THE EFFECT OF DIET ON THE IMMUNOCOMPETENCE OF MICE WHICH DEVELOP SPONTANEOUS MAMMARY TUMORS

AN HONORS THESIS

by

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INTRODUCTION

The purpose of this research was to determine whether a saturated fat diet influences the immune capability of Strong Strain A mice which develop spontaneous mammary tumors. It has been observed in our laboratory that mice on a saturated fat diet develop mammary tumors at a later age than mice on a stock diet. In order for tumor formation to occur, the tumor must survive attack by the host's immune system. The difference in the tumor development between the two groups of mice, then, may possibly be due to an alteration in the immune system allowing the animals on the saturated fat diet to exist a longer period of time without tumors.

REVIEW OF RELATED LITERATURE

The immune system is in contact with most, if not all, of the cells, tissues, and organ systems in the body. Therefore, any modification in the immune system would influence all other systems (1). It is well-documented that the efficiency of the immune system declines in an age-dependent manner after reaching a peak in adolescence (1-41). A strong relationship exists between the decline of the immune system and the emergence of diseases which can greatly affect many tissues (1).

The ability of certain unsaturated fatty acids to reduce cell-mediated immune reactions has been widely reported and has been shown to inhibit several lymphocyte activities, including response to antigen and mitogen stimulation in vitro (2,3,4,5,6). Several researchers have studied the influence of prostaglandin precursors, polyunsaturated fatty acids, on cellular immunity and have found that polyunsaturated fatty acids markedly suppress the blastogenic reaction of lymphocytes (2,4,7,8). Both linoleic (18:2) and arachidonic (20:4) acids have been found to interfere with blast formation in response to Phytohemagglutinin (PHA) (2,9). Offner and Clausen studied the effects of oleic acid, linoleic acid, arachidonic acid, prostaglandin E_1 and prostaglandin E_2 on normal lymphocytes. All of the unsaturated fatty acids studied caused a significant inhibition of the lymphocytes. Arachidonic acid had the highest inhibitory activity while oleic acid had the lowest inhibitory effect (9).

It has also been observed in several studies that high levels of polyunsaturated fatty acids enhance the incidence of chemically-induced tumors (10,11,12,13,14) and the growth rate of transplantable tumors (15,16,17,18,19). Hopkins, West, and Hard studied the effect of

polyunsaturated and saturated fatty acids on induced tumors in rats and they discovered that the mean tumor induction time among rats fed the diet high in polyunsaturated fatty acid was less than among rats fed the saturated fat diet (20). The results from this study agree with the findings of Gammal, Carroll, and Plunkett which showed that the incidence of palpable tumors was greater among rats fed a semisynthetic high corn oil diet rather than an isocaloric diet containing coconut oil (21).

It is well-documented that the occurrance of autoimmune disorders, infectious diseases, and cancers dramatically increase with age in all animals (22,23,24,25). This decline in the immune system which accompanies aging is due to alterations in the thymic-dependent lymphoid cell component of the immune system (T cells) (26). Thymic involution precedes the age-related decline in T cell function and results in a decreased capacity of the system to generate functional T cells (1). Thymic-dependent lymphoid cells are known to play a major role in cellular immunity and to serve as helper cells in certain types of humoral immune responses (27). Although Callard and Basten have reported that there is also a loss of spleenic B cell function associated with aging, T cell functions are most profoundly affected by aging (28,29,30,31). The helper function of T cells also declines with age and this decline has been demonstrated in intact animals as well as in <u>in vitro</u> assays (32,33,34,35). In the mouse, the decrease in certain T cell functions occurs upon reaching sexual maturity. It occurs later in the long-lived hybrids than the short-lived A,C57, or CBA strain (36).

The proliferative response of T cells to the mitogens, Phytohemagglutinin (PHA), Concavalin A (Con A), or to the stimulatory effects of allogeneic cells on the mixed lymphocyte culture all decrease markedly with age (37,38).

Other Tcell responses, which have been determined to decline with age, include such properties as theta bearing cell percentages, mixed lymphocyte reactions (MLR) and graft vs. host responses (GVH) (39,40).

One of the most widely used technique to study T cell function is the short-term culture of various lymphoid tissues. By stimulating T cells with specific mitogens or alloantigens, it is possible to determine the proliferative capacity of the T lymphocytes found in those tissues (41). By quantitating the T cells and examining the proliferative ability, it is possible to perform studies on animal and humans totally in vitro and to qualitate and to quantitate various changes related to age (41).

MATERIALS AND METHODS

Animals and Treatment Strong Strain A mice housed in a constant environment in CL 77 of Cooper Life Science Building were used for these experiments. One group of mice was fed a high fat diet containing 1 percent safflower oil and 14 percent stearic acid (95 percent pure). Another group of mice was fed a stock diet containing 4.5 percent fat. Both groups of mice received food and water ad libitum. The mice were placed on either the saturated fat diet or the stock diet at weaning. Fifteen mice on the stock diet at ages 5,7,11, and 13 months and nine mice on the saturated fatty acid diet at ages 3,6,8, and 14 months were sacrificed and tested for immunocompetence.

Lymphocyte Blastogenesis Mice were sacrificed by cervical dislocation and the spleens were removed surgically. The mouse was laid on his right side and his left side was saturated with 95 percent ethyl alcohol. The skin above the left back leg was held and a lateral cut was made. Special care was exercised to prevent touching the peritoneal lining. The skin was then pulled back so that the spleen could be seen through the peritoneal lining. The lining over the spleen was cut and the spleen was removed through the hole in the lining. The spleen was immediately placed in a sterile bottle which contained approximately 5 ml of Phosphate Buffered Saline (PBS pH 7.1). All surgical instruments were kept immersed in 95 percent ethyl alcohol before and after use. The remainder of the procedure was performed under a hood to prevent air flow and to reduce the risk of contamination. The spleen was washed by agitating the bottle containing the spleen and decanting the used PBS into a beaker. More PBS was added and this was repeated at least twice. Approximately 5 ml of PBS was added after the last

wash. The spleen was then poured onto a screen in a small beaker. The screen was held with hemostats while the spleen was homogenized with a syringe plunger. A single cell suspension which resulted from this procedure was drawn up into a 5 ml pipette, transferred to a sterile centrifuge tube, and centrifuged at 1200 rpm for 10 minutes. The supernatant was decanted and the pellet was suspended in 5 ml of PBS. Cells were resuspended by pipetting up and down, centrifuged, and resuspended in 2 ml of Eagle Hanks Amino Acid media (EHAA).

A mixture containing 0.1 ml of cells and 0.9 ml of 0.4 percent trypan blue was prepared and then transferred to a hemacytometer by a micropipetter. The cells were then counted with the hemacytometer. The original cell concentration was calculated by multiplying the number of cells counted x the dilution factor x 10^4. To obtain the required 3 ml of cell suspension which contained a concentration of 1.0 x 10^7 cells/ml, 3.0 x 10^7 cells total was needed. To determine the volume of cell suspension which contained 3.0 x 10^7 cells, the original cell concentration was divided from 3.0 x 10^7 cells. EHAA media was added to bring the cell suspension to 3 ml. Spleen cell suspensions were pipetted into microtiter plates in 0.1 ml aliquots. An equal volume of serially diluted Con A with final concentrations of 0.5 ug/ml, 1.0 ug/ml, 2.0 ug/ml, 4.0 ug/ml, and 8.0 ug/ml was added to the microtiter plate. Triplicate determinations were done for each concentration of Con A. Unstimulated values were determined by adding as equal amount of EHAA in place of Con A. Cells were incubated for 48 hours at 37° C in an atmosphere containing 5 percent CO_2 and 95 percent air. The cells were then radioactively labelled with tritiated (^3H) thymidine (2 uCi/well in 50 ul EHAA) for 24 hours and collected on filter paper with a microharvester. The harvested cells were placed in scintillation vials and air dried. Scintillation fluid (200 mg/4 l POPOP and 16 g/4 l PPO) was added and the

radioactivity associated with the cells was counted in a liquid scintillation counter. The counts per minute obtained from the uptake of tritiated-thymidine is related to the T cell proliferative ability and is thus a measure of immunocompetence. The net counts per minute were obtained by subtracting the average counts per minute of the 3-unstimulated cultures from the average counts per minute of the 3-stimulated cultures at Con A concentrations of 4 ug/ml and 8 ug/ml. The stimulation index (SI) was determined by dividing the average counts per minute of the 3-unstimulated cultures from the average counts per minute of the 3-stimulated cultures at Con A concentrations of 4 ug/ml and 8 ug/ml.

RESULTS AND DISCUSSION

It was noted that the animals on the saturated fat diet did respond to Con A in all cases. Six animals on the stock diet failed to produce a reaction (i.e. TN 3, TN 4, TN 5, TN 7, TN 10, TN 11) and three animals on the stock diet produced minimal reactions to Con A (i.e. TN 6, TN 14, TN 15). It was also found that when the net counts per minute are compared for the two groups of animals that the animals on the saturated fat diet which were 5 months old (TS 4 and TS 5) produced a greater response to the concentrations of Con A than did the animals on the stock diet which were 4 months old (TN 1 and TN 2). Higher net counts per minute were also obtained by saturated fat animals which were 8 months old (TS 7 and TS 8) than the net counts per minute obtained by the animals on the stock diet which were 7 months of age (TN 12 and TN 14). This finding can be observed for both the 4 ug/ml and 8 ug/ml concentrations of Con A. These data are also recorded in terms of the stimulation index (SI) since some researchers utilize SI as an indicator of proliferative ability.

It was hypothesized that the mice on the saturated fat diet would produce a higher response than mice on the stock diet to the mitogen, Con A; however, more research needs to be done to confirm this hypothesis. The findings mentioned above tend to support this hypothesis, but a trend was not discovered to verify that all of the data obtained conform to this hypothesis.

The technique used in this pilot study was new to our laboratory and thus some difficulty was experienced in adapting this procedure. This fact may account for some of the variability encountered in this experiment. The lymphocyte blastogenesis was performed on mice that were up to 12 months of

TABLE 1

EFFECT OF DIET ON T CELL RESPONSE TO CON A IN NET COUNTS PER MINUTE*

Animal Number Stock diet	Age	Con A concentrations 4 ug/ml	8 ug/ml
**TN 6	12 months	559.62	1,405.19
TN 9	10 months	23,113.16	3,328.00
TN 8	10 months	1,169.70	580.40
TN 13	10 months	1,810.10	1,657.83
TN 12	7 months	3,975.84	3,950.35
**TN 14	7 months	240.84	7,217.27
TN 1	4 months	23,557.55	38,332.82
TN 2	4 months	4,234.08	5,498.83
**TN 15	4 months	837.08	4,524.35

Animal Number Saturated fat diet	Age	Con A concentrations 4 ug/ml	8 ug/ml
TS 1	14 months	2,268.90	474.65
TS 2	14 months	10,882.01	9,741.04
TS 3	14 months	7,940.44	31,290.65
TS 7	8 months	13,320.01	7,141.62
TS 8	8 months	7,412.03	13,917.33
TS 9	8 months	2,736.87	8,186.00
TS 4	5 months	38,564.57	33,343.74
TS 5	5 months	17,174.66	8,851.58
TS 6	3 months	9,553.32	12,520.08

* Net counts per minute were obtained by subtracting the average counts per minute of the 3-unstimulated cultures from the average counts per minute of the 3 stimulated cultures at Con A concentrations of 4 ug/ml and 8 ug/ml.

** Minimal response

TABLE 2

EFFECT OF DIET ON T CELL RESPONSES TO CON A IN TERMS OF A STIMULATION INDEX*

Animal Number		Age	Con A concentrations	
Stock diet			4 ug/ml	8 ug/ml
**TN	6	12 months	2.30	4.27
TN	9	10 months	9.13	2.17
TN	8	10 months	2.52	1.76
TN	13	10 months	1.28	1.25
TN	12	7 months	4.73	4.74
**TN	14	7 months	1.07	3.10
TN	1	4 months	5.55	8.39
TN	2	4 months	3.91	4.79
**TN	15	4 months	1.14	1.74
Animal Number		Age	Con A concentrations	
Saturated fat diet			4 ug/ml	8 ug/ml
TS	1	14 months	1.21	1.04
TS	2	14 months	2.85	2.66
TS	3	14 months	1.53	3.07
TS	7	8 months	2.37	1.74
TS	8	8 months	1.74	2.38
TS	9	8 months	1.26	1.78
TS	4	5 months	12.24	10.72
TS	5	5 months	4.45	3.78
TS	6	3 months	3.57	3.06

* Stimulation index was determined by dividing the average counts per minute of the 3-unstimulated cultures from the average counts per minute of the 3-stimulated cultures at Con A concentrations of 4 ug/ml and 8 ug/ml.

** Minimal response

age but several of the experiments cited in the literature were performed on mice that were 2,4, and 6 weeks old. It should be mentioned that the mean life span of mice varies to a large extent between strains and even within a particular strain. The age of an animal is ambigious and can therefore be interpreted in many ways by different individuals. The age of peak response for a particular strain should first be determined before diet-related and various immunocompetence studies are performed. It is suggested that a study be performed to determine the age of peak response by testing the immunocompetence of very young and very "old" Strong Strain A mice before further diet-related studies are performed.

It is also suggested that a smaller gauge of screen wire be used or that a different method of homogenizing be used to insure that a single cell suspension is obtained. By keeping the cell suspension cold during the procedure, it might be possible to keep more of the lymphocytes alive. It is hoped that these suggestions might aid a researcher who wishes to investigate this area to a greater extent.

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APPENDIX

Preparation of Solutions

Phosphate Buffered Saline (PBS) For 10x final concentration:

55.2 grams $NaH_2PO_4 \cdot H_2O$
58.5 grams NaCl
12.2 grams NaOH

Add distilled water to make 1 liter and then autoclave at 15 lbs for 15 minutes. Before use, dilute 10X with distilled water. Autoclave.

Eagle Hanks Amino Acid (EHAA)

Sterile distilled H_2O	715 ml
Hanks balanced salt solution (10X)	100 ml
MEM essential amino acids (50X)	50 ml
MEM nonessential amino acids (100X)	40 ml
Nucleic acid precursors 100X (1 g/l of each)*	25 ml
MEM vitamins (100X)	20 ml
Sodium pyruvate (100mM)	25 ml
L-Glutamine (200mM)	20 ml
Pen-Strep (100X)	5-10 ml

Filter and freeze in 100 ml bottles.

Before use, thaw media and add per 100.0 ml media:

7.5% $NaHCO_3$	1.8 ml
Fetal Calf Serum	5.0 ml
HEPES Buffer (23.83g/100 ml)	1.0 ml

*(1 g/liter of each adenosine, cytidine, guanosine, uridine)

Note: If too acidic, solution turns yellow
If too basic, solution turns red
Solution should be orange.

Scintillation Fluid

POPOP	200mg/4 liters
PPO	16 g/4 liters