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Dietary Milk Proteins Inhibit the Development of Dimethylhydrazine-Induced Malignancy[1]

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Abstract. This study investigated the influence of two formula diets containing 20 g/100 g diet of either whey protein concentrate or casein, or Purina mouse chow on 1,2-dimethylhydrazine (DMH)-induced colon carcinoma in A/J mice. Four weeks after the 24th DMH treatment the incidence of tumour and tumour area in the whey protein-fed mice was substantially less in comparison to either the casein or Purina groups. The Purina group exhibited the greatest tumour burden. At the end of the experiment all animals continuously fed the whey protein diet were found to be alive, whereas 33% of those on the casein or Purina diet had died. Animals fed Purina diet for 20 weeks and then switched to either milk protein diet for a further 8 weeks exhibited a decrease in tumour burden as compared to those animals fed the Purina diet continuously. Body weights were similar in all dietary groups. In conclusion, a whey protein diet appears to significantly influence the development of chemically induced colon tumours and the short-term survival of mice.

Introduction

The current study was designed to evaluate the effect of whey protein in diets on the development of a chemically induced type of murine tumour. The 20 g net protein level/100 g diet was chosen because at this level most protein, including the two proteins in our test formula diets, supplies the minimum requirement of all indispensable amino acids for the growing mouse [1]. 1,2-Dimethylhydrazine (DMH) has been demonstrated [2, 3] to be a potent carcinogen which produces carcinomas of the colon in rodents in a reproducible manner. Fiber, fat, and level of dietary protein have been shown to be either protective [4, 5] or promotive [6, 7] in DMH-induced colon carcinoma. Tumours are characteristically located in the distal bowel and long-term exposure to the carcinogen is required before the lesions appear. The development of neoplasms is also

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Table 1. Vitamin and mineral content of formula diets

The vitamin mixture plus the vitamins contained in the base diet (Mead Johnson product 80056) provided *in milligrams per 100 g diet:*

ascorbic acid, 53.3; niacin, 5.1; riboflavin, 0.38; thiamin, 0.34; folic acid, 0.063; vitamin B-6, 0.26; biotin, 0.031; pantothenic acid, 1.93; choline, 44

and *per 100 g diet:*

retinyl palmitate, 1,295 IU, ergocalciferol, 260 IU; vitamin E (*dl*-tocopheryl acetate), 11.6 IU; vitamin B-12, 0.001 mg; and vitamin K (phylloquinone), 0.06 mg.

The mineral content of ions or cations (expressed in milligrams per 100 g diet) and the actual chemical compounds fed were:

Ca (whey protein diet: 400; casein diet: 350) ($CaHPO_4 \cdot 2H_2O$ and $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$); P (whey protein diet: 300; casein diet: 330) ($K_2HPO_2 \cdot 2H_2O$); Fe, 7.9 ($FeSO_4 \cdot 2H_2O$); Mg, 63.2 (MgO); Cu, 0.31 ($CuSO_4 \cdot 5H_2O$); Zn, 3.5 ($ZnSO_4 \cdot 7H_2O$); Mn, 0.48 ($MnSO_4$); Cl, 1108 ($C_5H_{14}ClNO$); K, 997 ($K_2HPO_4 \cdot 2H_2O$); Na, 232 (NaCl)

influenced by the genetic background of the animal [8] and susceptibility is related to the degree of DNA damage [9]. We therefore chose A/J mice since they are sensitive to DMH and their genetic background is well known. In addition, DMH-induced colon tumours appear to be similar to those found in humans as far as type of lesions and chemotherapeutic response characteristics are concerned [10, 11].

Materials and Methods

Mice

Sixty female, A/J strain mice (Jackson Laboratory) were segregated into equal groups of 12 individually numbered mice and housed in similar cages with 6 animals per cage. All mice were obtained at 6–8 weeks of age and then started on the test diets 3 weeks prior to commencing carcinogen treatment. Test diets were maintained throughout the duration of the experiment.

Carcinogen Treatment

DMH 2 HCl (Sigma Chemical Company) was prepared by dissolving the powdered chemical in 0.9% NaCl to a final concentration of 15 mg/100 ml with the pH adjusted to 6.9–7.0 using saturated NaOH. Carcinogen solutions were used on the same day they were prepared. Mice were injected subcutaneously with a weekly dose of 15 mg DMH/kg body weight for 24 weeks.

Tumour Assessment

The animals were killed 4 weeks after their 24th carcinogen injection. Colons were removed, opened longitudinally, fecal contents removed, and the colons then weighed and their length measured. Tumour burden was assessed both by the number of tumours and the sum of the products of the vertical and horizontal tumour diameters of all grossly visible tumours.

Survival Study

The number of surviving animals in the whey protein, casein and Purina groups was recorded at the time of sacrifice after a 28-week observation period.

Diets

The detailed composition of some common ingredients (vitamins and minerals) in the two defined formula diets is given in table 1. Diets are prepared in the following way: 20 g of selected net protein, 56 g of product 80056 protein-free diet powder containing corn syrup, corn oil, tapioca starch, vitamins and minerals (Mead-Johnson Co. Inc., USA), 18 g cornstarch, 2 g wheat fiber, 0.05 g Nutramigen vit-iron premix (Bristol-Meyers, Ontario, Canada), 2.65 g

Table 2. Effect of dietary milk protein on animal growth and tumour development in A/J mice treated with the carcinogen DMH

	Whey protein 28 weeks[a]	Casein 28 weeks[a]	Purina 28 weeks[a]	Purina/whey 20/8 weeks[b]	Purina/casein 20/8 weeks[b]
Initial weight, g	21.7±0.5[c]	21.5±0.7	21.9±0.8	21.9±0.4	22.0±0.7
Final weight, g	21.5±0.3	21.8±0.4	19.7±0.7	21.3±1.0	21.0±0.6
Number of tumours	8.4±1.5	24.7±3.0	35.9±2.6	15.1±3.2	21.7±4.3
Tumour area	38.8±6.4	90.9±10.6	160.0±11.4	47.9±10.4	77.7±10.9

[a] Mice treated with DMH for 24 weeks, and then sacrificed 4 weeks later.
[b] Mice treated with DMH for 24 weeks, and then sacrificed 4 weeks later. They were maintained on Purina mouse chow for 20 weeks and then switched to either whey protein or casein diet for the remaining 8 weeks.
[c] Mean ± SEM.

ANOVA: solid lines connect those means not significantly different ($p < 0.05$ for significantly different groups)

	Whey	Purina/whey	Purina/casein	Casein	Purina
Number of tumours		└──────	──────┘ └───	───┘	
Tumour area	└──────	──────┘	└──────	──────┘	

KCl, 0.84 g NaCl. The principal variable in the two purified diets was the type of protein. The formula diets contained 20 g/100 g diet of either whey protein concentrated or casein. Whey protein concentrate is made of proteins that remain soluble in 'milk serum' or whey after precipitation of casein at pH 4.6 and 20 °C, as in the manufacture of cheese. The whey protein concentrate used in our experiments was Lacprodan-80 (DanMark Protein) containing 80% protein received in 1987, with solubility of 94.5% at pH 6.4 in a 3% protein solution. Other animals were fed Purina mouse chow (estimated 23% protein from various sources). The amino acid distribution in whey protein concentrate and in casein was given in a previous article [12]. In addition, Lacprodan-80 contained 3.2% lactose, 8% fat, 2.5% ash and 4.9% moisture. The casein powder contained 80% casein, 1% lactose, 6% fat, 2.2% ash and 10% moisture.

Diets were continuously available in powder form from stainless steel feeders, 1.5 inches high and especially designed to reduce spillage and spoilage.

Statistical Analysis

Intergroup differences in tumour number and tumour area were analyzed by one-way ANOVA. The effect of milk protein on short-term survival was evaluated by χ^2 analysis.

Results

Growth

Body growth and the weight curves were similar for all dietary groups (table 2).

Tumour Development

The tumours exhibited histologic (fig. 1) evidence of adenocarcinoma (fig. 2) and biologic signs of malignancy. One hundred percent mortality in mice not sacrificed for tumour analysis was reached within 5 months

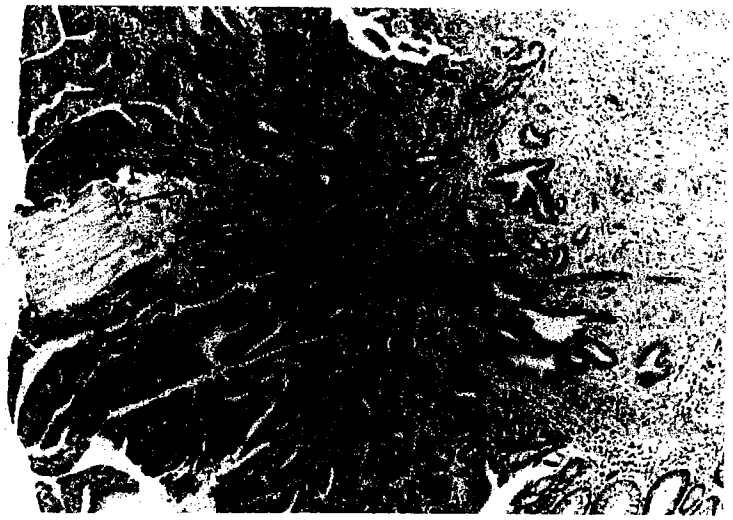

Fig. 1. Carcinoma invading colon submucosa in DMH-treated mice. HE. × 63.

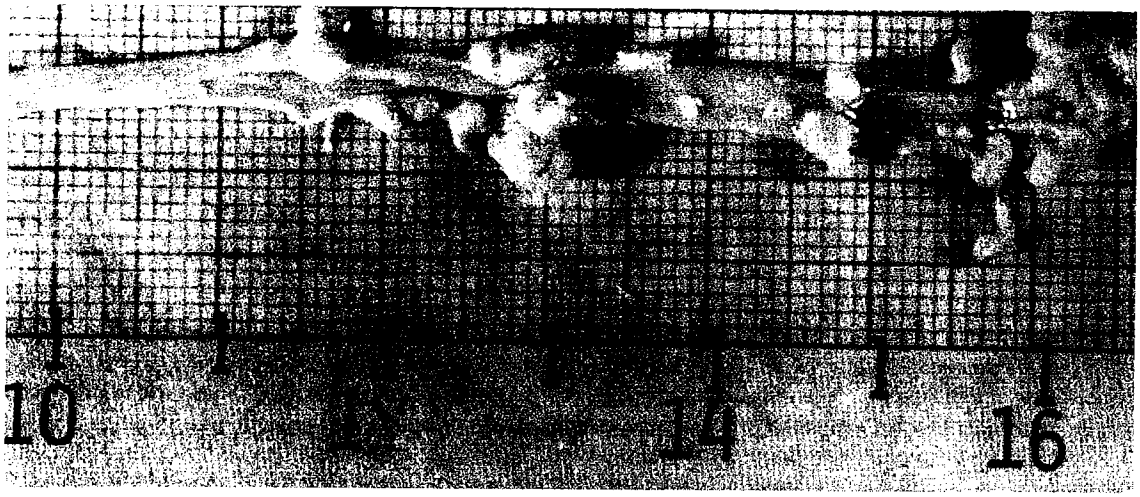

Fig. 2. Gross appearance of typical lesions in the luminal aspect of the colon of DMH-treated mice.

following the standard 24-week carcinogen treatment. At autopsy, multiple haemorrhagic and non-haemorrhagic lesions were seen in the colon, liver metastasis and ascites.

There were significant ($p < 0.05$) differences both in the final number of tumours and the total tumour burden (table 2). The animals fed the whey protein diet throughout the 28-week experiment developed an average of 8.4 ± 1.5 tumours per animal, whereas the corresponding casein-fed and Purina mouse chow-fed animals developed 24,7 ± 3.0 and 35.9 ± 2.6 tumours per animal, respectively. Moreover, tumour area development among groups was significantly

different with the whey protein-, casein-, and Purina mouse chow-fed animals having 38.8 ± 6.4, 90.9 ± 10.6, and 160.0 ± 11.4 mm^2 of tumour area, respectively. All intergroup differences were statistically significant. When animals were switched to the whey protein diet after 20 weeks of treatment, the average tumour number was 15.1 ± 32, not significantly different from the average number of 21.7 ± 4.3 in those switched to the casein diet at the same time. However, the tumour area development among the corresponding groups was significantly different with the Purina/whey and the Purina/casein having 47.9 ± 10.4 and 77.7 ± 10.9 mm^2 of tumour area, respectively.

Survival

Four weeks after the 24 weeks of carcinogen treatment most animal groups were sacrificed for tumour assessment. At that time, all mice continuously fed the whey protein diet were found to be alive; whereas, 33% of those on the casein or Purina diet had died (table 3).

Table 3. Effect of dietary milk protein on short-term survival in A/J mice treated with the carcinogen for 24 weeks

	Dietary group[a]		
	whey protein	casein	Purina
Mortality[b] at 28 weeks, %	0	33	33

[a] Originally 12 mice per group.
[b] Significance by χ^2 analysis: whey protein vs. Purina or casein $p < 0.05$.

Discussion

A 24–28% inhibition of Ehrlich ascites tumour cell count was reported in mice fed yogurt; the anti-tumour activity was found to be localized in the solid fractions. However, while yogurt effectively inhibited initial tumour growth, it did not retard long-term growth and had no significant effect on survival [13]. Similarly, a 32% inhibition of Ehrlich ascites tumour cells were obtained by others in mice fed yogurt [14]. Various types of cheese and yogurt were recently found to suppress the growth of several experimental tumours in mice in proportion to the duration of feeding. The tumor size was reduced anywhere from 17–70% depending on the type of tumour. Some minor differences in tumour-inhibiting effect was noted between various natural cheeses and yogurts. The principal protein component of these dairy products was casein, though small variable amounts of whey protein may be present. No increase in antibody response to sheep RBCs was observed [15]. Data in table 2 clearly confirm our earlier preliminary report on the inhibitory effect of whey protein on DMH-induced tumours [12]. The marked difference in the number and size of tumours between mice eating Purina throughout the entire 28-week experiment and those switched from Purina to a whey protein diet only during the final 8 weeks of study, may indicate an effect following tumour initiation. Though both milk proteins exhibit a tumour-inhibiting effect, it is apparent that the effect of whey protein is greatly superior especially when given throughout the entire experiment. The influence of milk protein feeding during the later stage of tumour development is substantiated by the observation that the tu-

mour area in mice fed the milk proteins only during the last 8 weeks is not significantly larger than the tumour area in mice fed the corresponding milk protein since the beginning of carcinogen treatment. The same holds true for the number of tumours in the casein-fed mice. However, the effect of whey protein feeding on the number of tumours is evident both in the initial and in the subsequent phase of carcinogen treatment. Our data do not clearly differentiate between the effect of diet on the activation of DMH or the carcinogenic process. Hence, we do not have a clear-cut understanding of the process leading to tumour inhibition. The whey protein diet may exert an inhibiting effect on the activation of DMH, induction and development of tumours as demonstrated by the severalfold difference in tumour number and size between the Purina-fed mice and those fed whey protein throughout the entire experiment. Several considerations point to the protein component of the diet as the crucial factor in the observed biologic effect.

With regard to the two formula diets, a major difference in the tumour-inhibiting effect of dietary treatment is seen when the principal variable is the type of protein. The two purified protein powders are vitamin free. Hence, the vitamin content of the two formula diets are the same. Mineral content is the same for both formulas with only minor differences in Ca and P. No major difference exists in the lipid content of the whey protein and casein powders. The mouse chow varies significantly from batch to batch. This will naturally weaken the validity of comparing the results between each of the two partially purified diets with the results with Purina mouse chow. However, in spite of these limitations, we have included data from the Purina group because most mice utilized for cancer research, including the progenitors of our mice, are fed Purina mouse chow. In spite of the great variations most Purina batches contain lower levels of fat and higher levels of fiber than the two formula diets. Fiber content in Purina is greater than 4 g% – substantially higher than the 2% content of our formula diet. Fibers have been shown in our laboratory to be protective against DMH-induced colon cancer in rodents [4]. In addition, the two formula diets contain 12.8 g corn oil/100 g diet. The fat component of Purina obtained from the manufacturer is 4.7%, mostly as beef fat. Recent experimental evidence indicates that increasing dietary fat in the form of corn oil enhances the promotion of chemically induced breast cancer in rats over a wide range of protein (casein) intake [16]. Polyunsaturated fat feeding promotes tumourigenesis [17] and polyunsaturated fatty acid incorporation into cell phospholipids can increase membrane fluidity by making it more 'sol-like' than 'gel-like' [18]. More specifically, in DMH-treated mice the highest tumour incidence and volume has been reported in animals fed corn oil as the only source of fat and was found to be lowest in mice fed beef fat as the only source of dietary lipid [19]. All these related observations obviate the likelihood that fat and fiber components of our formula diet may have exerted an anti-cancer effect in comparison to the Purina stock diet. The mechanism underlying this apparent anti-tumour effect of whey protein in the diet can only be a matter of speculation. With regard to the possible promotional effect of ammonia on cancer, studies in term infants, fed either whey-predominant or casein-predominant formula, demonstrate that, with similar total nitrogen intake, mean daily weight gain, total serum proteins, plasma ammonia

and urea nitrogen were similar [20]. These data suggest that, at least in humans, no difference is apparent between the two proteins in terms of digestibility and utilization. It would thus be unlikely that the amount of nitrogen reaching the colon would differ significantly in the two formula diet groups. Casein appears to exert some antitumour effect, particularly in terms of tumour area. It is possible that some of the most active anti-cancer components of the whey protein mixture may coprecipitate with the casein fraction during the manufacturing processes [21].

Our results indicate that the anti-cancer effect of the whey protein diet is substantially greater than the previously reported effect of cheese and yogurt on tumor growth. It is thus conceivable that the anti-cancer factor reported in the latter experiment was related to the milk protein moiety.

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