Proc. Nat. Acad. Sci. USA
Vol. 68, No. 9, pp. 2032–2036, September 1971

Pure-Strain and Genetically Mosaic Liver Tumors Histochemically Identified with the β-Glucuronidase Marker in Allophenic Mice

(gene control of neoplasia/hepatoma susceptibility)

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Communicated by André Lwoff, May 28, 1971

ABSTRACT **A histochemical procedure for β-glucuronidase has been used to make visible the cellular genotypes of liver tumors and of surrounding normal liver clones in allophenic mice. The animals had lifelong genetic mosaicism for cells with the allele for low β-glucuronidase activity (*g/g* genotype, C3H strain) and cells with the allele for high activity (*G/G* genotype, C57BL/6 or BALB/c strain). The former strain is also hepatoma-susceptible; both the latter are nonsusceptible. Of 12 "spontaneous" hepatomas examined, nine were entirely of susceptible-strain hepatic cells and one was of the nonsusceptible strain; the pure-strain tumors usually arose in a liver environment containing clones of each genotype. The cells therefore behave largely autonomously with respect to gene control of tumor susceptibility. However, two tumors with malignant cells of both genotypes were formed, which suggests some measure of intercellular transmission of tumor information. Alternatively, transformation might have occurred in two or more cells concurrently. Mosaic tumors in either case imply that even a hepatoma of one inbred strain, whether in a single-genotype animal or an allophenic mouse, may comprise diverse clones of transformed cells. Possibly many or all hepatomas may therefore be genetically complex entities.**

The occurrence of strain-specific malignancies in mice is strong evidence that significant aspects of tumor development are under genetic control (1). Allophenic mice (2, 3) are genetically mosaic animals that present unique possibilities for *in vivo* experimental analyses of the nature of genetic susceptibility to neoplasia. Each allophenic individual is derived from two genotypically different cleavage-stage embryos whose blastomeres are artificially aggregated *in vitro*. The resultant composite, after transfer to a pseudopregnant surrogate mother, gives rise to a viable mouse with permanent immunological tolerance (4) for its two cell strains. In such animals, cells with specific tumor susceptibilities can be made to coexist with nonsusceptible cells throughout life.

Liver tumors are among the malignancies already investigated by means of biochemical markers in allophenic mice originating from high-liver-tumor C3H (or C3Hf) strain cells combined with cells of the low-tumor C57BL/6 strain (5). Hepatomas occurred with high frequency and were predominantly of susceptible-strain cells despite the fact that, in some individuals, the neighboring normal liver tissue, and also other tissues in the body, were composed chiefly of C57BL/6 cells.

In the present study, hepatocarcinogenesis was analyzed *in situ* by applying to tissue sections from allophenic mice a histochemical method (6–8) for visualization of the enzyme β-glucuronidase. Previous biochemical work (9) showed that activity of this enzyme differs in inbred strains, depending on allelic differences at a single locus. Enzyme in normal livers from wild-type (*G/G* or +/+) strains (e.g., C57BL/6 or BALB/c) has substantially more activity than does C3H mutant-strain (*g/g*) liver enzyme, and is less readily heat-inactivated.

We therefore applied the histochemical procedure for β-glucuronidase to adult normal liver sections of these pure strains and found that *G/G* strains showed positive staining in *all* hepatic cells, whereas *all g/g* strain hepatic cells were negative. It thus became possible, in *g/g* ↔ *G/G* allophenics, to trace the normal clonal history of the liver, to diagnose the genotypic composition of liver tumors, and to observe directly the topographical relationships between tumor and normal cells of high- and low-susceptibility genotypes. We find that most hepatomas are composed of pure-strain (usually susceptible-genotype) cells, despite proximity to cells of the other strain. However, some genetically mosaic tumors are also formed, which possibly indicates intercellular effects.

MATERIALS AND METHODS

Allophenic mice

10 allophenic mice were chosen for study out of a larger population, because they showed liver tumors in autopsies conducted at 588–797 days of age. All were produced by methods reviewed in detail elsewhere (10). Three mice were obtained by aggregating blastomeres of the high-liver-tumor C3H strain (C3H/HeNIcr subline) and of the low-tumor BALB/c strain (BALB/cAnNIcr subline); in the remaining seven, C3H cells were combined with low-tumor C57BL/6 cells (C57BL/6JNIcr subline). All had ICR randombred (low-tumor) surrogate mothers. Partial hepatectomy was performed on animal 8 (Table 1) at 1 year of age. At autopsy after cervical dislocation, samples of liver tumors and of normal liver were fixed in glyoxal for histochemistry, fixed in neutral formalin for histology, and frozen in solid CO_2 for analysis of strain-specific electrophoretic variants of proteins.

Histochemical visualization of β-glucuronidase

Available methods (6–8) were used with little modification. Small pieces of tissue were fixed for 18–24 hr at 4°C in a solution of 5% glyoxal in 0.2 M sodium phosphate buffer with 0.3 M sucrose, at pH 6.5; rinsed for 3 hr to 2 days at 4°C in the same solution minus glyoxal; blotted; snap-

This is part II of a series entitled *Gene Control of Neoplasia*. Part I is ref. 17.

frozen, and immediately cut in a Slee-Pearse cryostat at 4 or 8 μm; and picked up on a cover glass. The method of Hayashi *et al.* (6) for enzyme visualization was followed, with naphthol AS-BI β-D-glucuronide as substrate and hexazotized pararosanilin as post-coupling agent, except that drops of reaction mixture were placed on the mounted sections instead of on floating sections. After incubation at 37°C for 30 min, the sections were rinsed in distilled water, counterstained in methyl green, dehydrated in ethanol, cleared in xylene, and mounted with Permount.

Biochemical genotypic analyses of liver proteins

NADP-dependent Malate Dehydrogenase (Mdh-1 Locus). Alleles at this locus produce electrophoretic variants of enzyme in tissue supernatant fractions (11); C3H and C57BL/6 are allelically different. Identification of these strain variants in allophenic livers and liver tumors was made in starch gel according to the procedure of Baker and Mintz (12). The enzymes in hepatomas of the separate control strains retain the electrophoretic characteristics that they possess in normal livers of these strains (5).

Glucosephosphate Isomerase (Gpi-1 Locus). This enzyme (EC 5.3.1.9) exists in allelically different electrophoretic forms (14); C3H and BALB/c strains have different alleles. Electrophoresis of supernatant fractions from tissue homogenates was performed overnight at 180 V in 13% starch gel with imidazole–EDTA buffer (pH 6.7 in gel, 7.3 in bridge) (R. Niece and B. Mintz, unpublished), and gels were stained as described previously (14). It should be noted that, while NADP-malate dehydrogenase is not detected in starch gels of blood cell lysates, glucosephosphate isomerase is plentiful in blood cells; therefore, the latter contribute a "contaminant" that must be taken into account in genotypic analyses of all vascularized tissues.

Major Urinary Protein (Mup-1 Locus). A major protein fraction normally excreted in mouse urine is manufactured in the liver and occurs in allelic, electrophoretically distinguishable forms (15, 16). We have found that results with this *Mup-1* marker in urine samples taken from allophenic mice before autopsy are in agreement with genotypic analyses obtained with the *Mdh-1* marker in livers of the same animals

TABLE 1. *Genotypes of liver tumors and normal liver regions in allophenic mice with histochemical and biochemical markers*

Animal no. and sex	Strain combined with C3H	Tumor type and lobe[a]	Genotype(s)[b] of tumor in % C3H:C57BL/6 or :BALB/c		Genotype(s)[b, c] of normal liver in % C3H:C57BL/6 or :BALB/c									
					L1		L2		L3		L4		L5	
			Histo.	Bio.	Histo.	Bio.	Histo.	Bio.	Histo.	Bio.	Histo.	Bio.	Histo.	Bio.
C3H (*g/g*) tumors														
1 M	BALB/c	Hep.,*p*,L3	100:0	C3H ≫ BALB/c				80:20	*80:20*					85:15
2 M	BALB/c	Hep.,*w*,L1	100:0		*80:20*		80:20	75:25						
3 M	BALB/c	Hep.,*w*,L1	100:0	C3H ≫ BALB/c	*100:0*	*95:5*	*100:0*		100:0	90:10		95:5		95:5
		Hep.,*w*,L2	100:0											
4 M	C57BL/6	Lymph.,L1	100:0[d]		*60:40*	67:33	90:10	55:45	0:100	C3H < C57BL/6	20:80		20:80	
5 F	C57BL/6	Hep.,*w*,L2	100:0	100:0			*100:0*		*100:0*	*100:0*				
		Hep.,*w*,L3	100:0	100:0										
6 F	C57BL/6	Hep.,*w*,L2	100:0	100:0	100:0		*100:0*	predom. C3H						
7 F	C57BL/6	Hep.,*p*,L3	100:0	100:0	100:0	100:0	100:0	100:0	*90:10*		100:0			
10 F	C57BL/6	Hep.,*p*,L2	100:0	100:0		100:0	*100:0*	100:0		100:0		100:0		100:0
C57BL/6 (*G/G*) tumor														
8[e] M	C57BL/6	Hep.,*w*,L3	0:100	10:90	←(partial hepatectomy)→				*100:0*	*100:0*	50:50	100:0	100:0	100:0
Mosaic (*g/g* + *G/G*) tumors														
9 M	C57BL/6	Hep.,*p*,L3	75:25	predom. C3H	50:50	45:55	50:50	67:33	*75:25*		20:80	40:60		85:15
10 F	C57BL/6	Hep.,*p*,L1	50:50	100:0										
		Lymph., secondary	95:5[d]			100:0	100:0	100:0		100:0		100:0		100:0

[a] Hepatomas (hep.) are classified as *p* (partially differentiated) or *w* (well differentiated). Lymph. indicates a lymphoma. Lobe numbers are designated L1 through L5, in order of decreasing size.

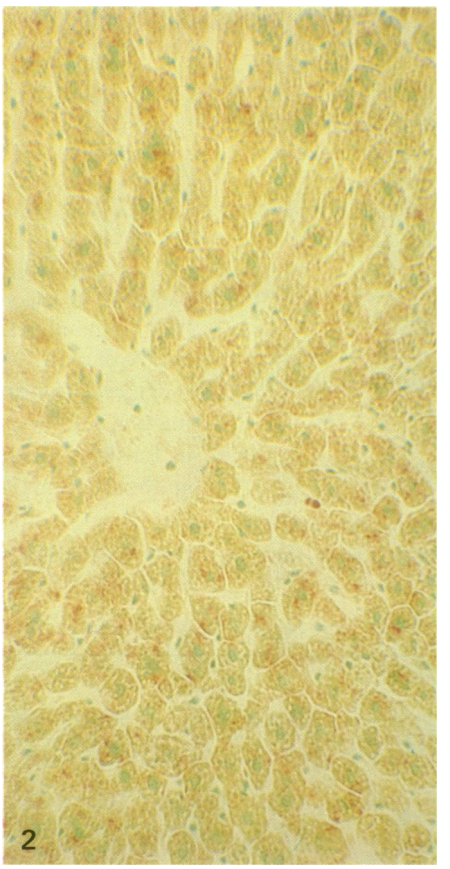
[b] The histochemical marker was β-glucuronidase (*g*-locus) in all cases. The biochemical marker was *glucosephosphate isomerase* (*Gpi-1* locus) in cases 1–3, and malate dehydrogenase (*Mdh-1* locus) in cases 4–10. The *Gpi-1* locus (but not the *Mdh-1* locus) is active in blood cells; see text.

[c] *Italicized* results are from normal tissue adjacent to the tumor.

[d] See text.

[e] Analyses of the major urinary protein variants (*Mup-1* locus) in this case gave the following results: before hepatectomy (age 338 days), 60:40, after hepatectomy (365 days), 70:30. Autopsy was at 638 days of age.


1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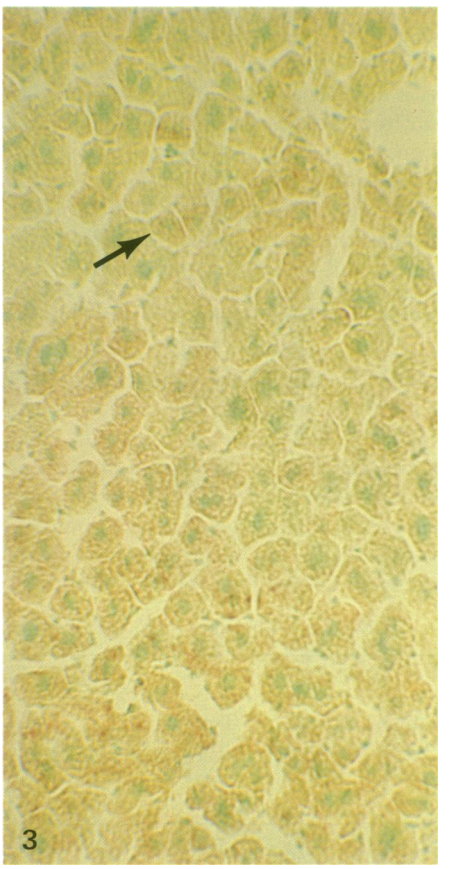
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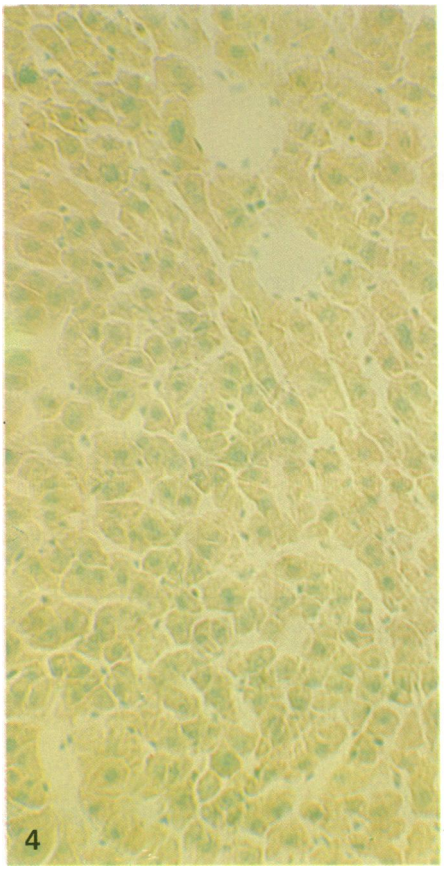
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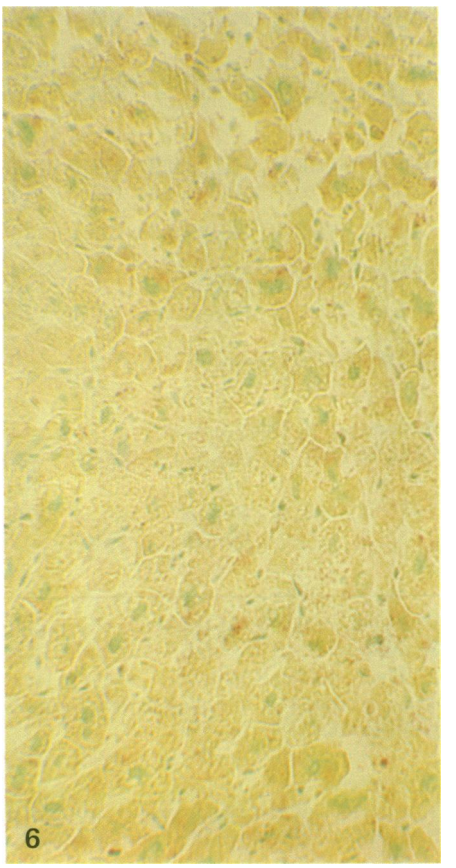
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at autopsy; therefore, *Mup-1* can be used to diagnose liver genotypes in living allophenic mice (W. Baker and B. Mintz, unpublished data). Urine was dialyzed against distilled water, lyophilized, redissolved in water to a 2% solution, electrophoresed overnight at 150 V in 13% starch gel with Tris-acetate buffer, pH 5.5, and stained with Buffalo-Black.

RESULTS

Hepatocytes and reticuloendothelial cells in adult C57BL/6 and BALB/c control livers stained for β-glucuronidase activity contained clusters of brilliant-red discrete cytoplasmic granules. All cells examined in serial sections were positive, with granules visible in almost every cell of single sections cut at the thicknesses employed here (Fig. 2). C3H liver cells processed in the same way failed to display the red stain (Fig. 1). Presumably the C3H liver enzyme, which is biochemically detectable (9), is totally inactivated by the fixation and (or) staining procedure; further inactivation of this relatively more heat-sensitive enzyme is therefore unnecessary and merely diminishes the visibility of the *G/G* enzyme.

Previous biochemical analyses of normal allophenic livers, with the NADP-malate dehydrogenase marker, have shown that each lobe can contain both cellular genotypes (13). This multiclonal origin of each lobe is now strikingly confirmed with the β-glucuronidase marker (Fig. 3). Small, variable-size patches of each genotype are seen. Some patches are presumably single clones or parts of clones from one genetically determined cell, others include adjacent clones of like genotype. Individual lobules of mixed genotypes are commonly encountered, despite their relatively small size. The liver clonal number may thus be rather large; this might provide a basis for functional specialization. Further data from normal mosaic livers will be presented in detail elsewhere and their interpretation will be discussed.

All allophenics had both component cell strains visible in the coat, indicating that in no individual had one genotype been completely eliminated. Nevertheless, in some cases (animals 5, 10) the liver was entirely of one strain (Table 1), as already found in other studies (13, 5).

14 liver tumors were found in the 10 allophenics at autopsy, including 12 hepatomas of various degrees of differentiation and two lymphomas (one a metastasis from the spleen). Tumor diagnoses were made from formalin-fixed sections stained with hematoxylin and eosin, as well as in the histochemically prepared material. Of the hepatomas, the large majority (nine tumors) had only unstained, and therefore presumably C3H, tumor cells (Fig. 4). That this lack of staining truly reflected cell genotype, and was not due to switching off of β-glucuronidase synthesis during tumorigenesis, was demonstrated by the biochemical results: all five hepatomas in the group analyzed with the *Mdh-1* marker (absent in blood cells) showed only the C3H electrophoretic variant. (Very minor BALB/c components in the two hepatomas analyzed for *Gpi-1* variants are attributable to vascular contamination.) Some of the hepatomas composed of C3H malignant hepatocytes also contained some positively-stained C57BL/6 or BALB/c reticuloendothelial cells that were ostensibly normal.

One hepatoma (animal 8) was entirely C57BL/6, judging from the variable but definite staining in all cells (Fig. 5). The biochemical analyses disclosed 10% C3H, which suggests either that normal surrounding tissue [seen histochemically (Table 1) to be C3H] accompanied the tumor sample, or that this tumor was really mosaic and that sections used for histochemistry passed through an exclusively C57BL/6 area.

Both histochemical and biochemical data on normal liver parts revealed that most of the pure-strain hepatomas arose in livers with some genotypic admixture of cells. Much more striking is the fact that five of these hepatomas (in animals 1, 2, 6, 7, and 8) arose in individual lobes containing cells of both genotypes, with both normal cell strains sometimes present at the very margin of the single-genotype tumor (Table 1). The contrast between genotypes of tumor and of surrounding normal cells is especially marked in animal 8 (Fig. 5).

The most surprising result was that both unstained and β-glucuronidase-stained cells, both apparently malignant, were found in two hepatomas (Figs. 6 and 7). It seems consistent with the pure-strain results to interpret the mosaically stained tumors as mixtures of C3H and C57BL/6 cellular genotypes. Animal 9 exhibited a high degree of mosaicism in every liver lobe. However, animal 10 had few histochemical samples taken; its liver (apart from the first-lobe tumor itself) may have been mosaic, but samples failed to include mosaic areas.

The tumor cells of mouse 9 showed rather poor developmental differentiation of both the stained and the unstained cells; the β-glucuronidase-stained cells were in the minority, usually arranged in irregular discrete patches surrounded by unstained tissue (Fig. 7). The first-lobe hepatoma of mouse 10 consisted of more extensively intermingled and better differentiated hepatic cells of both genotypes, and rather abundant, presumably nonmalignant, reticuloendothelial cells (Fig. 6). The fact that the biochemical analysis of this tumor disclosed only C3H rules out the possibility that the unstained tumor cells were merely a negative artifact, and implies that the degree of mosaicism varied from one part of the tumor to another.

Two lymphomas were found, for which only histochemical

FIGS. 1–6. All tissues are stained for β-glucuronidase activity, visualized as red dye deposit; methyl green counterstain. C3H cells appear negative for the enzyme; C57BL/6 or BALB/c cells are positive. (*1*) Normal liver from a C3H adult control. No enzymatic staining is visible (×240). (*2*) Normal liver from a C57BL/6 adult control. The plane of section passes through one or more clusters of red granules in almost every cell; a generalized diffuse cytoplasmic orange stain is also seen (×240). (*3*) Normal liver from a C3H ↔ C57BL/6 allophenic mouse. Cells of each genotype are seen in clusters. Note a row of C57BL/6 (red) cells (*arrow*) radiating from the center of a lobule, and another patch in the lower part of the figure; C3H (unstained) cells are in the middle (×240). (*4*) Tumorous and normal liver from allophenic animal 2 (C3H ↔ BALB/c). The hepatoma (*lower left half*) is composed of unstained C3H cells whose growth is compressing the surrounding normal BALB/c tissue (*upper right half*, stained) (×240). (*5*) Tumorous and normal liver from allophenic animal 8 (C3H ↔ C57BL/6). The hepatoma (*below*) is composed of C57BL/6 cells (vacuolated and stained red) and is surrounded by normal C3H (unstained) tissue (*above*) in which several intensely stained (C57BL/6) reticuloendothelial cells can be seen (×240). (*6*) Genetically mosaic hepatoma from the largest liver lobe of allophenic animal 10 (C3H ↔ C57BL/6). Both stained and unstained tumor cells are present (×240). (*Note added in proof: Much of the red-color component has been lost in reproduction.*)

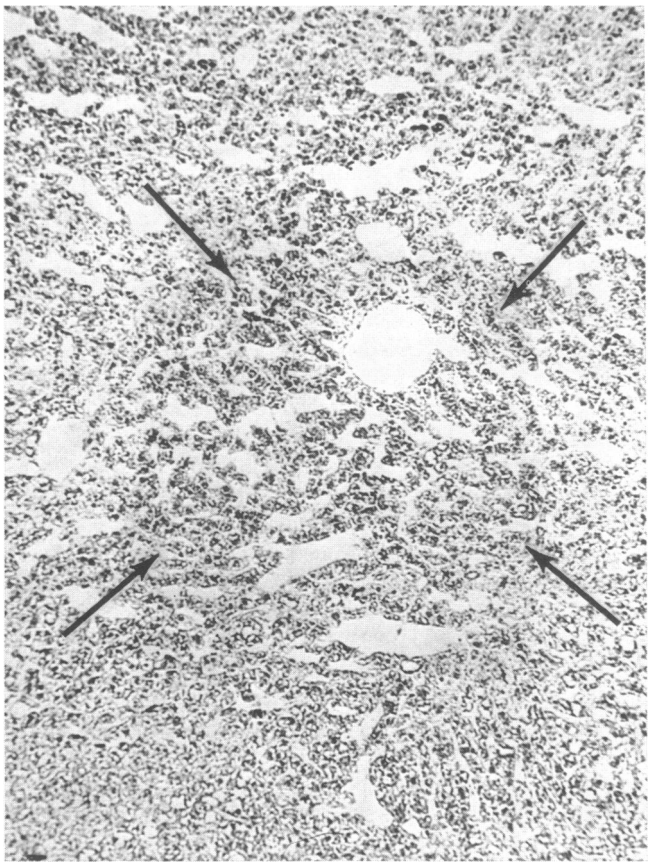

FIG. 7. Genetically mosaic hepatoma from allophenic animal 9 (C3H ↔ C57BL/6). A C57BL/6 (red-stained) core (*arrows*) is surrounded by C3H (unstained) tumor cells (×100).

data are available. Although they are classified in Table 1 as *g/g* and mosaic, respectively, according to staining properties, those diagnoses should be considered tentative, since we have found that, in normal C57BL/6 spleens, not all lymphocytes display positive β-glucuronidase staining.

DISCUSSION

The lifelong confrontation *in vivo* of tumor-susceptible and nonsusceptible cell strains appears, in most cases, to result in autonomous behavior of each strain in both mammary gland (17, 5) and liver (5). Moreover, the genotypes of tissues extrinsic to the potentially tumorous organ seem not to be pivotal (5). The present histochemical studies on hepatomas support the generalizations from the biochemical studies, by demonstrating prevalence of all-susceptible-strain tumors over nonsusceptible ones, and independence of tumor genotype from that of surrounding normal liver cells. These results suggest that susceptibility-controlling loci are chiefly expressed within the liver cells themselves, and that neither the phenotype produced by "susceptible" alleles (in C3H) nor by "nonsusceptible" alleles (in C57BL/6 or BALB/c) is transmitted intercellularly to any marked degree.

At the same time, the superior resolving power of the histochemical method reveals that some genotypically mosaic liver tumors do in fact form, and that they seem to be comprised of malignant cells of both genotypes. Thus, gene control of hepatoma formation may differ in some essential respects from that of mammary tumor formation. In mammary tumors of allophenic mice, C57BL/6 cells can be intermingled with C3H cells, but the former remain phenotypically normal while the latter become malignant (17). Hepatic carcinogenesis may involve a greater measure of cell interaction or intercellular transmission of tumor information. Conceivably, this might include transfer of some viral agent, although no virus has yet been identified as causal in hepatomas.

On the other hand, the mosaic tumors may signify independent occurrence of malignant transformation in two (or more) cells. The tumors might, then, have arisen from intercalation of two independently originating tumors, or individual tumors might indeed sometimes originate from two (or more) transformed cells.

In any case, the mere existence of mosaic tumors indicates that a hepatoma, even in a single-genotype animal, should be conceived of as a genetically complex entity that may well comprise disparate clones of transformed cells rather than a uniform pure clone.

This investigation was supported by U.S. Public Health Service grants HD 01646, CA 06927, and RR 05539, and by an appropriation from the Commonwealth of Pennsylvania. The first author (H.C.) was the recipient of a Damon Runyon Memorial Fund Postdoctoral Fellowship.

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