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MODIFICATION OF HERPES 2-TRANSFORMED CELL-INDUCED TUMORS IN MICE FED DIFFERENT SOURCES OF PROTEIN, FAT AND CARBOHYDRATE

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SUMMARY

The effects of different sources of protein (milk, soy, wheat, fish and beef), fat (corn oil and butter), and carbohydrate (dextrin and sucrose) on tumor development and on spleen characteristics were investigated in BALB/c mice injected subcutaneously with 5×10^5 herpes simplex virus Type 2-transformed cells (H238 cells). Low or high levels of protein and fat were used.

Several weeks post-injection results indicated that a high level of fat significantly enhanced tumor incidence. A high fat level was also associated with a lower spleen weight and a smaller proportion of mature granulocytes in the spleen. Butter, compared to corn oil, significantly restricted tumor volume. Among the most highly significant findings was the low tumor incidence in mice fed protein from either a milk or a fish source.

INTRODUCTION

In recent years, epidemiological data revealing strong positive correlations between breast cancer and total dietary fat intake and between colon cancer and meat intake [2,5,19] has sparked renewed interest in investigating the role of diet and carcinogenesis in laboratory animals. Some of these animal studies have shown that dietary fat influences spontaneous mammary tumorigenesis through promotion rather than by initiation [8,9], and that even transplantable mammary carcinoma is influenced by dietary fat [23,27]. Very few diet animal tumor studies have utilized more than one source of protein. However, one report indicates that with increased fat in the diets containing a high level of either meat or soy protein, the incidence

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of certain tumors increased [29]. The source of carbohydrate (CHO) has also recently been shown to influence tumorigenesis in both epidemiological studies [1,20] and in experiments using laboratory animals [21,24].

During the last decade, UV-inactivated herpes viruses (potentially oncogenic in humans) have been used to induce transformation of normal hamster cells into neoplastic cell lines [13,14,28]. Boyd and Orme [3] reported that inactivated herpes simplex virus Type-2 (HSV-2) induced the transformation of normal BALB/c mouse cells into established, malignant cells (H238 cells). In preliminary studies with mice injected with H238 cells we have found the relative spleen weight (RSW) to reach values of 190 in tumor-bearing mice at 7 weeks compared with RSW values of 40 for control animals [18]. As with some other mouse-tumor models, a significantly depressed lymphoproliferative response to phytohemagglutinin stimulation was found in the H238 cell-injected tumor-bearing mice at 7 weeks, at which time the stimulation index for spleen cells had dropped from a normal value of 50 to 3 [18]. We have also used the H238 cell-BALB/c mouse system in studies of changes in spleen morphology during tumor growth [26]. In other preliminary experiments with this tumor model, there were indications that source of dietary protein might affect tumor development and that diets containing milk protein might restrict tumor development [17]. In order to test these hypotheses, more extensive experiments were conducted, the results of which are presented here.

MATERIALS AND METHODS

Animals

Five-week-old male BALB/c mice, obtained from the Jackson Laboratory (Bar Harbor, ME), were randomized upon delivery into 15 groups and allowed to feed ad libitum on the powdered diets for a total of 22 weeks. Each animal was weighed individually on arrival and at weekly intervals thereafter. The amount of food eaten per cage of animals was recorded twice weekly.

Cultured cells for injection

H238 cells (BALB/c mouse embryo cells transformed by UV-irradiated HSV-2, Savage strain) were obtained from Dr. Ann Lewis Boyd (Frederick, MD) and stored in liquid nitrogen. The cells were passaged a constant number of times in vitro before injection into the mice. Dulbecco's MEM supplemented with 10% fetal calf serum and antibiotic was used for cell culture.

Composition of the diets

A summary of the diets is given in Table 1. All ingredients were obtained from United States Biochemical Corporation (Cleveland, OH) unless otherwise indicated. Each source of dietary protein contained different amounts of actual protein, fat and CHO. The typical analysis for each protein source

TABLE 1

DESCRIPTION OF THE DIETS[a], CALORIC INTAKE AND WEIGHT GAIN

Diet no.	Protein		Fat		Average kcals/ mouse/day[c]	Average weight gain (g)[d]
	Amount (%)	Source	Amount (%)	Source[b]		
1	11	Milk	5	Corn oil	10.3	8.7
2	11	Milk	30	Corn oil	10.6	9.5
3	33	Milk	5	Corn oil	9.6	9.3
4	11	Milk	30	Butter	11.0	10.4
5[e]	11	Milk	5	Corn oil	10.7	8.3
6	11	Soy	5	Corn oil	11.4	10.1
7	33	Soy	5	Corn oil	11.4	10.9
8	11	Wheat	30	Corn oil	9.9	9.3
9	33	Wheat	5	Corn oil	9.9	10.7
10	11	Fish	5	Corn oil	10.5	11.3
11	33	Fish	5	Corn oil	9.5	11.7
12	11	Beef	5	Beef fat[f] (3.55%) Corn oil (1.45%)	10.7	9.4
13	11	Beef	30	Beef fat[f] (3.6%) Corn oil (26.4%)	10.3	10.7
14	33	Beef	10.7	Beef fat[f] (10.7%) Corn oil (0.0%)	9.9	11.8
15[g]	22.5	Wheat Milk Soy	7.5	Corn oil Wheat Soy	13.2	11.3

[a] A salt mixture and a vitamin diet fortification mixture were added to the 14 experimental diets so that each one would contain 4% mineral mix and 2% vitamin mix.

[b] The amount of fat, brought up to the 5% or 30% total fat level with corn oil, also includes those small amounts of fat present in the protein source.

[c] Each number represents the average caloric intake obtained for each diet group during the entire course of the experiment.

[d] The weight gains shown are the averages obtained from the time the mice arrived (at 5 weeks of age) until injection of H238 tumor cells 15 weeks later.

[e] Sucrose was the CHO source in Diet 5; in the other experimental diets the added source was dextrin.

[f] The lean beef source contained 23% beef fat according to the analysis of the manufacturer.

[g] Old Guilford 96W/A, a commercially available mouse feed, was used as the control diet.

as well as for each of the other ingredients was obtained from the various manufacturers and was used when making all test diets equivalent with respect to total percentage of protein. Diets 1—5 contained protein supplied by powdered skim milk (Foremost-McKesson, Inc., Willows, CA). Vita-free casein was, however, added to Diet 3 so that the protein was composed of 50% casein and 50% powdered skim milk protein. Diets 6 and 7 contained soy protein (Textured Vegetable Protein, Archer Daniels Milling Company, Decatur, IL); Diets 8 and 9 contained wheat gluten (Do-Pep, Standard Flour Company, Los Angeles, CA) supplemented with 0.5% lysine and 0.2% L-threonine; Diets 10 and 11 contained fish protein; Diets 12—14 contained beef protein (pre-cooked, freeze-dried diced beef, Mountain House, Oregon Freeze Dried Foods Inc., Albany, OR).

Corn oil ('Mazola', Best Foods, Ingelwood Cliffs, NJ) was the source of added fat. The exceptions were Diet 4, in which 30% butter fat (Knudsen Dairy Products, Los Angeles, CA) was used, and Diets 12—14, which contained both corn oil and beef fat because the beef source of protein contained 23% beef fat. Dextrin, a modified cornstarch, was the CHO source in all of these diets except Diet 5 which had sucrose (C & H Sugar, San Francisco, CA) in place of dextrin, and Diet 3 which contained 23.9% lactose due to the lactose content of the skim milk. In order to keep the diets equicaloric (3.7 kcal/g), 'Celufil', a non-nutritive cellulose filler, was used. Vitamin Diet Fortification Mixture and Salt Mixture Hegsted were added at 2% and 4%, respectively. Old Guilford 96W/A (Emory Morse Company, Guilford, CT), a standard mouse feed, was used as the control diet. The diets were well-mixed in an automatic food mixer and frozen until fed.

Tumor induction

Fifteen weeks after arrival, 26—30 mice in each diet group were injected subcutaneously in the right thigh with 5×10^5 viable H238 cells. At this cell dosage 70% of the mice fed a standard mouse feed develop a progressively growing fibrosarcoma within 2 weeks. The animals remain alert and active for 6—7 weeks post-injection and exhibit no metastases. Non-injected mice (8—10/diet) were used as controls.

Tumor incidence and volume

H238 cell-injected mice were subdivided into 4 response categories at killing, 7 weeks after injection: Pr (mice with progressively growing tumor); RPr (mice in which tumor appeared soon after injection, regressed completely, but then re-appeared and grew progressively); R (mice in which tumor regressed completely); NVT (mice which developed no visible tumor after injection). Tumor measurements were done with Vernier Calipers and the length (L), width (W), and height (H) of the tumor were used in the following calculation [4].

$$\text{Tumor volume (in mm}^3) = LWH/2$$

Relative spleen weight

Each mouse was weighed immediately before killing and the spleens were weighed after removal. The RSW was calculated as follows [31]:

$$RSW = 10^4 \times \text{weight of spleen/weight of mouse}$$

Spleen cell differential counts

Spleen cell suspensions were streaked onto slides and stained with Wright's stain using an automated Ames Hema Tek stainer: 100—200 cells/spleen were identified as either small lymphocytes, medium to large lymphocytes, mature granulocytes, immature granulocytes, or blastocytes and immature RBCs.

Spleen cell size distribution

Light scattering of the laser beam in a Coulter model TPS-1 Cell Sorter (Coulter Electronics, Inc., Hialeah, FL) was used to obtain size distributions from spleen cell suspensions containing 1×10^6 cells/ml. The machine was set so that 1000 cells/sample were aspirated and sorted in 5 s. A computer program was used to calculate the relative mean cell size and the standard deviation of that mean for each cell preparation from the numeric readout of the cell sorter. Not all distributions were unimodal.

Statistics

An analysis of variance was done to determine the significance of the effect of dietary factors. Diet 4 (containing butter fat) and Diet 5 (containing sucrose) were excluded from this analysis. The Student's 2-tailed *t*-test was used in comparing results from mice fed these two diets and tumor incidence was analyzed using the Test for the Equality of Two Proportions.

RESULTS

Tumor incidence

Table 2 shows the tumor incidence for each diet group at 7 weeks post-injection. Lowest percentages of Pr mice and highest percentages of NVT mice were found with Diet 1 (the low milk—low fat diet) and with Diet 11 (the high fish protein—low fat diet). The values were significantly lower ($P \leq 0.05$) than those for Diets 2, 3, 7, 8 and 14.

The tumor incidence was analyzed with respect to weight gains and average food intake to see if any correlations might be found which would suggest that caloric intake had an effect on tumor development in these experiments. No such correlations were found. The caloric intake and weight gain averages for mice fed each of the various diets are included in Table 1.

Tumor volume

The source of dietary protein had a statistically significant effect ($P = 0.001$) on the size of the tumors with the diets containing milk or fish protein

TABLE 2

TUMOR STATUS IN MICE INJECTED WITH H238 CELLS

Protein source	Diet no.	Protein-fat level[a]	No. of mice	Percent			
				Progressors (Pr')[b]	Regressor-progressors (RPr')[c]	Regressors (R')[d]	No visible tumors (NVT')[e]
Milk	1	L-L	30	50.0[f]	0	3.3	46.7
Milk	2	L-H	28	75.0	0	3.6	21.4
Milk	3	H-L	26	76.9	0	3.9	19.2
Milk	4	L-H	30	56.7	3.3	6.7	33.3
Milk	5	L-L	29	69.0	3.5	0	27.6
Soy	6	L-L	29	69.0	0	6.9	24.1
Soy	7	H-L	28	89.3	0	3.6	7.1
Wheat	8	L-H	29	89.7	0	3.5	6.9
Wheat	9	H-L	29	65.5	0	13.8	20.7
Fish	10	L-L	29	55.2	3.5	10.3	31.0
Fish	11	H-L	30	43.3	6.7	13.3	36.7
Beef	12	L-L	29	62.1	6.9	6.9	24.1
Beef	13	L-H	30	73.3	0	10.0	16.7
Beef	14	H-L	29	79.3	0	3.5	17.2
Many sources, primarily wheat	15 (control)		26	69.2	0	23.1	7.7

[a]L-L: 11% protein—5% fat; L-H: 11% protein—30% fat; H-L: 33% protein—5% fat.

[b]Percentage of injected mice in which tumors progressed during entire experiment.

[c]Percentage of injected mice in which tumor appeared and grew for several days, regressed completely, but later appeared again and continued to grow progressively from then on.

[d]Percentage of injected mice in which tumors appeared and grew for several days, but then regressed completely and were absent from then on.

[e]Percentage of injected mice which never developed a visible tumor.

giving the smallest mean volumes. Maximum tumor growth was found in animals fed Diets 9 and 15, both of which contained wheat as the primary protein source. Figure 1 shows the mean tumor volume values in the Pr mice at 7 weeks after injection of the H238 cells. The mean volumes ranged from 2700 to 5550 mm^3 (excluding Diets 4 and 5).

Both source of protein and level of fat significantly increased ($P = 0.026$ and $P = 0.037$, respectively) the tumor volume attained in the R mice. These data were not included in Fig. 1 since the numbers of R mice were relatively low. As with the Pr mice, milk or fish protein diets resulted in less tumor

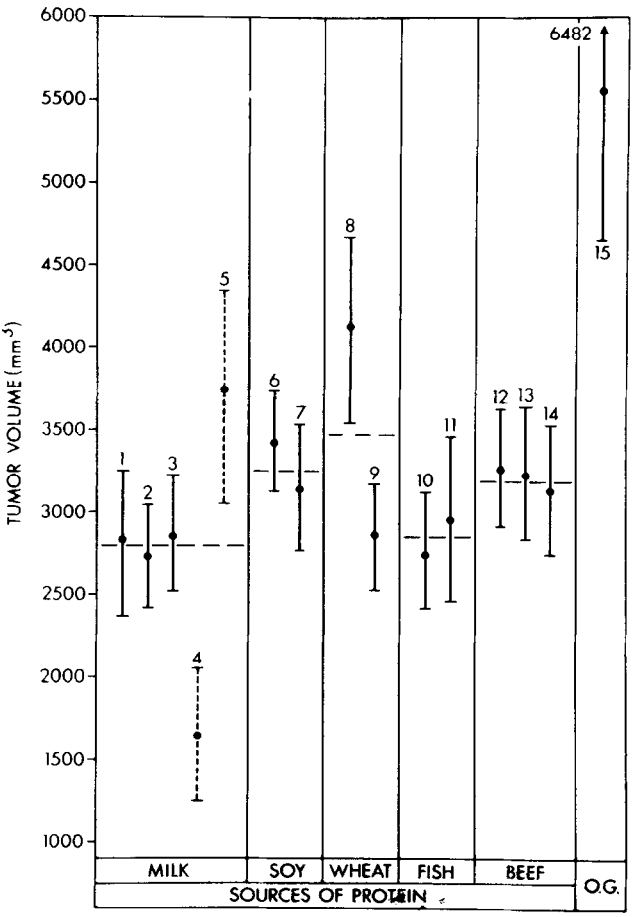

Fig. 1. Tumor volume obtained for mice fed different diets at 7 weeks after H238 cell injection. The mean and S.E.M. values for Diets 1—14 are grouped according to the source of protein. The diet number is shown above each of the values. Means obtained for each group of diets (excluding Diets 4 and 5) are represented by the broken horizontal lines. Results from mice fed Old Guilford (OG) 96W/A, the control diet, are shown in the right hand column.

growth than those fed wheat protein. High dietary fat resulted in increased tumor volume in these mice.

Relative spleen weight (RSW)

The level of dietary fat significantly affected ($P = 0.028$) the RSW at 7 weeks after H238 cell injection. Lower spleen weights were found in mice fed the high fat diets. Although statistical significance was not obtained, the groups fed the milk-containing diets had less splenomegaly (RSW = 152.0 ± 9.9) than those fed diets containing soy, wheat, fish or beef protein (RSW = 174.0 ± 9.0, 178.3 ± 13.1, 165.0 ± 9.5 and 175.5 ± 12.8, respectively). The range of RSW values for normal, non-injected mice was 30—40.

Spleen cell differential counts

Spleens from the Pr mice fed the high level of fat had a significantly lower ($P = 0.005$) percentage of mature granulocytes than did those from mice fed the low fat level at 7 weeks after H238 cell injection. The composition of the diet did not have a statistically significant effect on the proportions of the other cell types.

When all of the Pr mice (regardless of diet) were compared as a group with the pooled mean obtained for the control mice, they showed significant changes in cell populations associated with splenomegaly. The percentages of small lymphocytes, mature granulocytes, and immature granulocytes in the Pr mice and control mice (shown in parentheses) were 49.5 ± 4.3 (72.3 ± 4.5), 27.4 ± 3.5 (11.8 ± 4.0) and 4.7 ± 0.9 (0.8 ± 0.4), respectively.

Spleen cell size analysis

An increased heterogeneity in spleen cell size was evident in the Pr mice when compared to the corresponding non-injected mice at 7 weeks after H238 cell injection. The smallest standard deviations (13.3) in cell size, indicating the most homogeneity, however, were seen in the low milk protein—low fat diet group (Diet 1, see Fig. 2). The most heterogeneity was seen in the spleen cells from the beef-fed mice (Diets 12—14 gave an average standard deviation of 17.4). The mean spleen cell size values resembled those for the standard deviation and are not shown here.

Results from mice fed diets containing different sources of fat and CHO

The Pr mice fed a high level of butter fat (Diet 4) had the smallest tumor volumes and a significantly smaller ($P = 0.037$) tumor volume than those fed a comparable diet (Diet 2) in which corn oil replaced the butter (see Fig. 1). Other characteristics in Pr mice fed Diet 4 were: the lowest RSW (141.0 ± 13.8), the highest percentage of small lymphocytes (62.7 ± 3.8), and the lowest percentage of mature granulocytes (18.0 ± 2.8) when compared to those fed the other diets.

The H238 cell-injected mice fed sucrose as the CHO source (Diet 5) had a higher tumor incidence and a larger tumor volume than those fed a com-

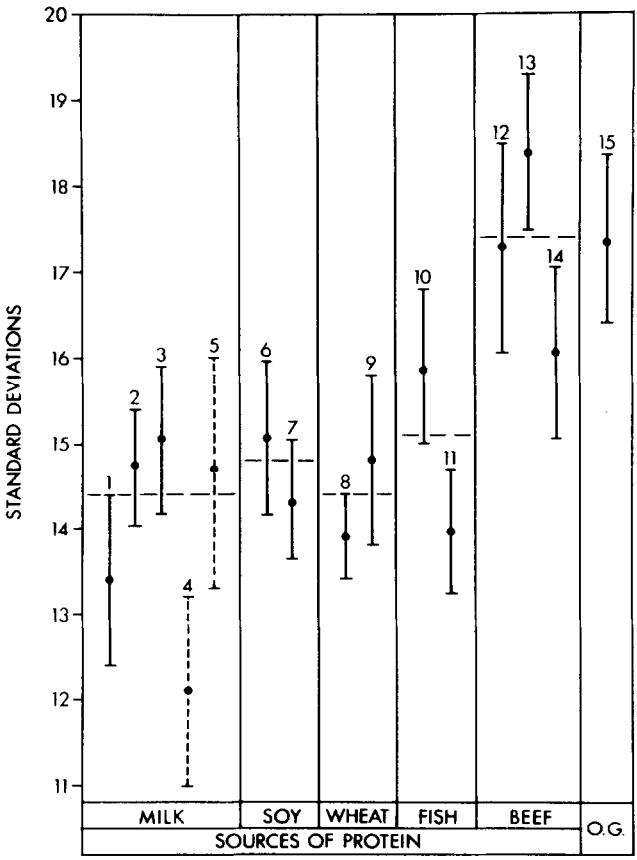

Fig. 2. Standard deviations in spleen cell size obtained by analysis with a Coulter TPS-1 laser beam cell sorter. See Fig. 1 for further explanation.

parable diet containing dextrin (Diet 1), but no statistical significance was obtained.

DISCUSSION

The great majority of diet studies have been conducted on chemically-induced or spontaneous tumor systems in which it is difficult to determine whether diet affects initiation, modification, or both of these aspects of tumor development. In the present study tumor-initiation had occurred in vitro, since virus-transformed cells were used, thereby limiting the observed effects of diet to modification only.

The modification of H238 cell-induced tumors by high-fat diets is demonstrated in our experiments in which a significant increase in tumor incidence was found with increased fat levels. At one time the hypothesis that dietary fat influenced tumorigenesis largely through initiation was emphasized [6].

Recent experiments, however, have shown that it acts primarily as a promoting agent [8,10]. Although fat has been shown to influence the growth of transplantable mammary carcinoma in host animals [23,27], it should be emphasized that our present findings with the H238 system indicate that tumors other than mammary tumors may also be influenced in their development (separately from their initiation) by fat in the diet.

Several theories have been advanced to explain increased tumorigenesis with increased fat intake including effects on the fluidity of the membrane [22] and suppression of cell-mediated immunity [11,12]. The complexity of the possible interactions is emphasized in the recent work of Giovarelli et al. [16] who report that the effects of dietary polyunsaturated fatty acids on the immune system and tumor proliferation are markedly dependent on the sex of the mouse and on non-H-2 strain background genes. Carroll and Hopkins [7] working with the effect of dietary fat on DMBA-produced mammary carcinogenesis in rats, discovered that 2 factors were necessary for the enhancement of tumor yield by fat. The first, already emphasized, was that the level of total fat in the diet be high. The second was that there was a minimum level of polyunsaturated fat required for mammary tumorigenesis. In comparing the effects of 2 different sources of fat at a high level on tumor development, our data supported their second finding. The Pr mice fed the diet containing butter (which had very little linoleic acid) exhibited much smaller tumors than those seen in mice on the equivalent corn oil diet, reminiscent of the rat mammary tumor system.

The literature shows no clear relationship between the level of protein and carcinogenesis. In our study the mice fed a high protein level had higher tumor incidence than those fed low protein, although no statistical significance was obtained. Epidemiological studies associate increased color tumors with increased meat, and particularly, beef, intake [15,32]. An extensive study of 2 human populations with 4-fold differences in colon cancer incidence has been reported [25]. Among the factors (including increased fiber) suggested to account for the decreased cancer incidence in the low-risk groups was the 'possible protective effect' of milk. Milk consumption was 4 times greater in the low-risk group. In our study, tumor incidence was found to be lowest in mice fed diets containing protein from milk (low-fat) or fish sources. Tumor volume was also relatively low in these mice.

In addition, our data show that spleens of tumor-bearing mice fed beef-containing diets exhibited a greater change from normal than the other mice. These observations may reflect the presence of more immature cells in the spleen. We have reported that a growing H238 cell-induced tumor resulted in a substantial increase in the compact myeloid tissue and reaction centers of lymphoid nodules in the BALB/c mouse spleen indicating increased hematopoietic activity [26]. Possibly a constituent of the beef diets further amplified this hematopoietic activity either directly or via increased production of a granulocytosis-promoting factor.

Differences seen in tumor development and host characteristics may be

due to the amino acid composition of the various dietary proteins and/or to differences in the kind and quantity of ingredients accompanying the proteins no matter how concentrated the protein sources may be.

A comparison of the results with Diets 1 and 5 suggests that sucrose enhances development of H238 cell-induced tumors. Recently, a positive correlation found between human breast cancer mortality and dietary sugar [20] has been confirmed in a rat study [21]. Other reports indicate that endometrial cancer in humans [1] as well as DMH-induced colon tumors [24] and Walker 256 carcinosarcoma [30] in rats may be enhanced by a high intake of sugar.

We have shown that mouse tumors produced by injection of herpes virus-transformed cells were modified by dietary manipulation. Results of experiments done in our laboratory designed to deal more specifically with the mechanisms which may be involved in the modification of tumor development by dietary components will be reported separately.

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