N-Nitrosodimethylamine Carcinogenesis in Nicotinamide-deficient Rats

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ABSTRACT

Special diets that were extremely low in protein (5.5%) and high in carbohydrate were used to test the effect of nicotinamide on N-nitrosodimethylamine-induced carcinogenesis in Holtzman albino rats. The level of nicotinamide in the three diets ranged from 0 mg/kg of food to 50 mg/kg to 500 mg/kg. During the treatment with these diets (5 weeks) and the carcinogen (18 days), the renal and hepatic concentrations of nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate-reduced nicotinamide adenine dinucleotide phosphate were measured. In the liver, the concentration of these coenzymes fell well below normal levels, and significant differences between the two extreme diets (0 and 500 mg/kg) were found in the hepatic content of nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide phosphate. In the kidney, the treatment with special diets and carcinogen had less effect. The content of nicotinamide adenine dinucleotide phosphate-reduced nicotinamide adenine dinucleotide phosphate remained normal, and the only significant drop in the concentrations of nicotinamide adenine dinucleotide-reduced nicotinamide adenine dinucleotide phosphate occurred in the animals on the nicotinamide-deficient diet.

After the treatment, all of the animals were returned to the same standard diet. During the remainder of the experiment, 85 weeks, it was found that the initial treatments had not affected tumor incidence levels or tumor type, but had altered the rate of tumor incidence. These differences could be seen by comparing the results for the rats that had been given the nicotinamide-deficient diet to the results for the animals receiving an excess of the vitamin.

INTRODUCTION

During the last 3 decades, a considerable body of evidence has accumulated that indicates that carcinogens can affect vitamin B3 (niacin and/or nicotinamide) metabolism. During this period of time, investigators (14, 35) have shown that some carcinogens can lower the NAD-NADH content of the tissues in which they produce tumors. Studies (9, 16, 29) with cells grown in culture have shown similar effects by a wider range of carcinogens. Other investigators (5, 6) have shown that a group of carcinogens can significantly increase within 12 to 24 hr the urinary excretion of nucleic acid and methylated nicotinamide derivatives. More recently, reports (2, 16, 33, 38) from a number of laboratories have indicated that a variety of carcinogens can stimulate poly(ADP-ribose) polymerase activity. This enzyme, which is found in the chromatin (25), uses NAD as a substrate (4). Several investigators have suggested that poly(ADP-ribose) polymerase may have a role in DNA repair (1, 3, 8, 15, 21, 22, 37).

It is not known if these different effects on nicotinamide and NAD metabolism are related, and the importance of these metabolic changes to the action of carcinogens remains a mystery. Still, the data do link a large number of carcinogens to this vitamin and this coenzyme and, in turn, raise the possibility that the dietary level of nicotinamide or the concentration of NAD in the tissues might modify the action of carcinogens. The results of 2 epidemiological studies, one in Mozambique (28) and the other in the Transkei of South Africa (41), provide support for this possibility. In one report, it was noted that the incidence of carcinoma of the liver was extremely high, while the other report concentrated on the high incidence of esophageal cancer in a region of South Africa. Both of the studies noted that the populations suffered from malnutrition and a dietary deficiency of nicotinamide. In both of these regions, pellagra is still prevalent. As expected, the authors found several problems in the diets of these people; however, in one case (41), the investigators provided evidence suggesting that the dominance of maize as the monostable crop might be increasing the susceptibility of the population to the action of carcinogens. Their evidence showed that most of the pellagrins suffered from ulceration and constriction of the esophagus. The data also demonstrated that pellagra became prevalent in this area after 1930 and that esophageal cancer became an acute medical problem after 1950. This type of time differential suggested that the vitamin deficiency might be acting as a modifying factor in the carcinogenic process.

To further test this possible link between carcinogenesis and the dietary level of nicotinamide, special diets were developed that were designed in part to duplicate the diets that are found in underdeveloped countries. The results of our first set of experiments with these special diets are the subject of this paper.

MATERIALS AND METHODS

For this study, 128 male albino rats initially weighing 80 to 100 g were purchased from the Holtzman Co. (Madison, WI). The animals were housed individually in suspended wire-mesh cages of stainless steel and were kept in a room maintained at 74°F. Tap water was furnished as needed.

After arriving, the rats were given 9 days to adjust to their new surroundings. During this time, the rats were fed Purina rat chow ad libitum. After this initial period of time, 121 animals were weighed, separated into 4 groups (average weight, 160 g/group), and placed on special diets. Each of 3 experimental groups contained 32 rats, while the size of the control group was 25. Seven animals were continued on Purina rat chow and were used as controls in assays for NAD-NADH and NADP-NADPH.

The 3 special diets were produced by Teklad Test Diets (Madison, WI), and were designed in part to mimic the low-protein, high-carbohydrate diets that are found in underdeveloped countries. The protein content of the 3 diets was 5.5% (normal protein content is 18 to 24%), and the only source of protein was a vitamin-free casein hydrolysate. This protein is deficient in tryptophan, and the inclusion of casein hy-
droysate in the special diets was designed to inhibit the metabolic pathway that is used to convert tryptophan into nicotinamide (13, 18). A second change in all 3 diets was that the carbohydrate content, starch plus sucrose, was raised from 67 to 81.5%. The other ingredients (mineral mix, vitamin mix, lipid, fiber, etc.), except for vitamin B₂, were kept at control levels.

The vitamin content of the 3 diets included vitamin A, 40 mg/kg; vitamin D₂, 2200 IU; vitamin E, 121 IU; menadione, 50 mg/kg; calcium panthothenate, 66 mg/kg; riboflavin, 22 mg/kg; thiamin HCl, 22 mg/kg; pyridoxine HCl, 22 mg/kg; folic acid, 2 mg/kg; inositol, 110 mg/kg; vitamin B₁₂, 30 mg/kg; biotin, 0.4 mg/kg; p-aminobenzoic acid, 110 mg/kg; vitamin D₂, 2200 IU; vitamin E, 121 IU; menadione, 50 mg/kg; calcium potassium sulfate, 19 mg/kg; sodium selenite, 0.35 mg/kg, and chromium potassium sulfate, 19 mg/kg. Other common components for the 3 diets included corn oil, 50 g/kg; choline dihydrogen citrate, 3.5 g/kg; pyridoxine HCl, 22 mg/kg; folie acid, 2 mg/kg; inositol, 110 mg/kg; pantothenate, 66 mg/kg; riboflavin, 22 mg/kg; thiamin HCl, 22 mg/kg; and ascorbic acid, 1.02 g/kg. The mineral content of the diets was calcium phosphate, dibasic, 19.7 g/kg; sodium chloride, 2.6 g/kg; potassium iodate, 0.35 mg/kg; sodium selenite, 0.35 mg/kg, and chromium potassium sulfate, 19 mg/kg. Other common components for the 3 diets included corn oil, 50 g/kg; choline dihydrogen citrate, 3.5 g/kg; and nonnutritive fiber (cellulose), 40 g/kg. The diets were given to the animals in pelleted form.

The only source of vitamin B₂ was nicotinamide, and the nicotinamide content of the 3 diets was the only thing that was varied. Rats carrying the code letter L (LC and L groups) were given a diet that was completely devoid of nicotinamide. Rats in the N group were placed on a diet containing a normal amount of nicotinamide, 50 mg/kg of food, while the animals in the H group received a diet containing a 10-fold excess of the vitamin, 500 mg/kg of food.

All of the animals were given 10 days to adjust to the new diets and, following this second period of adjustments, the rats in each group were given a series of injections. During the next 18 days, each animal received 12 injections (4 injections/week). The LC group is the control group (n = 25), and these animals were given injections of 0.85% NaCl. The L, N, and H groups are the experimental groups (n = 32), and these animals were treated with DMN. Data from this laboratory and others have shown that this procarcinogen can cause fragmented DNA (7), lower the NAD content of liver and kidney (35), increase the urinary excretion of methylated nicotinamide derivatives (6), and stimulate poly(ADP-ribose) polymerase activity (33). The DMN was diluted with 0.85% NaCl, and the dose was 5 mg/kg of body weight. The route of administration for all of the injections was i.p. The treatment with DMN is similar to the procedure of Jasmin and Riopelle (17). Once the injections had been completed, the rats were kept on the special diets for an additional week.

After this final period of time, all of the rats in each group were placed on the same standard diet, Purina rat chow. The rest of the experiment took 600 days to complete.

During the 5-week treatment period, rats from the 3 experimental groups were sacrificed, and the NAD-NADH and NADP-NADPH content of their livers and kidneys were determined. At the same time, rats that had been left on a normal diet, Purina rat chow, were sacrificed and used as controls. The cyclic assays of Nissenbaum and Green (26) were used for these analyses.

Throughout the experiment, the animals were monitored daily and examined every 7 to 10 days. At this time, they were weighed and checked for tumors. Whenever a rat died, it was autopsied. At necropsy, samples of the tumors and major organs were fixed in 10% neutral-buffered formalin, embedded, processed by routine histological techniques, and stained with hematoxylin and eosin.

DMN, thiazole blue, phenazine methosulfate, NADP⁺, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, and alcohol dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). A lithium salt of NAD⁺ was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN).

RESULTS

During treatment with special diets, the rats in all of the groups exhibited symptoms of extreme malnutrition. Food intake was not measured but appeared to be reduced, their coats became ruffled and thinned, the animals were lethargic, and they lost weight. The weight changes are given in Chart 1. The data for each group are plotted, and the line represents an average for the 4 groups. During the treatment, the average weight loss was 33 to 34%. The severity of the diets was also demonstrated by the fact that 6 animals died during this time; one in the LC group, 2 in the L group, and 3 in the N group. Since other animals were being sacrificed for coenzyme assays, the number of rats per group was reduced to 24 for the LC group, 23 for the L group, 22 for the N group, and 25 for the H group.

As can be seen in Chart 1, the weight loss patterns were similar. This indicated that the loss of weight was not due to the treatment with DMN or the different amounts of nicotinamide in the diets but was due to the reduced amount of protein and the poor quality of the protein that was present. The similarity in the weight data also meant that the groups given DMN received, on an average, the same amount of carcinogen. The average total intake of DMN for the animals in the L, N, and H groups was 6.82, 6.89, and 6.80 mg, respectively.

After the rats were returned to a normal diet, the weight loss pattern was reversed, and the overall appearance of the rats improved dramatically. This can be seen in the last set of points in Chart 1. The data were collected 5 days after the animals had been returned to a normal diet, Purina rat chow. The average weight gain for the rats in the 4 groups was 39 to 41 g.

Chart 2 shows the growth curves of the groups following the treatment with special diets. For approximately 20 weeks, the weight changes for the 4 groups were nearly identical. After this time, differences between the control and the 3 experimental groups were noted. The rats in the LC group continued to thrive and gain weight, while the animals in the groups receiving the carcinogen exhibited less growth and a more erratic weight gain pattern. The differences between the groups were not significant. Since the last animal in the L group died during the 60th week, the graph for the group was terminated at that time.

NAD and NADP Content. Preliminary studies (data not shown) indicated that extreme diets plus injections of DMN were needed to produce differences in the NAD-NADH content of the liver and kidney. During this experiment, rats from the 3 experimental
groups and the rats that were still being fed a normal diet were sacrificed, and the NAD-NADH and NADP-NADPH content of the livers and kidneys were measured. A total of 7 assays were performed and, in each assay, one animal from each of the groups was used. Five assays were run after the experimental animals have received 3, 4, 8, 9, and 12 injections of DMN. Since the injections were given in the afternoon, the experiments were done the next day. Two additional assays were run at the very end of the treatment period, Days 34 and 35. Average values were done the next day. Two additional assays were run at the very end of the treatment period, Days 34 and 35. Average values for the 7 separate determinations are given in Table 1.

As illustrated, the treatment with special diets and DMN mainly affected the hepatic concentrations of NAD-NADH and NADP-NADPH. For the 3 experimental groups, the average drop in the concentrations of NAD-NADH and NADP-NADPH was 52 and 57%, respectively. A t test was used to test the significance of the differences between the means for the L, N, and H groups and the corresponding values for the animals on the normal diet. In each of 6 separate comparisons, the probability value was less than 0.001. Tests between the experimental groups showed that the differences in NAD-NADH content for the L and H groups and the corresponding values for the animals on the normal diet. In each of 6 separate comparisons, the probability value was less than 0.001. Tests between the experimental groups showed that the differences in NAD-NADH content for the L and H groups were significant (p < 0.005). Similar comparisons of L versus N and N versus H were not significant. In the experimental groups, the hepatic concentrations of NADP-NADPH were very similar. The minor differences between the 3 groups were not statistically significant.

In the kidney, the treatments with special diets and DMN had considerably less effect on the concentrations of NAD-NADH and NADP-NADPH. The only numbers that fell significantly (p < 0.02) below normal levels were the NAD-NADH averages for the L group. The other 2 experimental groups showed a drop in the concentration of NAD-NADH, but these differences were not statistically significant when compared to either the values for the L group or the values for animals on normal chow. Minor differences were seen in the averages for NADP-NADPH, but these differences again proved to be insignificant.

Our earlier pilot studies with smaller groups of animals showed the same basic patterns for the hepatic and renal concentrations of these coenzymes. These studies also showed that the values for the coenzymes quickly returned (5 to 7 days) to normal levels after the animals were reintroduced to a normal diet.

Carcinogenesis. Rats fed the diet that was deficient in nicotinamide died with kidney tumors earlier than did rats fed the other 2 diets. Average life span (in weeks) after the animals were returned to the standard diet was 43.2 ± 1.8 (S.E.) for the rats in the L group (n = 23), 44.0 ± 3.1 for the N group (n = 22), and 47.5 ± 3.2 for the H group (n = 25). A t test to test the significance of the differences between 2 sample means was used to analyze the data. All of the analyses yielded probability values greater than 0.05.

After analyzing the probability curves (Chart 3) and considering the large S.E., especially for the N and H groups, we decided to eliminate outliers (animals that had died extremely early or late) and rerun the statistical tests. The number of outliers removed from the L group ranged from 1 to 6 animals. The range for the N and H groups was 4 to 8. This reduced the sample sizes to 17 to 22 for the L group, 14 to 18 for the N group, and 17 to 21 for the H group. When this was done, the L versus H comparisons yielded probability values between 0.05 and 0.02, indicating that there are significant differences between these 2 groups. Even after removing outliers, the L versus N and N versus H comparisons still proved to be insignificant.

The differences between the 2 groups, L and H, can be seen in Chart 3. These curves for cumulative probability of death show that very few animals were lost during the first 30 to 40 weeks. After this initial lag, the rate of attrition due to renal tumors increased. The break in the curves occurs at Week 34 for the L and H groups. When this was done, the L versus H comparisons yielded probability values between 0.05 and 0.02, indicating that there are significant differences between these 2 groups. Even after removing outliers, the L versus N and N versus H comparisons still proved to be insignificant.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>NAD-NADH (mg/g tissue)</th>
<th>NADP-NADPH (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Kidney</td>
</tr>
<tr>
<td>Control</td>
<td>0.53 ± 0.02</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>L</td>
<td>0.20 ± 0.02</td>
<td>0.35 ± 0.01</td>
</tr>
<tr>
<td>N</td>
<td>0.26 ± 0.02</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>H</td>
<td>0.30 ± 0.01</td>
<td>0.39 ± 0.02</td>
</tr>
</tbody>
</table>

a Rats receiving Purina rat chow.

b Mean ± S.E. of 7 separate determinations.

c Significantly less than the corresponding values for the control group.
d Significantly less than the corresponding value for the H group.

Chart 3. Probability of death with kidney tumor in rats treated with DMN. The cumulative probability of death was calculated according to the method of Saffiotti et al. (34).
and N groups and at Week 39 for the H group. During the next 12 weeks (Weeks 34 to 46), approximately 50% of the rats in each experimental group were lost. At the end of this time interval, the 3 probability curves tended to pinch back together. After Week 46, the rate of death increased again. This is easily seen in the data for the L group, while it is less apparent for the other groups. The last animal in the L group died during Week 60, while 2 rats, one from the N group and one from the H group, were sacrificed on Day 600 (Week 85). The autopsies showed that both of the animals had renal tumors.

Computer regression analysis of the data after the initial break (Week 34 or 39) showed that there was a significant difference (p < 0.005) between the slopes for the L curve and the slopes for the N or H curves. If the animals that died before the break are not considered, then the average life spans for the rats in the 3 groups are 44.1 ± 1.6 for the animals in the L group (n = 22), 47.9 ± 3.1 for the N group (n = 18), and 51.9 ± 2.8 for the H group (n = 21). The difference between the L and H groups is significant (p < 0.025) and remains significant (p < 0.05) when the animals that died extremely late are also removed from the calculations. Similar comparisons with the N group (L versus N and N versus H) were not statistically significant.

The average life span of the animals in the LC group (n = 24) was 82.9 weeks. Only 4 rats died before Week 85. The earliest death occurred during Week 64. The other animals died during Weeks 69, 78, and 79. In each case, the cause of death appeared to be due to respiratory problems. Autopsies on all of the animals in the control group showed that they were free of renal tumors.

Tumor Characteristics. All of the rats in each experimental group developed renal tumors. Macroscopic and microscopic examinations indicated that a large majority of these tumors were similar to the mesenchymal tumors described by Hard and Butler (11, 12). The spindle cell was the predominant cell type, and these cells were organized into sarcoma-like tissue. In each case, the tumor was extremely large but, from our preliminary examinations, showed that this was accomplished; and (b) to produce varying concentrations of NAD-NADH and/or NADP-NADPH in the livers and kidneys of the rats receiving DMN. It has been known for some time that such differences are difficult to produce in rats (18, 24), and our own preliminary experiments (data not shown) confirmed these earlier results. Nevertheless, by restricting the protein content of the diets to 5.5% and by using casein hydrolysate as the sole source of protein, differences in the concentration of NAD-NADH were produced. These changes were readily apparent in the liver and less apparent in the kidney.

The metabolic events underlying the basic observation on carcinogenesis are far from clear, and the complexity of the situation is compounded by the metabolic disturbances that the special diets must have produced. The differences in the concentration of nicotinamide plus the protein deficiency are bound to have altered innumerable aspects of metabolism including carcinogen activation and inactivation. With DMN, this has already been demonstrated. Swann and McLean (39) showed that rats fed a protein-free high-carbohydrate diet metabolized DMN at a reduced rate when compared to animals on a normal diet. Their experiments also showed that liver slices from animals fed the protein-free diet metabolized DMN at a reduced rate, while kidney slices from the same animals were unaffected. Since our test diets are similar to theirs, it is possible that our diets altered DMN metabolism in the same way. If this is true, then the dietary level of nicotinamide might have been modified even further these changes in DMN metabolism and, in turn, modified the rate of carcinogenesis. Such a possibility suggests that variations in liver metabolism may have contributed to the differences in renal carcinogenesis. One argument against this idea comes from the data on NAD-NADH and NADP-NADPH. Investigators (20, 23) have shown that NADP+, not NAD+, is required during the metabolism of DMN. Our data showed that the diets did not produce differences in the concentration of NADP-NADPH. The hepatic concentration of the coenzyme was depressed but to the same extent for the 3 experimental groups, while the renal concentration of the coenzyme was not affected by the diets or the carcinogen. Since the assays were always performed at the same time (18 hr after an injection of DMN), it is possible that transient changes in the concentration of the coenzyme were missed.

The data on the coenzymes suggest that fluctuations in the concentration of NAD-NADH are somehow related to the variations in renal carcinogenesis. The significant differences produced in the liver (L versus H) and kidney (L versus normal) showed that this coenzyme was more sensitive to the dietary level of nicotinamide during the 5-week treatment. Once again, a liver to kidney vector might be suggested, since the changes in the liver were greater than the changes that were found in the kidney. How these changes in the cellular concentration of NAD+ or NADH might affect DMN metabolism or be affected by the metabolism of DMN is not known, but a connection between the coenzyme and DMN is indicated by this data and data from other laboratories (33, 35).

As can be seen, there is no obvious explanation for the difference in response to DMN of rats fed low, normal, or nicotinamide-supplemented diets. However, the results add weight to the growing body of evidence connecting carcinogens...
to nicotinamide metabolism, and also provide support for the epidemiological evidence (41) linking a dietary deficiency of niacinamide to an increase in carcinogenesis. How important this support might be will depend on further experimentation using other carcinogens and other diets. In this regard, it is interesting to note that niacin supplementation of a lipotrope-deficient diet reduced the rate of death with hepatic tumors in rats treated with N-nitrosodimethylamine (30), and that nicotinamide supplementation of a basic grain diet suppressed the carcinogenic effects of bracken fern (27).

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