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Sprague-Dawley Rats Fed Milk or Beef Protein : Differences in Response to 1,2-dimethylhydrazine Carcinogenesis

Stanley W. L. Ng

Abstract

SPRAGUE-DAWLEY RATS FED MILK OR BEEF PROTEIN:

DIFFERENCES IN RESPONSE TO 1,2-DIMETHYLHYDRAZINE CARCINOGENESIS

by

Stanley W. L. Ng

Colon cancer is responsible for a high percentage of cancer deaths in developing countries, and there is convincing epidemiological evidence that meat protein and fat in the diet increase the incidence of this form of cancer. An animal tumor model which has been used by many investigators interested in colon cancer is the 1,2-dimethylhydrazine (DMH)-rat or DMH-mouse tumor model. In previous work done in this laboratory the DMH-BALB/c mouse tumor model was used to compare the production of colon tumors in mice fed a diet using non-fat powdered milk as the source of protein with those in mice fed beef as protein source. In contrast with epidemiological findings (and also contrary to results with other tumor models where milk was found to restrict tumor growth), colon tumor incidence was much greater in the milk-fed mice than in the beef-fed mice. Since dietary fiber studies in the DMH-mouse tumor model in another laboratory had also produced results contrary to human epidemiological findings, it was suggested that it would be well to investigate the DMH-rat tumor model with the hypothesis that it would more closely mimic the human and the epidemiological findings in humans. In order to investigate this possibility the current investigation was undertaken.

One hundred 5-week-old male Sprague-Dawley rats were divided into four diet groups: 1) a low (5%) fat, low (11%) protein diet with non-fat powdered milk as protein source, 2) a diet composed similarly except that beef was used as protein source and beef tallow replaced corn oil, 3) a control diet similar to the first diet except that casein (no lactose) was substituted for the milk, and 4) the standard recommended balanced diet for rats and mice (AIN-76A) which was similar to the third diet except that the protein level was moderate (about 19%). At 12 weeks of age, and for each of the 9 weeks following, each rat received an injection of DMH (10 mg/kg of body wt.). The 82 rats which were still alive were euthanized and necropsied at 52 weeks of age. Spleens and colons were removed and weighed, tumor counts were made and the tumors prepared for pathological examination. Spleen cells were removed and assays for cell-mediated immunity were performed: lymphocyte transformation test (LTT) with phytohemagglutinin (PHA) and concanavalin A (Con A) as mitogens, as well as assays for natural killer (NK) cell and cytotoxic T lymphocyte (CTL) activity.

The percentages of rats with tumors found at euthanization were: 1) low milk-fed rats - 44%, 2) low beef-fed rats - 79%, 3) low casein-fed rats - 64%, and 4) moderate casein-fed rats - 50%. Most of the tumors were in the colons. As in the previous study, colons were significantly heavier in rats fed the diet containing lactose, but this did not appear to enhance colon tumor growth. There was too much variation in the NK and CTL test results for any significance to be noted. In the LTT test, rats without tumors were more apt to have high

cell-mediated immunity than those with tumors. Also, with both mitogens, rats fed the AIN-76A control had the highest cell-mediated immunity.

The results with the DMH-rat tumor model more closely resemble the epidemiological findings in which beef protein tends to enhance the incidence of colon tumors, in contrast with non-fat powdered milk which tends to inhibit it.

LOMA LINDA UNIVERSITY

Graduate School

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by

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A Thesis in Partial Fulfillment

of the Requirements for the Degree

Master of Science in Microbiology

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Each person whose signature appears below certifies that this thesis, in his opinion, is adequate in scope and quality, as a thesis for the degree Master of Science.

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INTRODUCTION

The causes of large bowel cancer, one of the more common cancers in developed countries, are still largely unknown. Both epidemiological studies and experiments using animal models have identified a number of possible factors which may play a role, however. Intraluminal bowel constituents which have carcinogenic activity could play a role. A high intake of dietary fat may also be a factor. Large amounts of fat in the intestines have been found to affect the relative quantities of certain bacteria present which, in turn, may produce carcinogenic substances from the bile acids and neutral sterols (Hill et al. 1971; Reddy and Wynder 1973; Wynder and Reddy, 1973).

The rates of production of the substances modified by the intestinal microflora appear to be important in colon carcinogenesis. Certain products from the microflora-mediated reactions are likely to serve as colon tumor promoters or accelerators rather than as direct complete carcinogens. Animal experiments (Narisawa et al. 1974) have provided the evidence for bile acids as colon tumor promoters. Rats treated intrarectally with N-methyl-N'-nitro-N-nitrosoguanidine and taurodeoxycholic acid showed a remarkable increase in the development of adenomas (solid tumors) when compared to the group given only the N-methyl-N'-nitro-N-nitrosoguanidine, although bile acids themselves produced no tumors. Therefore, in this animal model, these bile acids present in high concentration in human stools, may act as colon tumor promoters. This suggests that the qualitative and quantitative

differences in luminal compounds such as bile acids and cholesterol metabolites may act as enhancing factors in colon carcinogenesis. These compounds are primarily derived from dietary factors and are eventually modified by the large bowel bacteria. Fat is among the most extensively studied of dietary factors related epidemiologically to cancer of various sites, including large bowel cancer. Dietary fat is present, along with proteins and other nutrients, in many foods consumed by humans. In the United States over 40% of the calories consumed by the average person are calories contributed by fat. In developing countries with lower fat consumption, incidence of cancers including those of the colon and breast are much lower than in the U.S.

Several reports indicate that meat consumption may be important. This may be because meat can be a significant source of dietary fat, especially saturated fat, but the source of protein may be important. Gregor et al. (1969) first reported a direct correlation between per capita consumption of animal protein and mortality from intestinal cancer. Howell reported a high correlation between colon cancer mortality and meat intake, especially beef, based on international per capita intake data (Howell 1975). Carroll and Khor (1975) observed strong correlations between the per capita consumption of dietary fat and age-adjusted mortality from cancer of the intestine (except rectum) in several countries. These results were confirmed by Armstrong and Doll (1975) who observed a strong correlation between the per capita intake of total protein, animal protein, as well as the total fat, and the incidence and mortality from colon and rectal cancer in both sexes.

In research conducted in our laboratory at Loma Linda University, 1,2-dimethylhydrazine (DMH) was used as a carcinogen to develop tumors, especially colon tumors, in animals fed different diets. Several reports (Locniskar et al. 1986; Reddy et al. 1977; Nauss et al. 1983; Rogers and Newberne 1973; Krauss et al. 1980, and others) have shown that DMH is effective in producing colon tumors in rodents.

Brief discussions of reports concerning certain dietary factors (i.e., fiber, selenium and protein) are presented in the following paragraphs since these substances have been used in studies of dietary factors affecting DMH-induced tumor production in rats and mice.

Fiber. A dietary supplement of 20% wheat bran was given to 48 male Sprague-Dawley rats fed defined diets for 31 weeks, either during and/or after administration of DMH. Tumor yield (both benign and malignant) was significantly greater in rats fed with bran during carcinogen administration than in the group fed a fiber-free diet. In rats fed bran after the completion of carcinogen exposure, the yield of benign tumor was reduced when compared with the group fed the fiber-free diet (Jacobs, L. 1983).

Selenium. A 40 ppm selenium supplement to the drinking water was provided concurrently with DMH treatment and continued until the death of the Sprague-Dawley rats or the time of their sacrifice (Jacobs, M. 1983). The rats were injected with 10 mg DMH/kg body weight weekly over a period of 10 weeks. All surviving animals were sacrificed at the 31st week after the tenth DMH injection. The cumulative colon tumor incidence for all animals found dead or sacrificed was significantly

reduced from 11 of 40 DMH-injected control animals to 3 of 40 DMH-injected, selenium-supplemented rats. The total number of colon tumors was reduced from 13 to 3, and the average number of tumors per rat from 1.2 to 1.0 by selenium supplementation.

Protein. Male BALB/c mice fed six different isocaloric diets were injected with DMH to induce the development of colon tumors (Nutter et al. 1983). Milk or beef, fed at low (11%) protein or high (33%) protein levels supplied the dietary protein. The diets were either low (5%) or high (30%) in fat which was supplied primarily by corn oil. The mice were injected with DMH (20 mg/kg mouse) or saline weekly for 11 weeks. At 59 weeks of age the milk-fed mice had a significantly higher tumor incidence than the beef-fed mice, 67 and 16% respectively, when the fat level was low. Level of fat did not seem to be important. The tumor volumes were not significantly higher in the mice fed 30% fat than in those fed 5% fat. In this BALB/c mouse study, then, the source of protein seemed to be of much greater importance than the level of fat. Tumor volume and colon weights in the milk-fed mice were also remarkably greater. In addition, the milk-fed mice had lower NK activity and higher serum blocking of antitumor cell activity. Furthermore, these mice also exhibited higher T-lymphocyte cytotoxicity against colon tumor cells.

The finding in this set of experiments, that the tumor incidence was four times greater in mice fed milk as a source of protein than those fed beef, was surprising in view of the fact that in previous diet-cancer studies performed in our laboratory with BALB/c mice the

animals fed milk or casein had fewer tumors and smaller tumors than those fed beef or other sources of protein (Nutter et al. 1983).

Until a short time ago it was customary in diet and tumor studies using experimental animals to use isocaloric diets, i.e., diets which had equal number of calories per gram of diet. This meant that diets higher in fat (with its 9:4 ratio of kilocalories/g in relation to protein or carbohydrate) required larger quantities of a non-nutritive filler such as Celufil (U.S. Biochemical Co., Cleveland, OH) than those with less fat to make the kilocalories/g remain the same. In the last two or three years concern has been expressed that effects produced might be due, at least partially, to the filler acting as fiber and not just to the different levels of fat. This has led Visek and his co-workers (Visek and Clinton 1983) to suggest that experimental diets should provide equal nutrient to calorie ratios rather than equal kilocalories/g. Nutrients include proteins as well as each individual vitamin and each individual mineral. Fat is added at the expense of carbohydrate and the diets do not have equal kilocalories/g. This discrepancy is compensated for by the observation that mice and rats tend to eat to satisfy their own caloric needs. The total calories consumed per day per animal will be the same, since the animals will eat correspondingly less of those diets which have higher fat levels and, thus, higher values of kilocalories/g (Clinton et al. 1984).

Several possible mechanisms by which diet may affect tumor development have appeared in the literature. It is likely that clinical manifestation of colo-rectal tumors involves a multi-step process

including initiation, promotion, and progression of tumor cells. Experiments relating to mechanisms of initiation suggest that initiation is a de novo induction phenomenon (Mondal and Heidelberger 1970; Boehn and Drahovsky 1983) in which the critical event may be the formation of adducts between DNA and carcinogen (Becker 1981; Farber 1981; Weinstein 1981; Neidle 1980). This may cause miscoding (Burnet 1978) or genetic transposition (Cairns 1981; Radman et al. 1982) resulting in malignancy. Chemical carcinogenesis inducing heritable changes in cellular DNA methylation patterns have been enhanced as shown in several recent experiments (Drahovsky and Boehn 1980; Razin and Riggs 1980; Razin and Friedman 1981). Decreased DNA methylation may alter gene regulation to the extent that oncogenic transformation is initiated. Further support for this has been reported in experiments in which many different tumor cells have been found to be highly hypomethylated compared to normal human cells (Diala et al. 1981, 1983; Lu et al. 1983). The broad range of susceptibility of the mouse and rat colon and rectum to DMH-induced neoplasia make these animal models particularly suitable for an investigation of the relationship between methylated adducts of DNA and the site-specific incidences of colon tumors (James and Autrup 1983). A miscoding DNA adduct, O^6-methylguanine, has been found to be correlated with the incidence of DMH-induced neoplastic change (as described above), using high performance liquid chromatography (HPLC), which, because of its high sensitivity, is able to detect and identify amounts far less than 1% of minor modified bases relative to the four common bases in DNA (Erick et al. 1983). The repair of carcinogen-induced

damage to DNA prior to replication may be enhanced in colon cells exposed to certain dietary factors in comparison to those cells exposed to other dietary factors.

Finally, there is evidence that the immune system plays a significant role in neoplasia, including colon-tumor development. Perhaps the main effect of diet is through modification (rather than through initiation) by its effect on cell-mediated immunity (Weisburger et al. 1977). This possibility is addressed in more detail in the Discussion.

Objectives of the Research

The main objectives of this study are to test the effects of milk and beef diets on the development of colon tumors produced in rats injected with DMH and to study the possible relationship of cell-mediated immunity. Four diets, numbered 6-9, have been used in the study, the first two experimental and the last two as control. Diet 6 is a low protein (11%), low fat (5%) diet with non-fat powdered milk as the source of protein. Diet 7 is similar except that lyophilized cooked beef is used as the protein source. Diet 8 is a control for Diet 6 and contains casein (no lactose), and Diet 9 is AIN-76A diet which is used as a control for both Diet 6 and Diet 7.

Various experiments have been set up to accomplish and fulfill the above two major objectives in this diet study. First and foremost we have aimed to determine if the incidence of tumors is greater in rats

fed the beef diet than in those fed milk as epidemiological studies in humans would indicate. In a previous study with BALB/c mice we found four times as many colo-rectal tumors in milk-fed mice than in those which were beef-fed (Nutter et al. 1983). Secondly, the tumors as well as the colons have been measured to determine if tumor size and colon weights from rats fed the milk diet are greater than in rats fed the beef diet, as was found to be the case in BALB/c mice (Nutter et al. 1983). We also have included the standard AIN-76A diet for comparison purposes. Furthermore, we have attempted to determine if casein-fed rats give the same results as powdered milk-fed rats. In other words, we have aimed to see if the lactose in the milk predisposes colon tissue to neoplasia in the presence of DMH, a possibility suggested by results in the previous study with DMH and BALB/c mice. Finally, we have attempted to determine if there are differences with diet in responses related to cell-mediated immunity in tumor-bearing and in non-tumor-bearing rats.

MATERIALS AND METHODS

Animals

The 100 rats used in these experiments were barrier-reared, male Sprague-Dawley rats (Sasco, Inc., Omaha, NE) purchased when they were five weeks of age. Their weights at arrival were 50-60 g. They arrived in groups of 25 per week over a four week period. Immediately upon arrival each week they were separated into four groups, and each of the four groups was fed one of the four diets described below.

The Diets and Their Preparation

The two experimental diets (Diet 6 and Diet 7) and one of the two control diets (Diet 8) were made to be low protein (11%) - low fat (5%) diets. Diet 6 contained milk as a source of protein with corn oil as the source of fat, whereas Diet 7 contained beef as a protein source with beef tallow as the source of fat. Diet 8 had casein as a source of protein rather than the non-fat powdered milk used in Diet 6. Diet 8, therefore, did not contain lactose as did Diet 6 and was designed to act as a control with respect to the effects of lactose in the rats. Diet 9, the other control diet, was Diet AIN 76-A recommended as a nutritionally balanced diet for both rats and mice by the American Institute of Nutrition (AIN) (Newberne et al. 1978). It has nearly 20% protein and 5% fat. All four diets followed the AIN-recommended

percentages of vitamins, minerals, fiber (Celufil), D,L-methionine and choline bitartrate. The 2:1 ratio of dextrin (cornstarch) to sucrose was adopted from diets used by Visek and Clinton (1983) and reflects the flexibility in the AIN-76A diet with regard to how much of the carbohydrate should be sucrose and how much cornstarch. The original AIN-76 diet contained 50% sucrose and 15% cornstarch. The compositions of the four diets are outlined in Table I.

It may be noticed that there are three different vitamin mixes and three different mineral mixes used in the four diets. In order to have equal nutrient to calorie ratios in all four diets (that is equal protein to calorie, equal individual vitamin to calorie, and equal individual mineral to calorie ratios) it was necessary to have each of the two protein sources, milk and beef, completely analyzed with respect to these ingredients. Since they were not 100% protein, and since they contained varying amounts of each vitamin and mineral, it was necessary to calculate how much of each was provided by the 11% protein included in the diets and then prepare two separate mineral mixes and two separate vitamin mixes in such a way that, when completely made up, all four diets contained the same amount of each vitamin and each mineral. The mixes made in our laboratory, then, had less than the commercial mixes had of most of the ingredients in order to allow for the amounts provided in the milk or in the beef in standardizing all the diets to the AIN-76A diet (vitamin and mineral) recommended levels.

A sample calculation to determine how much Ca++ should be included in the mineral mix used in Diet 6 is included below. The source of

TABLE I. COMPOSITION OF DIETS BY PERCENT WEIGHT

Diet	6	7	8	9
Powdered non-fat milk	31.52[a]	-	-	-
Beef	-	13.41[b]	-	-
Casein	-	-	12.14[c]	20.00
DL-methionine	0.30	0.30	0.30	0.30
Corn oil	4.72	-	5.00	5.00
Beef tallow	-	2.89	-	-
Dextrin	35.84	49.13	48.57	43.33
Sucrose	17.92	24.57	24.29	21.67
AIN-76A vitamin mix	1.00[d]	1.00[e]	1.00[f]	1.00[f]
AIN-76A mineral mix	3.50[d]	3.50[e]	3.50[f]	3.50[f]
Choline bitartrate	0.20	0.20	0.20	0.20
Celufil (cellulose)	5.00	5.00	5.00	5.00
	100.00	100.00	100.00	100.00

a In order for the level of protein to be 11% in this diet it was necessary to include 31.5% milk since the milk was analyzed to contain 34.9% protein (11/.349 = 31.52%). Analysis was performed by the Chemical and BioMedical Sciences Division of Hazelton Laboratories America, Madison, WI.

b In order for the level of protein to be 11% it was necessary to include 13.41% beef since it was analyzed to contain 82.0% protein (11/.820 = 13.41%). Analysis also was performed by Hazelton Laboratories.

c In order for the level of protein to be 11% it was necessary to include 12.14% casein which was analyzed to contain 90.625% protein (11/.90625 = 12.14%). Analysis was provided by United States Biochemical Corporation, Cleveland, OH.

[d] Our mix especially prepared for the milk diet.

[e] Our mix especially prepared for the beef diet.

[f] Commercially prepared mix for the AIN-76A diet.

calcium used in the mineral mix is calcium phosphate, dibasic ($CaHPO_4$). Its molecular weight is 136. The atomic weight of Ca++ is 40. The amount of calcium in the commercial AIN-76A mineral mix is calculated as follows:

$$\frac{40}{136} = \frac{X}{500 \text{ g/kg } CaHPO_4 \text{ (in AIN-76A mix)}} = \frac{X}{500}.$$

X = 147.1 g/kg Ca++ in the mix.

In Diet 6 there is 31.52% non-fat powdered milk which has 11.086 g/kg Ca++. The amount of Ca++ in Diet 6 due to the milk is, then, 0.3152 x 11.086 = 3.49 g/kg Ca^{++}.

In the AIN-76A diet the commercial mineral mix makes up 3.5%. The amount of Ca++ in this diet is, therefore, 0.035 x 147.1 = 5.15 g/kg Ca++. The amount of Ca++ needed to be provided by the mineral mix prepared especially to accompany the milk is 5.15-3.49 = 1.66 g/kg Ca++. The fraction of Ca++ needed to be added, therefore, is 1.66/5.15 = 0.32. This is also the fraction of $CaHPO_4$ needed in the special mineral mix for the milk diet (0.32 x 500 = 160) which means that the mineral mix has to contain 160 g/kg $CaHPO_4$.

A sample calculation is also included to indicate the method used in determining the composition of one of the vitamin mixes. In this case the calculation of the amount of nicotinic acid needed in the vitamin mix to accompany the beef diet (Diet 7) is described.

The amount of nicotinic acid in the beef was analyzed to be 112 μg/g = 112 mg/kg. The amount of nicotinic acid in Diet 7 due to the beef is, then, 0.1341 x 112 = 15.02 mg/kg. Since the amount of

nicotinic acid in the commercial AIN-76A vitamin mix is 3000 mg/kg and since this is used at the 1% level in preparing the AIN-76A diet, the amount of nicotinic acid in the standard diet would be 0.01 x 3000 = 30.00 mg/kg. The amount of nicotinic acid provided by the special vitamin mix made to accompany the beef diet would, therefore, be 30.00 - 15.02 = 14.98 mg/kg of diet. The fraction of nicotinic acid needed to be added would then be 14.98/30.00 = .50. This means that the amount of nicotinic acid included in the vitamin mix would be 1498 mg/kg.

The biochemical analyses of the milk and the beef protein sources were done by the Chemical and BioMedical Sciences Division of Hazelton Laboratories America, Inc., Madison, WI.

Feeding and Weighing the Animals

The rats were housed in half-stock cages in the rat room in the animal quarters in Alumni Hall for Basic Sciences. For the first month they were housed six to a cage (one cage per diet per shipment) and each of the six was individually identified with a picric acid-colored mark at a designated spot on the fur. Later the rats were housed three per cage (two cages, sub 1 and sub 2, per diet per shipment). The four shipments were designated as A, B, C and D. Each individual rat, then, could be identified in terms of diet, shipment, cage and marking number, for example: $7B_2$-5.

The rats were fed the powdered feed twice each week in jars with the food consumed being calculated at each feeding. The rats were weighed weekly until they were 25 weeks of age after which they were weighed monthly.

Tumor Induction

The procedures and methods for inducing colon tumors in the rats is based on the article by Maryce M. Jacobs (1983). Beginning at 12 weeks of age, 10 mg DMH per kg body weight was injected subcutaneously in the right hind thigh. The DMH was administrated weekly over a period of 10 weeks. A small portion of the rats in each diet group received no injection and served as controls. In Table II a calendar of the early events of the project are outlined. Thirty-one weeks after the tenth DMH injection, all surviving animals were euthanized, necropsied and their colons and small intestines examined under a dissecting microscope for tumors. Deaths, either nonspecific or due to tumor, were noted throughout the experiment.

PPD Footpad Testing

In vivo sensitization was done by subcutaneous injection of 0.75 ml complete Freund's adjuvant (killed Mycobacterium tuberculosis H37 Ra, Difco Lab., Detroit, MI) at five different dorsally-located sites two weeks prior to the date of sacrificing the animals (Lau et al. 1976).

TABLE II. CALENDAR OF EARLY EVENTS

5/29/85	26 Group A 5-week-old rats arrived	Divided into 4 diet groups 6A 1-7 8A 1-6 7A 1-7 9A 1-6
6/4/85	25 Group B 5-week-old rats arrived	Divided into 4 diet groups 6B 1-6 8B 1-6 7B 1-6 9B 1-7
6/11/85	25 Group C 5-week-old rats arrived	Divided into 4 diet groups 6C 1-6 8C 1-6 7C 1-6 9C 1-7
6/18/85	24 Group D 5-week-old rats arrived	Divided into 4 diet groups 6D 1-6 8D 1-6 7D 1-6 9D 1-6
7/17/85	Began DMH injection of all Group A rats (except the number 7 ones)	
7/24/85	Began weekly injection of all Group B rats (except the number 7 one)	
7/31/85	Began weekly injection of all Group C rats (except the number 7 one)	
8/7/85	Began weekly injection of all Group D rats	
9/18/85	Last DMH injection given to Group A rats	
9/25/85	Last DMH injection given to Group B rats	
10/2/85	Last DMH injection given to Group C rats	
10/9/85	Last DMH injection given to Group D rats	

Footpad testing was performed by injecting 0.025 ml sterile saline in the left hind footpad and an equal volume containing 50 µg purified protein derivative (PPD; Connaught Laboratories, Toronto, Canada) in the right footpad at 1 week after sensitization. Footpad swelling was measured with Vernier calipers at both 24 h and 48 h after the injection of PPD (Lau 1976). The percent increase in footpad swelling of the PPD-injected over the saline-injected footpad was calculated as follows:

$$\frac{\text{mm (diam.) PPD-injected footpad - mm (diam.) saline-injected footpad}}{\text{mm (diameter) saline-injected foot pad}} \times 100 = \% \text{ footpad swelling.}$$

Relative Spleen Weights and Relative Colon Weights

At necropsy the relative spleen weights (RSW) were calculated by using the formula: $$RSW = \frac{\text{spleen wt. (g)}}{\text{rat wt. (g)}} \times 10^4.$$

Likewise, the relative colon weights (RCW) were calculated by means of the formula: $$RCW = \frac{\text{colon wt. (g)}}{\text{rat wt. (g)}} \times 10^4.$$

Lymphocyte Transformation Test (LTT)

The LTT was performed according to the method of Dean et al. (1975) but with some modification. In order to obtain a single cell suspension, the spleens were mashed with the blunt end of a flat-bottom test tube. The cells were washed once with TBS (Tris-buffered saline) and once with RPMI-1640. They were then suspended in 1.1 ml of medium and the number of viable cells was counted in a hemacytometer. One drop of 0.4% Erythrosin B was added to 0.1 ml of the cell suspension and 0.9 ml of the diluting fluid in order to ascertain viability of the cells. The spleen cells were adjusted to 1×10^6 viable cells/ml. From each cell suspension, 0.1 ml aliquots were dispensed with an automatic pipettor (Oxford Lab., Inc., Foster City, CA) into wells of No. 3040 Microtest II culture plates (Falcon Plastics, Oxnard, CA). Next, 0.1 ml of a 1:40 dilution (1 µg/0.1 ml) of commercial phytohemagglutinin (PHA-P, Wellcome Lab., Triangle Park, NC) or 0.1 ml of 1:480 dilution (0.21 µg/0.1 ml) of commercial concanavalin A (Con A, Sigma Chemical Company, St. Louis, MO) were added to each test well. Control wells received 0.1 ml of medium instead of mitogen and the plates were incubated for 43 hours in 5% CO_2 at 37°C. Tritiated thymidine (ICN, Irvine, CA) was added at a concentration of 1 µCi/0.05 ml per well and the plates were reincubated for an additional 5 hours. After labeling, the cells were harvested with a Brandel M24-S cell harvester (Brandel, Gaithersburg, MD) on glass fiber filters (Whatman, Inc., Clinton, NJ). The glass fiber discs were placed into counting vials containing 2.0 ml

CytoScint (WestChem, San Diego, CA). The amount of ^{3}H-thymidine incorporated into DNA was counted in a model 3801 liquid scintillation counter (Beckman Instruments, Los Angeles, CA) for 2 minutes or to 0.2 percent error. Mean disintegrations per minute (dpm) were obtained from triplicate samples. Since the counting vials were washed and reused, background counts were always done before adding the glass fiber discs. Vials with background counts greater than 100 were washed again and recounted before use.

Stimulation Index (SI)

Spleen cells of all rats were stimulated with both PHA and Con A according to the lymphocyte transformation method described previously. The SI values were calculated as follows:

$$SI = \frac{\text{mean dpm in test wells - mean dpm in control wells}}{\text{mean dpm in control wells}}.$$

^{51}Chromium Labeling Procedure for YAC-1 (a Maloney T-cell lymphoma) and MAM Cells (a rat colon tumor cell)

YAC-1 cells were transferred from a tissue culture flask into a centrifuge tube. MAM cells were trypsinized and then transferred into another centrifuge tube. The cells were centrifuged at a 100 x g for 5 minutes. The cells were resuspended with 2-3 ml medium (RPMI 1640 for YAC-1; Dulbecco for MAM) and counted with the aid of a hemacytometer

using trypan blue exclusion to determine viability. Each cell type was adjusted to a concentration of 1 to 5 x 10^6 cells/ml. The YAC-1 and MAM cells were labeled separately by adding 0.1 ml ^{51}Cr solution (1 mCi/ml, 200-900 Ci/g) to 1 ml of cell suspension. The cells were incubated at 37°C in a 5% CO_2 incubator for 2 hours during labeling. They were then centrifuged at 400 g for 5 minutes. The cells were washed 4 times with medium and the last washing solution was checked to see that most of the radioactivity in the supernatant was absent. The cells were resuspended with 1-5 ml medium and counted as described previously. The cells were then diluted to 2 x 10^6/ml for use in the microcytotoxicity assays.

Microcytotoxicity Assay (NK and CTL)

Splenic cell cytotoxicity was measured against two tumor cell lines: YAC-1 (a Maloney mouse T-cell lymphoma) for the NK assay because it can also be used for rat cell activity (Locniskar et al. 1986), and MAM (a rat colon tumor cell) for the CTL assay, according to the procedure of Kumar et al. (Kumar 1979).

In each well of microtiter plates, 100 µl volumes of the ^{51}Cr-labeled YAC-1 or MAM cells were pipetted. The spleen cells from each rat were added to the wells in 100 µl volumes. Four effector-to-target (E/T) ratios were used (25:1, 50:1, 100:1, 200:1). There were three wells used for each target cell type for each E/T ratio for each rat, and spleen cells were added to these. For each target cell type, labeled target cells were placed in six wells with medium

only (no spleen cells added). Three of these were for use as non-specific (spontaneous) lysis control wells and three (to which 100 µl of 10% sodium dodecyl sulfate was added to completely lyse the cells after incubation just prior to harvest) as maximum available cpm wells. The plates were incubated for four hours at 37°C in a 5% CO_2 incubator. Subsequently, 100 µl volumes of the supernatant fluid from each well were collected by means of a multiple automated sample harvester and the amount of ^{51}Cr radioactivity was counted in a gamma-counter (Model 1193 Gammatrac, TM Analytic, Inc., Elk Grove Village, IL). The mean percent specific cytotoxicity was calculated according to the following formula.

$$\% \text{ specific cytotoxicity} = \frac{\text{experimental cpm - spontaneous cpm}}{\text{maximum (total) cpm - spontaneous cpm}} \times 100 =$$

% lysis.

Spontaneous lysis (cpm) = ^{51}Cr cpm in wells containing labeled tumor cells but no effector cells.

Maximum lysis (cpm) = ^{51}Cr cpm released by lysis of the target cells with 50 1 per well of sodium dodecyl sulfate (SDS).

RESULTS

Food Consumption and Weight Gain of the Rats

In Table III the average food consumption of the rats (grams/rat/day) is presented for four different successive three week periods. Rats fed Diet 9 had the highest average weekly consumption followed by Diet 8, 6 and 7 in descending order of amount of food eaten. The rate of average food consumption (grams/rat/day) increased at first and then decreased after a peak was reached in the 30-31st week period. The rats gained weight quite constantly from week 5 through week 11 (see Fig. 1). During the period from week 12 through week 21 the weight gains decreased but then increased at a greater rate from week 22 on. As will be mentioned in more detail later, it was from week 12 through week 21 that all but four of the rats were injected each week with DMH. It is interesting to note that rats fed Diet 9 gained weight the most rapidly, followed by those fed Diet 8, Diet 6 and Diet 7, in the same order as for the food consumed.

Results of the Food Pad Test

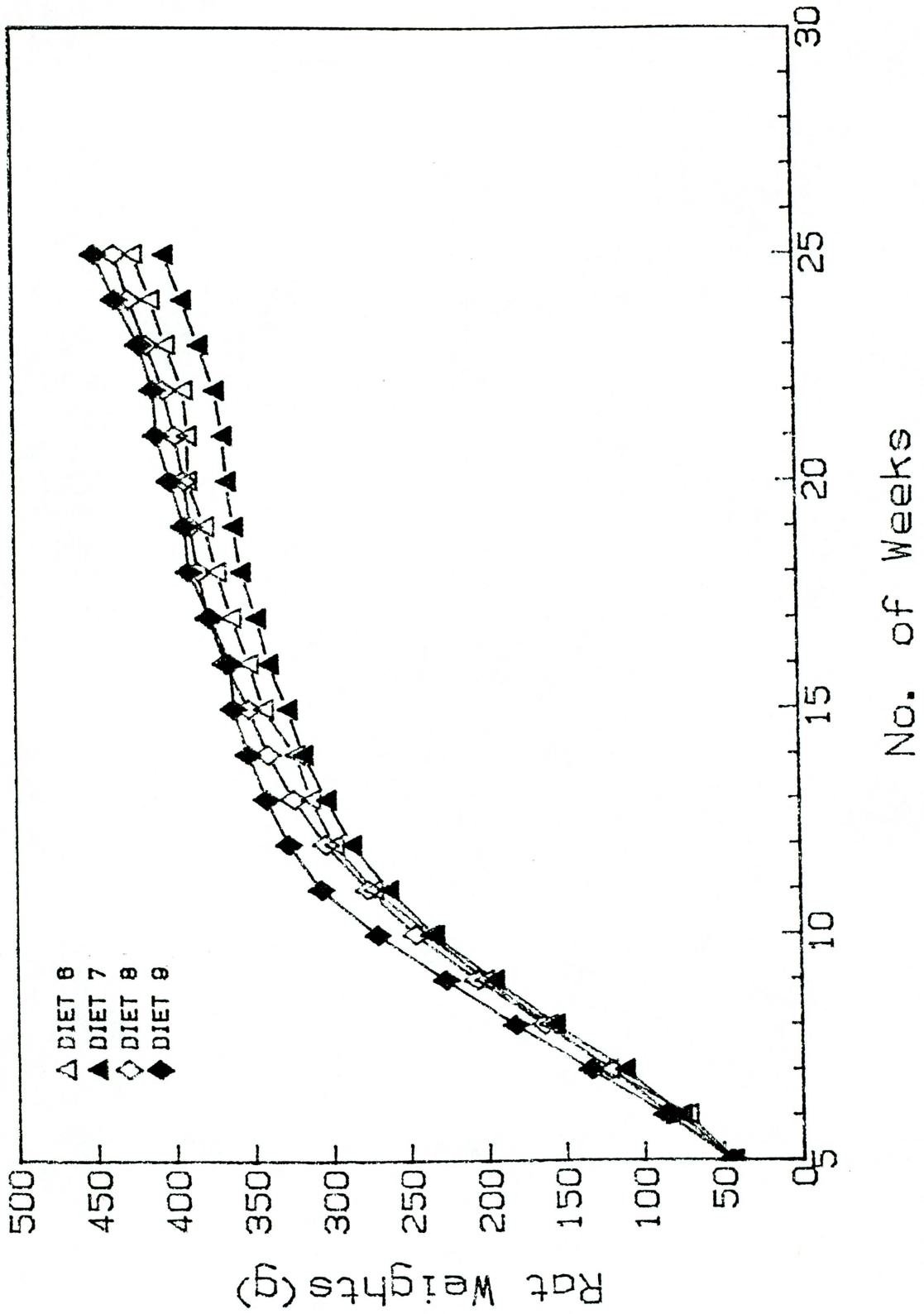
Foot pad thicknesses were measured on two successive days a week after the PPD and TBS injection. We have chosen the day (of the two) giving the highest number differences (in general) between the right foot (injected with PPD) and the left foot (injected with TBS). The

TABLE III. FOOD CONSUMPTION IN GRAMS PER RAT PER DAY

Week No.	Diet 6	Diet 7	Diet 8	Diet 9	Average
3 - 4	15.2	15.1	18.0	16.2	16.1
4 - 5	17.7	15.9	18.1	19.0	17.7
5 - 6	17.2	18.0	19.6	20.7	18.9
Average	16.7±0.76	16.3±0.87	18.6±0.52	18.6±1.31	17.6±0.81
15 - 16	17.8	17.3	19.3	19.3	18.4
16 - 17	16.8	17.7	18.9	18.3	17.9
17 - 18	17.7	18.1	18.2	19.4	18.4
Average	17.4±0.32	17.7±0.23	18.8±0.32	19.0±0.35	18.2±0.17
28 - 29	18.0	16.6	17.9	18.3	17.7
29 - 30	20.9	19.8	20.0	22.1	20.7
30 - 31	23.2	19.5	19.7	21.0	20.9
Average	20.7±1.51	18.6±1.02	19.2±0.66	20.5±1.13	19.8±1.04
40 - 41	17.9	17.8	17.2	18.1	17.8
41 - 42	16.2	18.3	17.8	19.6	18.0
42 - 43	15.2	16.4	17.0	19.6	17.1
Average	16.4±0.79	17.5±0.57	17.3±0.24	19.1±0.50	17.6±0.27
Overall Average	17.8±0.65[a]	17.5±0.40[b]	18.5±0.29	19.3±0.44[a,b]	18.3±0.39

[a] Significantly different by the Student's t Test ($p < 0.04$).

[b] Significantly different by the Student's t Test ($p < 0.004$).


Rat Weights (g)
500
450
400
350
300
250
200
150
100
50
0
5
10
15
20
25
30
No. of Weeks
DIET 6
DIET 7
DIET 8
DIET 9

averages for all the rats in a given category have been tabulated. Results of the test are shown in Table IV. Responses were fairly similar for all diet groups with Diet 8 rats tending to have smaller values. In Diet 7 and Diet 8 rats, the response seemed to be more depressed in the rats without tumors.

Tumor Counts (Tumor Incidence)

Between Jan. 1, 1986 and the conclusion of the experiment, at which time the rats were 52 weeks of age, 18 of the 100 rats died. A few of them died over a weekend and were not noted and/or not necropsied, but 12 of the 18 were necropsied. The information available on these rats is shown in Table V.

The number of rats which were found to have tumors are listed according to diet group in Table VI. A description of which organs were found to have tumors is also included for each tumor-bearing rat.

The percentage of rats with tumors of any organ (colon, small intestine, etc.) at the time of killing are shown in the first portion of Table VII. Rats fed Diet 7, the diet containing beef and beef tallow had the highest percentage of tumors followed by Diet 8, 9 and 6 (the milk-containing diet) in descending order. The total percentage of rats with tumors of any organ (including those rats which died and were necropsied before being euthanized) are shown in the mid-portion of the table. The order ranking from the highest percentage to the lowest is the same as mentioned previously. The last section of Table VII shows

TABLE IV. PERCENTAGE INCREASES IN FOOTPAD THICKNESS DUE TO SWELLING[a]

Diet Number	Rats With Tumors	Rats Without Tumors
6	9.33%	9.00%
7	9.16%	7.00%
8	7.50%	4.83%
9	10.00%	9.80%

[a] No significant differences by the Student's t Test.

TABLE V. DATES OF RAT DEATHS BEFORE EUTHANIZATION[a]

Diet 6	Diet 7	Diet 8	Diet 9
	1/26/86 Rat 7A-2 Necropsied Colon tumors Hemorrhage		1/4/86 Rat 9D-3 No necropsy possible 1/26/86 Rat 9C-1 Necropsied No tumors Jaw abscess
	2/23/86 Rat 7A-4 Necropsied Hemorrhage		2/17/86 Rat 9B-6 No necropsy possible
3/1/86 Rat 6B-2 No necropsy possible 3/18/86 Rat 6A-5 Necropsied Colon tumors Abscess 3/29/86 Rat 6C-6 No necropsy possible 3/30/86 Rat 6B-5 Necropsied No visible tumors	3/13/86 Rat 7A-5 Necropsied No visible tumors	3/7/86 Rat 8D-5 Necropsied No visible tumors 3/14/86 Rat 8B-2 Necropsied 2 small tumors in mesentery	

TABLE V. CONTINUED

Diet 6	Diet 7	Diet 8	Diet 9
3/31-4/21/86 Rat 6A-7 Non-injected No necropsy possible			3/31-4/12/86 Rat 9A-6 No necropsy possible
4/8/86 Rat 6D-1 Necropsied No visible tumors			
4/13/86 Rat 6D-4 Necropsied Hemorrhage			
	5/5/86 Rat 7C-2 Necropsied Large tumor in small intestine		
	5/11/86 Rat 7D-5 Necropsied No visible tumors		

[a] The remaining 82 of the 100 original rats were euthanized and necropsied at 52 weeks of age as follows:

Group A - 4/22/86 - 20 rats
Group B - 4/29/86 - 21 rats
Group C - 5/06/86 - 22 rats
Group D - 5/31/86 - 19 rats.

TABLE VII. SUMMARY OF TUMOR DATA

Diet No.	Fraction & Percentage of Rats With Tumors at Euthanization		Fraction & Percentage of Tumors in All Rats Autopsied		Total No. of Colon Tumors Found in Autopsied Rats
6	8/18	44.4%[a]	9/22	41%[b]	8
7	15/19[c]	78.9%[a,d]	17/24	71%[b,e]	12
8	14/22	63.6%	15/24	63%	8
9	10/20[f]	50.0%[d]	10/21	48%[e]	9

[a] Significantly different by the χ_c^2 test ($p < 0.01$).

[b] Significantly different by the χ_c^2 test ($p < 0.01$).

[c] + 1 non-injected.

[d] Significantly different by the χ_c^2 test ($p < 0.02$).

[e] Significantly different by the χ_c^2 test ($p < 0.05$).

[f] + 2 non-injected.

TABLE VI. TUMOR INCIDENCE IN EACH DIET GROUP AT EUTHANIZATION

Diet No.	Descriptions	Total No. of Rats With Tumors
6	6 - colon only 2 - small intestine only	8
7	6 - colon only 3 - colon + small intestine 5 - small intestine only 1 - kidney + lung	15
8	6 - colon only 1 - colon + small intestine 5 - small intestine only 1 - kidney + lung 1 - liver + stomach	14
9	5 - colon only 2 - colon + small intestine 1 - colon, small intestine, liver, muscle and metastasis 2 - small intestine only	10

the total number of colon tumors for each diet group. Diet 7 also shows the highest number among all four diets.

Relative Spleen Weights (RSW) and Relative Colon Weights (RCW)

Although, at first glance, it appears that the RSW of the tumor rats are larger than for the corresponding non-tumor rats in the case of Diet 7 and 9 (see Table VIII), the values are not significantly different. The RCW of the tumor-bearing rats in diet groups 6, 7 and 9 appear to be larger than for the corresponding non-tumor-bearing rats even though colons of rats with colon tumors were not included in the determination of the mean values. The mean RCW value for tumor rats fed Diet 6 was 45.8 whereas that for tumor rats fed Diet 8 was 35.0. The mean RCW value for non-tumor rats fed Diet 6 was 41.7 whereas that for rats without tumors fed Diet 8 was 34.4. These values for Diet 6 are significantly higher than the corresponding values for Diet 8 with $p = 0.003$ and $p = 0.017$ respectively.

Lymphocyte Transformation Test (LTT)

Tables IX and X present the data from PHA and Con A LTT tests. Both PHA and Con A are stimulators of T cells and the T cell has a cell-mediated effect on tumor development. The two tables compare the SI values of all 4 diets with tumor-bearing rats and non-tumor-bearing rats tabulated separately.

TABLE VIII. RELATIVE SPLEEN WEIGHTS (RSW) AND RELATIVE COLON WEIGHTS (RCW) ACCORDING TO DIET GROUP

Diet No.	Tumor		Non-tumor	
	RSW	RCW	RSW	RCW
6	18.4 ± 1.947	45.8 ± 0.895[a]	17.7 ± 1.146	41.7 ± 2.212[b]
7	20.7 ± 4.416	36.7 ± 1.824	12.5 ± 0.829	33.0 ± 5.308
8	16.4 ± 1.279	35.0 ± 1.822[a]	15.3 ± 1.267	34.4 ± 2.128[b]
9	23.7 ± 3.798	38.9 ± 2.111	19.7 ± 1.461	33.8 ± 2.037

[a] Significantly different by the Student's t Test ($p < 0.003$).

[b] Significantly different by the Student's t Test ($p < 0.017$).

TABLE IX. MEAN STIMULATION INDEX VALUES IN THE LYMPHOCYTE TRANSFORMATION TEST WITH PHA AS MITOGEN[a]

	Group	Diet 6	Diet 7	Diet 8	Diet 9
A	Tumor-bearing	11.41	166.71	79.4	98.0
	Non-tumor-bearing	23.6	29.8	32.8	56.07
B	Tumor-bearing	38.28	18.6	8.7	62.37
	Non-tumor-bearing	34.7	71.16	11.8	26.1
C	Tumor-bearing	9.1	4.4	3.9	-0.22
	Non-tumor-bearing	13.6	-	9.0	42.4
D	Tumor-bearing	46.2	34.5	46.0	71.1
	Non-tumor-bearing	107.7	45.2	-	213.7

[a] Significant values were not numerous enough to make their inclusion in the table practical.

TABLE X. MEAN STIMULATION INDEX VALUES IN THE LYMPHOCYTE TRANSFORMATION TEST WITH CON-A AS MITOGEN[a]

	Group	Diet 6	Diet 7	Diet 8	Diet 9
A	Tumor-bearing	17.35	232.1	169.8	161.6
	Non-tumor-bearing	68.5	99.3	112.3	89.04
B	Tumor-bearing	40.02	31.1	7.0	172.17
	Non-tumor-bearing	45.2	122.98	10.9	48.8
C	Tumor-bearing	4.9	1.3	1.0	-0.26
	Non-tumor-bearing	9.9	-	3.2	9.1
D	Tumor-bearing	70.1	49.4	102.0	139.0
	Non-tumor-bearing	200.74	64.8	-	407.58

[a] Significant values were not numerous enough to make their inclusion in the table practical.

In 14 available comparisons with tumor and non-tumor rats in the PHA LTT, 9 of the 14 had SI values higher for non-tumor rats whereas 5 had them higher for tumor-bearing rats. In looking at the effect of diet without regard to tumor or non-tumor: the SI values were highest, overall, among the four diets for cells from rats fed Diet 9, next highest for Diet 6, next for Diet 7 and last for Diet 8 (see Table XI). In 14 available comparisons with tumor and non-tumor rats in the Con A LTT, 10 of the 14 had SI values higher for non-tumor rats, whereas 4 had them higher for tumor-bearing rats. In looking at the effect of diet without regard to tumor or non-tumor: the SI values were highest, overall, among the four diets for cells from rats fed Diet 9. Cells from rats fed Diets 6 and 7 were about the same and Diet 8 was last (see Table XII).

The NK and CTL Tests Results

The counts in assays of cells from rats in groups C and D were too low to be meaningful. For some unknown reason the cells did not take up sufficient quantities of ^{51}Cr label. The results of the percent NK and percent CTL assays for spleen cells from rats in groups A and B are shown in Table XIII. Few significant differences were noted. Although rats which were not injected with DMH (7A-7 and 9B-7) had fairly high % NK values and moderate to low % CTL values (47.9 and 23.2 for 7A-7 and 27.2 and 4.1 for 9B-7, respectively), rats without tumor did not have consistently higher % NK values nor lower % CTL values than rats with

TABLE XI. SUMMARY OF DATA IN TABLE IX

Diet 9	5 out of 8 had highest Stimulation Index of the 4 diets.
	1 out of 8 had second place.
	1 out of 8 had third place.
	1 out of 8 had last place.
Diet 8	0 out of 7 had highest Stimulation Index of the 4 diets.
	1 out of 7 had second place.
	3 out of 7 had third place.
	3 out of 7 had last place.
Diet 7	2 out of 7 had highest Stimulation Index of the 4 diets.
	1 out of 7 had second place.
	2 out of 7 had third place.
	2 out of 7 had last place.
Diet 6	2 out of 8 had highest Stimulation Index of the 4 diets.
	4 out of 8 had second place.
	0 out of 8 had third place.
	2 out of 8 had last place.

TABLE XII. SUMMARY OF DATA IN TABLE X

Diet 9	3 out of 8 had highest Stimulation Index of the 4 diets.
	2 out of 8 had second place.
	2 out of 8 had third place.
	1 out of 8 had last place.
Diet 8	1 out of 7 had highest Stimulation Index of the 4 diets.
	2 out of 7 had second place.
	1 out of 7 had third place.
	3 out of 7 had last place.
Diet 7	2 out of 7 had highest Stimulation Index of the 4 diets.
	2 out of 7 had second place.
	1 out of 7 had third place.
	2 out of 7 had last place.
Diet 6	2 out of 8 had highest Stimulation Index of the 4 diets.
	2 out of 8 had second place.
	2 out of 8 had third place.
	2 out of 8 had last place.

TABLE XIII. PERCENT LYSIS OF LABELED TARGET CELLS BY NATURAL KILLER CELLS (NK) AND BY CYTOTOXIC T-LYMPHOCYTES (CTL)

Rat #	Tumor Status	% NK Lysis	% CTL Lysis
6A-1	No tumor	41.7	23.3
6A-2	Tumor (colon)	45.2	24.7
6A-3	No tumor	35.6	20.8
6A-4	No tumor	47.1	35.1
6A-6	No tumor	28.1	27.8
7A-1	Tumor (colon)	24.6	29.6
7A-3	No tumor	37.5	29.6
7A-6	Tumor (colon)	23.8	54.8
7A-7	Non-injected (no tumor)	47.9	23.2
8A-1	Tumor (colon)	32.8	30.3
8A-2	Tumor (colon)	42.5	26.8
8A-3	No tumor	31.5	28.9
8A-4	No tumor	37.2	24.3
8A-5	Tumor (colon)	34.0	23.3
8A-6	Tumors (kidney + lung)	0.0	68.6
9A-1	2 tumors (small intestine)	33.1	27.7
9A-2	No tumor	45.4	14.9
9A-3	Tumor (colon)	31.1	14.4
9A-4	Tumor (colon)	36.7	0.0
9A-5	2 large tumors (small intestine)	43.2	0.0
6B-1	No tumor	41.6	9.4
6B-3	No tumor	33.2	11.4
6B-4	1 tumor (small intestine)	34.6	12.1
6B-6	No tumor	21.2	13.4
7B-1	3 tumors (small intestine)	7.0	13.4
7B-2	Tumors (kidney + lung)	21.0	7.4
7B-3	Tumors (colon + small intestine)	28.8	12.6
7B-4	Tumors (colon + small intestine)	46.8	14.6
7B-5	1 tumor (small intestine)	28.8	13.4
7B-6	No tumor	20.0	9.1
8B-1	1 tumor (small intestine)	17.4	17.0
8B-3	Spots in liver Large bubble in stomach	15.0	17.0
8B-4	No tumor	21.6	13.3
8B-5	No tumor	23.2	9.7
8B-6	No tumor	16.2	11.7
9B-1	No tumor	22.6	15.9
9B-2	No tumor	25.2	15.9
9B-3	No tumor	26.2	10.3
9B-4	1 tumor (colon)	15.4	11.1
9B-6	No tumor	20.0	9.1
9B-7	Non-injected (no tumor)	27.2	4.1

tumors. There were few significant differences with respect to diet as well. Results are shown in Table XIV.

TABLE XIV. NATURAL KILLER (NK) AND CYTOTOXIC T-LYMPHOCYTE (CTL) TEST RESULT AVERAGES

Diet No.	Rats With Tumors		Rats Without Tumors	
	% NK	% CTL	% NK	% CTL
6	39.9	18.4	35.5	20.2
7	25.8	20.8	35.1	20.4
8	23.6	22.9	25.9	17.6
9	31.9	10.6	27.8	11.6

DISCUSSION

In comparing the weight gains of the rats with respect to the composition of the diets, rats fed Diet 9 (the AIN-76A control diet) were found to gain most rapidly, with rats fed Diet 8, Diet 6 and Diet 7 following in order of decreasing weight gain, although the weight gains were very close together for all four diets. It was interesting to note that when the amounts of food consumed for each diet in grams per rat per day were calculated, the order was exactly the same: the rats fed Diet 9 consumed the most followed by those fed Diet 8, Diet 6 and Diet 7. Two conclusions can be drawn as a result of analyzing these data. First, the diets were extremely equivalent, as they were meant to be, with respect to their caloric content. Second, Diet 9 must have been the most palatable of the diets, possibly because it had a higher protein content than the others. Diet 8 may have been second because of the higher sugar content in the form of lactose. Diet 7 was last, perhaps because the beef and beef tallow it contained were not as palatable to the rats as the other ingredients. The fact that the weight gains for the rats on all four diets were very close to one another makes it possible to conclude that the differences observed between diets with respect to tumor incidence could not have been due to differences in caloric intake.

In previous studies in our laboratory, several mouse tumor models have been used to reflect the effect of diet on tumor development and on cell-mediated immunity. The source as well as the level of protein and

the level of fat were all found to be important in modifying the growth of the tumors. In several of our tumor systems a low protein, low fat diet, in which the protein was non-fat powdered milk or casein, was found to give the greatest enhancement of cell-mediated immunity and the greatest restriction of tumor growth. However, in one of the research projects done in our laboratory using DMH-injected BALB/c mice, the tumor incidence was four times higher in the mice fed the milk diet than in those fed the beef diet (Nutter et al. 1983), a result quite unexpected after the previous results with the other tumor systems. Neither did this result match the epidemiological data in humans. On the other hand, interestingly in the present study, the rats fed the beef diet had a higher tumor incidence approximately twice that of those fed the milk diet, contrary to the results of the above-mentioned tumor study.

Before further discussion of the tumor findings, it is appropriate, perhaps, to mention again that 82 of the original 100 rats survived the full year until they were taken at the time of necropsy. Available details on the 18 which died appear in Table V. The first rat died on January 4, 1986. It had been fed Diet 9 and had been dead long enough to not allow it to be necropsied. The first rat to die with tumors, discovered at necropsy, was a rat fed Diet 7 which died January 26. Obvious colon tumors were visible. Two rats, one of which was euthanized on January 26, contracted jaw abscesses. Surgery had been performed on the latter by Dr. Charles Kean, Director of Animal Care at Loma Linda University, but the abscess had reoccurred. Most of the 18

rats were necropsied; some were found to have tumors and some not. The tumors were large enough to have blocked off the function of vital organs in some cases. In others, hemorrhaging seemed to be the cause of death.

The data on the 82 rats which were euthanized appear in Tables VI and VII. Most of the tumors discovered were tumors of the colon, but there were many tumors of the small intestine in addition to a few at miscellaneous sites. The percentage of rats with tumors which were fed Diet 7, the beef diet, was significantly greater than that for rats fed the AIN-76A control diet, Diet 9. This was in contrast to the tumor burden in rats fed Diet 6, the milk diet, where the percentage was even lower than for Diet 9. These findings add evidence to the idea that the DMH-injected rat-tumor model more closely reflects the carcinogen-produced colon tumor picture in humans and serves as a better model for studies on colon cancer than the DMH-injected mouse-tumor model (Nutter et al. 1983).

Several tests of immune status were performed in connection with the project. The results from the footpad testing reflecting the cell-mediated immunity were not very consistent and had considerable variation. Therefore, the results were not significantly different from each other in terms of statistical meaningfulness. The best one could conclude would be only in terms of the trends indicated in the Results section.

In the previous study in this laboratory using DMH-injected mice, the colons of the mice fed milk as the protein source were about two

times the weights of those fed beef as the protein source. There was speculation that the weight increase might be due to the lactose in the milk. Therefore, when the present rat-tumor experiments were designed, Diet 8 was included as a control diet with casein (with no lactose) substituted for the non-fat powdered milk. The primary goal was to see if removing the lactose would lower the relative colon weights. The RCW values for tumor or non-tumor mice compared separately in the 7, 8 and 9 diet groups do not appear to be significantly different from one another. This suggests that the lactose in Diet 6 may be responsible for the increase in colon weights in the mice fed that particular diet since the colons of the rats fed Diet 6 were significantly heavier than those fed Diet 8, for example, even when tumor-bearing and non-tumor-bearing rats were considered separately.

There was also some speculation in the previous study (Nutter et al. 1983) that there might be an association between the increase in colon weight and the increase in tumor incidence in the colons of the milk-fed mice. Consideration was given to the idea that perhaps the lactose increased the weights of the colons and thereby predisposed them to developing tumors. The results of the present study do not support that idea. The inclusion of Diet 8 as a no lactose control for Diet 6 did help to confirm that the lactose was probably responsible for the increased colon weights, but since the rats fed Diet 6 had the least tumor incidence of all four diets, there was no evidence for a relationship between increased colon weight due to lactose and increased tumor incidence.

In the NK and CTL tests, the last two experiments were not useful at all because of the insufficient uptake of ^{51}Cr by the target cells (YAC-1 as well as MAM). However, for the first two experiments, there were few significant differences. Although rats which were not injected with DMH (7A-7 & 9B-7) had fairly high % NK values and moderate to low % CTL values (47.9 and 32.2 for 7A-7 and 27.2 and 4.1 for 9B-7 respectively), rats without tumor did not have consistently higher % NK values nor lower % CTL values than rats with tumors. There are few significant differences with respect to diet as well.

It has been recognized that the lymphocyte transformation test is one of the best methods for determining the level of cell-mediated immunity. It has been increasingly recognized, however, that there is great variability between tests run on different days even though the conditions and cells are kept as similar as possible. Some of the reasons for this variability are: differences in trauma to the cells due to handling, differences in cell counting, differences in culture media and sera, differences in mitogen concentration, etc. from one time to the next. Since this variability exists one cannot safely compare SI values for similar animals in tests run on different days. For this reason, the data in the LTT tests performed in connection with this project were analyzed in such a way that the SI values for each euthanization day were analyzed separately with the results from the four diets compared with one another. Also the SI values for the rats in a particular group (A, B, C or D) and fed a particular diet were

separated into tumor-bearing and non-tumor-bearing subgroups which were then compared with one another.

In the 14 comparisons with tumor and non-tumor rats in the PHA lymphoproliferation tests, 9 of the 14 had SI values higher for non-tumor rats whereas 5 had them higher for tumor-bearing rats. Similarly, when Con A was used, 10 of the 14 had SI values higher for non-tumor rats. These comparisons seem to favor the immunosuppression theory that non-tumor-bearing animals should have very little or no cellular immunosuppression compared to the tumor-bearing animals which have immunosuppression.

Although because of the great variability in the SI values in the lymphoproliferation assays, significant differences with diet could not be seen, cells from rats fed Diet 9 most consistently had the highest SI values when compared with those from rats fed the other diets and euthanized on the same day. This was true for both PHA and Con A assays. The order of decreasing SI values for the other diets was also similar for the two mitogens. Whether it is possible to apply meaning to these trends is not known. It is interesting, though, that Diet 9 was the standard AIN-76A diet with the moderate protein level.

While immunological data in this study were too variable to adequately measure significant differences between the treatment groups, one important fact can be concluded about test models. Based on tumor incidence, perhaps the most important test parameter measured, the DMH-rat tumor model appears to more closely resemble the human epidemiological finds than the DMH-injected mouse model.

SUMMARY

One hundred 5-week-old male Sprague-Dawley rats were divided into 4 diet groups: 1) a low (5%) fat, low (11%) protein diet with non-fat powdered milk as protein source, 2) a diet composed similarly except that beef was used as protein source and beef tallow replaced corn oil, 3) a control diet similar to the first diet except that casein (no lactose) was substituted for the milk, and 4) the standard recommended balanced diet for rats and mice (AIN-76A) which was similar to the third diet except that the protein level was moderate (about 19%). At 12 weeks of age, and for each of the 9 weeks following, each rat received an injection of DMH (10 mg/kg of body weight). The 82 rats which were still alive were euthanized and necropsied at 52 weeks of age. Spleens and colons were removed and weighed, tumor counts were made and the tumors prepared for pathological examination. Spleen cells were removed and assays for cell-mediated immunity were performed: lymphocyte transformation test (LTT) with PHA and Con A as mitogens, as well as assays for natural killer cell and cytotoxic T lymphocyte activity.

The percentage of rats with tumors found at euthanization were in the descending order: 1) Diet 7 - 79%, 2) Diet 8 - 64%, 3) Diet 9 - 50% and 4) Diet 6 - 44%. The percentage of rats with tumors among those fed Diet 7 were significantly higher than the percentages among those fed Diets 6 and 9.

Injected rats fed Diet 6, regardless of whether they developed tumors or not, had significantly heavier colons than rats fed Diet 8

which contained no lactose. This lends support to the theory that lactose may be responsible for the increased weight. Since the rats fed Diet 6 had fewer tumors than those fed the other diets (including Diet 8), there is no evidence of an increase in tumor incidence associated with an increase in colon weight.

In footpad testing, the swelling of footpads injected with PPD was significantly greater than the swelling in footpads injected with saline only, but there were no significant differences between diets or the presence or absence of tumors. There is not any significance to be found in the NK and CTL test results, since there was too much inconsistency in the testing. In the LTT test, rats without tumors inclined to have higher cell-mediated immunity than those with tumors. In addition, with both mitogens, rats fed the AIN-76A control had the highest cell-mediated immunity.

In this project we found that the results with the DMH-rat tumor model more closely resemble the human epidemiological findings than DMH-injected mice fed similar diets.

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