percentage fraction directly reflect alterations in the total cell population of all AHF in the liver. Since the reversible expansion of the initiated cell population under the influence of the promoter is the principal operational component of the process of promotion, this parameter is most useful in determining the extent and effectiveness of the stage of promotion during hepatocarcinogenesis. The volume percentage fraction, especially when monitored as a function of time, may also fairly accurately reflect the growth rate of the population of AHF as a whole. Alternatively, one may determine the volume percentage fraction occupied by a specific phenotypic population of AHF. Previous studies have demonstrated that, in general, those AHF that are most altered from normal liver in their expression of genetic information have the most rapid rate of growth (18, 83). Furthermore, as expected and as has been shown in other studies (84, 85), the volume fraction or number of cells in the AHF populations continues to increase until carcinomas arise, and areas of necrosis and tissue destruction confine such measurements even though the number of AHF reaches a plateau after a long period of promotion.

DISTRIBUTION OF PHENOTYPES OF AHF The biochemical phenotype of each individual AHF is the third critical parameter in evaluating AHF; it can be readily analyzed and reflects the major characteristic of the stage of promotion, i.e. the alteration of gene expression (86). Although more than 40 different "markers" (Table 1), most of which reflect the action of specific genes, are altered in their expression in AHF from that seen in normal hepatocytes, most studies with AHF as an endpoint have employed only a single marker for identification and quantitation of these lesions. Studies from this (87) and other laboratories (36, 56, 88) have demonstrated the effectiveness of one or more markers over others in identifying the greatest number of AHF. At present, GST-P (56, 87) is the most efficient marker for scoring the largest number of AHF by itself. On the other hand, even this highly efficient marker does not score all AHF, as evidenced from the fact that when multiple

markers are used together with the GST-P marker, at least 10% more AHF may be identified (87) than are scored by GST alone. Furthermore, although both the GST and the GGT markers are most effective when phenobarbital (PB), TCDD (89), or other promoting agents are employed (90, 91), most AHF cells do not express these markers abnormally during carcinogenesis with peroxisome proliferators (92, 93). At least one promoting agent, PB, can increase the level of GGT in cells of AHF (94). On the other hand, the promoting agent, C. I. Solvent Yellow, induces AHF that have extremely low levels of GGT, but most of which express high levels of GST (95). Therefore, the phenotype of the AHF induced by the same initiating agent appears to be significantly dependent on the molecular nature of the promoting agent itself and on other factors as well (Y. Xu & H. C. Pitot, unpublished observations).

INDUCTION OF ALTERED HEPATIC FOCI

As noted above, AHF in relation to murine hepatocarcinogenesis were first observed more than two decades ago after administration of chemical hepatocarcinogens. During the last decade, however, several model protocols have been developed, especially in the rat, to examine qualitatively and quantitatively the natural history of hepatocarcinogenesis. There have been several reviews of such models in the rat (19, 96, 97), but relatively few comparable studies in other murine species. Mouse protocols using neonatal animals for the initiation of carcinogenesis have been described (98, 99). Ward et al (100) reported the successful promotion of AHF to hepatic neoplasms when initiation in mice was carried out at 4 weeks of age. A possible reason for the retarded development of multistage hepatocarcinogenesis protocols in the mouse relates to the high level of spontaneous hepatomas in many strains of mice (101) and the high level of endogenous promoting activity in some mouse stains (102). Moreover, PB can "promote" spontaneous hepatomas in C3H mice (103) and induce hepatomas in CF-1 mice (104), but not in BALB/c mice (105). Furthermore, administration of PB to year-old C3H/HeNCr mice results in rapid tumor induction, but at earlier ages PB actually inhibits the process of carcinogenesis (106, 107). Female mice of the B6C3F1 strain do show promotion of hepatocarcinogenesis by PB, whereas males show the opposite effect (108). In the hamster, however, PB and p,p'-dichlorodiphenyltrichloroethane (DDT) exhibit no promoting activity, whereas CCl_4 does serve to promote hepatocarcinogenesis (109).

Survey of Protocols for Multistage Hepatocarcinogenesis in the Rat

The numerous protocols developed for the study of multistage hepatocarcinogenesis in the rat and to a lesser degree in the mouse can be divided into two general categories. The first involves a format very similar to the original

initiation-promotion format in mouse skin (3) and administers a single dose of an initiator (usually a complete carcinogen at a subcarcinogenic dose) in association with a mitotic stimulus, either physiological in the neonate (83) or a partial hepatectomy in the adult (17). Variations have used either a relatively short continuous feeding (3–7 weeks) of a complete carcinogen (110, 111) or the administration of a large carcinogenic dose of a complete carcinogen (16, 72) for the process of initiation. In this latter case, hepatic necrosis with a subsequent proliferative response of hepatic parenchyma occurs and is presumably the mechanism of "fixation" (112). On the other hand, induction of DNA synthesis and mitosis by several chemical agents, including some hepatic promoting agents, does not cause the fixation of the initiation of hepatocytes by initiating agents (113). Furthermore, the methylation of DNA by two carcinogenic nitrosamines to form O^6-methylguanine indicates that the formation of the methylated DNA adduct, although necessary, is not sufficient for initiation of AHF (114).

A second type of protocol involves the "selection" of initiated cells by the administration of an agent, usually a complete carcinogen, that inhibits mitotic activity in normal hepatocytes but, owing to the relative lack of metabolism to its ultimate form in altered hepatocytes, allows them to grow (16, 115). Although AAF is the most common selective agent, other chemicals used include CCl_4, polychlorinated biphenyls, orotate, and a diet deficient in choline and methionine (116). All these selecting agents except orotate are carcinogenic. In particular, choline-devoid diets (116) or purified diets deficient in choline and methionine (117) induce the formation of hepatocellular carcinomas in rats without any added initiating agent.

ADVANTAGES AND LIMITATIONS OF MULTISTAGE HEPATOCARCINOGENESIS PROTOCOLS Several of the protocols referred to above have been used to develop relatively short-term bioassays for carcinogenic agents. The most extensively studied is that of Ito and his associates (73). However, for the distinct separation and characterization of the stages of initiation, promotion, and progression, certain protocols may be more useful than others. In particular, the use of high, necrogenic doses of complete carcinogens as initiators apparently leads to significant chromosomal damage in such protocols (118). Furthermore, the continued administration of a complete carcinogen as an initiating event is complicated by the fact that promotion will occur during the prolonged feeding of the complete carcinogen, even at low doses. Such a situation may prevent the identification of weak promoting agents whose activity may be less than the promoting effect of the "initiation event".

The selection protocols can be quite useful in identifying initiating agents, since the protocol is usually short and the endpoint of AHF can be discerned within only a few weeks. However, because the selection procedure usually

involves an intense promotion (119), identification of promoting agents by these protocols is less effective. Protocols that use subcarcinogenic, non-necrogenic doses of complete carcinogens for initiation followed by the chronic administration of putative promoting agents allow the most sensitive assays for the identification of initiating and/or promoting agents in rat hepatocarcinogenesis, although these too have disadvantages. Protocols wherein neonatal animals are initiated (83) suffer from the fact that many chemical carcinogens require metabolism to their ultimate form before their carcinogenic activity is effective. Because of the low activity of xenobiotic metabolism of neonatal liver, some chemicals may not be activated and thus not scored as having initiating activity. The use of a partial hepatectomy to induce mitotic activity, while relatively physiological, does involve significant intervention in the animal and theoretically could complicate the situation.

Chemical and Physical Induction of Altered Hepatic Foci

Many chemical and physical agents have been associated with increases in the numbers of AHF after administration of such agents either alone or followed by a variety of promoting agents. However, just as in chronic bioassays for carcinogenesis, when inducing altered hepatic foci it is important to distinguish background numbers of AHF to insure that the agent under consideration actually causes an increased number of AHF, thus inducing changes in previously normal hepatocytes. This becomes expecially important in monitoring very small AHF. Moore et al (120) have identified single hepatocytes that can be scored with the ubiquitous marker, GST-P, and suggested that such cells are themselves initiated. While many such altered cells may be the result of the action of the initiating agent, only those cells capable of giving rise to AHF may be considered as initiated by our present understanding of multistage carcinogenesis. Furthermore, the enumeration of single altered cells becomes very difficult by the quantitative stereologic techniques described above, and other satisfactory methodologies have not been devised.

If one stipulates that an inducer of AHF must increase the total population of AHF in the liver and not simply increase the size of existing or potential spontaneous AHF, then not all chemical agents that reportedly induce AHF fulfill this requirement. A complete list of agents inducing or enhancing the appearance of AHF is beyond the scope of this review, but the reader is referred to several studies with different protocols in which a number of chemical agents induced and/or enhanced the appearance of AHF (73, 121–123). Of the physical agents studied, Kitagawa et al (124) reported that X-irradiation of young rats 8 or 22 days of age resulted in numerous ATPase-deficient AHF by 22 weeks of age when PB was administered in the diet following weaning until sacrifice. These AHF were small, GGT-negative,

and actually demonstrated little further growth response to PB, with no development of hepatomas. Somewhat similar results were reported later by Peraino et al (125). Vinyl chloride, but not trichloroethylene, resulted in increased numbers of AHF following exposure for several weeks (126). Several chemicals exhibiting little or no mutagenic activities have been reported to induce AHF in single or prolonged doses (127, 128). Thus, a variety of agents may induce the appearance of AHF in liver, although it has not always been unequivocally demonstrated that such induction represents the appearance of new AHF and not the expansion of those previously present.

Dose Response Studies

Because AHF are clonal in origin (79–82) and their appearance clearly linked to the development of hepatic neoplasms (see below), it is appropriate that some studies should deal with the relation of the dose of the inducing agent administered to the number of AHF that appear in response to such administration. Because the induction of AHF over the background level of such lesions probably reflects newly initiated cells, the dose-response characteristics can give valuable information on the effectiveness or potency of an agent as an initiator as well as other toxicological characteristics of the test agent.

The first demonstration of the quantitative relation between the number of AHF induced in rat liver and the dose of the inducing agent was reported by Scherer & Emmelot (129). On a log-log basis, these authors demonstrated the linear relationship between the dose of diethylnitrosamine (DEN) administered and the appearance of ATPase-deficient AHF. However, this relation was maintained only up to a dose of 30 mg/kg administered following a 70% partial hepatectomy. Beyond this dose, linearity was lost and animals developed hepatocellular carcinomas within the time frame of the study, whereas at doses below 30 mg/kg no neoplasms developed. Using a similar protocol, Pitot et al (130) reported a similar effect. In the inbred Fischer-344 female rat with subsequent promotion by PB, the loss of linearity was seen at doses above 10 mg/kg DEN. In this study three different markers, ATPase, GGT, and G6Pase, were used in scoring AHF. Préat et al (131), using only the GGT marker, demonstrated a relatively linear increase with dose in AHF for the carcinogens DEN and N-nitrosomorpholine (NNM) between 10 and 250 mg/kg. Malignant neoplasms were obtained only at doses of 100 mg/kg or more under these conditions. Klaunig et al (132) reported that DEN administration to mice at 15 days of age resulted in a dose-dependent increase in hepatic adenomas only when they were subsequently treated with PB and monitored 24 weeks later. Essentially no dose-response relation was seen when PB was not given following DEN.

Other studies using continuous administration for varying periods of time have also related total dose of the carcinogenic agent to the appearance of

AHF. Using three-dimensional analyses, Kunz et al (133) have demonstrated the linear relation between the daily dose of NNM and DEN in both volume and number of AHF. Moore et al (134) had earlier reported a similar effect with the continuous administration of NNM at varying doses. Swenberg and his associates (135, 136), in some elegant experiments with GGT as the AHF marker, reported significant differences in the numbers of AHF induced by continuous administration of DEN at several doses in three different lobes of the liver (135) and also a correlation between the O^4-ethylation of thymidine in DNA and the exposure to DEN over a period of at least 30 days (136). Using two-dimensional analysis in a complex series of experiments with a modified Solt-Farber (16) protocol, Tamano et al (137) reported dose responses to AAF, 3'-MDAB, or ethionine given as the selective agent. Using the area of AHF, which reflects the volume percentage fraction (75), Zerban et al (138) reported a linear relationship of this parameter with dose of *N*-nitroso-diethanolamine and the appearance of G6PD-positive AHF. On the other hand, Laib et al (139) reported a similar relation for ATPase-deficient AHF following the administration of low doses of vinyl chloride by atmospheric exposure for varying periods of time. Although the relation of continuous to single dosing is not clear for the reasons already specified, Herren-Freund et al (140) compared the effect of a single dose of DEN with the same dose split over a period of time in relation to the appearance of GGT-positive AHF. Rats of several different ages were used, and the authors concluded that when this chemical was administered to juvenile rats, the dose responses to single or multiple split doses were similar, exhibiting neither a threshold nor a plateau under the conditions of the experiment. The analyses were three-dimensional and suggest that dose-response curves for certain chemical agents, especially those with highly effective initiating activity and relatively low efficiency as promoting agents, may be effective under either conditions.

NATURAL HISTORY OF ALTERED HEPATIC FOCI IN RELATION TO THE PROMOTION STAGE OF HEPATOCARCINOGENESIS

Despite considerable evidence that most if not all AHF represent the progeny of single initiated hepatocytes, it is quite reasonable to argue that it is the later stages, promotion, and/or progression in hepatocarcinogenesis in which the growth of AHF by replication of the progeny of initiated cells occurs. Furthermore, at present most is known about the stage of promotion, which immediately follows initiation.

Spontaneous Altered Hepatic Foci—Rat and Mouse

The incidence of spontaneous hepatocellular neoplasms in rats and mice used in experimental investigations has been well documented (101, 141, 142). In

general, spontaneous hepatomas in mice are relatively frequent in animals 100 weeks of age or older, with 55% or more incidence in some strains (101). On the other hand, the incidence of spontaneous hepatocellular neoplasms in rats is usually of the order of 1% or 2% (141, 142) except in germ-free animals obtained after the age of 30 months, where liver tumors may exceed 90% incidence in the Wistar strain (143). Thus, given the characteristics of AHF and their potential role in the development of hepatocellular neoplasms, one would expect to find a significant number of spontaneous AHF in livers of both mice and rats.

Although the appearance of spontaneous AHF has been best studied in rats, a recent provocative study by Lee et al (144) suggested that epithelial cells cultured from normal C3H strain mouse liver that express an immortal phenotype may represent the precursor population for spontaneous hepatocellular carcinomas in this high-incidence strain. Several studies (145–147) have demonstrated the occurrence of AHF in the livers of aging rats of at least two different strains. In two of these studies, the numbers of AHF calculated either by two-dimensional analysis (145) or quantitative stereology (147) increased with the age of the animal, especially beyond 18 months of age. Mitaka & Tsukada (148) have confirmed and extended these investigations, demonstrating that the increases in AHF in a related strain of rats were greater in males than in females. This latter investigation used two markers, GGT and GST-P, whereas the earlier studies employed either GGT alone (145, 146) or H & E histologic identification of AHF (147). Moore et al (120) have reported the presence of single GST-P-positive hepatocytes both in control untreated rats and in animals treated with PB or several different complete carcinogens. Studies in our laboratory have confirmed these earlier reports demonstrating an increase in both number and volume percentage fraction of spontaneous AHF in both sexes with four different markers (149). These data indicate that at weaning the incidence of spontaneous AHF is similar in male and female animals, but thereafter their appearance increases more dramatically in males than in females in this strain of rats, in agreement with the findings of Mitaka & Tsukada (148).

In view of the spontaneous occurrence of hepatocellular carcinomas in control animals, usually after 2 years of age, when a promoting agent acts chronically on a population of AHF that numerically exceeds 1,000 per liver (see ref. 147, 149), it is not surprising for an occasional hepatocellular carcinoma to arise. This concept has been discussed both by our laboratory (150) and by others (151). Rossi et al reported in 1977 (152) that the long-term administration of PB to rats resulted in a significant number of hepatic tumors (up to 59% of males). None of the nodules could be classified as hepatocellular carcinomas, but a number were designated as hepatic adenomas and nodules of regenerative hyperplasia. More recently, Harada et al (28) have described extensive morphologic characteristics of spontaneous AHF in

the Fischer 344 strain of rats. Although the cause of the development of such spontaneous lesions and neoplasms has generated considerable speculation, among the first data to demonstrate potential genetic changes associated with the spontaneous incidence of AHF and hepatomas were those of Randerath et al (153). These studies used the technique of ^{32}P-postlabeling of DNA to demonstrate that the genome of the liver and other tissues contains derivatives not detected in DNA of newborns, and that such adducts markedly increase with age. Therefore, in long-term chronic bioassays, chemicals given at maximally tolerated doses would be expected to induce neoplasms from spontaneous AHF or initiated cell populations in other tissues when the agent itself exhibited only promoting activity. By this mechanism, chemicals with no known mutagenic or DNA-damaging activity may induce the appearance of benign and malignant neoplasms in such assays.

Phenotypic Effects of Promoting Agents

In general, the phenotypic effects of promoting agents can be divided into those acting on normal hepatocytes and those affecting cells of AHF. In considering the former, it may be further generalized that promoting agents effective in hepatocarcinogenesis stimulate the rates of DNA synthesis (154, 155), cell replication (154), cell death (apoptosis) (156), and the expression of specific genes within hepatocytes (157). By and large, promoting agents for hepatocarcinogenesis do not produce acute signs of toxicity in the liver but do induce liver growth by either hyperplasia and/or hypertrophy (see ref. 157). The exact roles of hypertrophy and hyperplasia in the action of several promoting agents are not altogether clear. For example, although phenobarbital administration does stimulate DNA synthesis and cell replication in rat liver in vivo (154), there is no measurable increase in the activity of DNA polymerase α (158). Whereas single administrations of PB to male rats cause a transient increase in hepatocyte mitotic activity, they subsequently stimulate a marked decrease in this activity to well below control levels 3 days after administration of PB, with a return to normal levels 48 hours later (159). In mice, PB administration stimulates DNA polyploidization to a significant degree. On cessation of PB administration, those cells containing the high level of ploidy are eliminated, presumably by the process of apoptosis (160). Administration to mice of the hepatic promoting agent dieldrin (161) is reported to produce a similar effect. Likewise in rats, administration of the hepatic promoting agent, cyproterone acetate, induces hyperplasia in rat liver, and on withdrawal there is a marked increase in apoptosis of individual hepatocytes (162).

Just as TPA is the most extensively studied promoting agent for epidermal carcinogenesis in the mouse, the model agent PB is used most in studies on the mechanism of action of hepatic promoting agents. The similarities be-

tween the actions described for the two include the induction of ornithine decarboxylase in liver (163, 164), followed by an increase in spermidine concentration (165), an interaction with protein kinase C (166), and the ability to alter the structure of gap junctions of rat hepatocytes (167) as well as decrease the mRNA for gap junction protein in rat liver (168). Other effects of PB on hepatocytes include an increase in lipid peroxidation in vitamin E-deficient rats (169), an induction of the repair of O^6-methylguanine in hepatic DNA (170), and a decrease in receptors for insulin and epidermal growth factor, but not glucagon (171, 172).

EFFECTS OF PROMOTING AGENTS ON AHF In general, the effects of hepatic promoting agents on cells of AHF are similar to those reported for hepatocytes, but with varying degrees of exaggeration. Schulte-Hermann et al (173) described an enhanced proliferation of AHF cells following administration of several different hepatic promoting agents, including PB, steroids, organic halides, and a peroxisome proliferating agent. Tsai et al (174) presented data suggesting that the stimulation of hepatic DNA synthesis by PB and other hepatic promoting agents may be dependent on adrenergic effects. Using the model system described by Ito and his associates (73), Tatematsu et al (175) demonstrated that AAF, but not PB or butylated hydroxyanisole, while enhancing the proliferation of hepatocytes in AHF to varying degrees, showed either an intense inhibition (AAF), a slight inhibition (PB), or no effect (butylated hydroxyanisole) on the proliferation of hepatocytes surrounding the AHF. These data further support the selective action of AAF and the relative lack of such action by PB and butylated hydroxyanisole in model systems of multistage hepatocarcinogenesis.

Just as tumor-promoting agents can alter the rate of apoptosis (control cell) in normal hepatocytes (156, 162), PB (176) and orotic acid (177) appear to alter this process in cells of AHF. The potential role of apoptosis regulation in the promoting action of such agents is of considerable interest, but not yet understood. After studying such effects, several investigators (118, 178, 179) have demonstrated that the preponderance of cells in AHF are diploid in contrast to normal hepatocytes, which are predominantly tetraploid or of higher ploidy in the adult rat. Danielson et al reported a similar shift in ploidy during hepatocarcinogenesis in mice (180), but concluded that such alterations are not essential for the process of hepatocarcinogenesis. Following a 70% partial hepatectomy, more cells within AHF demonstrate an increase in DNA synthesis than in cells of the surrounding liver tissue (181). Furthermore, DNA synthesis in cells of AHF fails to return to a baseline level following stimulation of cell proliferation by partial hepatectomy, unlike hepatocytes in the surrounding liver (182). In accord with these findings, Rotstein et al (183) have concluded from kinetic labeling studies that the S

phase of cells of AHF is approximately twice as long as that in regenerating control liver.

THE EFFECT OF PROMOTING AGENTS ON PHENOTYPES OF AHF Quantitative studies on the phenotypic distribution of AHF with multiple markers have demonstrated considerable heterogeneity (17, 83), except for the finding by Farber and his associates (88) that most AHF phase I enzymes of xenobiotic metabolism showed generally decreased activity, while those of the phase II pathways, which would include GGT and GST-P, were generally increased in activity over that seen in normal hepatocytes. This is also true in GGT-positive hepatocytes of AHF isolated from hepatic cell suspensions (184, 185). However, when inducers of phase I enzymes were administered, a heterogeneous pattern of alterations in phase I enzymes was seen in AHF, further substantiating the generalization of phenotypic heterogeneity of gene expression within AHF (186). Whereas these and other studies (84, 187) argued for the stability of AHF phenotypes as long as the promoting agent was being administered, later studies demonstrated that several markers were less stable under other conditions. Schulte-Hermann & Timmermann-Trosiener (188) showed that in the absence of PB, AHF lost a significant amount of the GGT-staining characteristic. Later, Sirica et al (94) and Herren-Freund & Pereira (189) reported that PB administration induced GGT activity in AHF of the Solt-Farber protocol (16). Subsequently, a number of investigations have demonstrated that a variety of hepatic promoting agents can increase GGT activity in normal hepatocytes, with the potential for such an effect in cells of AHF (190–193). Schulte-Hermann et al (194) demonstrated that at least one cytochrome P-450 isozyme was inducible by PB within AHF induced by several different regimens.

In further support of the effects of promoting agents on the phenotypes of AHF, Reddy and his associates (195), as well as Glauert et al (93), have demonstrated a lack of or marked deficiency in GGT as well as GST-P (195) staining in cells of AHF. Furthermore, Yeldandi et al (196) have presented evidence to indicate that such changes are relatively permanent, since treatment with AAF following ciprofibrate administration did not stimulate the appearance of GGT within such GGT-negative AHF. Studies from this laboratory (95) with C. I. Solvent Yellow 14 as a promoting agent showed the resultant AHF, while exhibiting high levels of GST-P, showed very low levels of GGT. Thus, whatever the mechanism, it is apparent that the phenotypic distribution reflecting expression of specific genes within AHF is to a significant degree a function of the chemical nature of the promoting agent used. Furthermore, several studies (18, 197) have shown that AHF with different phenotypes exhibit different thymidine-labeling patterns of individual cells. Taken together, the studies described in this review, as well as

many others whose description space does not allow, demonstrate the numerous molecular effects of promoting agents and the variability of responses of preneoplastic AHF during the stage of promotion in multistage hepatocarcinogenesis.

Dose-Response Studies of Promoting Agents

Because the characteristics of promoting agents are significantly different from those of initiators (198), one might expect that the dose-response relation in the two stages would be different. The first studies that reported on such a relation in hepatocarcinogenesis in the rat with "hyperplastic nodules" (199) and "tumors" (110) both demonstrate a threshold or no-effect level of PB when administered chronically after a short dose of AAF, sometimes given slightly before a single dose of CCl_4 (199). Subsequently, however, Mochizuki et al (200) were unable to demonstrate a threshold at 0.01% PB given in the diet following 3 or 5 weeks' administration of DEN. Later, using a similar 3-week feeding period of 3'-Me-DAB, Kitagawa et al (201) were also unable to detect a no-effect level of PB, although administration of DDT did exhibit a no-effect level at 10 ppm. In this study the response was measured as numbers of AHF/cm^2. Using three-dimensional analyses, Goldsworthy et al (84) with the protocol described by Pitot et al (17) demonstrated both a threshold level for PB at 0.005% dietary concentration, as well as a maximal induction of numbers of AHF at 0.05% PB in the diet. Driver & McLean (202) also reported a no-effect level of 100 ppm in the PB promotion of hepatocellular carcinomas in rats, with dimethylnitrosamine (15 mg/kg) as an initiator. Interestingly, at doses in which no promoting action was noted, the induction of xenobiotic metabolizing enzymes still occurred; this suggests that these two functions of almost all hepatic promoting agents could be divorced. Schröter et al (203) studied the dose-response relation of the isomers of hexachlorocyclohexane as promoters following a single dose of NNM. By both numerical estimation and determination of area percentage occupied by AHF, these promoting agents exhibited no-effect levels that were approximately the same for the α-, β-, and γ-isomers.

In a study designed to use the dose-response relation to compare potencies of different initiating and/or promoting agents during hepatocarcinogenesis, Pitot et al (130) demonstrated no-effect levels for both PB and TCDD. Furthermore, at one- or two-dose levels below the threshold, the numbers of AHF were actually less than the number at zero concentration of the promoting agent. The mechanism for this effect, which had also been reported earlier (84), was not explained. However, on the basis of quantitative stereologic calculations, these authors proposed parameters to estimate the relative potency of chemicals as initiating or promoting agents; these were termed the initiation index and promotion index, respectively (130). Determination of values for these indices for a variety of chemicals showed considerable

variation, with TCDD having the highest promotion index, by several orders of magnitude, and DEN in the Sprague-Dawley strain of rats the highest initiation index. The dose of the initiating agent did not significantly affect the value of the initiation indices as long as no toxicity or cell death resulted from the doses administered. Similarly, promotion indices did not change significantly (204) between a threshold or no-effect level and a maximal effect level (196). Thus, within specific constraints based on the dose-response relation of AHF, the relative potencies of individual agents as initiators and/or promoters may be determined in at least one model of multistage hepatocarcinogenesis in the rat.

Stability (Persistence) of Altered Hepatic Foci in the Presence and Absence of Exogenous Promoting Agents

The stage of tumor promotion in hepatocarcinogenesis is characterized by instability, "reversibility," and alterations in most physiologic parameters as influenced by various environmental factors (198). Since AHF cells are in the stage of promotion in many models of multistage hepatocarcinogenesis, the effects of environmental changes on the population as well as the phenotypes of AHF have been extensively investigated, especially in the rat. Because promoting agents serve both to increase DNA synthesis and cell replication in AHF cells to a greater degree than in hepatocytes (173) and also to inhibit the turnover or death of cells within AHF, their removal results in a dramatic decrease in DNA synthesis and cell replication as well as an increased rate of apoptosis (176, 177). From these facts one would expect changes in the overall population of AHF following both administration and withdrawal of promoting agents during this stage. In fact, all models of multistage hepatocarcinogenesis in the rat exhibit both a decrease in size (volume percentage) and a loss of the number of AHF when the promoting stimulus is withdrawn (198). Reuber (205) was among the first to describe the lack of continued growth of AHF when a carcinogen is discontinued after a relatively short period of treatment. One of the first quantitative characterizations of this phenomenon was described by Williams & Watanabe (206), who used AAF as the carcinogen and a deficiency-of-iron staining to score AHF. A similar effect was noted on short-term administration of NNM (207), and those remaining AHF exhibited an internal heterogeneity in both morphology and histochemistry. Similarly, with the Solf-Farber model (208, 209) and that described by Ito and his coworkers (210), the great majority (75% or more) of AHF regressed and disappeared within a short time after the selection. Following chronic administration of several different promoting agents and regimens, a choline-deficient diet (211), clofibrate (212), or PB (213), cessation of administration resulted in a dramatic and rapid decrease in the number of AHF present in the livers of animals treated in this manner. In mice,

cessation of administration of the promoting agent, hexachlorocyclohexane, after 20 weeks of administration resulted in the loss of most tumors and hyperplasias, at least some of which seem to be compatible with AHF (214). Lipsky et al (215) reported that cessation of safrole administration after periods of administration up to 52 weeks resulted in a decrease in the number of AHF present. On the other hand, Ruebner et al (216) have pointed out that most hepatic lesions in mice, whether spontaneous or induced, do not characteristically regress and/or disappear. This may be the result of the strong endogenous promoting action of the mouse internal environment in specific strains (102).

On the basis of our knowledge of the action of promoting agents on cell replication and turnover (see above), the most logical mechanism for the loss and decreased volume of AHF on removal of the promoting stimulus is the rapid cell turnover (apoptosis) that results when the promoting stimulus is removed. This has been proposed as a mechanism by Schulte-Hermann and others (157, 177). Another possible mechanism for the loss of AHF on removal of the promoting stimulus is the redifferentiation or remodeling of AHF and nodules as proposed by Farber and his associates (217). Such a mechanism appears most applicable to the selection protocols in which there is a rapid growth and loss of AHF (16, 73). Whatever the mechanism of the loss of number and volume of AHF and since the process of initiation is irreversible, a critical question is whether reinstitution of the promoting stimulus will result in a regain of both number and volume of AHF. Hendrich et al (218) demonstrated that such is the case in at least one model system following withdrawal and readministration of PB; they showed quantitatively that both loss of AHF and their reappearance occurred within a relatively short period following withdrawal and readministration of the promoting agent. However, in this and other experiments, withdrawal of the promoting agent does not lead to a loss of all AHF. Persistent or promoter-independent AHF are particularly observed after initiation with relatively large, toxic doses of complete carcinogens (219, 220). The exact mechanism for the persistence of such promoter-independent AHF is not clear, but several potential mechanisms are now evident. Both endogenous and exogenous promoting agents include sex steroids (221, 222), pituitary hormones (223), and endogenous bile acids (224). Exogenous promoting factors usually not controlled for in animal experiments include both known dietary constituents such as selenium (225), choline deficiencies (116), unsaturated fat (226), dietary tryptophan (227), or sucrose (228). Furthermore, the crude, cereal-based diets commonly used in carcinogenesis experiments with rodents may have promoting action and/or promoter-enhancing activities themselves (229). Therefore, the persistence of many AHF may in fact be due to uncontrolled promoting activities, both endogenous or exogenous. Alternatively the promoter-independent AHF may reflect a qualitatively different population from the

promoter-dependent AHF. If so, then such a population is likely to consist of those lesions already in the stage of progression.

THE RELATION OF ALTERED HEPATIC FOCI TO THE DEVELOPMENT OF HEPATOCELLULAR CARCINOMA

From the earliest and subsequent quantitative stereologic estimations of the numbers of AHF in livers of animals initiated with various doses of complete carcinogens, both at subcarcinogenic and carcinogenic levels (17, 129), the marked discrepancy between the numbers of malignant neoplasms resulting from the multistage process and the numbers of AHF induced was quite evident. The two numbers differed by two or three orders of magnitude, a fact that led Peraino et al (83) to suggest that possibly AHF and malignant neoplasms develop as separate entities by actions of chemical agents at different genetic loci. However, these workers granted the possibility that malignant neoplasms may develop from AHF as other investigators had proposed (230–232), based on the correlation of dose-time relations with the appearance of the two different types of lesions. Drinkwater and his associates (102) showed a similar relation between these two types of lesions in mice. Thus, although it is reasonable to argue that a precursor product relation exists between AHF and hepatocellular carcinomas, it is clear that within the lifespan of the animal very few AHF proceed to malignant neoplasia, especially during model protocols that use nontoxic, subcarcinogenic doses of carcinogens for the initiation process (129). Therefore, one must consider additional mechanisms to account for this extreme discrepancy in population sizes when substantial evidence for a precursor-product relationship exists. The most likely explanation for this relation is that another alteration, which is most favored to occur within cells of AHF, is induced or occurs spontaneously and results in a new population of cells developing within preexisting AHF. Circumstances in which the initial administration of a carcinogen induces within the same cell both the changes required for initiation and those for the second event, herein termed progression, may be seen at very high, toxic doses of the chemical agent. In this circumstance, the final result, hepatocellular carcinoma, is the same, but the multistage nature of cancer development can be demonstrated only when initiation is cleanly separated from the final stage of progression. The characterization of progression can best demonstrate the relation of AHF to the development of hepatocellular carcinoma.

The Stage of Progression and Its Characteristics in Multistage Hepatocarcinogenesis

In the classical two-stage carcinogenesis in mouse epidermis, tumor promoters were strictly effective only when chronic treatment resulted in the appear-

ance of malignant neoplasms. This concept was inherent in such studies despite the fact, at least in the epidermal system, that benign tumors or papillomas were the endpoint in most experiments (233). Therefore, the same rationale has predictably been applied to analogous stages in hepatocarcinogenesis in the rat regardless of the fact that nodules of benign-appearing hepatocytes have been monitored as endpoints in several models (97). With the overwhelming evidence of the instability of the stage of promotion, especially in hepatocarcinogenesis (198), and the defined stability and irreversibility of malignant neoplasms, one may define the final stage in the development of malignant neoplasia, i.e. progression, quite differently from the stage of promotion.

MORPHOLOGIC EVIDENCE OF PROGRESSION Numerous examples of intermediate, premalignant lesions have been described in both the human and animals in various histogenetic types of neoplasms (234). Similarly, in rat liver with a model system patterned after the initiation-promotion-initiation format first proposed by Potter (235), Scherer and his associates (236, 237) have demonstrated that focal carcinomas can be induced to arise in preexisting AHF or nodules. These workers have designated such lesions as foci-in-foci. These investigators as well as preliminary experiments from our own laboratory (234) have indicated that following the second initiation there is a significant increase in the foci-in-foci when the alkylating agent, ethylnitrosourea, is used as the second initiating agent or "progressor" agent. Although the quantitation of numbers and volume percentage fraction of foci-in-foci has not yet been refined, these studies do indicate that phenotypically heterogeneous AHF, when seen either directly with the light microscope or by the use of multiple phenotypic markers, may reflect the conversion of cells in the stage of promotion to the stage of progression. Similar conclusions have been drawn by Estadella et al (238) using a similar initiation-promotion-initiation protocol. Even at the strictly light microscopic level, Harada et al (29) have proposed that specific morphologic types of AHF are more usually predictive of malignant hepatic neoplasia.

KARYOLOGIC EVIDENCE OF THE STAGE OF PROMOTION Boveri (239) was one of the first to recognize that significant karyotypic abnormalities are seen in most human and animal malignant neoplasms. Today it is clear that essentially all malignant neoplasms in the human exhibit some degree of karyotypic abnormality if one uses the modern techniques of banding and/or premature chromosome condensation (240).

Recently, in multistage epidermal carcinogenesis in the mouse, Aldaz and his associates (241) demonstrated that the karyotype of early appearing papillomas after initiation and promotion with TPA showed normal banded

karyotypes. However, as promotion continued with frequent applications of TPA, karyotypic abnormalities appeared with increasing complexity. Recent studies in our laboratory (118) showed that cells isolated from altered hepatic foci in the Peraino protocol (83) exhibit essentially no chromosomal abnormalities. However, hepatocytes from altered hepatic foci induced by the Solt-Farber protocol (16) demonstrated that the majority of hepatocytes from the foci exhibited significant chromosomal abnormalities, indicative that the toxicity resulting from the extremely high dose of diethylnitrosamine and the selection procedure employed in this protocol caused significant karyotypic damage. This is in line with the fact that the Solt-Farber protocol rapidly induces hepatocellular carcinomas when animals are promoted with PB (242), whereas the Peraino protocol takes considerably longer (83). Because earlier studies showed that virtually all hepatocellular carcinomas in the rat exhibited chromosomal abnormalities (243, 244), it is reasonable to propose that the development of such changes is the result of genetic alteration, different from those that produced the initiated cells and their progeny in the stage of promotion. In more recent studies (245), we have demonstrated that in the initiation-promotion-initiation protocol more than half of the karyotypes derived from cells of AHF also exhibit gross chromosomal changes, further validating this method of studying the stage of progression and also characterizing this stage as one of karyotypic abnormalities and karyotypic instability.

Although both morphologic and karyologic alterations are characteristic of the stage of progression, both the quantitation of these effects and the molecular mechanisms that are the basis for this stage of neoplastic development have yet to be elucidated. Some studies (234) have suggested that the transcriptional activation of proto-oncogenes within AHF may be important markers, indicating that AHF cells are in the stage of progression. In view of the frequency of proto-oncogene activation, both transcriptional and mutational in malignant neoplasms, such a finding is quite reasonable, but requires considerably more verification.

CONCLUSIONS

Although AHF represent only one biologic class in the variety of pathologic lesions occurring during the development of hepatocellular carcinoma in the rodent, this lesion does offer a tool both to quantitate changes occurring during the three stages of hepatocarcinogenesis—initiation, promotion, and progression—and also to study mechanisms involved in their development that ultimately lead to a rational picture of carcinogenesis in rodent liver, with potential applications to the human. In a more immediate and practical sense, however, AHF may be useful as an adjunct to the chronic bioassay presently

employed in the determination of potential carcinogenicity of chemical agents and their risk to the human. As briefly discussed in this review, several model systems have been proposed (73, 121–123) with AHF as an endpoint for the determination of both complete carcinogenicity and the action of specific agents as initiators or promoters. In addition, the use of changes in quantitative parameters of AHF has been employed to determine relative potencies of individual chemical agents as promoting and/or initiating agents in rat liver. Although many promoting agents exhibit clear tissue specificity, many chemical agents have exhibited promoting activity in hepatocarcinogenesis in the rat. Furthermore, because of the extensive capacity for xenobiotic metabolism by hepatocytes, the initiating potential of many complete carcinogens may be analyzed in the liver.

Before regulatory agencies are willing to use multistage models in regulatory decisions and risk estimation in the human, considerably more experimentation will be required. However, one area in which multistage hepatocarcinogenesis can be quite useful is that of fitting mathematical models to the biological situations in this system. Most applicable is the incidence function derived by Moolgavkar and Knudson (see ref. 246) in which two events, presumably genetic and irreversible, are interrelated by an expanding, intermediate cell population, such relations being mathematically defined. This mathematical model is closely related to the initiation-promotion-initiation biological model discussed above (235). Recently, studies from this laboratory (247) have used some experimental values as well as values from the literature in relation to populations of AHF to determine whether one may predict the mathematical relation of the incidence function. Although in their infancy, such studies do offer the potential that the determination of certain biological parameters when placed into an appropriate mathematical model may be predictive not only of the carcinogenic potency of a specific chemical, but also the stage(s) during carcinogenesis in which the chemical exerts its principal effects. The application of mathematical models relating experimental biologic parameters to risk estimation in the human may then be placed on a much firmer basis.

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