On Relationships between Induction of Liver Cancer with Azo Dyes and Changes in Nuclear RNA Metabolism During Carcinogenesis

(34390)

DONALD E. KIZER AND JOSEPH A. CLOUSE
Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73401

Aromatic amines (or metabolic derivatives) bind to DNA, RNA, and proteins during induction of liver tumors in rats (1). These interactions frequently were accompanied by changes in the metabolism of these cellular components. For instance, the content of rat liver nuclear RNA decreased during carcinogenesis with 3′methyl-4-dimethylaminoazobenzene (3′-Me-DAB) (2, 3), or with *N*-hydroxy-2-acetylaminofluroene (3), but these depletions were absent when weakly carcinogenic or noncarcinogenic derivatives of DAB were administered (2, 3). The observation of similar depletions with aflatoxin B_1 led Sporn and Dingman (3) to suggest that this unique alteration of nuclear RNA metabolism might be a characteristic shared by diverse chemicals capable of inducing liver tumors. On the other hand, Kizer and Clouse (4) reported diverse patterns with 3′-Me-DAB, 4′-fluoro-DAB (4′-F-DAB) and thioacetamide, and concluded that changes in nuclear RNA metabolism were irrelevant to liver tumor induction. In their experiments (4), carcinogens were fed in a semisynthetic diet (5) which contained moderate levels of riboflavin. Subsequently, it was shown (6) that induction of liver tumors was retarded when 4′-F-DAB was fed in that diet (5). Tumors were readily induced (6) if 4′-F-DAB was fed in a low riboflavin diet (7). Therefore, the purpose here was to determine whether patterns of nuclear RNA synthesis and content during tumor induction in rat liver with 4′-F-DAB differed from patterns seen when animals were protected from 4′-F-DAB carcinogenesis. Since tumors were readily induced with 3′-Me-DAB, whether fed in the moderate (5) or low (7) riboflavin diets, patterns of hepatic nuclear RNA metabolism during 3′-Me-DAB feeding served as an internal control.

Materials and Methods. Female Holtzman rats were fed 0.06% 4′-F-DAB or 3′-Me-DAB for 6 weeks in a semisynthetic diet described by Farber (5) or one described by Medes *et al.* (7). Liver tumor incidence after feeding 0.06% 4′-F-DAB for 12 weeks in these diets was reported (6) as being: (a) "Farber's diet," 0/24; (b) "Medes' diet," 10/11. Similar regimes using 0.06% 3′Me-DAB consistently resulted in essentially 100% hepatic tumor incidence, irrespective of diet.

Animals were injected with orotic acid-6-^{14}C, 3.3 μCi/rat, or adenine-8-^{14}C, 5 μCi/rat, and the isotope was allowed to incorporate 30 min. Animals were killed by cervical fracture and livers were quickly removed and perfused sequentially with ice-cold 0.9% saline and 0.25 *M* sucrose (8); and were homogenized in 0.25 *M* sucrose. Nuclei were separated by centrifugation at 1250*g* (9) and were purified by centrifugation in 2.2 *M* sucrose (10). Nuclear RNA was subfractionated into a non-nucleolar nuclear fraction (NNN) and a nucleolar fraction by a neutral salt extraction procedure (11). These subfractions had inadequacies when judged by cytologic criteria (12), but possessed markedly different rates of precursor incorporation (13). Nucleoproteins from these 2 fractions were extracted by the method of Steele *et al.* (8). Methods used for quantitative determinations were: RNA by reaction with orcinol (14) using yeast RNA as a standard; protein by reaction with phenol

TABLE I. Effect of Diet upon Incorporation of Orotic Acid-6-^{14}C into Nuclear Ribonucleic Acid (RNA) Subfractions during Carcinogenesis in the Liver.[a]

	(dpm/mg of protein)	
	NNN[b] RNA	Nucleolar RNA
Additions to ''Medes' diet''		
None	(9) 366 ± 47[c]	(9) 1627 ±522
4′-F-DAB	(8) 383 ± 56	(9) 458 ± 67[d]
3′-Me-DAB	(8) 200 ± 88	(8) 260 ±129[d]
Additions to ''Farber's diet''[e]		
None	(10) 755 ±101	(10) 2948 ±214
4′-F-DAB	(12) 928 ±180	(12) 3219 ±388
3′-Me-DAB	(7) 384 ±121[d]	(7) 1621 ±395[d]

[a] Animals were fed 0.06% 4′-F-DAB or 3′-Me-DAB in a low riboflavin diet (''Medes' diet'')(7) or in a high riboflavin diet (''Farber's diet'')(5) for 6 weeks. Orotic acid-6-^{14}C, ca. 3.3 μCi/rat, was injected intra-abdominally and allowed to incorporate 30 min. Nuclei were separated from liver homogenates by centrifugation (9) and purified by centrifugation through 2.2 *M* sucrose (10). Non-nucleolar nuclear (NNN) and nucleolar subfractions were obtained essentially by neutral salt extraction procedure (11). Other experimental details are given in the ''Materials and Methods'' section.

[b] NNN, non-nucleolar nuclear.

[c] Values are averages ± SE; number of animals given in parentheses.

[d] Values different from corresponding ''none'' values at a probability level of 0.1 or greater.

[e] Data from this dietary regimen were depicted diagrammatically in a previous publication (4).

reagent (15) using crystalline bovine albumin as a standard; and isotope by liquid scintillation counting in a xylene–dioxane–cellosolve counting cocktail (16) with quenching corrected by the channels ratio method (17).

Results and Discussion. Incorporation of orotic acid-6-^{14}C into nuclear RNA subfractions after 6 weeks on diets containing either 4′-F-DAB or 3′-Me-DAB is recorded in Table I. When the 2 dyes were fed in "Medes' diet," the only appreciable change was a decrease in the deposition of label in the nucleolar fraction. When both dyes were fed in "Farber's diet," appreciable changes were observed only among animals fed 3′-Me-DAB. Here, deposition of label into both nucleolar and NNN RNA were decreased. Tumor induction would have been expected from 3′-Me-DAB when fed in either diet, but from 4′-F-DAB only when fed in "Medes' diet" (6). Therefore, decreased incorporation of labeled orotic acid into nucleolar RNA appeared to be correlated with the onset of cancer induced in rat liver by azo dyes. Since decreased incorporation of labeled orotic acid into NNN RNA was seen only among animals fed 3′-Me-DAB in "Farber's diet," this lesion apparently was associated with dietary influences, not with the onset of cancer. Thus, this experiment suggested that decreased synthesis of nucleolar RNA was symptomatically associated with the onset of liver cancer induced by azo dyes.

Since changes in precursor pools and precursor interconversions influence incorporation patterns into cellular components, adenine-8-^{14}C also was used as a precursor of nuclear RNA, and the data are summarized in Table II. Here, both 3′-Me-DAB and 4′-F-DAB, irrespective of diet, stimulated incorporation of labeled adenine into NNN RNA. Incorporation of adenine-8-^{14}C into nucleolar RNA was not appreciably affected by either dye or either diet. Thus, this experiment suggested that both azo dyes stimulated NNN RNA synthesis, but this stimulation was not symptomatically associated with the onset of liver cancer.

The content of RNA in the 2 nuclear subfractions was determined and the data are recorded in Table III. Except for the group fed 3′-Me-DAB in "Medes' diet," neither diet nor azo dye addition caused appreciable change in the RNA content. Thus, this experiment suggested that the amount of RNA in the 2 nuclear subfractions was unrelated to the onset of liver cancer induced by either of these dyes.

We reported previously that a common pattern of nuclear RNA synthesis and content was not seen among animals fed 3′-Me-DAB and 4′-F-DAB, but this conclusion was compromised by the use of "Farber's diet," which retarded tumor induction with 4′-F-

TABLE II. Effect of Diet upon Incorporation of Adenine-8-^{14}C into RNA Subfractions during Carcinogenesis in the Liver.[a]

	(dpm/mg of protein)	
	NNN[b] RNA	Nucleolar RNA
Additions to "Medes' diet"		
None	(10) 212 ± 28[c]	(10) 678 ± 80
4'-F-DAB	(9) 424 ± 41[d]	(13) 656 ± 93
3'-Me-DAB	(13) 412 ± 47[d]	(9) 529 ± 94
Additions to "Farber's diet"[e]		
None	(10) 185 ± 16	(10) 801 ±157
4'-F-DAB	(10) 253 ± 30[d]	(12) 924 ±121
3'-Me-DAB	(10) 313 ± 64[d]	(11) 868 ±165

[a] Adenine-8-^{14}C, ca. 5 μCi/rat, was injected intraabdominally 30 min before the rats were killed. Other experimental details were as described in Table I.

[b] NNN, non-nucleolar nuclear.

[c] Values are averages ± SE; number of animals given in parentheses.

[d] Values different from corresponding "none" values at a probability level of 0.1 or greater.

[e] Data from this dietary regimen were depicted diagrammatically in a previous publication (4).

DAB. In present experiments, tumor incidence with 4'-F-DAB was deliberately retarded or enhanced by dietary means, but the tumor incidence with 3'-Me-DAB was not affected. Under these conditions, when orotic acid was the labeled precursor, nucleolar RNA synthesis was inhibited in correlation with expected tumor incidence; when labeled adenine was the precursor, NNN RNA synthesis was stimulated, irrespective of tumor incidence; and the amount of RNA in either fraction was not appreciably changed, irrespective of expected tumor incidence. Thus, there appeared to be no consistent relationship between liver tumor induction with these 2 azo dyes and changes in nuclear RNA metabolism during the onset of cancer.

Incorporation patterns in Tables I and II were appreciably altered by factors apart from those assoicated with the synthesis of nuclear RNA. Whether these differences arose from differences in pool sizes or differences in interconversion rates, can not be determined here. But it was interesting that deposition of labeled orotic acid into nucleolar RNA was correlated with the cancer risk of the animals. Although studies on the interconversion of orotate, viz., reaction with pyrophosphorylase, decarboxylase, and kinase (18), in precancerous rat liver have been reported (19), further studies seem appropriate.

If treatments which potentiated or retarded the incidence of liver tumors also potentiated or retarded biochemical effects exerted by the inducing agents during carcinogenesis, then Reid (20) considered that the biochemical effects were likely to be key changes in oncogenesis. By this criterion, changes in the synthesis and content of nuclear RNA would not appear to be key changes. Since both transfer RNA (21) and ribosomal RNA (22) interact with aromatic amines, failure to detect correlations here may be associated

TABLE III. Effect of Diet upon the Concentration of RNA in Nuclear Subfractions during Carcinogenesis in the Liver.[a]

	(μg/mg of protein)	
	NNN[b] RNA	Nucleolar RNA
Additions to "Medes' diet"		
None	(18) 32 ± 2[c]	(20) 42 ± 3
4'-F-DAB	(22) 30 ± 3	(25) 38 ± 2
3'-Me-DAB	(15) 41 ± 2[d]	(16) 42 ± 2
Additions to "Farber's diet"[e]		
None	(10) 28 ± 4	(10) 38 ± 3
4'-F-DAB	(11) 33 ± 2	(12) 40 ± 3
3'-Me-DAB	(7) 20 ± 3	(7) 32 ± 2

[a] RNA was determined by reaction with orcinol (14) using yeast RNA as a standard. Protein was determined by reaction with phenol reagent (15) and crystalline bovine albumin was the standard. Other experimental details were as described in Table I.

[b] NNN, non-nucleolar nuclear.

[c] Values are averages ± SE; number of animals given in parentheses.

[d] Values different from corresponding "none" values at a probability level of 0.1 or greater.

[e] Data from this dietary regimen were depicted diagrammatically in a previous publication (4).

with heterogeneity of RNA fractions isolated.

Summary. The purpose was to determine whether changes in hepatic nuclear RNA synthesis and content were correlated with tumor incidence in rats fed 4′-F-DAB or 3′-Me-DAB. Rats were fed 4′-F-DAB for 6 weeks in diets that potentiated ("Medes' diet") or retarded ("Farber's diet") hepatic cancer induction. Other rats were fed 3′-Me-DAB in "Medes' diet" or "Farber's diet," but, risks for hepatic cancer induction were essentially identical. RNA synthesis was adjudged by incorporation of orotic acid-6-^{14}C or adenine-8-^{14}C into 2 nuclear RNA subfractions obtained by neutral salt extraction. Results were: (a) incorporation of labeled orotic acid into nucleolar RNA was inhibited by both azo dyes in correlation with expected tumor incidence, incorporation into non-nucleolar nuclear (NNN) RNA was inhibited by 3′-Me-DAB in one diet, but not by 4′-F-DAB; (b) incorporation of labeled adenine into NNN RNA was stimulated by both azo dyes but not in correlation with expected tumor incidence, incorporation into nucleolar RNA was not changed; and (c) RNA content of both fractions was not appreciably changed. It was concluded that no consistent relationship appeared to exist between liver tumor induction with these 2 azo dyes and changes in these aspects of nuclear RNA metabolism during carcinogenesis.

---

1. Farber, E., Cancer Res. 28, 1859 (1968).

2. Kizer, D. E., Howell, B. A., Shirley, B. C., Clouse, J. A., and Cox, B., Cancer Res. 26, 822 (1966).

3. Sporn, M. B. and Dingman, C. W., Cancer Res. **26**, 2488 (1966).

4. Kizer, D. E. and Clouse, J. A., Cancer Res. **28**, 502 (1968).

5. Farber, E., Cancer Res. **16**, 142 (1956).

6. Kizer, D. E., Cox, B., Howell, B. A., and Shirley, B. C., Cancer Res. (1969), in press.

7. Medes, G., Friedman, B., and Weinhouse, S., Cancer Res. **16**, 57 (1956).

8. Steele, W. J., Okamura, N., and Busch, H., Biochim. Biophys. Acta **87**, 490 (1964).

9. Schneider, W. C. and Hogeboom, G. H., J. Biol. Chem. **183**, 123 (1950).

10. Chauveau, J., Moule, Y., and Rouiller, C. H., Exptl. Cell Res. **11**, 317 (1956).

11. Allfrey, V. G. and Mirsky, A. E., Proc. Natl. Acad. Sci. U. S. **43**, 821 (1957).

12. Busch, H., Byvoet, P., and Smetana, K., Cancer Res. **23**, 313 (1963).

13. Kizer, D. E., Shirley, B. C., Cox, B., and Howell, B. A., Cancer Res. **25**, 596 (1965).

14. Volkin, E. and Cohn, W. E., *in* "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 1, p. 287. Interscience, New York (1954).

15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

16. Bruno, G. A. and Christian, J. E., Anal. Chem. **33**, 1216 (1961).

17. Bush, E. T., Anal. Chem. **35**, 1024 (1963).

18. Mahler, H. R. and Cordes, E. H., *in* "Biological Chemistry," p. 714. Harper & Row Publishers, New York (1966).

19. Reid, E., Nature **194**, 1153 (1962).

20. Reid, E., Cancer Res. **22**, 398 (1962).

21. Agarwal, M. K. and Weinstein, I. B., Proc. Am. Assoc. Cancer Res. **10**, 2 (1969).

22. Warwick, G. P. and Roberts, J. J., Nature **213**, 1206 (1967).

Received May 15, 1969. P.S.E.B.M., 1969, Vol. 132.