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T-CELL FUNCTION IN THE OFFSPRING OF LIPOTROPE- AND PROTEIN-DEFICIENT RATS

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Summary. The progeny of mothers subjected to marginal lipotrope or moderately low protein diets exhibit deficient humoral immune responses. Serum antibody titres and numbers of antibody-forming cells to sheep erythrocytes (SRBC) are lower in the offspring of mothers whose diets were limited in either percentage protein or percentage lipotropes than in offspring whose mothers were fed a complete diet. Immune lymphocyte stimulation by SRBC antigens was marginal and not measurably different between the progeny of malnourished mothers and controls.

The *in vitro* response of spleen cells from offspring of nutritionally deprived mothers to the T-cell mitogen phytohaemagglutinin (PHA) is considerably less than that of controls. Thymus cells from marginal lipotrope offspring respond well to PHA, suggesting that PHA-reactive cell migration may be inhibited in these animals.

These results, obtained using a T-dependent antigen (SRBC) and a T-dependent mitogen (PHA), support the conclusion that maternal dietary deficiency can adversely affect T-cell function in rat offspring.

INTRODUCTION

Dietary insufficiency can lead to immunological deficiences in malnourished animals (Harmon, Miller, Hoefer, Ulrey and Leucke, 1963; Jose and Good, 1973; Kenney, Roderbuck, Arnrich and Piedad, 1968; Kenny, Magee and Piedad-Pascual, 1970; Mathur, Ramalingaswami and Deo, 1972; Nalder, Mahoney, Ramakrishnan and Hendricks, 1972; Smythe, Brereton-Stiles, Grace, Mafoyane, Schonland, Coovadia, Loening, Parent and Vos, 1971), and evidence that both thymus-independent (B-cell) and thymus-dependent (T-cell) lymphocyte systems are affected by protein deprivation has recently been reported (Mathur *et al.*, 1972). Thus, mice and rats subjected to low protein diets do not produce normal levels of antibody to sheep erythrocytes (SRBC), a T cell-dependent antigen in these species. Restoration of humoral immunity to SRBC in protein-deprived mice may be accomplished with T cells from normal mice, although evidence suggests that protein malnutrition also affects the mitotic potential of B cells (Mathur *et al.*, 1972).

It has been observed that protein–calorie malnutrition leads to depressed cell-mediated

immune responses which predispose humans toward infections normally dealt with by T cells (Smythe *et al.*, 1971). In contrast, cell-mediated lysis of tumour cells, presumably a T-cell function, was not affected by several amino acid-deficient diets in mice, although blocking antibody levels were significantly reduced (Jose and Good, 1973). It is, thus, evident that additional study is necessary to establish the effect of dietary restriction on T- and B-cell populations in various animals.

The model system for this study has been previously reported (Newberne, Wilson and Williams, 1970). Female rats whose diets have been limited in either lipotrope or protein concentration produce offspring more susceptible than normal progeny to *Salmonella typhimurium* infections; they also have lower than normal immunoglobulin and white blood cell levels in their peripheral blood (Newberne *et al.*, 1970). In this study the T-cell responses of progeny of nutritionally deprived mothers have been evaluated.

MATERIALS AND METHODS

Animals

Female Sprague–Dawley rats were divided into three groups, and from weaning they were fed one of the diets listed in Table 1. After approximately 3 months on these diets, the females were mated with male rats fed the complete diet.

TABLE 1

COMPOSITION OF COMPLETE, LIPOTROPE- AND PROTEIN-DEFICIENT DIETS

Ingredients	Diet*		
	Complete	Marginal lipotrope	Low protein
Alpha protein	18·0	18·0	2·0
Corn starch	23·6	23·6	39·6
Sucrose	42·5	42·5	42·5
Cottonseed oil	9·0	9·0	9·0
Salt mixture	4·5	4·5	4·5
Vitamin mixtures †	1·8	1·8	1·8
DL-methionine	0·3	0·1	0·3
Choline‡	0·3	0·1	0·3
Vitamin B_{12}§	4·5	4·5	4·5

* Percentage of diet (except for vitamin B_{12}).

† Vitamin mixture complete for rat except for choline and vitamin B_{12}.

‡ Choline chloride added to diet as a water solution; 200 mg/ml at the time of mixing diet.

§ Vitamin B_{12} added to diet as a water solution; 10 μg/ml at time of mixing diet.

The offspring of mothers fed marginal lipotrope or low protein diets weighed significantly less than those of mothers fed the complete diet; they achieved normal size and weight, however, when reared solely on the complete diet (Newberne *et al.*, 1970), as in this study. All progeny used in this study were 4 months old and were fed the complete diet *ad libitum*. Twelve rats (both sexes) in each group (control, marginal lipotrope, and low protein), from at least three different litters, were used in this study.

Immunization

A 10 per cent suspension of sheep red blood cells in saline was used to stimulate specific antibody production. Each rat received 0·5 ml suspension i.v. 4–5 days prior to testing for

antibody production, antibody-forming cells, and antigen-sensitive lymphocytes. Some animals were tested at later time intervals (8, 12, and 16 days), but because the response to a single SRBC injection was found to have dropped in both normal and nutritionally deprived offspring at these later times, only the 4- to 5-day results are given here.

Antibody titration

Rats were bled prior to sacrifice via cardiac puncture. Haemagglutinating and haemolytic antibody titres were determined in a standard microtitration system. Sera were heat-inactivated by heating at 56° for 30 minutes. Guinea-pig complement, diluted 1:5, was added to each well of the microtitre plate for quantification of the haemolysin titre.

Antibody-forming cells specific for SRBC

A modified version of the Jerne–Nordin technique (Jerne and Nordin, 1963) for enumeration of antibody-forming cells (AFC's) was used. Base layers of 1·4 per cent agar plus 0·5 mg DEAE dextran per ml RPMI-1640 culture medium (Grand Island Biological Company, Grand Island, New York) were poured into 60 × 15 mm plastic Petri dishes. Immediately prior to pouring the second agar layer, the Petri dishes were floated on a 45° water bath. At the same temperature 1 ml agar–dextran solution and 1 ml of a 4 per cent SRBC suspension in RPMI with 10 per cent foetal calf serum (FCS) were mixed together in 13 × 10 mm glass tubes. Measured volumes of rat spleen cell suspensions (obtained as outlined below) were placed directly on the surface of the floating Petri dish, followed by the contents of a single glass tube. The Petri dish was rapidly rotated to facilitate even distribution of the components of the upper layer and then placed on a level surface until the second layer had gelled. Three ml of RPMI–10 per cent FCS mixture were carefully pipetted onto each plate, and the plates were incubated at 37° for 1½ hours. Following incubation the medium was replaced by 2 ml of a 1:5 dilution of guinea-pig complement; the plates were reincubated for an additional 15–30 minutes. Following this second incubation the complement was poured off and the plaques counted.

Cell culture

The culture procedures used herein were adapted from those used for mouse lymphoid cells reported elsewhere (Konda, Nakao and Smith, 1972). Briefly, whole spleens were minced on sterile 60-mesh wire screens which had been wetted with RPMI-1640 culture medium. The cell clumps passing through the screen were collected in a syringe fitted with a 19-gauge needle. A single cell suspension was obtained by expressing the fluid through 23- and 25-gauge needles.

The cells were washed at least once in RPMI-1640 culture medium and then suspended in RPMI-1640 supplemented with 10 per cent FCS and 1 per cent penicillin–streptomycin mixture (Grand Island Biological Company) to a concentration of 2×10^6 cells per ml. Each sterile culture tube (number 2063, Falcon Plastics, Oxnard, California) received 0·5 ml cells in complete medium. Two microlitres of phytohaemagglutinin-P (Difco Laboratory, Detroit, Michigan) were added to cultures to assess cellular response to this mitogen. Control cultures received no mitogen. After completing the additions to quadruplicate sets of tubes, they were incubated at 37° in a 5 per cent CO_2–air atmosphere for 72 hours. Eighteen to 24 hours before the cultures were terminated, 1 μl [^{3}H]thymidine (1·9 Ci/mmole; Schwartz-Mann, Orangeburg, New York) was added to each.

Cellular DNA was prepared for scintillation counting by washing each tube once

with 4 ml cold saline, 4 ml cold 5 per cent trichloroacetic acid (TCA), and 4 ml cold methanol. After drying, the TCA precipitates were solubilized in 0·1 ml NCS solubilizer (Amersham-Searles, Arlington Heights, Illinois) and transferred to scintillation vials. All scintillation counting was done in an LS-250 liquid scintillation counter. The results in the Tables and Fig. 1 are reported as the mean ± standard error (SE) for at least four replicate cultures.

RESULTS

SERUM ANTIBODY TITRES AND ANTIBODY-FORMING CELLS TO SRBC

Twenty-four animals (eight per group: control, marginal lipotrope, and low protein) were tested for humoral immunity to SRBC in four different experiments. The results in Table 2 were selected as examples of the data from a typical experiment. Clearly, the

TABLE 2

ANTI-SRBC RESPONSES OF RATS BORN TO LIPOTROPE- OR PROTEIN-DEPRIVED MOTHERS

Cell and serum source	Antibody-forming cells/ 10^6 spleen cells *	Serum antibody level†	
		Haemolytic titre	Haemagglutinating titre
Normal offspring	275 ± 13	1:2,048	1:256
Marginal lipotrope offspring	37 ± 8	1:32	1:8
Low protein offspring	58 ± 4	1:4	1:2

* The values given represent the mean and standard error of counts obtained from four separate assay plates. The total number of spleen cells per animal was not found to be significantly different in any of the experiments. For this experiment the cell numbers were: normal, 400×10^6; marginal lipotrope offspring, 392×10^6; low protein offspring, 411×10^6.

† The values given were obtained using serum samples from individual rats of each type. Five other experiments involving two animals of each type (normal, low protein, and marginal lipotrope) per experiment yielded similar data.

offspring of mothers fed the marginal lipotrope or low protein diet did not respond as well as the normal age-matched rats to SRBC's. Serum antibody titres (haemolytic and haemagglutinating) were lower in the progeny of nutritionally deficient mothers. This difference in antibody titre and number of antibody-forming cells could not be accounted for on the basis of total spleen cell numbers in the animals tested. The normal rat spleen yielded 400×10^6 gells (±SE of 30 for eight animals); this number was not significantly different from the spleen cell yields of the offspring of malnourished mothers (compare footnote, Table 2). These data, of course, only answer the question of quantitative differences or similarities and tell nothing about qualitative differences in cell populations of the animals studied.

In vitro STIMULATION OF LYMPHOID CELLS BY PHYTOHAEMAGGLUTININ

In initial experiments the PHA reactivity of splenic lymphocytes from twelve rats (four normal, four marginal lipotrope, and four low protein) was measured. These animals were from the same group immunized with SRBC. When tested over a wide range of doses and at different times in culture, it was found that spleen cells from offspring of nutritionally deprived mothers were less well stimulated by PHA compared to cells from control rats. A typical experiment demonstrating lower PHA reactivity of offspring of undernourished mothers is shown in Table 3. The data shown were selected to show

TABLE 3

COMPARISON OF PHA RESPONSES OF SPLEEN CELLS FROM NORMAL RATS AND PROGENY OF MOTHERS RAISED ON MARGINAL LIPOTROPE OR LOW-PROTEIN DIETS*

Origin of spleen cells	[^{3}H]Thymidine incorporation†	
	Control	PHA
Normal progeny	5,686 (1,171–9,819)	65,380 (49,573–74,725)
Marginal lipotrope progeny	3,348 (991–5,266)	6,154 (2,368–9,110)
Low protein progeny	4,773 (3,640–5,647)	27,295 (13,450–35,394)

* In this experiment $1{\cdot}0 \times 10^6$ spleen cells in 0·5 ml medium were incubated with or without PHA for 48 hours; [^{3}H]thymidine was added and the experiment terminated at 72 hours.

† The values given represent the means and ranges of eight replicate cultures from two separate experiments involving two different rats in each group.

maximal PHA responses achieved by both experimental and control animals; they are consistent with results obtained in several other experiments in which the quantity of PHA and length of culture varied.

In subsequent experiments the PHA reactivity of both spleen and thymus cells from nonimmune normal rats and offspring of nutritionally deprived mothers was tested. Four animals of each type were used in these studies, and the results of one such experiment are shown in Fig. 1. Most striking was the contrast between PHA responsiveness of

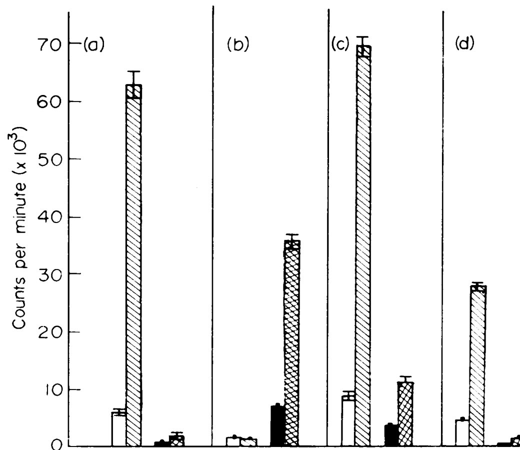

FIG. 1. PHA responses of spleen and thymus cells from normal rats and offspring of nutritionally deprived mothers. In this experiment 1×10^6 spleen cells in 0·5 ml medium were incubated (▧) with or (□) without PHA for 48 hours; [^{3}H]thymidine was added and the experiment terminated at 72 hours. Thymus cells incubated (⊠) with or (■) without PHA. (a) Control marginal lipotrope. (b) Marginal lipotrope. (c) Control low protein. (d) Low protein.

spleen and thymus cells from control rats and that of offspring whose mothers had been fed the marginal lipotrope diet. Thymus cells from the normal Sprague–Dawley rat typically react poorly but significantly to PHA. In contrast, thymus cells from offspring of mothers fed only the marginal diet responded very well to PHA. Typically, this response was better than the thymus cell response of the normal rat. Both spleen and thymus cells from offspring of mothers fed the low protein diet generally gave poorer responses to PHA (Fig. 1).

Attempts to demonstrate the sensitivity of immune lymphocytes to SRBC antigens *in vitro* using [^{3}H]thymidine incorporation as a measure of reactivity gave variable results, not significantly different among the different groups of animals studied. The absence of differential stimulation by erythrocyte antigens in animals which exhibited clear-cut differences in humoral immunity is unexplained at present, although this apparent paradox could conceivably be a reflection of the specific type of defect caused by maternal nutritional deficiency.

DISCUSSION

Decreased immunological potential, particularly of the cell-mediated immune system, is associated with several disease states in man and other organisms. Heavily parasitized organisms (Svet-Moldovsky, Shaghijan, Chernakhovskaya, Mkheidze, Litovehenko, Ozeretskovskaya and Kadaghidze, 1970; Faubert and Tanner, 1971), those bearing neoplasms in advanced stages of growth (Sutherland, Inch and McCredie, 1971; Adler, Takiguchi and Smith, 1971), and individuals with certain oral disorders (Lehner, 1972) have been shown to possess deficiencies in their cell-mediated immune capacities. The precise causes of cellular immune deficiency are not known, although some studies have demonstrated the existence of serum factors which inhibit lymphocyte responsiveness (Faubert and Tanner, 1971; Lehner, 1972).

Inhibition of a fully competent and mature T-cell system in a diseased organism may bear no direct parallel to the T-cell deficiency seen in the progeny of malnourished mothers, although it is interesting to speculate on the possible convergence of these observations. A greater similarity is likely to exist between the mechanism responsible for immune deficiency in the offspring of deprived rats and the mechanism responsible for cell-mediated immune deficiency and thymic dysplasia seen in undernourished children (Smythe *et al.*, 1971). Regardless of whether the deficiency is acquired through a disease process or through malnutrition, the precise explanation for the defect is unknown.

The experiments reported here suggest that maternal diet can affect the normal development and maturation of the T-cell system of offspring. In both groups of progeny tested, those born to either lipotrope- or protein-deprived mothers, the spleen cell response to the T-cell mitogen PHA (Meuwissen, Van Alten and Good, 1972) was significantly lower than that of normal spleen cells. The fact that thymus cells from progeny of lipotrope-deficient mothers responded significantly better than thymus cells from either normal rats or progeny of protein-deficient mothers suggests that perhaps the PHA-responsive cells in these rats have remained in the thymus and not migrated to the spleen. Retarded maturation and/or migration might also explain the relatively poor antibody response of these animals to SRBC, a probable T-dependent antigen in rats (Borum, 1972).

The results obtained with both groups of progeny are also compatible with a quantitative deficit of PHA-responsive cells, although it is possible that the cells are present in normal numbers but cannot respond to PHA because of some internal defect. Only in cases of severe maternal malnutrition (not reported here) are gross and histological deficiencies apparent in the spleen and thymus of the offspring.

Further study of possible B-cell deficiencies in the offspring of undernourished mothers is required. Offspring of nutritionally deprived mothers have fewer antibody-producing cells following immunization with SRBC. Assuming this antigen is T-dependent in the dose used (Borum, 1972), the apparent antibody-forming cell (B-cell) deficiency may

actually reflect a helper T-cell deficit in the progeny of malnourished mothers. This suggestion is supported by results obtained with the reputed B-cell mitogen lipopolysaccharide (LPS) (Greaves and Janossy, 1972; Yoshinaga, Yoshinaga and Waksman, 1972). It was found (Gebhardt and Newberne, unpublished observations) that the progeny of lipotrope- and protein-deprived mothers and normal rats have equivalent numbers of LPS-responsive cells in their spleens and that this cell population is not greatly expanded in the normal or experimental groups by oral infection with *S. typhimurium*, the source of the LPS. Additional studies are in progress to pinpoint the nature of T-cell deficits in offspring of nutritionally deprived mothers.

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