Effects of Riboflavin Deficiency upon Concentrations of Riboflavin, Flavin Mononucleotide, and Flavin Adenine Dinucleotide in Novikoff Hepatoma in Rats

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SUMMARY

The concentrations of riboflavin and of the two coenzymes derived from riboflavin, flavin mononucleotide and flavin adenine dinucleotide (FAD), were determined in Novikoff hepatoma grown i.p. and in host liver of normal and riboflavin-deficient male Holtzman rats. Riboflavin deficiency significantly prolonged the 50% survival time of tumor-bearing animals from 5.7 to 10.8 days. The growth of Novikoff hepatoma did not alter hepatic riboflavin, flavin mononucleotide or FAD concentrations in either normal or riboflavin-deficient rats. In tumor from riboflavin-deficient rats, the concentration of FAD was nearly identical to that in tumor from animals on a normal diet. By contrast, in tumor from deficient animals, the flavin mononucleotide concentration was significantly lower than that in tumor from control animals. The free riboflavin concentration was decreased even further and was undetectable in one-third of the tumors from riboflavin-deficient animals. The FAD pyrophosphorylase activity was increased in liver but not in tumor from riboflavin-deficient animals.

INTRODUCTION

An effect of riboflavin upon tumor growth was first noted by Morris and Robertson in 1943 (18). Dietary deficiency of this vitamin led to slower growth and smaller size of spontaneous mammary tumors in mice, whereas restoration of riboflavin to the diet of deficient animals resulted in accelerated tumor growth (17, 18). Other investigators have also observed the inhibitory effects of dietary riboflavin deficiency or treatment with structural analogs of riboflavin upon tumor growth (1, 10, 21, 25). Some evidence exists that riboflavin deficiency has antitumor effects in man and may possibly be of value in the treatment of polycythemia vera and lymphoma (14, 15).

These studies were undertaken to determine whether Novikoff hepatoma differs from normal tissue in its adaptation to riboflavin deficiency and whether the presence of this tumor alters the hepatic concentrations of riboflavin and its coenzyme derivatives, FMN and FAD. The Novikoff hepatoma in rats (4, 19) was selected as a model system for investigation because of its undifferentiated structure and rapid growth, generally leading to death of the host within 5 to 10 days after transplantation (12). These studies indicate that the presence of Novikoff hepatoma in the peritoneal cavity of the rat does not accelerate the depletion of hepatic FAD, FMN, and riboflavin concentrations in animals receiving a riboflavin-deficient diet, and that tumor tissue is more resistant than host tissue to riboflavin deficiency.

MATERIALS AND METHODS

Animals. Male Holtzman rats (The Holtzman Co., Madison, Wis.) were used in all experiments. Riboflavin deficiency was produced experimentally by placing weanling animals in individual wire-bottom cages and by feeding them ad libitum a diet deficient in riboflavin (Nutritional Biochemical Corp., Cleveland, Ohio, or General Biochemicals Corp., Chagrin Falls, Ohio). Diets were fed for varying periods of time. As in previous studies, (8, 11) riboflavin-deficient animals lost weight and developed inanition, alopecia, reddening of the skin, and cataract.

Animals used as controls in these experiments were of the same strain, sex, and age as the deficient animals, and were generally littermates. Control animals were fed ad libitum Purina chow, which according to the manufacturer’s specifications contains approximately 22 times the minimum daily requirement for riboflavin. No attempt was made in these experiments to pair feed deficient animals with normal controls because previous studies in this laboratory (24, 26) have shown that hepatic flavin levels and activities of several flavoprotein enzymes, both basal and induced by hormones, are nearly identical in animals fed ad libitum and in those subjected to severe food restriction.

Preparation of Tissue Samples. Samples of Novikoff hepatoma were obtained from animals in which the tumor line has been maintained by serial passage on a weekly basis for several years. Original specimens of tumor were ob-
tained from Dr. Jay Roth, Department of Biochemistry, University of Connecticut, and their histology has been confirmed by Dr. Jacob Furth, Department of Pathology, Columbia University. Under these conditions, the tumor has remained largely confined to the peritoneal cavity without distant metastases. The absence of metastases to liver in particular by either gross or microscopic examination was demonstrated by Dr. Jacob Furth. To prepare for transplantation, viable specimens were removed immediately after sacrifice of the tumor-bearing animal. The tumor was minced in 0.9% NaCl solution, passed through a sieve 0.9 mm in diameter, and suspended in 3 volumes of iced 0.9% NaCl solution. A suspension of 0.4 to 0.5 ml was injected into the peritoneal cavity of each recipient animal. In studies of tissue coenzyme concentrations and enzyme activities, animals were sacrificed 5 to 19 days after transplantation of the tumor. In studies of mortality rates of normal and riboflavin-deficient animals bearing tumor transplants, suspensions of tumor were injected similarly, but animals were observed until the time of death.

To obtain samples of liver and tumor for the analysis of riboflavin, FMN, and FAD concentrations and for the assay of FAD pyrophosphorylase activity, animals were sacrificed by a blow to the head followed by decapitation and immediate exsanguination. Animals were generally sacrificed between 8 and 10 a.m.

**Enzyme and Coenzyme Assays.** Fresh samples of tumor and liver weighing 2 to 3 g each were used for assay of FAD pyrophosphorylase. In approximately 10% of the animals, tumors were too small to be analyzed individually; samples from 2 or 3 animals were pooled, and the results were considered as a single sample for statistical analysis of the data. Tissues were homogenized with 5 volumes of iced 0.25 M sucrose, followed by centrifugation at 100,000 x g for 1 hr. Assays were performed by a spectrophotometric method, as previously described (23). The enzyme from tumor did not differ from the hepatic enzyme with respect to pH optimum, magnesium requirement, and concentrations of FMN and ATP required for optimal activity. The assay previously described for liver (23) was suitable for assay of the tumor enzyme. Enzyme activity is expressed in this report as nmoles FAD synthesized per g tissue per hr.

The hepatic and tumor concentrations of FAD, FMN, and riboflavin were simultaneously determined in fresh tissue by the fluorometric method of Burch (5). Concentrations were expressed as µg/g fresh weight of tissue. Liver was selected as the sole basis for comparison with tumor because, of the several organs, its flavin concentration best reflects the nutritional status of the animal (6). In addition, the liver appears to be the main storage site for flavins, and the depletion of flavins in liver during riboflavin deficiency is quantitatively greater than that observed in other organs (6, 20). Concentrations of FAD, FMN, and riboflavin were each expressed as µg/g tissue weight in order to permit comparisons to be made between the total flavin content of tumor and liver. Protein concentrations were measured in duplicate, with the biuret reagent (9).

**RESULTS**

The mortality rates of normal and of riboflavin-deficient animals that had been given i.p. transplants of Novikoff hepatoma are shown in Chart 1. At 18 days following transplantation, nearly all animals from both groups had died. The 50% survival time of control tumor-bearing animals of 5.7 days was similar to that usually observed in this laboratory. In animals that received transplants of tumor after being on a riboflavin-deficient diet for 71 to 77 days, survival was significantly prolonged; the 50% survival time was recorded at 10.8 days following transplantation. This effect of riboflavin deficiency in extending the mean survival time of tumor-bearing animals is especially noteworthy in view of the high death rate (19%) observed during the same 18-day experimental period in rats which were similarly riboflavin deficient but which were not bearing tumors.

The concentrations of FAD in liver, in host liver (liver of tumor-bearing animals), and in Novikoff hepatoma from normal and riboflavin-deficient animals are shown in Chart 2. As in previous studies (6, 8, 13, 16), the concentration of FAD was reduced to approximately one-third of normal in livers of deficient rats. The presence of the Novikoff tumor did not alter the overall hepatic concentration of FAD of rats with adequate riboflavin stores, nor did it alter the overall hepatic FAD concentration of deficient rats. A further analysis of the data revealed that at no time during the course of riboflavin deficiency did tumor-bearing and non-tumor-bearing animals differ either in the hepatic concentration of FAD or in the rate at which FAD was depleted after the onset of riboflavin deficiency.

In Novikoff hepatoma, concentrations of FAD were less than one-tenth that of liver expressed per wet weight of tissue. In contrast to the results in liver, riboflavin deficiency did not decrease FAD concentrations in Novikoff hepatoma, as shown in Chart 2. Even in tumor from animals in the terminal stages of riboflavin deficiency, from 91 to 132 days after onset, there was no change in FAD levels.

Results of measurements of FMN concentrations in the same samples of liver, host liver, and Novikoff hepatoma...
Chart 2. Concentrations of FAD in liver, host liver (liver of tumor-bearing animals), and Novikoff hepatoma from normal and riboflavin-deficient animals. Data are expressed as the mean ± S.E.; numbers in parentheses, number of animals in each group. Deficient animals had been receiving a special diet for 25 to 132 days, with an average of 72.2 days.

from normal and riboflavin-deficient animals are given in Chart 3. In riboflavin-deficient rats, the depletion of FMN concentrations in liver was proportionately greater than that of FAD, as noted in previous studies (6, 8, 13, 16). FMN levels in deficient rats were decreased to 26% of control levels, whereas FAD concentrations were decreased to only 39% of control levels. FMN concentrations in host livers from both normal and deficient animals appeared to be slightly lower than those of animals without tumors, but these differences were not statistically significant (p > 0.05). FMN concentrations in samples of tumor were approximately one-tenth as great as in liver and exhibited relatively wide variability. In deficient animals, FMN concentrations in tumor, unlike those of FAD, were clearly reduced (p < 0.001) below the levels observed in tumor from control animals. In riboflavin-deficient animals, the relative decrease in FMN concentrations was greater in the host liver (to 20% of control) than in the tumor (to 36% of control). The differences were highly significant (p < 0.001).

The magnitude of the depletion of hepatic-free riboflavin concentrations in deficient animals was similar to that of FMN, as shown in Chart 4. In previous studies of riboflavin deficiency, free riboflavin levels have decreased either similarly or to a greater degree than did those of FMN and, in each instance, to a greater degree than those of FAD (6, 8). Free riboflavin concentrations in host liver did not differ from those in liver in either normal or deficient animals. Free riboflavin concentrations in tumor were approximately one tenth as great as those in liver. Much wider variability in the results of riboflavin measurements was obtained in tumor than in liver. In tumor from deficient animals, free riboflavin levels were at the lower limits of sensitivity of the method and, in 13 of the 32 specimens of tumor from deficient animals, no riboflavin could be detected at all. In tumor from deficient animals, the concentration of free riboflavin was reduced to 16.2 ± 4.8% of that observed in tumor from animals on a normal diet. The mean concentration of free riboflavin in the hepatoma was decreased proportionately more than that of either FMN (35.5 ± 3.6% of control) or FAD (87.5 ± 6.4% of control).

Hepatic protein concentrations in riboflavin-deficient animals bearing tumors (274.4 ± 8.1 mg/g) did not differ significantly from levels found in tumor-bearing animals fed a normal diet (263.9 ± 9.5 mg/g). The protein concentration in Novikoff tumor was approximately one-half that in
liver and was nearly the same in samples of tumor grown in riboflavin-deficient (153.4 ± 8.2 mg/g) and normal animals (157.1 ± 7.6 mg/g).

To elucidate possible mechanisms involved in the differential responses of liver and Novikoff hepatoma to riboflavin deficiency, measurements were made of the activity of FAD pyrophosphorylase, which catalyzes the conversion of FMN to FAD. As in previous reports from this laboratory (8), FAD pyrophosphorylase activity was significantly increased (p < 0.001) in liver from riboflavin-deficient animals (Chart 5). This adaptive response to riboflavin deficiency was also demonstrable in livers from tumor-bearing rats, in which the proportional increase in enzyme activity was nearly the same as that of deficient animals without tumors. Enzyme activity in the Novikoff hepatoma expressed per g of tissue did not differ in magnitude from that obtained from normal animals.

To determine whether FAD pyrophosphorylase activity in the Novikoff hepatoma had any relation to the length of time after transplantation, enzyme activity was determined in tumors from normal and deficient animals at 7 to 19 days following transplantation. As shown in Chart 6, enzyme activity did not vary in any systematic fashion in either normal or deficient animals during this time period. Concentrations of FAD, FMN, and riboflavin also did not appear to be influenced by the interval between transplantation and sacrifice.

DISCUSSION

This study demonstrates that, in response to experimental riboflavin deficiency, Novikoff hepatoma and liver differ in the relative changes that occur in the concentrations of riboflavin, FMN, and FAD. It is well known that when normal animals become riboflavin deficient, concentrations of all 3 flavins in liver decline (6). The fact that the decrease in hepatic FMN concentration in riboflavin-deficient animals is relatively greater than that of FAD has also been demonstrated in several laboratories (6–8, 13, 16), but the significance of this finding has not been well appreciated (20). We have previously presented evidence that the greater decline in FMN than in FAD concentrations in liver in riboflavin deficiency may be due at least in part to an adaptive increase in the activity of FAD pyrophosphorylase, which converts FMN to FAD. Enhanced FAD pyrophosphorylase activity appears to represent a physiological mechanism for conserving the vital supplies of FAD, at the expense of the more dispensable supplies of FMN and free riboflavin (8).

The fact that the highly malignant Novikoff tumor grown in the peritoneal cavity of the rat did not accelerate the development or increase the severity of riboflavin deficiency in the host, or alter the activity of FAD pyrophosphorylase in host liver, suggests that in animals which become riboflavin deficient, the liver can maintain its normal metabolism of riboflavin even with the increased demands made upon it by a large and rapidly growing neoplasm. In these experiments, the total FAD content of the tumor in riboflavin-deficient animals was approximately 15% that of liver. The results obtained in the host livers are similar to those of Aptekar and Ganetskaia (2, 3), who transplanted 5 types of tumors into rats and noted that, in 4 of the 5 groups of recipient animals, no change occurred either in the hepatic concentrations of FAD or of FMN and riboflavin recorded together.

In contrast to liver of tumor-bearing animals, the Novikoff tumor exhibited a remarkable degree of resistance to riboflavin deficiency with respect to the flavin coenzymes.
With limited reserves of dietary riboflavin, Novikoff hepatoma retained a relatively greater proportion of FMN and especially of FAD than did host liver. Both tumor and liver exhibited greater decreases in FMN than in FAD concentrations in riboflavin deficiency, but only in liver was an increase in FAD pyrophosphorylase activity demonstrable. An increase in the net uptake of flavin from blood, an increase in the number of binding sites for FAD in neoplastic tissue, an increase in the affinity of binding for FAD, or a decrease in the rate of enzymatic degradation in tumor tissue could also account for greater retention of FAD by the Novikoff hepatoma. Unfortunately, information on these points is not currently available.

Novikoff hepatoma also differed from liver in the relationship between the magnitude of the concentration of FAD and the activity of the FAD biosynthetic enzyme, FAD pyrophosphorylase. Expressed per g wet weight, FAD concentrations in tumor were less than one-tenth that of liver, but the activity of FAD pyrophosphorylase was nearly the same in both tissues. Per mg protein, FAD concentrations in tumor were approximately one-fifth as great as in liver, and FAD pyrophosphorylase activity was greater than in liver. This relationship suggests active flavin biosynthesis and/or turnover in the tumor. Preliminary observations in this laboratory of the administration of riboflavin-14C to tumor-bearing animals indicate that the rate of FAD synthesis from riboflavin in vivo in Novikoff hepatoma is indeed greater than that in liver.

A further point of interest in the results observed is that, of the 3 flavins measured in tumors from deficient animals, the greatest percentage decrease was in the free riboflavin fraction. This observation may indicate that, in tumor from riboflavin-deficient animals, the small storage pool of free riboflavin, amounting to only a small percentage of the total flavin, is nearly completely utilized in the synthesis of the coenzyme derivatives.

The considerable prolongation of survival of riboflavin-deficient animals bearing tumors has been observed with mammary cancers, lymphosarcoma, and Walker carcinoma (1, 7, 14, 17, 18, 21, 25). Riboflavin deficiency has been produced either by the use of a deficient diet or with structural analogs of the vitamin. The mechanism of tumor inhibition by riboflavin deficiency and its possible application to the treatment of human tumors require further clarification.

REFERENCES

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