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## DIETARY WHEY PROTEIN INHIBITS THE DEVELOPMENT OF DIMETHYLHYDRAZINE INDUCED MALIGNANCY

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**Abstract**—This study investigates the influence of two formula diets containing 20 g / 100 g diet of either whey protein concentrate or casein or Purina mouse chow, on the humoral immune responsiveness and dimethylhydrazine induced colon carcinogenesis in A / J mice. After 20 weeks of dimethylhydrazine treatment, the number of plaque forming cells per spleen, following intravenous inoculation with  $5 \times 10^6$  sheep red blood cells, was nearly three times greater in the whey protein-fed group than in the casein-fed mice although both values were substantially below normal. After 24 weeks of dimethylhydrazine treatment the incidence of tumors in the whey protein-fed mice was substantially lower than that in mice fed either the casein or Purina diet. Similarly, the tumor area was less in the whey protein group in comparison to either the casein or Purina groups, with some difference between casein and Purina groups. Body weight curves were similar in all dietary groups.

In conclusion, a whey protein diet appears to significantly inhibit the incidence and growth of chemically induced colon tumors in mice.

**Résumé**—Nous évaluons l'effet de diètes apportant 20 g / 100 g de protéines sous forme de protéines de petit lait, de caséine, ou de diète commerciale (PURINA) sur la réponse immune humorale et sur la colonisation carcinogénique du colon induite par le diméthylhydrazine chez la souris A / J. Après 20 semaines, le nombre de cellules de la rate formant des plaques post-incubation avec des globules rouges de mouton est deux fois plus élevé chez les animaux nourris au petit lait qu'à la caséine, quoique les deux valeurs demeurent sous la valeur témoin. Après 24 semaines de diméthylhydrazine, l'incidence et la surface d'invasion tumorale sont plus basses chez les souris recevant du petit lait que dans les autres groupes. Les courbes de croissances pondérales sont semblables dans tous les groupes. Nous concluons que les protéines de petit lait semblent inhiber l'incidence et la croissance de tumeurs induites par des carcinogènes.

**Key words:** dietary whey proteins, tumor growth, 1,2-Dimethylhydrazine.

### INTRODUCTION

IT HAS LONG BEEN RECOGNIZED that protein calorie malnutrition depresses host resistance to infections [1]. Experimental [2, 3] and clinical [4] evidence has since substantiated the concept that protein intake restriction adversely affects various components of the immune system. Our interest in the effect of dietary amino acids on the immune system was prompted by the observation that changes in the amino acid profile of the diet can influence the immune response without having any significant effect on the nutritional status of the host [5]. It was subsequently discovered that indeed the type of protein (i.e. amino acid profile) in nutritionally adequate and similar diets can influence the intensity of the immune response. The humoral immune response (number of plaque forming cells to sheep red blood cells) of mice fed 20 g whey protein concentrate / 100 g diet was found to be significantly

higher than that of mice fed formula diets of similar nutritional efficiency containing 20 g / 100 g diet of any other type of food protein such as casein, soy, wheat, corn, egg white, fish, beef protein, *Spirulina maxima*, *Scenedesmus* algae protein or Purina mouse chow [6, 7]. Moreover, mice fed a 20 g whey protein / 100 g diet showed improved survival after intravenous infection with *Streptococcus pneumoniae* type 3, as compared to mice fed a 20 g casein / 100 g diet of similar nutritional efficiency [6]. The immunoenhancing property of whey protein was maximal at 20% concentration [8]. It was found that raising the protein level of either whey protein, casein, soy or wheat protein in the diet above 20% failed to enhance the immune response of the host beyond the values observed with the 20 g protein / 100g diet [9].

The current study was designed to evaluate the effect of whey protein in diets on the development of a chemically induced type of murine tumor. The 20 g net protein level / 100 g diet was chosen for the above described reasons. In addition, at this level most protein, including the two proteins in our test formula diets, supplies the minimum

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requirement of all indispensable amino acids for the growing mouse [10]. 1,2-dimethylhydrazine has been demonstrated [11, 12] to be a potent carcinogen which produces rodent carcinomas of the colon in a reproducible manner. Fiber, fat, and level of dietary protein have been shown to be either protective [13, 14] or promotive [15, 16] in dimethylhydrazine induced colon carcinogenesis. Tumors 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 influenced by the genetic background of the animal [17] and susceptibility is related to the degree of DNA damage [18]. We therefore chose A / J mice since they are sensitive to dimethylhydrazine and their genetic background is well known.

## MATERIALS AND METHODS

### Mice

Thirty female, A / J strain mice (Jackson Laboratory) were segregated into 3 equal groups of individually numbered mice and housed in similar cages with 5 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

1,2-Dimethylhydrazine (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 dimethylhydrazine / kg body weight for 24 weeks.

### Tumor 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. Tumor burden was assessed both by the number of tumors and the sum of the products of the vertical and horizontal tumor diameters of all grossly visible tumors.

### 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., U.S.A.), 18 g cornstarch, 2 g wheat fiber, 0.5 g Nutramigen vit-iron premix (Bristol-Meyers, Ontario, Canada), 2.65 g

TABLE 1. VITAMIN AND MINERAL CONTENT OF TEST DIETS

The vitamin mixture plus the vitamins contained in the basal diet (Mead Johnson product 80056) provided in milligrams per 100 g diet: ascorbic acid, 65.0; niacin, 9.2; riboflavin, 0.69; thiamin, 0.63; folic acid, 0.12; vitamin B-6, 0.36; biotin, 0.058; pantothenic acid, 3.38; choline, 76 and per 100 g diet: retinyl palmitate, 1439 IU; ergocalciferol, 360 IU; vitamin E ( <i>dl</i> -tocopheryl acetate), 9.0 IU; vitamin B-12, 0.54 mg; and vitamin K (phylloquinone), 90 µg. The mineral content of ions or cations (expressed in milligrams per 100 g diet) and the actual chemical compounds fed were:	
Ca, 378 (CaHPO <sub>4</sub> · 2H <sub>2</sub> O and Ca <sub>3</sub> (C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> ) <sub>2</sub> · 4H <sub>2</sub> O); P, 208 (K <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O); Fe, 7.7 (FeSO <sub>4</sub> · 2H <sub>2</sub> O); Mg, 44 (MgO); Cu, 0.38 (CuSO <sub>4</sub> · 5H <sub>2</sub> O); Zn, 2.5 (ZnSO <sub>4</sub> · 7H <sub>2</sub> O); Mn, 0.63 (MnSO <sub>4</sub> ); Cl, 840 (C <sub>5</sub> H <sub>14</sub> ClNO); K, 1050 (K <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O); Na, 245 (NaCl).	

TABLE 2. AMINO ACID COMPOSITION OF COW MILK PROTEINS (IN G / 100 G PROTEIN)<sup>a</sup>

Amino acid	Casein <sup>b</sup>	Whey <sup>c</sup>
Phenylalanine	5.3 ± 0.2	3.4 ± 0.3
Tryptophan	1.4 ± 0.2	2.1 ± 0.0
Glycine	2.0 ± 0.1	2.0 ± 0.2
Serine	6.2 ± 0.5	5.2 ± 0.4
Leucine	10.0 ± 0.4	10.4 ± 0.7
Isoleucine	6.0 ± 0.6	6.1 ± 0.8
Valine	7.1 ± 0.3	5.8 ± 0.8
Methionine	2.9 ± 0.2	2.1 ± 0.3
Cysteine	0.3 ± 0.1	2.3 ± 0.3
Aspartic acid	7.3 ± 0.1	10.7 ± 0.7
Glutamic acid	22.9 ± 0.3	18.8 ± 0.7
Histidine	3.0 ± 0.1	2.0 ± 0.2
Tyrosine	6.0 ± 0.1	3.0 ± 0.4
Proline	11.6 ± 0.4	6.1 ± 0.7
Arginine	4.0 ± 0.1	2.8 ± 0.3
Alanine	3.1 ± 0.3	4.9 ± 0.4
Lysine	8.2 ± 0.1	9.2 ± 0.5
Threonine	4.6 ± 0.3	6.8 ± 1.3

<sup>a</sup>Value expressed as Mean ± S.D. of data from reliable sources.

<sup>b</sup>References 19, 20, 21.

<sup>c</sup>References 19, 22, 23, 24.

KCl; 0.84 g NaCl. The only variable in the two purified diets was the type of protein. The formula diets contained 20 g / 100 g diet of either whey protein concentrate 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. Other animals were fed Purina mouse chow (estimated 23% protein from various sources). The amino acid composition of bovine whey protein concentrate and casein is given in Table 2, which shows the grand mean of all data from reliable sources including the samples used in our study [19–24].

### Immunization for plaque assays

The diet-fed mice were immunized by an intravenous injection of 5 × 10<sup>6</sup> washed sheep red blood cells, obtained weekly from Institut Armand-Frappier, Laval des Rapides, Quebec, Canada.

TABLE 3. EFFECT OF DIETARY PROTEIN REGIMEN ON BODY GROWTH AND TUMOUR DEVELOPMENT IN 1,2-DIMETHYLHYDRAZINE TREATED A / J MICE<sup>a</sup>

Variable	Dietary treatment		
	Whey protein	Casein	Purina
Body weight <sup>b</sup>			
Initial (g)	21.06 ± 1.32	23.94 ± 2.49	22.13 ± 1.36
Final (% initial)	108.0 ± 7.7	108.9 ± 10.2	110.7 ± 9.70
Number of tumours <sup>c</sup>	8.50 ± 3.87	13.8 ± 4.83	16.9 ± 9.85
Tumour area <sup>d</sup>	32.18 ± 13.69	47.35 ± 14.02	78.15 ± 31.19

<sup>a</sup>Mean of 10 mice per group ± Standard Deviation.

<sup>b</sup>Among dietary groups there was no statistically significant difference in initial body weight or in the body weight reached after 28 weeks.

<sup>c</sup>Whey protein vs casein  $p = 0.0138$

Whey protein vs Purina  $p = 0.0208$

Purina vs casein  $p = 0.3868$

<sup>d</sup>Whey protein vs casein  $p = 0.0236$

Whey protein vs Purina  $p < 0.001$

Purina vs casein  $p = 0.0104$

#### Plaque forming cell assay

The method used for assaying IgM plaque-forming cells was essentially the one described by Cunningham and Szenberg [25] with certain minor modifications [5]. The mice were injected intravenously (i.v.) with  $5 \times 10^6$  sheep red blood cells and assayed for plaques on day 5 when the response was shown to peak [5].

#### Statistical analysis

Statistical evaluation of difference between dietary groups was done by the Student's t-test.

## RESULTS

#### Growth

Our previous studies [6, 9] have shown that mice fed the above described 20 g protein / 100 g formula diets and Purina mouse chow increased in body weight by approximately the same amount, with similar food consumption. In the current studies body growth and the weight curves were similar for all dietary groups (Table 3).

#### Plaque-forming cells assay

After 20 weeks of dimethylhydrazine treatment, the total number of plaque-forming cells per spleen in whey protein fed mice and in casein fed mice were  $13 \pm 1.6$  (Mean ± SE) and  $5 \pm 1.3$  ( $p < 0.005$ ) respectively. Eight weeks later, with increasing tumor burden, the number of plaque forming cells in response to sheep red blood cells had further declined to marginal levels:  $1.77 \pm 0.9$  for the whey protein group and  $0.34 \pm 0.1$  for the casein group.

#### Tumor development

There were significant ( $p < 0.05$ ) differences, both in the

final number of tumors and the total tumor burden (Table 3). The whey protein-fed animals developed an average of  $8.50 \pm 3.87$  tumors per animal, whereas the casein-fed and Purina mouse chow-fed animals developed  $13.80 \pm 4.83$  and  $16.90 \pm 9.85$  tumors per animal respectively. Moreover, tumor area development among groups was significantly different with the whey protein-, casein-, and Purina mouse chow-fed animals having  $32.18 \pm 13.69$ ,  $47.35 \pm 14.02$ , and  $78.15 \pm 31.19$  mm<sup>2</sup> of tumor area respectively. All intergroup differences were significantly different, except for the number of tumors between the casein-fed and the Purina mouse chow-fed groups.

## DISCUSSION

Our previous studies have shown that feeding whey protein to mice enhances the humoral immune response of the animals to heterologous erythrocytes in comparison to the other commercially available purified food protein [6–9, 26, 27]. The immunoenhancing effect of dietary whey protein has been observed in several unrelated strains of mice, namely C3H / HeN, DBA / 2, C57BL / 6, BALB / C, and CBA / N [6–9, 26, 27]. They ranged in age from 7 weeks [6–9, 26, 27] to 6 months [27]. This effect was found to reach its peak after 2 weeks and to persist as long as dietary treatment is continued (tested up to 2 months) [7]. In the current study the plaque forming cell response to sheep red blood cells was found to be higher in the whey protein-fed mice in comparison to the casein fed-mice, after 20 weeks of carcinogen treatment. These values are about ten times lower than the corresponding values previously observed in healthy mice fed the same two diets [6–9, 26, 27] presumably because of the immunodepressing effect of dimethylhydrazine treatment and tumor burden. It is noteworthy, however, that the difference in immune responses between whey protein and casein-fed, carcinogen treated mice is comparable to that previously reported in healthy, untreated mice fed the same two diets [6, 9].

The casein diet differed from the whey protein test diet only in the type of protein. We selected casein as control because this good quality protein is the most commonly used food protein both in laboratory and clinical settings. As mentioned earlier, at 20% concentration bovine casein supplies all the amino acid requirements for the growing mouse [10]. Purina was included in our studies as a second reference control because most mice, including the ancestors of the current mice, are Purina-fed. In addition, to the extent that dietary protein type influences the humoral immune response, the presence of varying amounts of beef, fish, and whey protein in Purina rodent chow may explain the relatively high plaque forming cell response to sheep red blood cells previously reported in our Purina-fed mice. These values were found to be second only to those noted in whey protein fed mice and very close to the corresponding values in beef and in fish protein-fed mice [6–9].

The similarity in body weight curves among the three dietary groups, consistent with studies in other mouse strains [6–9, 26, 27] is striking and appears to rule out conventional nutritional factors for the observed differences in the development of tumors.

The difference in the incidence of tumors and tumor area between whey protein-fed and casein-fed mice is particularly

significant in view of the fact that the two diets are identical except for the protein type. Similarly there is no difference in body growth, colon weight, or length between the two dietary groups.

It is well known that the incidence and size of tumors are influenced by the immune system. In the advanced phase of disease in which our plaque forming cell measurements were made, the humoral immune response was greatly reduced in all dietary groups. These measurements, nevertheless, reflected the pattern of humoral immune response in relation to food protein type noted in our previous studies on healthy mice [6–9, 26, 27]. It is, hence, conceivable that, particularly in the early phase of tumor development, the protein-related difference in immune reactivity among the dietary groups may have influenced the observed difference in tumor development.

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