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Spleen and thymus histology and proliferative response of splenic cells in rats fed raw and cooked *Phaseolus vulgaris* beans

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SUMMARY. Histological studies of the spleen and thymus of rats fed raw black beans (*Phaseolus vulgaris*) show an atrophy of both lymphoid organs. Decrease in relative thymus weight was most marked. All histological organization of this organ appeared altered. An evident decrease in cell number was also observed in both organs. Proliferative response of splenic cells stimulated in vitro with Concanavalin A was increased as compared to that from animals fed the control diet. It is likely that histological changes observed in the spleen and the thymus are due mainly to a protein caloric deficiency, although the possibility that toxic factors present in the raw diet have an effect on the inmune system of the rat can not be overruled. KEY WORDS: diet; *Phaseolus vulgaris;* rat; lymphocyte response; spleen; thymus; histology.

RESUMEN. Estudio histológico del bazo y el timo y respuesta proliferativa de las células esplénicas en ratas alimentadas con frijoles negros crudos y cocidos. El estudio histológico del bazo y el timo de ratas alimentadas con frijoles negros crudos (*Phaseolus vulgaris*), muestra una atrofia de ambos órganos linfoides. La disminución en el peso relativo del timo fue más marcada, apareciendo alterada toda la organización histológica de este órgano. La respuesta proliferativa de las células esplénicas estimuladas «in vitro» con Concanavalina A, resultó aumentada al compararla con la de los animales alimentados con la dieta control. Es probable que los cambios histológicos observados en el bazo y el timo se deban principalmente a una deficiencia calórica proteica, aunque no se puede excluir la posibilidad que factores tóxicos, presentes en la dieta cruda, tengan un efecto sobre el sistema inmune de la rata. PALABRAS CLAVES: dieta; *Phaseolus vulgaris*; rata; respuesta linfocitaria; bazo; timo; histología.

INTRODUCTION

Legumes are an important item in the diet of many underdeveloped countries. However several toxic factors have been reported in legume seeds which affect the nutritional value of these foods (1-5). Their toxicity is drastically abolished after cooking (6).

Rats and mice fed raw *Phaseolus vulgaris* beans display a decrease in spleen weight (6). The same result is observed when animals eat soybeans *(Glycine max)* (7,8), winged beans *(Psophocarpus tetragonolobus)* (9), or a purified globulin fraction from *P. vulgaris* (10). Such spleen atrophy is also encountered in rats fed a protein-fre ediet (6, 11).Accompanying the size change is a decrease in cell number (11, 12), and an alteration in the humoral response (11, 13-15).

On the other hand, there are only a few reports on the immune response of animals fed plant proteins (15, 16).

In the present work we studied the spleen and thymus histological changes in rats fed raw and cooked beans, as well as the in vitro response of splenic cells stimulated with Concanavalin A (Con A).

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MATERIAL AND METHODS

Rats. Sprague-Dawley male rats weighing 45 g (3 weeks of age) were used at the start of the experimental diets.

Dietary treatment. Animals were divided in 6 groups of 6 rats each group. A basic isocaloric diet was prepared following the recommendations of A.O.A.C., 1980 (17). Protein (10% of whole diet) was provided in Groups 1 and 6 by casein, in Group 2 by ground raw black beans *Phaseolus vulgaris* (cultivar Tacarigua obtained from the Faculty of Agriculture, Maracay, Venezuela), and in Group 3 and 5 by cooked black beans from the same cultivar. Seeds, 2 kgs., were soaked in 4 l. of water and autoclaved for 30 minutes at 121° and 14 Psi. The grains were separated with a strainer, dried and grounded. The broth was lyophilized and the resulting powder mixed with the ground beans. Diets of groups 2, 3 and 5 were supplemented with 0.3% of D-L-methionine. Animals of Group 4 received no protein at all. All animals were kept in individual screen-bottom cages, received food and water ad libitum, and were weighed every other day. Food consumption was measured daily. Group 1-4 were fed the corresponding diet for up to 28 days. Intake of animals of Groups 5 and 6 was regulated by restricted feeding, so that the animals ingested as much cooked beans or casein respectively as the rats fed the raw beans. All animals of each group were killed every seventh day. One half of the animals were used for histological studies, and the other half for lymphocyte cultures. Dietary nitrogen was measured by the Micro-Kjeldahl procedure (18) using 6.25 as the nitrogen to protein conversion factor.

Histological studies. Spleen and thymus were removed, weighed, and fixed immediately in Bouin fixative (19).

Sections were cut from paraffin-embedded tissues (Histosec, Merck Lab.) and stained with haematoxilyn and eosin (19). Observations were done under a light microscope (Photomicroscope Zeiss, West Germany). Microphotograhs were taken with Kodak Panatomic X film.

Mitogen responses. All procedures were carried out under sterile conditions. The spleen tissue of each animal was dispersed with the aid of 2 bent needles. The cell suspension was pelleted in a clinical centrifuge for 5 minutes at 220 x g. The cells were washed 3 times with 10 ml of phosphate buffer solution (PBS) and the final pellet was resuspended in RPM I-1640 medium (Microbiological Associates, Maryland). The cell concentration was adjusted to $2x10^6$ cell /ml and 200 µ l were plated in each well of microculture plates (Dynatech Lab. Inc.). Cells were cultured as described previously (20) using RPM I-1640 medium adjusted to pH 7.4, and supplemented with L-glutamine (2 mM/ml), penicillin (100 U/ml), streptomycin (100 g/ml) and sodium bicarbonate (2 mg/ml). For each experiment Con A concentrations of 0.6-80 µ g/ml were used. Cells were kept in a CO_2 wet-chamber at 37° for 48-56 hours after which time 0.2 µCi of ^3H thymidine (sp. act. 6.7 Ci/mmol; New England Nuclear Corp. Boston, M.A.) was added to each well. Cells were harvested 20 hours later with an automatic cell harvester (Mash II, Microbiological Associates, Maryland) and the radioactivity was counted in a Packard Tr-Carb, model 3300.

Statistical anaysis. The following tests were performed in order to detect statisticaly significant differences between the experimental groups:

a) t-Student tests to compare two sample means.

b) One-way analysis of variance to compare more than two sample means.

Both tests were performed when the sample variances were homogeneous. The homogeneity was checked by applying the F-max-test (21).

c) The Kruskall-Wallis test (21) to compare more than two sample means. This is a nonparametric test which was used when the sample variances were not homogeneous.

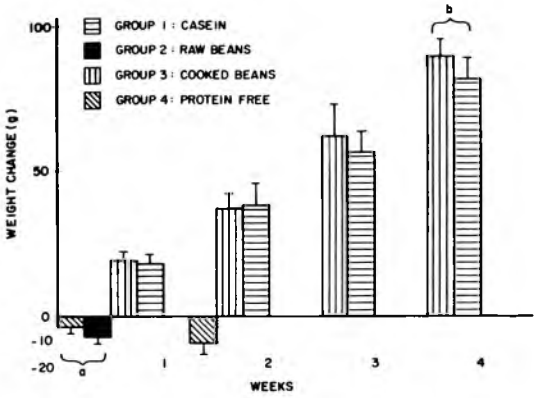
RESULTS

Growth and food consumption. Rats fed a raw black bean diet as the sole source of protein (Group 2) showed a marked decrease in body weight during the first week of experimentation (Figure 1). Animals not receiving any protein in the diet also lost weight, although their loss was significantly lower. Most animals of Group 2 died between 7 and 14 days (Figure 1). Rats fed raw black beans ate less food (50%) per day than those fed casein or cooked beans (Table 1). Furthermore, the food consumption of animals of Group 2 was lower than that of animals deprived of protein in the diet.

Spleen and thymus weight and cellularity. The effect of dietary conditions on spleen and thymus weight is shown in Table 2. Both lymphoid organs decreased in weight in rats fed the raw beans. The decrease in relative thymus weight was most marked. Animals receiving casein or cooked beans in the diet for 4 weeks did not show any significant change in the spleen and thymus relative weights.

Concomitantly with the weight loss in the lymphatic organs observed in animals fed the raw diet, there was an evident decrease in cell number (Table 3). Spleen cells were diminished by a factor of 8 in animals of Group 2 as compared to animals in Groups 1 and 3. This reduction was more dramatic in the thymus reaching a value 25 times lower than in control animals (Table 3).

FIGURE 1
Weight change during 4 weeks of rats fed the different diets.



Each bar represents the mean ± SD
a = difference statistically significant (p <0.05)
b = difference statistically non significant (p <0.05)

TABLE 1
DAILY FOOD INTAKE AND WEIGHT GAIN IN RATS FED THE DIFFERENT DIETS

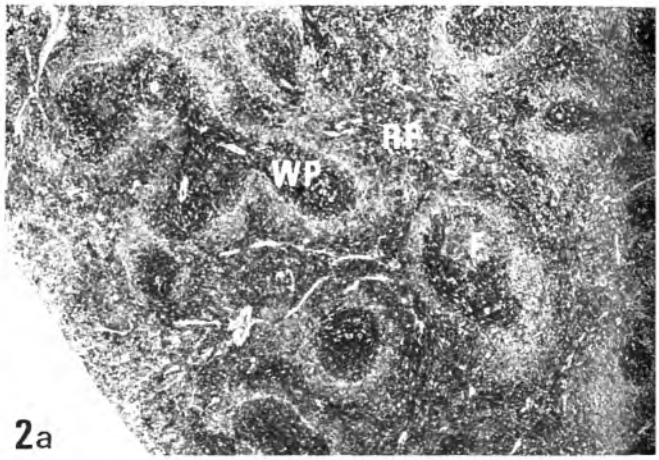
Diet	Daily Food Intake per Animal (g)	Weight Gain In one Week (g)
Raw *P. Vulgaris* (Group 2)	3.9 ± 1.2	-10.2 ± 2.4
Cooked *P. Vulgaris* (Group 3)	9.2 ±2.1	21.3 ±4.1
Protein Free (Group 4)	5.4 ± 1.4	- 4.6 ±1.9
Casein (Group I)	8.1 ± 3.0	20.1 ± 3.5

Values are Means ± SD

The spleen and thymus atrophy of rats of Group 2 could not be attributable only to the lower food intake, since paired fed animals (Groups 5 and 6) and rats fed a diet free of protein (Group 4) did not show this marked effect on the lymphoid organs (Table 2).

Histological studies. As mentioned previously, rats fed the raw beans exhibited atrophy of the spleen. This is clearly shown when the spleen of these animals is compared to those of controls (Figure 2). The most striking difference was observed in the white pulp. The periarteriolar lymphatic sheet (PALS) was reduced, due mainly to a decrease in the number of small lymphocytes and reticular cells. The PALS periphery appeared diffuse and there was no clear difference between white and red pulp (Figure 3).
Such changes were absent in the spleen of animal fed the cooked beans, except for a moderated atrophy of this organ (Figure 4).

FIGURE 2
Spleen of rats fed casein diet (a) and raw black beans (b) for one week. RP: Red pulp; WP: white pulp; F: Folicle. Haematoxylin and Eosin stain (H/E)x 39.



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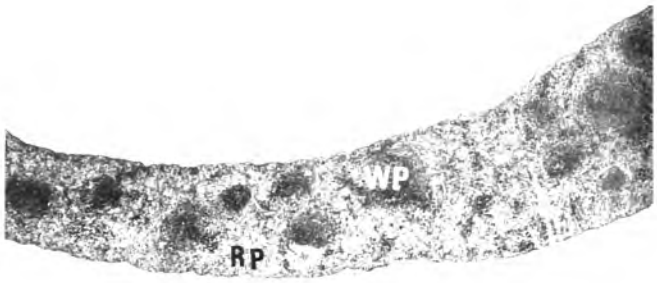

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FIGURE 3
Spleen of rats fed casein diet (a) and raw black beans (b) for one week. RP: Red pulp; MZ: Marginal zone; A: central arteriole; PALS: Periarteriolar Lymphatic Sheet. (H/E) x 390.

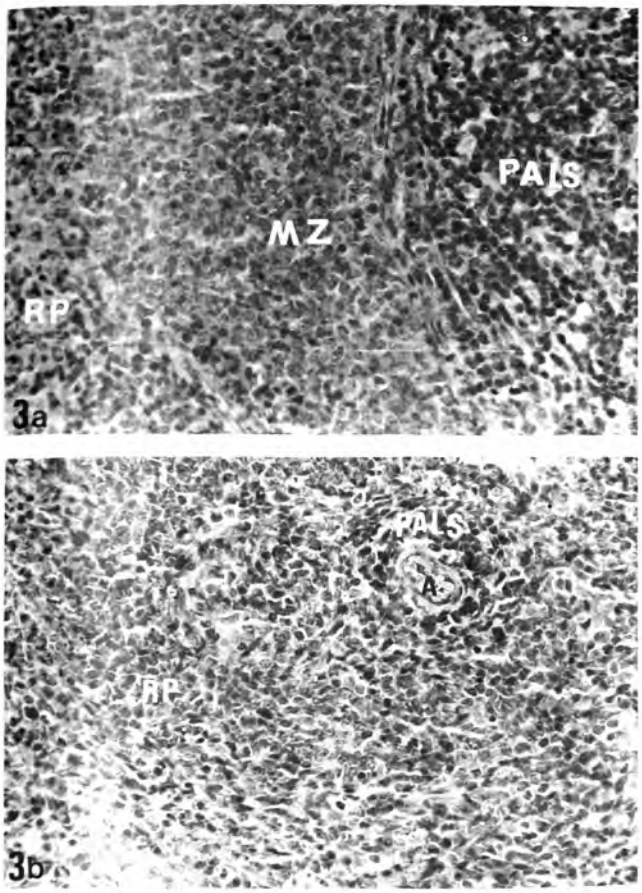
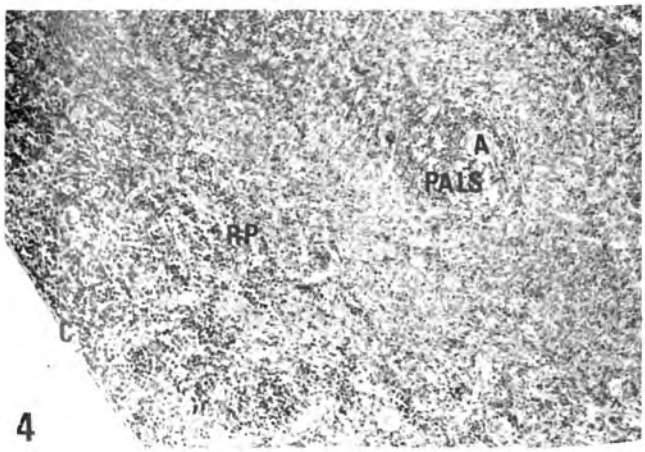


FIGURE 4
Spleen of rats fed cooked black beans diet for one week. A: central arteriole; C:Capsule; RP: Red pulp; PALS: Periarteriolar Lymphoid Sheet; (H/E) x 156.



A histogical analysis of the spleen of the rats fed the protein free diet. (figure not shown), also reveals an absence of the limiting zone and a slight reduction of the PALS as compared to the controls. Atrophy was evident, although not as severe as in the animals of Group 2.

In the thymus, the atrophy was severe. All histological organization appeared altered. Lobulation was non existent and the connective tissue was more evident. Differences between cortex and medulla wene not evident and the former was drastically reduced in size (Figure 5). A reduction in size of this lymphoid organ was observed in the rats fed the protein free diet, but no histological alterations were present (Figure 6).

FIGURE 5
Thymus of rats fed casein diet (a) and raw black beans (b) for one week. Co: Cortex; Me: Medulla; C:Capsule. (H/E) a: 39 x; b: 63x.

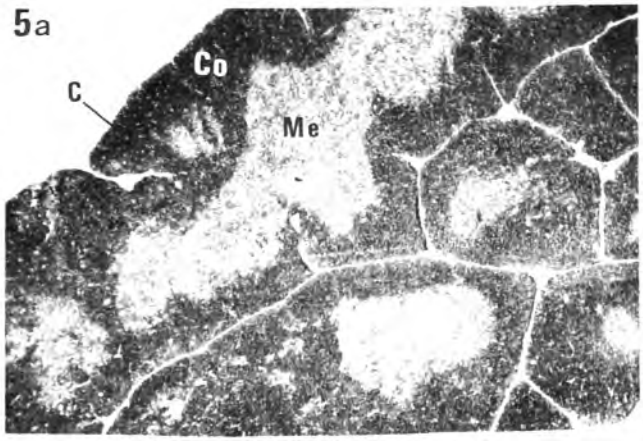


TABLE 2
RELATIVE WEIGHT OF SPLEEN AND THYMUS OF RATS FED THE DIFFERENT DIETS

Diet	SPLEEN WEIGHT/BODY WEIGHT x 100 WEEKS					THYMUS WEIGHT/BODY WEIGHT x 100 WEEKS				
	O¥	1	2	3	4	O¥	1	2	3	4
Raw *P. Vulgaris* (Group 2)	0.17[a1] ± 0.04	*	—	—		0.10[a1] ± 0.04	*	—	—	
Cooked *P.Vulgaris* (Group 3)	0.27[b1,2] ± 0.04	0.23[2] ± 0.04	0.27[2] ± 0.07	0.24[2] ± 0.05		0.33[b1,3] ± 0.07	0.23[3] ± 0.01	0.32[3] ± 0.01	0.24[3] ± 0.03	
Casein (Group I)	0.31[b1,3] ± 0.05	0.26[3] ± 0.05	0.26[3] ± 0.06	0.24[3] ± 0.02		0.34[b1,2] ± 0.07	0.28[2] ± 0.01	0.35[2] ± 0.05	0.25[2] ± 0.03	
	0.31 ± 0.04					0.34 ± 0.02				
Protein Free (Group 4)	0.22[c] ± 0.03					0.26[c] ± 0.05				
Cooked Paired Fed (Group 5)	0.20[c] ± 0.04					0.20[c] ± 0.07				
Casein Paired Fed (Group 6)	0.28[b] ± 0.06					0.31[b] ± 0.03				

*: Animals died after one week of diet
¥: Weaning time: 23± 2 days
Values are means ± SD

Different letters are values statistically different
1: Means were compared by the student T test ($p<0.05$)
2: Statistically significant ($p<0.05$, anova)
3: Statistically significant (Kruskall-Wallis Test)

TABLE 3
SPLEEN AND THYMUS WEIGHT AND CELL NUMBER OF RATS FED THE DIFFERENT DIETS FOR ONE WEEK

Diet	Spleen		Thymus	
	Weight g x 10^{-2}	Cell Number x 10^6	Weight g x 10^{-2}	Cell Number x 10^6
Raw *P. Vulgaris* (Group 2)	6.25 ± 1.70	16.5 ±10.78	3.79 ± 1.91	16.4 ± 11.2
Cooked *P. Vulgaris* (Group 3)	18.78 ± 3.79	122.2 ± 30.4	23.76 ± 5.64	409.0 ± 173.3
Casein (Group I)	21.70 ± 3.79	146.2 ± 51.4	32.70 ± 1.70	418.8 ± 207.0

Values are Means ±SD

FIGURE 6
Thymus of rats fed cooked black beans diet (a) and protein free (b) for one week. Co: Cortex; Me: Medulla. BF: Brown fat. (H/E)x39.

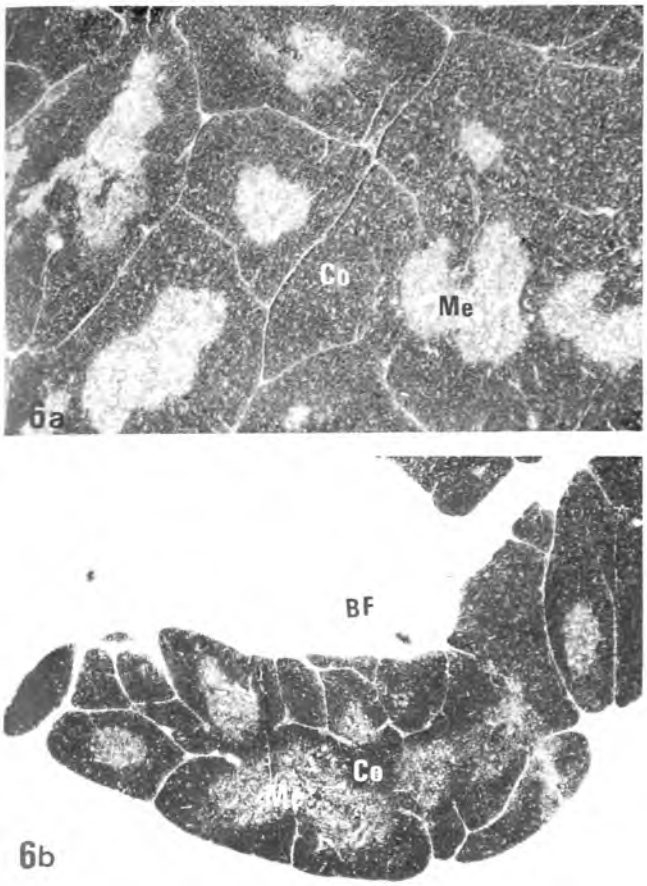
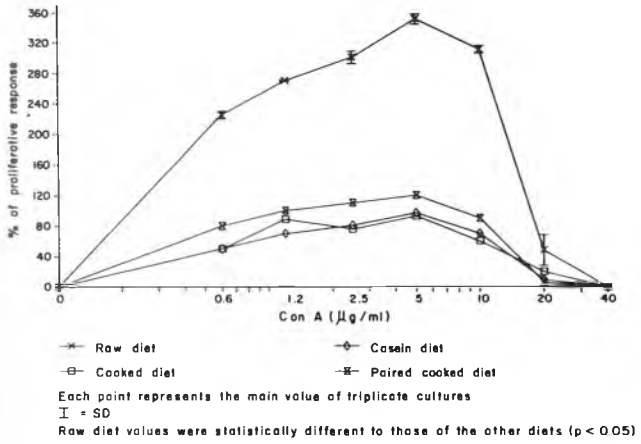


FIGURE 7
Proliferative response of splenic cells to Con A from rats fed the different diets for one week.



In vitro proliferative response. Spleen cells from rats fed the raw beans (Group 2) incorporated more thymidine at each concentration of Con A than cells from rats fed casein diet ad lib (Group 1). In both cases the optimal dose was 5 µg/ml (Figure 7). The proliferative responses of cells from animals fed the cooked and the paired fed diets were identical to those of the control cells.

DISCUSSION

The loss of corporal weight in animals fed raw seeds from *P. vulgaris* observed in this study agrees with previous reports (22-24). At the same time spleen atrophy was confirmed and this effect was also observed in the thymus. According to our results, atrophy of the lymphoid organs cannot be attributable solely to a protein caloric deficiency. This is infered from the low ingestion in the animals fed the raw beans. Results of studies on protein caloric deficiency support this interpretation (12, 24). Additionally, crude or purified proteins from legume seeds exhibit a low digestibility both in vivo and in vitro, contributing to the poor growth of the animals (6). Moreover, thymus and spleen relative weight values were higher in paired fed animals than in animals fed the raw diet, even though the former were fed casein or cooked beans as the sole source of protein.

It is noteworthy the short period of time in which atrophy of the lymphoid organs appeared. This result differs from previous reports in which a more delayed manifestation of such condition was observed when animals were fed a low protein diet of animal origin (12, 24, 25).

A possible explanation for this early atrophy may be the nature of the raw bean diet. Legumes contain antinutritional factors such as lectins and protease inhibitors (26). These factors may contribute along with the protein low digestibility (10) to the precarious condition of the animal, accelerating the damage of the lymphoid organs.

Animals not receiving protein exhibit spleen and thymus atrophy one week later than animals fed the raw beans, although they eat more food daily. When both groups received the same amount of food (data not presented) atrophy appeared at the same time, suggesting once more that caloric deficiency was involved in the observed phenomenon.

Our results showed that atrophy was accompanied by a drastic decrease of thymic and splenic cells. This finding is in good agreement with the reported response of these organs in studies on maturation and protein defiency in rats (11, 24, 27).

Spleen and thymus atrophy associated with a marked decrease in cell number is a common finding in animals exposed to protein caloric deficiency (11, 24, 27).

The observed effect on cellularity may be caused by the high turnover of spleen and thymus cells (28), which

implies greater nutrient requirements for cell growth and differentiation. Thymic cells possess a higher proliferative rate than splenic cells (28). This could explain the greater sensitivity of the thymus to the dietary conditions.

Another factor to be considered is the effect of adrenocortical hormones, specially the corticosteroids. Under stress conditions, the levels of these hormones are elevated (29). The same is true in animals with a protein caloric deficiency. A marked decrease of lymphoid tissue is observed the thymus being the more affected organ (30, 31, 32).

The main change observed in the thymus is the loss of the cortex. Immature thymocytes present in the cortex are more sensitive to the corticosteroid effect (12, 31, 33, 34), therefore experimental conditions used in the present work may have produced a similar effect.

As in the thymus, splenic histological changes seemed to be restricted to certain regions of this organ. Our findings are similar to those described in animals with protein caloric deficiency (12, 35, 36). One of the essential functions of the thymus is to produce T cells, requiring a continuous supply of precursor cells from the bone marrow (28). Protein caloric deficency causes a decrease in haematopoyetic stem cells in addition to the effect on the thymic mass cell (12). Therefore, splenic white pulp would be affected by changes occurring in both lymphocyte sources (28, 37).

Results on the effect of protein caloric deficiency on the functional activity of splenic cells are variable and contradictory (14). The increase in the proliferative response observed by us with respect to a comparison between the animals fed raw beans and those fed diets containing cooked beans or casein, coincides with the results of other authors (13, 14, 31, 38, 39). It is plausible that the elevated response is associated with a particular cell population less susceptible.

The possibility that toxic factors present in the raw diet have an effect on the immune system of the animal cannot be overruled. Black beans contain antinutritional factors, namely enzyme inhibitors, lectins, tannins and fitic acid (41). Tannins inhibit several enzymes reducing protein and other nutrients digestibility. They also reduce food intake, and are lethal to rats when fed at high concentrations (42). In rats feeding trials, tannin content was negatively correlated with net protein ratio, a measure of protein quality (43).

It has been demonstrated that rats fed on with raw seeds of *P. vulgaris*, with high hemagglutinating titers, lose weight (44). The net protein utilization of rats fed on a 5% casein-containing diet was strongly depressed by the addition of the pure lectin to the diet (45). Internalization of lectins from *P. vulgaris* by duodenal and jejunal cells has been reported more recently (46). More over, the inclusion of purified kidney bean lectins in egg albumin-based rat diets induced thymus atrophy (47). However, more detailed studies are required to understand the contribution of each nutritional factor to the immune response.

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