PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 463-468 (1976)

New Aldehyde Dehydrogenase Isozymes in Chemically Induced Liver Tumors in the Rat[1] (39419)

ROBERT N. FEINSTEIN, R. J. MICHAEL FRY, ERMA C. CAMERON, CARL PERAINO, AND HAROLD P. MORRIS

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439; and Department of Biochemistry, Cancer Research Unit, Howard University, Washington, D.C. 20059

We have earlier reported (1, 2) that liver tumors induced in Sprague–Dawley rats by the feeding of acetylaminofluorene (AAF) will exhibit new isozymes of aldehyde dehydrogenase (Ald D). These new forms can be distinguished from those of normal rat liver by several criteria: (a) normal Ald D is NAD-specific on polyacrylamide gels, while tumor Ald D can use either NAD or NADP; (b) normal Ald D isozymes have pI values in the range of 6.2–6.4, whereas those of liver tumors have a neutral pI (pH 6.9–7.2); (c) the new forms are all cytoplasmic, whereas normal liver Ald D is distributed between cytoplasm and particulate fractions; and (d) the tumor isozymes are more stable than the normal forms to heat, pH extremes, guanidine, and urea. The purpose of the present communication is to describe the relationship of the new Ald D forms to species, strain, tissue, and carcinogenic agent. Liver tumors discussed in this paper range from those sometimes called "hyperplastic nodules" (3) to malignant adenocarcinoma; it was found that neither the Ald D isozyme pattern nor the total enzyme activity correlated with the degree of cellular differentiation.

Materials and methods. Animals used were weanling male Sprague–Dawley and Buffalo rats. They were fed the diets described by Peraino *et al.* (4): (a) normal diet (30% casein); (b) normal diet containing 0.05% phenobarbital; or (c) normal diet containing 0.02% AAF or DAB (dimethylaminoazobenzene). Three dietary schedules were employed to induce liver tumors: (a) AAF or DAB diet for 18 days, then normal diet thereafter; (b) AAF for 18 days, then phenobarbital diet thereafter; or (c) four cycles, each consisting of 4 weeks on AAF diet, followed by 1 week on normal diet. This last regimen resembles that described by Reuber (5).

Liver tumors were also induced with ethionine in a manner similar to that described by Epstein *et al.* (3). Weanling Sprague–Dawley male rats were fed a diet containing 0.25% ethionine for 5 weeks, then a diet containing 0.5% ethionine for 3 weeks, and finally a diet containing 0.8% ethionine for 8 weeks, followed by a normal diet thereafter.

Ald D activity was assayed as described earlier (1); essentially, this assay measures the increase in A_{340} due to reduction of NAD or NADP to the reduced form, as benzaldehyde is oxidized. Polyacrylamide gel electrophoresis (PAGE) was done using the Canalco apparatus and reagents, and isoelectric focusing on polyacrylamide gel slabs was performed with the Multiphor apparatus (LKB Produkter, Bromma, Sweden), modified for greater flexibility as decribed elsewhere (6).

Results. In Sprague–Dawley (S.-D.) rats we were able to induce liver tumors by AAF or DAB by any of the dietary regimens of Peraino described above (Table I), and the tumors invariably resulted in the production of the new isozyme forms of Ald D. In Buffalo rats liver tumors could be produced by AAF only by the four-cycle regimen of Reuber (5); however, all liver tumors produced in a Buffalo rat by AAF invariably showed the new Ald D isozyme forms having the characteristics described in the introduction.

After ethionine feeding, considerably fewer tumors developed. These tumors, with a single exception, were lower in Ald D activity than normal liver, and showed none of the characteristics of the Ald D of AAF

[1] Work supported by the U.S. ERDA and USPHS Grant No. CA 10729.

or DAB tumors. The single exception was a late developing hepatocellular carcinoma which showed Ald D isozymes of high activity and characteristics similar to those in AAF- or DAB-induced tumors.

Table II indicates that the high Ald D activity observed in AAF- or DAB-induced liver tumors is not found in some other liver tumors tested, including several Morris hepatomas, or in a variety of tumors of other tissues.

Table III describes the Ald D activity of nontumorous rat liver. Particularly noteworthy is the fact that, unlike the more common situation with other isozymes in liver tumors, the Ald D activity of the tumor does not recapitulate the fetal liver pattern. Not only is the total Ald D activity of fetal liver much lower than in the AAF and DAB liver tumors, but the fetal enzyme has a specific requirement for NAD and has a pI like that of the normal liver isozymes.

Figure 1 compares the isoelectrically focused Ald D isozyme patterns of AAF, DAB, and ethionine tumors, and of normal liver, all with NAD as coenzyme. It is of interest to note that (a) the lower pI isozymes (pH 6.2–6.4) of normal liver are still expressed by the tumors induced by AAF and DAB; (b) the AAF and DAB tumor isozymes appear qualitatively similar (pI 6.9–7.2), although in this particular instance there is some quantitative difference; and (c) the ethionine tumor (a hepatocellu-

TABLE I. ALDEHYDE DEHYDROGENASE ACTIVITY OF RAT LIVER TUMORS AS RELATED TO RAT STRAIN AND CARCINOGEN FED.[a]

Rat strain	Tissue	Dietary regimen	Ald D activity[b]		
			Number	Mean ± SD	Range
S.-D.	Normal liver	Normal diet	15	65 ± 14	39–88
S.-D.	Liver tumor	AAF, then normal	6	215 ± 106	74–355
S.-D.	Liver tumor	AAF, then phenob.	10	239 ± 94	91–390
S.-D.	Liver tumor	AAF, 4 cycles	33	187 ± 348	14–2010
S.-D.	Liver tumor	DAB, then phenob.	3	374 ± 128	228–436
S.-D.	Liver tumor	Ethionine	28	17 ± 13	0–48
S.-D.	Liver tumor	Ethionine	1[c]	152	
Buffalo	Normal liver	Normal diet	3	68 ± 6	62±73
Buffalo	Liver tumor	AAF, 4 cycles	58	129 ± 168	0–658

[a] Abbreviations used: SD, standard deviation; S.-D., Sprague–Dawley; phenob, phenobarbital.
[b] Ald D activity units are defined as (change in A_{340}/minute/milligram protein) (1000), corrected for non-Ald D activity.
[c] This single ethioinine-induced liver tumor is tabulated apart from the other ethionine tumors, not only because its Ald D was more active than that of the others, but because the Ald D of this one tumor exhibited biochemical characteristics we have heretofore found only in AAF- or DAB-induced tumors (see text).

TABLE II. ALDEHYDE DEHYDROGENASE ACTIVITY IN SOME OTHER TUMORS.[a]

Species	Strain	Carcinogen	Tissue	Ald D activity		
				Number	Mean ± SD	Range
Rat	S.-D.	AAF	Lung tumor	1	9	
			Intestinal tumor	1	2	
			Salivary gland tumor	2	1 ± 1	0–1
Rat	Buffalo	MBAF[b]	Morris hepatoma 9618B	1	0	
		FPA[c]	Morris hepatoma 7777	3	3 ± 3	0–5
		FPA	Morris hepatoma 5123tc	3	11 ± 13	2–26
		FPA	Morris hepatoma 7800	3	26 ± 1	25–27
		FPA	Morris hepatoma 7794A	1	49	
Mouse	BCF_1	None	Normal liver	3	19 ± 20	5–42
		None	Liver tumor (spontaneous)	3	13 ± 17	1–32
Mastomys		None	Normal liver	1	20	
		None	Liver tumor (spontaneous)	1	0	

[a] Ald D activity is defined as in Table I legend.
[b] α-(4′methyl-)benzoylaminofluorene.
[c] *N*-2-fluorenylphthalamic acid.

TABLE III. ALDEHYDE DEHYDROGENASE ACTIVITY IN NONTUMOROUS SPRAGUE-DAWLEY RAT LIVER.

Liver	Number	"Tumor" isozymes[a]	Ald D activity	
			Mean ± SD	Range
Normal adult (normal diet)	15	0	65 ± 14	39 ± 88
Regenerating				
16 hr postpartial hepatectomy	1	0	51	
2 days postpartial hepatectomy	1	0	50	
5 days postpartial hepatectomy	1	0	57	
Fetal				
16 days postconception	3	0	3 ± 1	2-12
20 days postconception	3	0	6 ± 2	5-8
"Normal" after AAF feeding[b]				
No tumor macroscopically evident				
AAF, then normal diet	1	0	56	
AAF, then phenobarbital	2	0	85 ± 35	60-109
Tumor elsewhere on liver				
AAF, then normal diet	3	1	59 ± 5	54-63
AAF, then phenobarbital	5	2	70 ± 15	53-87
AAF, four cycles	5	5	57 ± 10	44-72

[a] "Tumor" type isozymes are defined as multiple bands appearing on PAGE gels at the appropriate location under standard conditions. Numbers given are the numbers of sections examined, and those found to contain these forms.

[b] These are macroscopically and histologically normal liver sections, taken at sacrifice at least 6 months after initiation of a diet of AAF followed by phenobarbital, or after four cycles of AAF, or at least 12 months after start of a diet of AAF followed by normal diet. These are the times at which grossly macroscopic tumors were first detected in this experiment.

Ald D activity is defined as in Table I legend.

lar carcinoma) showed even less activity than did the normal liver. It is unfortunate that the single active ethionine tumor was not isoelectrically focused; the tissue was totally used up in routine assays and PAGE gels before it was realized that it exhibited an anomalous Ald D activity.

Figure 2 shows the same tissues, using NADP as coenzyme. It is clear that the normal type (lower pI) isozymes, in either normal liver or AAF- or DAB-induced tumors, cannot use NADP as coenzyme; the neutral pI isozymes characteristic of these tumors used NADP approximately as effectively as NAD.

Discussion. The liver tumors studied in this work were assayed and tested for isozyme pattern by one individual, and evaluated histologically for degree of differentiation by another. No correlation could be noted between the biochemical and the histological characteristics. For example, the single ethionine-induced hepatocellular carcinoma with a high Ald D activity had a strong reaction on PAGE gels with either NAD or NADP, while other hepatocellular carcinomas induced by ethionine showed very low Ald D activity, faint or absent PAGE banding with NAD, and no PAGE banding with NADP. AAF-induced tumors also exhibited no correlation between biochemical and histological characteristics.

The reason for the wide quantitative variation in tumor Ald D activity is unknown. However, in the case of AAF- or DAB-induced liver tumors, the isozyme pattern we associate with tumor Ald D is invariably present, although faintly in tumors of low total Ald D activity.

This sort of faint, but recognizable tumor isozyme pattern has also been observed occasionally in the macroscopically and histologically normal sections of livers of rats that had been fed AAF many months earlier and that exhibited tumors elsewhere in the liver. In other words, the biochemical change has upon occasion been observed in areas where no histological change is yet observable. It was unexpected that the isozyme pattern found in the hepatocellular carcinomas should also be found in lesions in which hypertrophy, hyperplasia, and altered glycogen storage were the only indications of neoplasia. It was even more surprising to detect the isozyme changes in the apparently normal livers of rats treated with car-

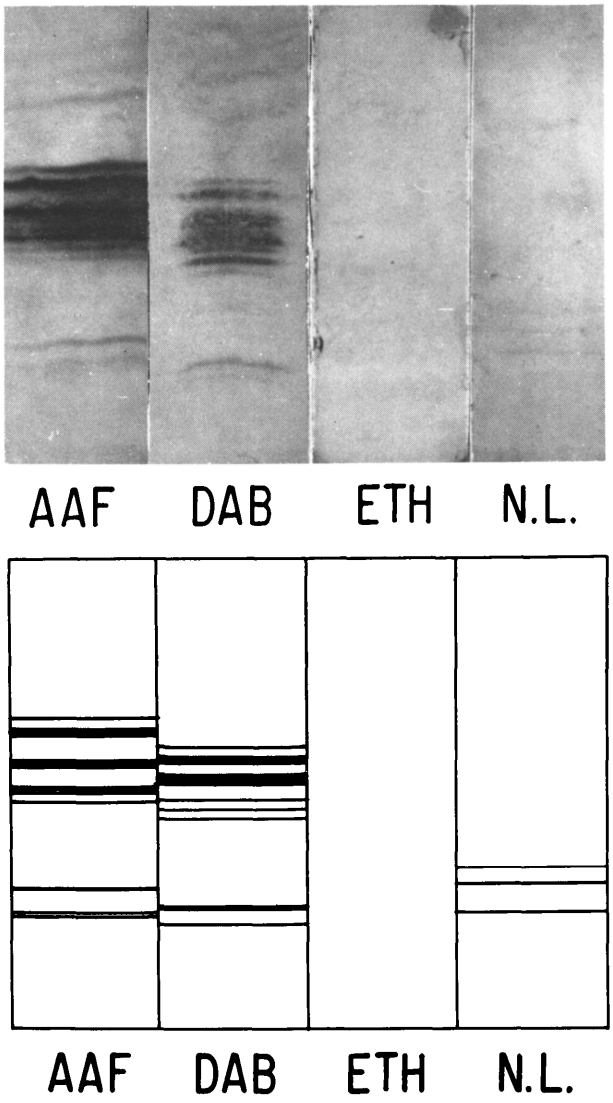

FIG. 1. Photographs and drawn counterparts of isoelectrically focused Ald D activity of rat liver tumors induced by AAF, DAB, or ethionine (ETH), or of normal rat liver (N. L.). Substrate was benzaldehyde; coenzyme was NAD. The variability of location of the AAF and DAB tumor bands is no greater than seen in a series of AAF tumors. At least part of the variation is due to slight day-to-day shifts in the location of the pH gradient. The strong bands always have a pI range of 6.9–7.2, while the weaker bands, found also in normal liver, have a pI of 6.2–6.4. Cathode is at top, anode at bottom.

cinogens. While an extensive examination was made, serial sections were not cut, and as it is not known how many altered cells have to be present for the detection of the characteristic isozyme pattern of the tumor, the interpretation of this result must be made with caution.

The role of Ald D in the economy of normal liver or of liver tumors is not clear. The enzyme has been studied by others chiefly with regard to alcohol metabolism, but ethanol is hardly to be considered an important natural metabolite. The suggestion has been made (7) that Ald D may be of importance in the metabolism of aldehydes resulting from the oxidative deamination of biogenic amines; this would seem to be a trivial consideration in the tumor econ-

omy. The new Ald D isozymes of these tumors are of interest to us chiefly as a convenient device for studying the mechanism whereby the carcinogenic transformation is linked to the production of a new molecular species of protein.

In connection with this mechanism the chemical specificity of the carcinogen is of interest. Liver tumors induced with AAF or DAB invariably show the same new Ald D isozymes; liver tumors induced with ethionine do not. The qualitative constancy of the AAF and DAB effect argues against the possibility of a mutation on the structural gene biosynthesizing the new Ald D; on the other hand, a change in the control of the expression of a structural gene for Ald D could lead to the invariant production of identical isozymes. The results for the ethionine-induced tumors suggest that there is no correlation between tumorigenesis and the alteration in the control of the structural gene for Ald D. The oncogenic property of AAF and ethionine indicates a commonality in some of the sites altered by these compounds, whereas the difference in the isozyme patterns of the Ald D suggests that, in addition, these compounds affect sites that are not in common.

The fact that the five Morris hepatomas tested—ranging from very fast to very slow growing, from poorly differentiated to well

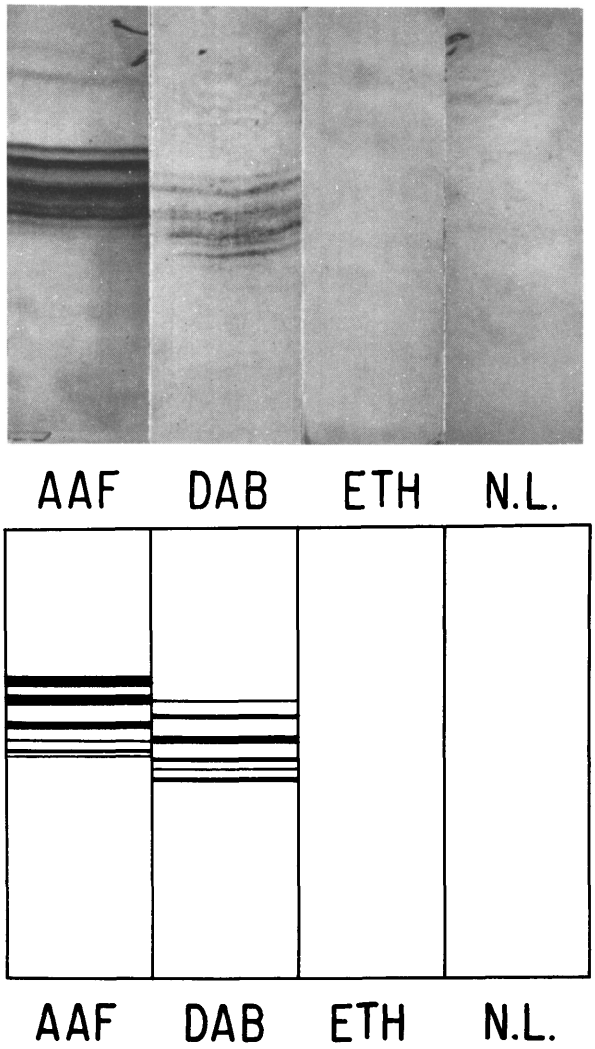

FIG. 2. Legend as for Fig. 1, except that coenzyme was NADP.

differentiated—all showed very low Ald D activity and none of the PAGE banding typical of AAF tumors, could be attributed to one of three factors: rat strain (Morris hepatomas are carried in Buffalo rats), nature of the carcinogen used, or the fact that the Morris hepatomas were all multigenerational transplants, while the other liver tumors we have studied were autochthonous. The Ald D of tumors induced (with difficulty) by feeding Buffalo rats with AAF resembles the Ald D of liver tumors in AAF-fed Sprague-Dawley rats. The critical test for choosing between the other two alternatives would be the observation of Ald D isozymes in transplanted AAF tumors; such a transplantation, however, we have not yet been able to achieve. Because of the difference in effect of ethionine, as opposed to AAF,or DAB, we feel the most likely reason for nonactivity in the Morris hepatomas tested lies in the nature of the carcinogen initially used.

The last point that we wish to emphasize is that the tumor Ald D isozymes do *not* repeat the fetal pattern. This is in contrast to Potter's suggestion (8) of "oncogeny as blocked ontogeny," and to a host of observations on other liver isozymes [see for instance (8)]. Because of this lack of recapitulation of the fetal situation, we consider the tumor Ald D production to be a possible instance of an "archeogene" (9), a gene ability repressed for untold ages and now released under the influence of certain specific stimuli.

Summary. All liver tumors induced in rats by AAF or DAB, but only one out of 29 tumors induced by ethionine, exhibited new isozymes of aldehyde dehydrogenase. These new isozymes, which differed from those of normal liver with regard to activity, pI, stability, cellular distribution, and coenzyme specificity, were not found in fetal or regenerating rat liver, nor in spontaneous liver tumors of the mouse or mastomys, nor in several other tumors appearing in rats bearing AAF-induced liver tumors, nor in any of several transplanted Morris hepatomas. The activity of the new isozymes did not correlate with histological criteria of differentiation, and the new forms were sometimes detectable in the liver of AAF-fed rats in areas where no tumor was macroscopically or histologically visible.

1. Feinstein, R. N., and Cameron, E. C., Biochem. Biophys. Res. Commun. **48,** 1140 (1972).
2. Feinstein, R. N., *in* "Isozymes" (C. L. Markert, ed.), Vol. III, Developmental Biology, p. 969 (1975).
3. Epstein, S., Ito, N., Merkow, L., and Farber, E., Cancer Res. **27,** 1702 (1967).
4. Peraino, C., Fry, R. J. M., Staffeldt, E., and Kisieleski, W. E., Cancer Res. **33,** 2701 (1973).
5. Reuber, M. D., J. Natl. Cancer Inst. **34,** 697 (1965).
6. Feinstein, R. N., Anal. Biochem., in press.
7. Duncan, R. J. S., and Sourkes, T. L., J. Neurochem. **22,** 663 (1974).
8. Potter, V. R., Canada. Cancer Conf. **8,** 9 (1969).
9. Anderson, N. G., and Coggin, Jr., J. H., "Proc. First Conf. and Workshop on Embryonic and Fetal Antigens in Cancer," p. 7 (1971).

Received March 15, 1976. P.S.E.B.M. 1976, Vol. 152.