THE POSSIBLE ROLE OF RIBOFLAVIN DEFICIENCY IN EPITHELIAL NEOPLASIA. II. EFFECT ON SKIN TUMOR DEVELOPMENT

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Temporary riboflavin deficiency in young mice tends to enhance chemically-induced skin carcinogenesis. When mice are kept on a normal diet, the injection of riboflavin before initiation or promotion does not affect the tumor induction by the two-step mechanism of chemical carcinogenesis.

RIBOFLAVIN DEFICIENCY IN MICE CAN LEAD TO atrophy and hyperkeratosis of the epithelium of the upper alimentary tract as well as to hyperplasia of the skin.[16] These changes led to investigation of the response of this altered epithelial tissue to a carcinogen.

One of the earliest links of riboflavin to carcinogenesis came from its inhibitory effect on the induction of liver tumors in rats by butter yellow. Kensler et al.[6] showed that yeast or riboflavin with casein added to the diet of rats tended to protect the animals against the carcinogenic effect of the azodye. Riboflavin was thought to be an essential constituent of the enzyme systems that detoxify butter yellow.[5] Its protective influence was found to be less when the liver carcinogen 2′- or 3′-methyl-4′-dimethylaminoazo-benzene was given.[3]

Tannenbaum and Silverstone[11] reported that vitamin B complex administered in high concentrations did not affect skin tumor induction in mice. Boutwell et al.[1] found little change in tumor yield when benzo(a)pyrene was applied to the skin of animals on diets containing varying amounts of vitamin B complex. Mice kept low on all B vitamins had a relatively low tumor yield. After repeated applications of 3,4-benzo(a)pyrene, Roe[7] found no beneficial effect of a dietary supplement of riboflavin although injected riboflavin resulted in a slightly, but not significantly, reduced tumor induction.

These studies dealt with the long-term effects of vitamins on chemical carcinogenesis. In the present report, the effect of temporary riboflavin deficiency on mouse skin tumorigenesis is reported.

METHODOLOGY

Female Swiss ICR mice (Millerton Research Farms), 3–4 weeks old and approximately 13 g in weight, were used. Ninety-nine mice were divided into 4 groups and fed a vitamin B_2-deficient diet (Nutritional Biochemicals Corp., Cleveland, Ohio, consisting of 18% vitamin test casein, 68% sucrose, 10% vegetable oil, and 4% U.S.P. salt mixture) for 4 weeks, after which the animals were returned to a normal diet (Purina Lab Chow). On the 21st, 24th, 28th, or 29th day of the experiment, mice in one of the groups were initiated on their shaved backs with a fresh 0.15% solution of purified 7,12-dimethyl-benzanthracene (DMBA, 75 μg) in spectrograde acetone. Twice weekly for 18 weeks, 100 μl of 0.5% croton oil in acetone was pipetted on the initiated skin of the mice, beginning 10 days after they were returned to a normal diet. The controls (129 mice) were kept on a normal diet for 4 weeks before being initiated with DMBA and 10 days later were promoted with croton oil twice weekly.

Another experiment investigated whether administration of the synthetic diet containing the vitamin B complex might affect tumor development. Three groups of mice were initiated with 75 μg of DMBA after being put on either a fortified vitamin B complex diet (similar in composition to the vitamin B_2-deficient

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diet but with riboflavin added) for 4 weeks (70 mice), a normal diet for 4 weeks (40 mice), or, a vitamin B_2-deficient diet for 5 weeks (26 mice). Immediately after initiation, all mice were given a normal diet and, beginning 10 days later, were painted with 1% croton oil in acetone twice weekly for 12 weeks.

Another control group of 30 mice was put on a calorie-restricted diet (1 to 1½ g of normal diet a day) for 4 weeks. The body weight was checked regularly to ensure that their weight gain paralleled that of mice on the vitamin B_2-deficient diet. After 4 weeks, they were also initiated with 75 μg of DMBA and food given *ad libitum*. Beginning 10 days later, they were promoted by pipetting 100 μl of 0.5% croton oil on to their backs twice weekly for 18 weeks.

The effect of an excess of riboflavin on tumor yield was also investigated. Four groups of 40 8-week-old mice were initiated with 75 μg of DMBA, and 100 μl of 0.5% croton oil was pipetted on twice weekly for 15 weeks. Riboflavin was then given intraperitoneally in the following manner: Group 1: 350 μg 3 hours before application of DMBA; Group 2: 350 μg 3 hours before each application of croton oil; Group 3: 350 μg 3 hours before application of DMBA and again before each application of croton oil; Group 4: none.

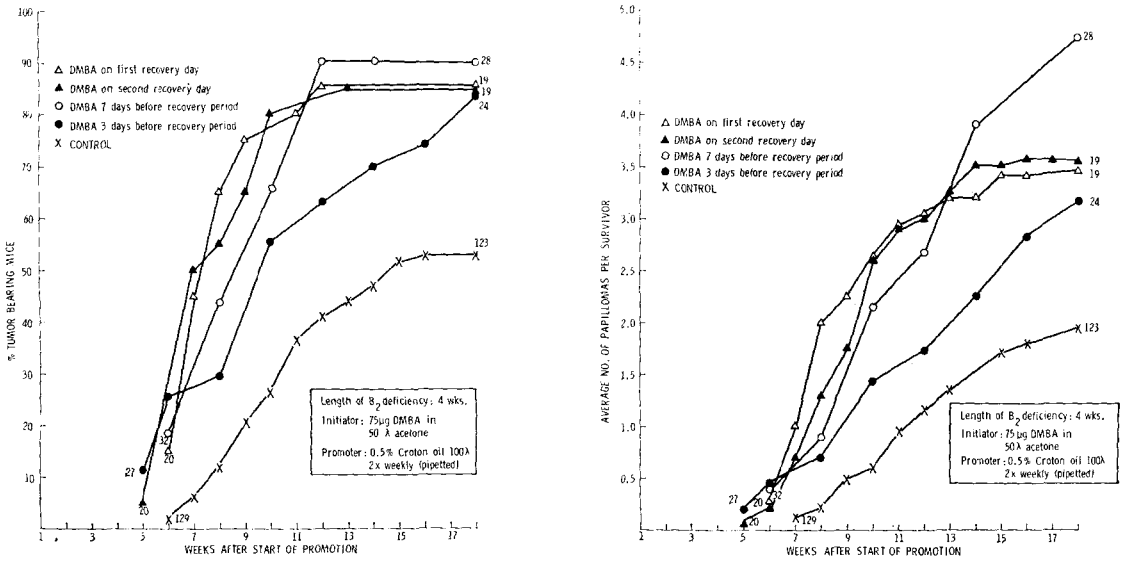
All animals were checked regularly and tumors larger than 1 mm in diameter were recorded weekly. All the registered tumors persisted and continued to grow in size throughout the entire period of observation.

RESULTS

All 4 groups of mice recovering from a 4-week vitamin deficiency and initiated with DMBA had a tumor yield significantly greater (χ^2 test, $p < 0.01$) than that of controls kept on a normal diet (Figs. 1, 2). The deficient mice developed tumors earlier and in greater numbers. There was no difference in tumor yield between the 4 groups of mice on the 4-week riboflavin-deficient diet initiated at various times.

Histologically, the tumors were all papillomas of the types described by Shubik et al.[8] Classification into morphological types was not attempted.

There were no differences in tumor yield between mice on a normal diet and mice which had received the synthetic diet fortified with vitamin B_2 for 4 weeks (Fig. 3) indicating that the accelerated tumor development noted with mice recovering from riboflavin deficiency was not related to the synthetic diet. In addition, Fig. 3 shows that accelerated tumor development also occurred in mice initiated after 5 weeks of deficient diet, and



FIGS. 1 *(left)* and 2 *(right)*. Cumulative tumor incidence in female Swiss mice (3 weeks old) either on a riboflavin-deficient diet for 4 weeks then returned to normal diet thereafter, or controls on normal diet throughout. The riboflavin-deficient mice were initiated with DMBA at the time as shown and the controls at 7 weeks of age. Croton oil was pipetted on all mice twice weekly beginning at age 8½ weeks.

then painted with 1% croton oil twice weekly after being put back on a normal diet.

The animals kept on a calorie-restricted diet for 4 weeks had a similar tumor yield to the controls. Thus, the phenomenon observed in the 4-week riboflavin deficient animals does not appear to be a direct result of retarded growth.

In those experiments in which mice on a normal diet were injected with riboflavin before initiation and/or promotion, the tumor yield did not differ from that of the controls. This indicates that the riboflavin treatment per se does not counteract the initiating effect of DMBA, nor the promoting effect of croton oil as given in these experiments.

DISCUSSION

Much of this experimental work originated from epidemiological observations of the relationship between Plummer-Vinson's syndrome, or excessive consumption of alcohol, to cancer of the upper alimentary tract; this suggested that nutritional deficiencies might facilitate the malignant transformation of certain types of epithelial cells.[14, 15]

In a preliminary experiment carried out to determine the point at which riboflavin deficiency reaches its peak in affecting mouse skin tumor induction, it was found that mouse skin tumor development was enhanced more after 4 or 5 weeks' than 3 or 7 weeks' deficiency. Certainly at 3 weeks, the "optimal" degree of deficiency for chemical carcinogenesis seems not to have been reached, while at 7 weeks it is too far advanced.

Several factors may explain the findings of the present study. Twenty-four to 30 hours after initiation, a larger amount of the applied DMBA could be extracted from the skin of a mouse recovering from riboflavin deficiency than in the skin of a normal mouse.* After 4 weeks of riboflavin deficiency, the epithelium undergoes atrophy and is accompanied by a reduced rate of tritiated thymidine uptake (labeling index) in the interfollicular basal cells. Since the higher the labeling index the more cells are initiated,[2] it could be argued that deficiency should reduce the number of cells that become initiated. On the other hand, with the epithelium atrophied, more of the carcinogen applied during initiation might come into contact with the surviving basal

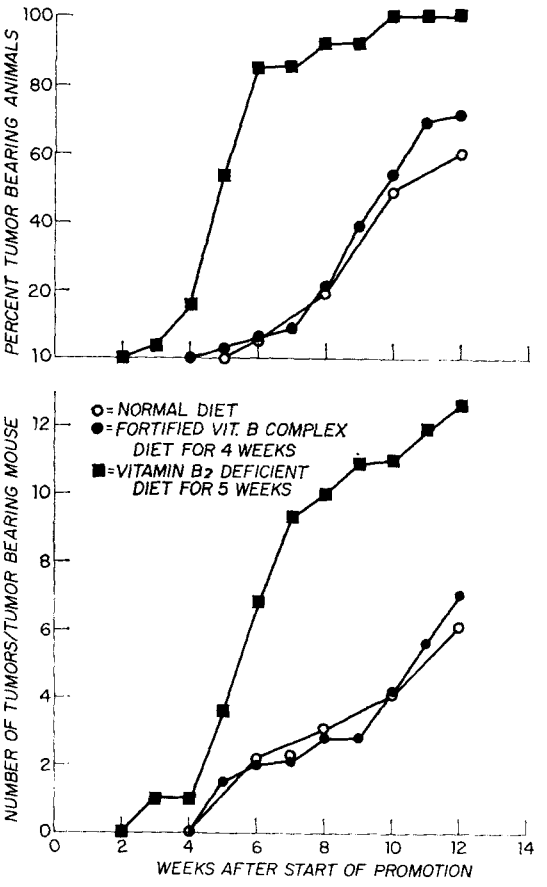


FIG. 3. Cumulative tumor incidence in female Swiss mice (3 weeks old) initiated with DMBA after being put on a normal diet for 4 weeks, a fortified vitamin B complex diet for 4 weeks, or a vitamin B_2-deficient diet for 5 weeks. All mice were put on normal diet after initiation and painted with 1% croton oil twice weekly beginning 10 days after initiation.

cells of the epidermis. After returning to a normal diet, an increased labeling index in the basal cells and a gradual return to normal appearance of the epidermis was noted.* This increased tritiated thymidine uptake, coupled with the prolonged presence of DMBA in the skin, might enhance the initiation phase of skin tumor development.

Wilk and Wynder[12, 13] have shown that not only do carcinogenic hydrocarbons tend to interfere with mitochondrial respiration but croton oil and its most active metabolite, phorbol ester, do as well. Abnormal mitochondrial changes have been observed in the livers of riboflavin-deficient mice.[9, 10] Croton oil is particularly toxic when applied to the skin of riboflavin-deficient animals and often leads to death. Mitochondrial function in mice recovering from riboflavin deficiency in our ex-

* Chan, P. C., and Wynder, E. L.: Unpublished data.

perimental setting may be related to tumor acceleration. Experimental evidence implicating the mitochondria in neoplastic transformation has been reported by Graffi.[4]

Although riboflavin deficiency seemed to enhance the yield of mouse skin tumors, vitamin B_2 supplements do not cause a sparing effect. However, such negative findings may result from the possibility that riboflavin injected into an animal that does not lack the vitamin will be readily metabolized and/or excreted unchanged before it can affect individual skin cells. Unless the application of DMBA and croton oil increases the demand of skin epithelial cells for riboflavin, the specific transport to, and deposition of, the vitamin in dermis and epidermis, appear unlikely.

REFERENCES

1. Boutwell, R. K., Brush, M. K., and Rusch, H.P.: Influence of vitamins of B complex on induction of epithelial tumors in mice. *Cancer Res.* 9:747–752, 1949.

2. Frei, J. V., and Ritchie, A. C.: Diurnal variation in the susceptibility of mouse epidermis to carcinogen and its relationship to DNA synthesis. *J. Nat. Cancer Inst.* 32:1213–1220, 1964.

3. Giese, J. E., Clayton, C. C., Miller, E. C., and Baumann, C. A.: The effect of certain diets on hepatic tumor formation due to m-methyl-p-dimethylaminoazobenzene and o'-methyl-p-dimethylaminoazobenzene. *Cancer Res.* 6:679, 1946.

4. Graffi, A.: Ist die Ursache der malignen Entartung eine Mutation der Mitochondrien? *Zbl. Gynaek.* 28: 945–958, 1968.

5. Kensler, C. J.: The influence of diet on the ability of rat liver slices to destroy the carcinogen N, N-dimethylaminoazobenzene. *Cancer* 1:483–488, 1940.

6. Kensler, C. J., Sugiura, K., Young, W. F., Halter, C. R., and Rhoads, C. P.: Partial protection of rats by riboflavin with casein against liver cancer caused by dimethylaminoazobenzene. *Science* 93:308–310, 1941.

7. Roe, F. J. C.: Effect of massive doses of riboflavin, and other vitamins of the B group, on skin carcinogenesis in mice. *Brit. J. Cancer* 16:252–257, 1962.

8. Shubik, P., Bazerga, R., and Ritchie, A. C.: The life and progression of induced skin tumor in mice. *Brit. J. Cancer* 7:342–351, 1953.

9. Tandler, B., Erlandson, R. A., Smith, A. L., and Wynder, E. L.: Riboflavin and mouse hepatic cell structure and function. II. Division of mitochondria during recovery from simple deficiency. *J. Cell Biol.* 41, 2:477–493, 1969.

10. Tandler, B., Erlandson, R. A., and Wynder, E. L.: Riboflavin and mouse hepatic cell structure and function. I. Ultrastructural alterations in simple deficiency. *Amer. J. Path.* 52:1:69–95, 1968.

11. Tannenbaum, A., and Silverstone, H.: Nutrition in relation to cancer. *In* Advances in Cancer Research, Jesse P. Greenstein and Alexander Haddow, Eds. New York, Academic Press, 1953; pp. 451–501.

12. Wilk, M., and Wynder, E. L.: Uber die Wirkung polycyclisher, aromatischer Kohlenwasserstoff auf isolierte Mitochondrien der Mauseleber. *Z. Naturforsch.* (B) 21B(2), 1966.

13. ———, and ———: Uber die Wirkung von Cocarcinogenen aus Crotonoil auf isolierte Mitochondrien der Mauseleber. *Z. Naturforsch.* (B) 21B(10), 1966.

14. Wynder, E. L., Bross, I. D. J., and Feldman, R.: A study of the etiological factors of cancer of the mouth. *Cancer* 10:1300–1323, 1957.

15. Wynder, E. L., Hultberg, S., Jacobsson, F., and Bross, I. D. J.: Environmental factors in cancer of the upper alimentary tract. A Swedish study with special reference to Plummer-Vinson (Paterson-Kelly) syndrome. *Cancer* 10:470–482, 1957.

16. Wynder, E. L., and Klein, U. E.: The possible role of riboflavin deficiency in epithelial neoplasia. I. Epithelial changes of mice in simple deficiency. *Cancer* 18:167–180, 1965.