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Hepatic neoplasia: reflections and ruminations

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"We must be wary of the ever-present occupational risk of the cancer researcher: generalization." [188]

Tumorigenesis in the liver is one of the most difficult and most fascinating topics in experimental and clinical hepatology. Although all cellular elements of the liver can give rise to tumours, the emphasis has largely been placed on the numerically dominant epithelium-derived neoplasms to the relative neglect of the mesenchymal tumours, and this trend will also be followed here. Hepatocellular carcinoma (HCC), exacting a death toll of up to 1,000,000 cases a year [90, 281] is now considered to be one of the most common [27, 49, 123, 124], if not the most common [90], cancers in the world, at least in males [281]. Parkin et al. however, in their study of the worldwide incidence of cancers in 1985, point to carcinoma of the lung as being "by far the most common cancer of men" [245]. These seeming discrepancies can perhaps be attributed to the "extreme" difficulties in arriving at a "reliable assessment of the secular trends in the incidence and mortality of primary liver cancer" which Stuver and Trichopoulos have mentioned [314]. It is interesting to note in this context that these authors in their study have also pointed out that "converging data suggest that tobacco smoking can cause hepatocellular carcinoma independently" of infection by hepatitis B virus (HBV) or hepatitis C virus (HCV), whose role in the development of HCC has attracted much attention, as will be shown.

The clinical importance of HCC has led to the malignant form of hepatic tumours being put into the spotlight. It must however not be forgotten that, as Huff et al. pointed out in their analysis of the meaning of "benign" and "malignant", these convenient terms "simply define different stages of the spectrum of a single parameter – biological behavior" [169]. Maronpot, in his basic survey of "chemical carcinogenesis", considered it "... probable that any malignancy ... is a consequence of the same stimulus that produced the benign proliferative lesions, interacting with other critical intrinsic or extrinsic factors [217]". The sequence of histochemical and cytological changes (see [20, 29, 33, 38, 359]) that take place in the preneoplastic and neoplastic lesions of the liver can serve as a visual illustration of this view. Experimental hepatocarcinogenesis – see, for instance, the discussion by Goldfarb and Pugh [145] and by Jang et al. [176] of hepatic neoplasia in the mouse – has shown that the dividing line between "benign" and "malignant" can be as blurred and as ill-defined as the dividing lines of the postulated stages in routine studies of the development of an experimental hepatic tumour; the possible role of "benign" hepatocellular tumours as precursor stages of malignancy (for example [25]) is now also being discussed in clinical medicine [10, 24, 71, 94, 241, 278, 281, 317, 323]. This is an indication of the increasing awareness of the "complex and perverse biology of cancer" [244].

This complex character of hepatocarcinogenesis, involving, as it does, "considerable interaction between various biological variables" [69], has led to the realization that "the sequence of cellular events leading to hepatocellular carcinoma is not yet understood" [90], that "it is not possible at the moment to assemble all information into one general functional concept that can explain the mechanisms of tumorigenesis and malignant transformation" [97] and that, to cite another example, Weinberg's enthusiastic conclusion that the "oncogene paradigm ... has proved to be particularly powerful in generating an explanation of cancer on the molecular level" [345] has to be somewhat tempered by the warning that "critical gene changes in hepatocellular carcinoma remain to be identified" [202]. It is, as Baserga et al. pointed out, "somewhat disconcerting how fast proto-oncogenes, only a few years ago hailed as the key to the understanding of cancer biology, have been supplanted by the tumour suppressor genes in the consideration of the scientific community [39]." Despite our "giant steps towards under-

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standing liver cancer ...", there are still "... many questions to be answered before morphological and molecular biological studies can be merged into one consistent theory of hepatocarcinogenesis" [281]. The very nature and interpretation of hepatocarcinogenesis becomes one of these questions if we contrast the widely held view of neoplastic transformation as "an anarchic alteration of cell development" [214] and of cancer as the expression of a progressive sequence of damaging genetic changes (see for instance [315]) leading "rebellious cells" [189] to violate "the social contract with other cells, proliferating and spreading in an unfettered way" [49], with the other hypothesis of the response of hepatocytes to the administration of carcinogens as an "adaptation to a changed environment" [98, 229], with clonal adaptation to the hazards of the environment an important phase, the trigger [97] of the process of hepatocarcinogenesis [106, 108, 109, 111]. Cancer can be represented as "perhaps only incidental", a "deviant of adaptation", a "manifestation of the imperfection of the adaptive process" [97, 117, 249]; "adaptation" here is seen as the "selection of the fittest" [283], the early appearance of a "distinctive pattern of differentiation" [60] and a "new physiological cell population" [108, 109] resistant to environmentally-induced cell damage and expressing a "physiological adaptative response" [155] "beneficial to the host in a hazardous environment" [120]. It may also be an expression of a new pattern of genes, a "ubiquitous genetic program for cellular adaptation ..." [97] entailing however "disabilities as well as abilities", as Rous and Kidd have pointed out in their discussion of rabbit skin tumours [276].

It is the very complexity of this phenomenon, and "the lack of understanding of the basic biology of neoplasia ..." [64] with its resulting uncertainty of interpretation, that contributes so much to its fascination and intellectual challenge. When the concept of primary carcinoma of the liver gradually emerged in the middle of the last century – Eggel's classical review was published only in 1901 [93] – the facts then available were embellished by a number of more or less plausible sounding interpretations and theories of currently largely historical interest. In 1958 Ackerknecht spoke of cancer as "... a notion comparable in its vagueness and all-embracing character only to the expressions 'fever' or 'plague' as they were used hundreds of years ago" [2], and Willis referred to cancer as "a variety of diseases" [352]. A few decades later when we are, in contrast, faced with an explosion of information on various aspects of tumorigenesis – see, for instance, the list of advances in the paper by Harris [153] – we still perceive, uncomfortably, a lack of a comprehensive understanding of the cellular and molecular events involved in multistage carcinogenesis [348]. "No unitary concept can give a satisfactory explanation of the intimate nature of cancer" [64, 237, 333], for cancer is such a complex entity that "probably no one model can fully describe it, and it will be difficult to find a single theory to explain the mechanisms of carcinogenesis in different systems" [198]. One is remined of Hieger's conclusion that "while we have at present a number of *theories* of carcinogenesis, there is as yet no *theory* of carcinogenesis; that these hypotheses have scarcely proved more than re-statements of the facts of experiment or observation; and that the very tentative nature of these ideas is a measure of the difficulties of our formidable and wonderful problem" [160].

These somewhat pessimistic assessments have their roots in part in a loosely used, almost arbitrary, nomenclature [6, 64, 81, 92, 100, 126, 176, 334] with "noncommittal terms" [145] and definitions coined by pathologists of the last century which "for some strange reason have failed to become modified by the last fifty years of experimental pathology" [301]. Altmann's recent comments on the "number of misinterpretations, terminologic inventions and rechristenings with ensuing and often irremediable confusion" to be found in the "recent flood of publications" on benign tumours of the liver bears out this view [10]. It was, however, largely the uncertainty that surrounds the individual components that has contributed to our lack of understanding of the process itself. Harris has described multistage carcinogenesis as "... driven by carcinogen-induced genetic and epigenetic damage in susceptible cells that gain a selective growth advantage and undergo clonal expansion as the result of activation of protooncogens and/or inactivation of tumor suppressor genes [153]". Each facet of this definition, randomly chosen from one of the recent papers, has given rise to a very large number of publications which cannot be reviewed here (see Appendix). They have, regrettably, not succeeded in removing "the present state of indecision and uncertainty ..." [206]. For one aspect that is characteristic of our present interpretations of hepatocarcinogenesis is the fact that for every generalization there have been found exceptions, and every explanation attempted has been met by counterarguments. The persisting debate about the part played by aflatoxins, notably aflatoxin B_1 – the "... most potent ubiquitous natural chemical carcinogen" [211] – and/or by viral hepatitis in the development of hepatic malignancies can serve as an example.

Aflatoxins have come to be viewed as possible, even probable, human liver carcinogens [61, 354, 355]. Wigley lists aflatoxins as chemicals "with proven carcinogenic activity in humans" [349] and Stuver and Trichopoulos consider the evidence concerning aflatoxins as "very strong" [314]. In a number of hepatic cancer patients who were exposed to a high dietary intake of aflatoxin there is a "characteristic" [80, 241] or "specific" [138, 157, 194, 246, 281, 336, 360] mutation at a "mutational hotspot" at codon 249 of the tumour suppressor gene *p53* [123, 154, 242, 281, 305]. This suppressor gene, whose "emerging picture" has been reviewed by Selter and Montenarh [295], is stated to undergo mutations in "about half of almost all types of cancer arising from a wide spectrum of tissues" [154] or, according to Soussi et al., in "the majority of human tumors" [306]. In hepatic cancer the early reports described the characteristic changes of the tumour suppressor gene as guanine-

to-thymine (G-to-T) transversions [144, 306] that occurred with a "remarkable ... uniformity" in a "monotonous pattern of mutations" [154].

These reports, however, came from areas where the population was not only exposed to a high dietary intake of aflatoxin, but where there was also a high incidence of hepatitis B. Gerbes and Caselmann viewed the specific G-to-T transversion in codon 249 as reflecting directly the extent of the exposure of aflatoxin B_1 [138]; it was apparently not present in hepatocarcinoma patients from areas where aflatoxin B_1 was not a risk factor [14, 332]. Cohen and DeRose likewise consider the possible link between aflatoxin and selective mutations at codon 249 of the *p53* gene [61], although they also point out that mutations in the *p53* gene are not necessary for the experimental induction of hepatocarcinogenesis by aflatoxin in non-human primates and rats. According to a recent Japanese study HBV "without aflatoxins was not sufficient to promote the specific mutation of *p53* ...". The carcinogenesis in Japan, especially with the HCV, was viewed as being different from the process in areas where "... other factors such as aflatoxins prevail" [319]. Some earlier critics, however, have pointed out that the association between human HCC and aflatoxin had not been consistently demonstrated [11, 360]; assessments based on ecological correlation studies rather than on – which is rather more difficult – individual assessments [360] were seen as rather crude [351], ignoring a possible role of other environmental factors [71, 123] or of an association of changes in the *p53* gene in HCC with HBV or HCV infections [144, 167], an association that has recently been questioned [138, 299, 335]. From their analysis of 80 HCC from three continents Unsal et al. suggested that codon 249 mutations may characterize a particular, distinct type of HCC [332]. The complexity of the problem is reflected in a parallel conclusion by Harris and Hollstein [154] and Goldblum et al. [144] that, if the incidence of hepatic cancer around the world is considered, these patients can be divided into two groups – one set from areas with a high incidence of tumours that presents the "specific" mutations of the tumour suppressor gene described, and another one from low tumour incidence regions, presenting a mutation spectrum resembling other tumour types – an indication of the genetic heterogeneity of HCC [332]. It may be of interest in this context to point to the recent study by Diamantis et al. who, on the basis of their observations on HCC in Taiwanese patients, concluded that exposure to aflatoxin was "not a major co-factor for the development of HCC in Taiwan" [85], in contrast to other regions such as China or South Africa. They did, however, discover in 12 of 38 of these Taiwanese patients "a new mutational hot-spot codon of the *p53* gene" in codon 166, and tentatively suggested that "the characteristic of hot-spot-specific mutations of the *p53* gene is liver specific". The meaning of this "liver-specific clustering of *p53* point mutations" and a possible role of infection by the HBV need to be studied further.

To these arguments must be added the "compelling" [200], "strong, specific and consistent" [71] evidence pointing to the important part played in hepatocarcinogenesis by hepatitis virus infections in the experimental animal (for examples see [1, 74, 132, 281]) and in man [40, 41, 42, 43, 54, 56, 123, 157, 164, 209, 297, 305], including the hepatitis D virus (HDV) [50] and the "important and hitherto hidden contribution" [336] of infection by the HCV [99, 182, 240, 296, 299, 330] in HBV-negative patients. These views helped to underline the doubts expressed concerning a primary role of aflatoxins in human hepatocarcinogenesis [51, 71, 312], leading Campbell et al. to conclude that "aflatoxin contributes little or no primary liver cancer risk" [61].

This rather apodictic statement is in turn open to question, for experimental studies in infected woodchucks with a superadded HDV infection [50], or in ducks with congenital duck hepatitis virus infection exposed to aflatoxin [73, 74] ("not a promising model for the human situation" [289]!), as well as in human HBV carriers exposed to aflatoxin or other agents [83, 123, 129, 181, 194, 210, 212, 281, 294, 349, 360], have led to the conclusion that viruses "can be viewed most accurately as cofactors in the etiologies of human cancers" [305], exemplified by the "synergy between HBV and AFB" [148a], and to an emphasis on the "probable interaction between viral and chemical agents ..." in the multifactorial origin of primary liver cancers [354] in man as well as in the experimental animal [185, 294, 327]. Here, however, we must realize that "despite the plausibility of an interaction, from epidemiology and the studies in transgenic mice, no strong evidence of a mechanism of interaction" has been produced [350], that "the role of HBV in the etiology of HCC differs among individual tumors" [305], that experimentally in the HBV transgenic mouse, for instance, "exogenous, chemical cofactors are not required for hepatocarcinogenesis" [91] and that in multistep hepatocarcinogenesis "the contribution of each step to primary liver cancer may vary among HBV-infected human populations" [123].

An even more striking example of our uncertainty – intimately linked to histochemical and immunohistochemical studies – is the basic question of the very existence and nature of the hypothetical stem cells of the liver. This has been the subject of much debate, reviewed in some detail by Aterman [17], but there is still no agreement on the "susceptible cells" [153] which give rise to the primary liver tumours. "The exact origin of cancerous hepatocytes is still unclear" [45]. We find, for instance, in the monograph on "The role of cell types in hepatocarcinogenesis" Thorgeirsson and Evarts maintaining that "the existence of hepatic stem cells in adult normal rat liver has been well established" [326] and Sirica et al. [303] corroborating this view, whereas Marceau et al. consider that the question of progenitor or stem cells in the liver is still unanswered [215]. Hixson et al. [163] speak of the "elusive" stem cell that in the experimental animal has still not been identified [309] and whose existence in the human liver too, according to van Eyken and Desmet, is "at present entirely speculative ..." [103]. Farber, however, takes issue with the very concept

of a distinct "stem cell" by pointing out that there is "direct visual, incontrovertible evidence that a few original hepatocytes can be altered during initiation and that some of these altered hepatocytes are the sites of origin for foci, and in turn nodules" [113]. In keeping with this view Hixson et al., who consider that a significant percentage of primary rat HCC are derived from stem cells, also agree that the tumours in certain experimental models may be derived from differentiated hepatocytes [163]. The debate goeson [328], and the fact that papers having a bearing on one or the other aspect of this intricate problem continue to appear in significant numbers (for example [45, 47, 48, 59, 63, 70, 94a, 102, 104, 134, 140, 149, 165, 170, 177, 222, 225, 226, 243, 279, 281, 290, 291, 292, 300, 304, 309, 318, 325] merely underlines the regrettable diversity of opinions about such a fundamental question of the pathology and carcinogenesis of the liver.

There is similarly no agreement on the interpretation of "malignancy as the outcome of step-like deviations", as Rous and Kidd [276] had formulated this forerunner of the now widely-held concept of carcinogenesis as a "multistage" [248, 358] or "multistep" [217] process. In this "radical departure from previous concepts" [105] it is postulated by Dragan et al. [89], Pitot and Dragan [256] and others that a stage of "initiation" is followed by a stage of "promotion" [131] and then of "progression" to malignancy. While this view has its roots in chemical carcinogenesis, it has by now become a generalized concept as, for instance, the recent paper by Cucinotta and Wilson [78] on tumorigenesis by high-charge and energy radiations shows. A short description of this generalization may help in the evaluation of the divergent views published. In this context Maronpot's survey of the nomenclature and of the definitions of the terms generally used will be found useful [217]. While Miller has recently presented a brief historical account of chemical carcinogenesis [223], the morphological and biological characteristics of the stages in this process have been tabulated by Pitot and his associates [252, 253, 255, 256, 258]. They are largely based on the results obtained in the various experimental models that have been reviewed and described repeatedly [33, 44, 87, 112, 120, 147, 248], but whose diversity has contributed to the difficulties in arriving at a uniform nomenclature.

Initiation in chemical carcinogenesis is seen as being determined by the presumptive [118, 120] covalent binding of the "ultimate carcinogen" [76, 224] to cellular DNA [339] with a subsequent irreversible and heritable mutation after replication [109, 204, 313, 353]. "Cancer begins as a carcinogen-induced genetic change in a single cell" [358]. In viral hepatocarcinogenesis it is the integration of HBV DNA into the cellular genome and its subsequent derangement that matters [262, 263, 264, 265, 336, 337]. Of interest here, however, is that such an integration has so far not been observed with the HCV that is also known to be involved in hepatocarcinogenesis in man. According to Kew "there is no evidence that the replicative intermediates [of HCV RNA] become integrated into host DNA", so that "... insertional mutagenesis could be excluded as a pathogenic mechanism" [182]. It may perhaps be relevant to point out in this context that Dunsford et al., who acknowledged the presence of integrated HBV DNA sequences in HBV-related HCC, also suggested that "HBV may be oncogenic in humans by virtue of its ability to induce chronic liver disease rather than by insertional mutagenesis" [91].

Initiation, to continue, is considered to be irreversible and not dependent on a threshold level of the carcinogen. Although its actual mechanism is not yet fully understood [88], the postulate that initiation "without exception ... implies alterations of cellular DNA at one or more sites in the genome" [217] as the cardinal event [336] has become axiomatic [348] and "well accepted by most investigators" [256], and has led to the classification of initiators as "genotoxic carcinogens" capable of inducing mutations. The unqualified emphasis on mutations – see, for instance, Gallagher's "carcinogens are mutagens" [136] – was, however, modified by Wigley [349] and has been questioned again by Farber and Sarma [118], Strauss [313] and Prehn [266]; it was held up by Lijinsky [205] as an example of the blurring of concepts, in this case of the possible distinction between a carcinogen and a mutagen. While Guengerich stressed the importance of mutation at all stages of chemical carcinogenesis [150], Maronpot pointed out that "it has not been unequivocally demonstrated that mutation is a universal, sufficient or necessary prerequisite of all cancers" [217], and Pitot and Dragan presented a partial list of "nonmutagenic carcinogens" [256]. Like Farber [110], Dragan and Pitot maintained that it was "likely that the formation of no single adduct or its ensuing mutation(s) results in the initiation of a hepatocyte that can expand clonally into altered hepatic foci in the presence of a promoting agent", for the "adduct formation and the mutagenesis ... may not be the only molecular change that leads directly to the malignant phenotype" [88]. The general validity of the mutation hypothesis had been questioned already by Willis [352] and by Friedrich-Freksa et al. [132].

The next stage, the stage of *promotion,* is even less well characterized than the preceding stage of initiation [88]. Certain substances – "promoters" – that do not react directly with the cells' DNA can induce cellular changes that, in contrast to the changes in cellular initiation, are threshold-dependent and reversible. This reversibility has, however, been questioned [329]. Some aspects of the action of "hepatopromoters" have been reviewed by, for instance, Moore and Kitagawa [229]. The significant manifestation of the promoting stage is the clonal expansion [95, 118, 119, 141, 186, 229, 254, 260, 269, 346, 348] of a subset of – intentionally or endogenously [255], that is spontaneously (fortuitously) [256, 258, 260] – initiated cells to phenotypically altered "foci" and "nodules". Since we are still hearing some loud echoes from the past it may be permissible to consider in some detail the question of "foci" and "nodules", and of their significance. On reading some of the many publica-

tions on this topic it is not always clear whether a distinction between these new formations has been, or should be, made. Are we to take the notion of the "step-like" development literally or are we dealing with a continuum? While, for instance, Squire and Levitt [308], Stewart et al. [311] and Peraino et al. [248] clearly spoke of "foci or areas of cellular alteration" and of "neoplastic nodules" and Moore and Kitagawa [229], like Farber [107, 110a], pointed to a "sequence of development from small foci of cells ... to nodular lesions", Pitot et al. [260], it seems, used the term "altered hepatic foci" – "the first morphologic lesions seen during the process of multistage hepatocarcinogenesis" – to include both of these "preneoplastic" lesions. To complicate matters further the preneoplastic nature of the altered foci, as opposed to nodules, has been questioned by Farber and his associates [118, 120, 121] on the grounds that in most experimental models the so-called "preneoplastic" foci are not persistent and tend to "mature", "differentiate" or "remodel", so that only very few of them endowed with the "resistance phenotype" and "persistence" progress to form nodules. It is this persistence that "... sets the stage for the truly carcinogenic sequence" [121]. Bannasch et al. [37], however, point to the strong statistical correlation between foci of altered hepatocytes and hepatocellular tumors" and to the fact that "the early emergence of foci of altered hepatocytes (FAH) seems to be a general phenomenon of hepatic carcinogenesis, no matter how this process has been elicited" [359]. The reversibility of preneoplastic lesions stressed by Farber's group appears to be a function of the model involving particularly the high doses of carcinogens used, and is not encountered, at least not to the extent reported in these studies, in models such as the "stop" experiment with low toxicity used by Bannasch and his co-workers [341, 342, 343].

The studies of this group have led Bannasch et al. to "propose that the majority of foci represent different stages in an ordered sequence of cellular changes" leading to the formation of hepatocellular tumours [35], thereby justifying the designation of foci as "preneoplastic". Preneoplasia in this context has been defined by this group of workers [36] as the state of a phenotypically altered cell population not presenting any signs of a preneoplastic nature, but facing a higher probability than the surrounding normal parenchyma of progressing to a benign or malignant neoplasm. "Preneoplastic lesions are believed to have a high propensity to neoplasia" [217], and "foci" are considered to be an early response to carcinogenic agents in a number of species and strains under varied experimental conditions. Foci consist characteristically of "altered cell populations [that] are perfectly integrated into the architecture of the liver parenchyma and to not show any expansive growth" [36] or, at most, cause only "slight compression" [218]. This description is in marked contrast to the features characterizing the "focal proliferations of hepatocytes" [107], also called "hepatocyte nodules" [121] or "hyperplastic nodules, neoplastic nodules, adenomas etc." [108]. These nodules are "discrete proliferations ... sharply demarcated from surrounding liver by compression and tinctorial staining differences ... with loss of normal lobular architecture ... [and] often an increased mitotic index" [218]. The hepatic plates are usually two or three cells thick, tend to impinge at an angle on the surrounding liver plates [218], and there is a change in nodular perfusion with a decrease in the portal, and a normal or increased arterial, blood supply [108]. These changes are, of course, the result of a gradual development and it is therefore not surprising that Farber should distinguish between "early, persistent and ultimate precancerous nodules" [113], agreeing in essence – but not in terminology – with those workers who consider that "foci of cellular alteration, hepatocellular adenoma and hepatocellular carcinoma ... represent a spectrum of changes that comprise the natural history of neoplasia" [218] and form the "early, intermediate and late stages of hepatocarcinogenesis ... morphologically defined as focal hepatic preneoplasia, benign nodular neoplasia (adenoma) and malignant neoplasia/carcinoma" [36].

The frequent use of the term "adenoma" in these descriptions should be noted, since the term "neoplastic nodule" has not only "become synonymous with benign hepatocellular neoplasia or adenoma" [218], but is now apparently being replaced by it [32, 36, 359]. It is interesting to note in this context that in his comments on the early phase of development of the adenoma in the human liver – a tumour known to most pathologists as a single, fairly large and apparently non-malignant mass – Altmann points to the occurrence of smaller, protruding, clearly circumscribed hepatic nodules that can be interpreted as "true microadenomas, an equivalent of the 'preneoplastic nodules' observed in experimental animals" [10]. While these early, precursor, stages in the human have so far not received much attention, experimental studies in a variety of animals have shown abundantly that the progression from foci to benign or malignant tumours involves a number of – frequently sequential – changes in the phenotype of the "altered cells". The cytological aspects of this sequence – involving at least seven different types of foci [27] – have, after a simple start [30, 31] been described in detail and with ample illustrations in a series of papers [23, 32, 33, 35, 36, 220, 341, 342, 343, 359]. Details of the growth properties for instance of liver nodules [97] and of histochemical reactions – a reflection of the major characteristic of the stage of promotion, that is the alteration of genetic expression [257] – have been listed by Pitot [254] and his associates [256, 260] and by Bannasch and his associates [27, 30, 31, 32, 33, 35, 36]. Earlier Peraino et al. had published a helpful account of the histochemical characteristics of the cells of the altered hepatic foci, and of their significance in hepatic neoplasia [248].

It should be noted in this context that, despite the extensive investigation of these potential precursor lesions in the experimental animal, we still face some uncertainty and controversy concerning the biological significance of such altered hepatic foci [221]. Already in 1959 Farber and Ichinose had maintained that nodular hyperplasia

was a lesion "constantly" found during the premalignant phases of experimental hepatocarcinogenesis in the rat [116], and three decades later Dubois et al. described in transgenic mice a "classical sequence of events: hyperplasia, dysplasia, adenoma and carcinoma" [90]. Such a sequential development in transgenic mice was also reported by other workers [91, 137, 327] – a reminder of the optimistic prediction by Yuspa and Poirier [358] that the transgenic mouse (and now also the transgenic rat [170]) – "promises to be the technological advance which may ultimately ... elucidate the precise role of individual genes or modifying factors in each stage of tissue-specific carcinogenesis". A distinct sequence of foci, nodules and cancer in seemingly spontaneous hepatocarcinogenesis in the LEC rat – a mutant strain of Long-Evans rats with a high incidence of spontaneous hepatitis – has been described by Enomoto et al. [96]. In their review of rat liver carcinogenesis Ericksson and Andersson also maintain that "... in all models intermediate focal prestages, nodules, are generated" [97], but since Hirota and Williams, for instance, had not detected any transition of nodules to carcinoma [162] they, like Williams earlier [351], suggested that "the neoplastic nodule is not an obligate precursor to carcinoma". Similarly Bannasch [29] who, with his associates [33, 359] and others, has systematically explored the "sequential cellular and molecular changes during the emergence and progression of ... focal preneoplastic lesions" has pointed to some findings that "suggest that malignant neoplasia may also directly arise from preneoplastic lesions without going through a benign intermediate stage". Goodman et al. consider the preneoplastic focus as a "key stage" also in mouse liver tumorigenesis [148], but in two different strains of mice the prolonged administration of phenobarbitone only produced an increase in the number of hepatic – mostly eosinophilic – nodules which was not accompanied also by an increase in the number of HCC [100, 101].

The difficulty in generalizing is illustrated by some apparently contradictory findings reported in studies of experimental hepatocarcinogenesis in the Syrian hamster. The term "apparently" is used here to point to the possibility, or probability, that these contradictory findings may have been determined by differences in the experimental arrangements rather than by more fundamental differences in the tumour development process itself. As Weinhouse has pointed out, "much of the confusion in cancer research is due to the unfortunate, but understandable, tendency to make generalizations based on limited tumor types" [347]. Hamsters have not been used extensively in such experiments, but the results presented here are indicative of the difficulties in arriving at definite conclusions. Moore et al., for instance, found that aflatoxin B_1, which in the rat primarily damages the parenchymal cells and induces neoplastic nodules and hepatocellular carcinomas, in the Syrian hamster produces cholangiofibrosis and cholangiocarcinomas, but not hepatocellular nodules and HCC [231]. (Biliary proliferation is apparently a feature of aflatoxin exposure also in the duck [73, 74]). In the light of repeated statements (for examples see [21, 22, 23, 25]) that the development of cholangiofibrosis and cholangiofibromas is apparently the result of high, damaging, doses of carcinogens and can be avoided by the use of "relatively low doses" [26, 27], one may well ask whether these different responses of rat and hamster (or duck) to aflatoxin are the result of doses that are "high" for the one, but not for the other species. Perhaps one should here point to the observation by Peterson that phomopsin, a mycotoxin contaminant of lupin plants and seeds, can in *low* doses induce in *rats* not only nodular cirrhosis, but also extensive biliary hyperplasia progressing to cholangiomas and cholangiocarcinomas [251]. He interprets this effect as being due to the "greater bias towards biliary than to parenchymal tumor production by phomopsin" compared to other hepatotoxins. (Of interest here, however, is the fact that the untreated older control animals used in this study – an inbred strain regarded to be Long-Evans rats – also developed spontaneous, age-related foci of bile duct proliferation in variable numbers, progressing in a few animals to cholangiofibrosis.) Are we seeing in these instances differences in the response determined by the species or strain of the experimental animals, by certain specific propensities of the carcinogen, or merely by the size of the dose? When Moore et al. used a carcinogen other than aflatoxin, they could induce in hamsters glycogen-storing and homogeneously basophilic nodules, positive for placental glutathione transferase staining, as well as cholangiofibrotic lesions and cholangiocarcinomas [230]; Thamavit et al. in yet other systems similarly obtained high yields of cholangiocellular carcinomas as well as hepatocellular nodules of the glycogen-storing or of the basophilic type [320, 321], whereas Oberley et al. in their system succeeded in inducing large trabecular HCC, but "hyperplastic (preneoplastic) nodules ... as described for chemical carcinogenesis in the rat, were not observed in any liver section at any time" in the Syrian hamster [239]. Similarly "... no liver nodules and tumors were observed in Syrian hamsters", in contrast to rats fed, like the hamsters, the peroxisome proliferators nafenopin or Wy-14,643 [201]. Coe et al. likewise did not find any preneoplastic lesions in the liver of Armenian hamsters with subcutaneously implanted pellets of diethylstilbestrol [66], although HCC developed after comparatively short intervals. The invariable development of nodular precursor lesions in tumorigenesis in the liver has been questioned by other workers [16, 17], including now also some researchers who previously had firmly adhered to the generalization of the precursor role of nodules in hepatic cancerogenesis. Farber, for instance, who at one time had maintained that so far no model existed in which "hepatocellular cancers occur without a prior appearance of hepatocyte nodules" [118, 120], later corrected this view by pointing out that in a study of 39 small tumours in humans, in which he participated, 4 such carcinomas had been found that "... appeared to arise de novo without a preexisting nodule in close proximity" [60]. In another study Farber also remarked that

foci or islands of altered hepatocytes do occur in carcinogenesis by peroxisome proliferations, but they occur "in very late stages" [112]. Premalignity represents "a poorly defined and broadly constructed area" [244], and "direct evidence demonstrating that foci lead to cancer is lacking" [221]. Farber maintains that such a view may have been based on the overlooked fact that new focal proliferations may grow more quickly than their precursors and thereby destroy them [113].

In the human too "premalignant lesions and the cellular alterations preceding fully developed hepatocellular carcinoma have remained speculative" [281] and the question is "unsettled" [241]. The lack of rigorous, consistent and practicable criteria for the diagnosis of early or borderline hepatocellular neoplasms, recently stressed by Ferrell et al. [126], can undoubtedly account for much of this uncertainty. The difficulties in this area and the need for stringent diagnostic criteria have also been emphasized by Altmann in his detailed morphological treatise on tumorigenesis in the human liver [10]. Of interest here, however, is Altmann's reference to the occasionally documented transition of such benign hepatocellular newformations as "regenerative macronodules" or "adenomas" – but apparently not of "focal nodular hyperplasia" – to malignant variants [10]. Although Wanless in his study of nodular regenerative hyperplasia has not encountered any unquestionable neoplastic changes [338], Altmann points out that such regenerative nodules are exposed to the risk of increasing cellular turnover and its possible consequences [10]. In his description of foci, "heteromorphic" nodules and "microadenomas", the equivalents of the experimental foci and nodules of "phenotypically altered hepatocytes", Altmann points out that "in our own material they [the "microadenomas" or "heteromorphic nodules"] were found particularly often in livers with manifest carcinomas in another area. They resemble exactly the 'storage foci' known from animal experiments ..." [10]. A possible relation between adenoma and carcinoma of the human liver had already been postulated by Heukelom in 1894 [159], when he – a century before Ferrell et al. [126] again had to stress the need for strict criteria – pointed to the difficulty in distinguishing clearly between these newformations. Sixty years later Payet et al. maintained that "l'adénome paraît précéder regulièrement la cancérisation" [247], and another few decades later Schirmacher et al. modified this, still tentative, conclusion by pointing out that while "adenomas seem to progress to HCC's frequently, they do not constitute obligatory precancerous lesions" [281]. Bannasch and Klinge considered it likely that adenomas rich in glycogen had the potential of changing ultimately into rapidly growing tumours [30]. One such case may well have been the "cancer avec cirrhose (adénome)" reported already in 1891 by Martin-Durr who, impressed by the "still very strong" iodine reaction for glycogen in the adenomatous nodules in his case, concluded that this developmental process ("ce processus évolutif") did not seem to impair the glycogenic function of the hepatocyte [219]. A century later Bannasch and associates again had occasion to point to the "predominance of glycogen-rich clear or ground glass (acidophilic) cells" in adenomatous hepatic hyperplasia, adenomas, and some other lesions [27, 28, 36], and suggested that the sequence of phenotypic cellular changes in hepatocarcinogenesis was, in principle, similar in man and experimental animals [34]. Farber and Cameron were similarly impressed by the "probable basic similarity in etiology, pathogenesis and pathology between experimental and human hepatic carcinoma" [115]. Altmann has repeatedly drawn attention to a possible role of the adenoma of the human liver as a precursor of HCC [5–9] and Kharsa et al. [183], like Limmer et al. [207], suggested that HCC always or almost always was the result of the malignant transformation of a hepatocellular adenoma, at least in glycogen storage disease. Okuda, however, who accepted the possible transition of adenomas to malignant tumors in certain metabolic disorders, maintained that otherwise there was "no evidence" of such a malignant transition [241]. Other workers similarly concluded that hepatocellular "adenomas" – the quotation marks here are intended to draw attention to ambiguities in the blurred nomenclature (see for example [235]) – were not premalignant [192, 317] or that malignant transformation was "exceptionally rare" [137, 172]. Evidence is now, however, accumulating that such lesions as macroregenerative nodules with [12, 235, 322] or without [10, 323] cirrhosis, or with the atypical form of adenomatous hyperplasia [233, 234, 235, 278], frequently though not invariably may progress in man and in the experimental animal [137, 359] to malignancy and may already represent transformed or neoplastic lesions [278] whose malignant nature had not been recognized. However, the possibility of HCC developing de novo – without a *demonstrable* nodular precursor lesion – cannot be excluded, as has already been pointed out [125, 278]. This view is supported by the increasing number of studies on small tumours of the human liver [6, 94, 135, 190, 191, 192, 193, 240a, 241], made possible by better diagnostic procedures and by the availability of specimens removed at liver transplantation [60]. Kin et al. [184], for instance, have recently pointed to some small HCC that, at the stage at which they were examined, did not show the changes in the blood supply that several authors [13a, 108a, 109, 118–120, 277] have described in the proliferating hepatocellular nodules.

As in initiation the mechanism by which the selective clonal expansion of the initiated hepatocyte takes place is still not completely resolved [186]. Promoters may act indirectly by influencing growth signals in the surrounding tissue or directly by stimulating proliferation of initiated cells, or by a combination of these factors [158]. In recent years considerable attention has also been paid to changes in the rate of programmed cell death or apoptosis [180] in the altered hepatic foci and nodules [58, 90, 121, 139, 203, 255, 284, 285, 286, 287, 340] – possibly one of the functions of *p53*, the tumour suppressor gene [55, 80, 295, 310]. While the concept of apoptosis itself has become yet another subject of debate [3, 55, 114,

331], there is apparently "no direct evidence for a role of apoptosis in the pathogenesis of carcinogenesis" [331], although Wyllie suggested that "cancer can be conceived as a disease resulting from deficiency in apoptosis" [356]. As far as experimental hepatocarcinogenesis is concerned, however, attention should be drawn to the conclusions of Zerban et al. [359] whose results, somewhat in contrast to an earlier finding by Farber [110, 121] on "cell loss or cell death as a quantitatively important property" of the early persistent nodules, "do not support the concept that cell death (apoptosis) plays a major role in counterbalancing cell replication in foci of altered hepatocytes, but rather suggest – in agreement with Farber et al. [121] – "that cell death occurs more frequently in the course of heptocarcinogenesis the more neoplastic development advances". At any rate, because of the postulated clonal expansion of initiated hepatocytes during promotion, the older theory of the multicentric origin of hepatocellular tumours was replaced by the concept of unicentric genesis [161] – brusquely dismissed by Willis in 1967 [352] as "false" – and the clonal nature of neoplasia [269, 270, 271] has now come to be seen as the cornerstone of multistage carcinogenesis [153, 316, 346, 358], although it has been noted that "at high doses of carcinogen, polyclonal tumours occur" [268]. This polyclonality had earlier been attributed to the probable coalescence of adjacent primary tumours [358]. Maronpot too, although he thought the clonal theory to be "generally true" [217], pointed out that there were certain specific neoplasms that had been shown to have a multicellular and multifocal origin – recalling Willis's earlier "field theory" of cancer [171, 352] which has again been invoked for hepatocarcinogenesis by Bannasch [20, 26, 27, 35] and which finds some experimental support in older studies, cited by Higgins [161], and in the recent studies by Dubois et al. [90] and by Cullen et al. [79] on hepatic carcinogenesis in transgenic mice expressing SV40 T-antigens. Clinical findings also point in this direction [60, 125, 168]; on the basis of their study of surgically resected human liver specimens Sheu et al., for instance, concluded that "multiple hepatocellular carcinomas frequently have different clonalities ..." and stressed the "'field defect' in the development of multiple HCCs [298]". It should be noted in this context that these considerations about the clonal origin of hepatocellular tumours are largely based on studies with genotoxic carcinogens, for "there appear to be no studies on the clonality of tumours induced by non-genotoxic carcinogens" [268].

The next stage, the stage of *progression,* is seen again as being characterized by a combination of irreversible genetic changes in neoplasms and in the "preneoplastic" formations described above. These changes lead to the marked heterogeneity, noted already in the phase of promotion [254], of morphological and behavioural features indicative of malignancy: "Progression refers to malignant conversion"[148]. The currently known characteristics of the stage of progression have been presented by Pitot's group [253, 254, 259]. Although Pitot has stressed the contrast between the instability of the stage of promotion and the stability and irreversibility of established malignancy [254], the distinction between promotion and progression is not always easy and clear cut [57], and the molecular mechanisms underlying this phase of carcinogenesis require further work. It had, however, ben recognized comparatively early in experimental studies that there was one rather consistent feature of the sequence of events described (hyperplasia, benign neoplasia, malignant neoplasia, with dysplasia – now also again emphasized in man [361] – as a questionable intermediate feature): a certain uniformity in that the "neoplastic systems are remarkably similar" [198] and the metabolic patterns in the different models "remarkably uniform" [64]. Others described "an overall seemingly basic pattern of liver cancer development" [121], "a tissue response ... with independence of the original etiologic agents" [60], recalling the "commonality" of Farber [108, 109] and of Eriksson and Andersson [97], and the "common endogenous pathway for hepatocarcinogenesis (CEPH)" activated by "viral, degenerative, mitogenic and chemical carcinogenic effectors" of Geller et al. [137], to which we now can add irradiation [78, 238] and, as pointed out earlier, the development seen in transgenic animals. One should, however, point to the view of Lea who remarked that "there is no reason to believe that there is a single mechanism for hepatocarcinogenesis, but some common controlling elements are being elucidated" [202], and to the comment by Cullen et al. on the "limited morphological repertoire for hepatocytes and biliary epithelium during the process of neoplastic transformation" [79], – the "beschränkten Reaktionsformen" (limited responses) of the liver emphasized already by Thomas [324].

The conceptual importance of the original interpretation of carcinogenesis as developing in two stages, later widened to the current view of it as a multistage process, did not prevent, indeed probably stimulated, critical analysis of various aspects. Willis considered the two-stage theory to be "almost wholly speculative" [352], and some later workers, for instance Marks and Fürstenberger, expressed serious "doubt that there exists already something like a two-stage theory" [216], although they were equally convinced that this approach could lead to "a more generalized concept of carcinogenesis". Iversen, however, was concerned about the "weakness" of the paradigm of the two-stage theory [174], and Weinberg also pointed to the "regrettable" fact that faith in the model of initiation, promotion and progression was "not reinforced" by the data that had been accumulated in the last few years [344]. The "electrophilic-genotoxic theory of carcinogenesis" could not explain all instances of chemically induced cancer [14]. Cohen and Elwein likewise pointed to the confusion of the terminology of initiation, promotion and progression [68, 69]. The fact that many agents did not fit into this model, and the difficulty of defining the term "carcinogens" led these and others workers (such as Emmelot and Scherer [95], Conway et al. [72] and Purchase [268]) to propose instead a division

into "genotoxic" and "non-genotoxic" chemical agents – "mindful that cancer is in all cases a genetic disease" [14]. Genotoxic agents or their metabolites interact, as has already been pointed out, *directly* with DNA, whereas "non-genotoxic" chemicals are substances that do not form DNA adducts, do not induce DNA repair and give negative results to certain in vivo or in vitro tests of mutagenicity [143, 221] – the "indirect" carcinogens of Purchase [268]. Jackson et al. [175], like Ashby [14], however, cite certain studies that have shown that although the primary action of these putative non-genotoxic chemicals does not involve a reaction with DNA, they may "yield genotoxic events as a secondary result of other induced toxicity ...", and point to a group of substances, the peroxisome proliferators, seen as a "new group of carcinogens" by Rao et al. [273, 274] that, according to some, lead to oxidative damage to the genome by an increase in the volume of peroxides now produced [143, 272]. Kraupp-Grasl et al. have, however, pointed out that the peroxisome proliferators "should not necessarily be considered as a uniform group" [195]. The mechanism of cell injury by activated oxygen species has been discussed by Farber et al. [122], Guyton and Kensler [151] and by Kehrer [179] according to whom, however, only an "equivocal" answer can be given to the question whether changes mediated by free radicals are a major cause of tissue injury and human disease. Purchase shares these doubts [268]; there is no "unequivocal evidence of DNA damage after peroxisome proliferation". Perhaps it should be pointed out here that these studies are all based only on experiments with rodents whose hepatocytes respond to peroxisome proliferators; human hepatocytes apparently do not develop this response [268].

The question is still further complicated by the fact that it had been known for some time that the prolonged administration of certain putative non-genotoxic "promoter" substances – and that includes "almost all" of the then known chemical promoters [283] – may lead to the emergence of malignant tumours. This effect had been explained tentatively by the promoting action of these substances on endogenously initiated cells [195, 196, 208, 228, 255, 329], the results of the "enormous DNA damage rate by normal endogenous oxidants" [12]. The concept of "endogenous initiation" has, however, been viewed by Perera et al. only as an interesting hypothesis and not a fact [250], and by Farber as "scientifically indefensible" [112], "unproven speculations that so far are untestable" [142]. An interesting aspect of this problem is the fact that an "extensive" re-examination by Jackson et al. of such putative non-genotoxic carcinogens has shown that, if adequately tested, many of these substances do induce gene or chromosome mutations or aneuploidy [175], although it is "not clear whether the mutagenicity of these compounds plays an important role in their carcinogenicity ...". The conclusion by Rao and Reddy, that the role of peroxisome proliferators as initiators has now been "clearly established" and negates the old concept that they are "selective tumor promoters" [272], should be seen in the context of these studies. Such uncertainties do not detract from the importance of the concept of non-genotoxic carcinogens, for "the term embraces carcinogens with a number of known mechanisms and some where the mechanism has yet to be elucidated" [268]. As Ashby and Paton have stressed in their extensive study of carcinogenesis by a number of experimental and some fewer human carcinogens, there occur "mechanistically distinct processes" depending on the nature of the carcinogen [15]. While the carcinogenicity of "structurally-altering [genotoxic] chemicals resides mainly with DNA-reactivity of the test chemical or its metabolites ... the stimulus for non-alerting [non-genotoxic] chemicals is a function of a potentially idiosyncratic response of the test species to protracted exposure to the agent".

It should not be difficult to expand this, admittedly selective, account of ambiguities and uncertainties in our current knowledge of hepatocarcinogenesis. If, as seems likely, the reflections presented here will strike some readers as being rather pessimistic, the answer (as pointed out already in the introductory remarks) can only be that much of the intellectual stimulation and challenge of the problem of tumorigenesis in the liver resides in the very complexity of this question, and that we must continue, in Karl Popper's phrase, to "falsify" our hypotheses. Only that way can the pessimist retain his reputation of being a well-informed optimist.

Appendix

For reference purposes a short list of arbitrarily selected papers on various aspects of carcinogenesis is appended. The introductory chapters of "Cancer: Principles and practice of oncology" 4th edition, volume 1, edited by V.T. DeVita Jr, S. Hellman and S.A. Rosenberg and published by J.B. Lippincott Company, Philadelphia (1993) present a very helpful general survey. Problems in experimental and human hepatocarcinogenesis are discussed in "Introduction to the cellular and molecular biology of cancer" edited by L.M. Franks and N.M. Teich, published by Oxford University Press (1986), "Cancer Biology" by Ruddon (1987), "Theories of carcinogenesis" edited by O.A. Iversen, published by Hemisphere Publishing Corporation, Washington (1988), "Liver cell carcinoma" edited by P. Bannasch, D. Keppler and G. Weber (Falk Symposium 51) published by Kluwer Academic Publishers, Dordrecht (1989) and "Hepatocarcinogenesis in the rat" by R. Hasegawa and N. Ito in "Carcinogenesis" edited by J.P. Waalkes and J.M. Ward published by Raven Press, New York (1994) pages 39–65. Additional references are: for carcinogenesis, nomenclature and problems see [15, 46, 62, 69, 95, 96, 118, 119, 120, 148, 153, 169, 206, 217, 228, 283, 293, 358]; for initiation, promotion and progression see [64, 72, 88, 89, 148, 158, 248, 254, 255, 288, 329]; for peroxisome proliferators and oxidative stress see [11, 12, 18, 62, 72, 82, 112, 122, 128, 146, 152,195, 196, 228, 268, 272, 275]; for experimental models see [87, 110, 113, 118, 119, 120, 121, 173, 241, 254]; for membrane biochemistry and intercellular communication see [97, 127, 186, 187, 197, 213, 348]; for viral hepatitis see [71, 77, 123, 124, 167, 178, 194, 202,232, 236, 262, 263, 264, 265, 267, 281, 282, 305]; for human hepatocellular cancer see [19, 71, 86, 110, 178, 181, 190, 191, 193, 199, 207, 232, 235, 241, 278, 280, 322, 323, 344, 348]; for genes and genetics see [13, 18, 49, 52, 53, 65, 69, 75, 79, 84, 130, 144, 154, 158, 166, 202, 227, 303, 307, 345].

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