Total Dietary Restriction and Thymus, Spleen, and Phenotype and Function of Splenocytes in Growing Mice

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Abstract

To study the immunological effect of total dietary restriction, BALB/c mice were limited to reduced body growth or maintained at practically constant body weight between 21 and 61 days of age by giving them 70% (R70%) or 55% (R55%) of the average daily food intake of the control group fed ad libitum. Thymus and spleen weight were decreased, but thymus size was maintained in proportion to body weight in R70% mice, whereas the ratio of thymus weight to body weight was significantly decreased in R55% mice. In restricted mice, splenocytes showed a lower percentage of B lymphocytes and a higher percentage of T lymphocytes. Results of stimulation showed that proliferation capacity was increased for B lymphocytes and decreased for T lymphocytes in restricted mice. Our data show the importance of studying the thymus over a period, because normal thymus growth was first greatly impaired by dietary restriction, and subsequent thymus involution was delayed, showing that an isolated comparison between the thymus of growing mice is not enough. Our data show the interest of determining the percentage of lymphocyte populations parallel to their response to mitogen stimulation. *Nutrition* 1992;8:pages 258–265

Key words: weanling mice, total dietary restriction, growing mouse thymus and spleen, splenocyte phenotype and function

Introduction

Human populations suffering from malnutrition often exhibit greater susceptibility to infection and depressed immunity.[1-5] Thymus and other lymphoid tissues react to nutrition deficiency more rapidly than most other organs.[3] Impairment of cell-mediated immunity, which is mainly dependent on thymus-derived T lymphocytes, has been reported in protein-energy malnutrition,[3,6] particularly marked atrophy of the thymus and thymus-dependent areas of the spleen and lymph nodes,[7] diminished number of T lymphocytes in peripheral blood,[8,9] and alterations in the proportion of lymphocyte subsets[8,10] and of T-lymphocyte subpopulations.[5,11] Results in mildly and moderately malnourished children also provide evidence for a reduction in cell-mediated immunity function.[12]

Experimental animal models have not always yielded results consistent with a reduction in cell-mediated immunity in malnutrition. The reduction in dietary protein in a mouse model significantly influences the immunoregulatory cells or their products,[13] but moderate reductions in dietary protein only, although lowering lymphoid cell numbers, often results in increased immune responsiveness.[14] These observations suggest that dietary protein reduction alone may not be the cause of immunosuppression in malnourished humans. Malnutrition is usually a composite syndrome of multiple nutrient deficiencies, and simplified models of pure deficiencies in controlled animal experiments cannot be directly extrapolated. Rodents fed a low-protein diet did not experience an energy deficiency[13,15] and were, for example, only marginally zinc deficient.[16]

Studies with experimental animals have shown different results according to the age of initiation of the nutrition defect and its severity and quality.[3] We used a model in which growing animals were fed a nutritionally adequate diet in restricted quantities, i.e., 70 and 55% of control consumption, initiated at weaning. We studied the effects of diet restriction on body weight and length, thymus and spleen weight, and splenocyte phenotype and function in male BALB/c mice at different times.

During the study period, mouse thymus had a natural evolution, being large in young mice, reaching its maximum size at sexual maturity, and then decreasing with age, that could be modified by the restriction. To compare the thymus weight of control and restricted animals during the growing phase, results were required over a period and not on a sporadic basis. In addition to measurement of the proliferative response of splenocytes to specific mitogens, measurement of the percentage of B- and T-lymphocyte populations led to conclusions similar to those observed in malnourished children.

Materials and Methods

Inbred male BALB/c mice were obtained from Iffa Credo (L'Arbresle, France). At 21 days of age (weaning), mice were caged individually and randomly allotted to one of three groups. The animals were housed in a room controlled for temperature (22°C), humidity (40–50%), and light (alternate 12-h periods of light and dark). All animals had access to tap water ad libitum. Mice were fed a pellet diet from UAR (Villemoisson-sur-Orge, France), composed of 12% water, 23.5% proteins, 5% lipids, 49.8% carbohydrates, 4% cellulose, and 5.7% minerals and vitamins, that provided 13,388 kJ/kg (3200 kcal/kg). Mice in the control group were fed ad libitum. A quantity of pellet was weighed and then distributed among the control mice so that they were given, ~15 g of food per mouse once daily (which is more than needed). After 24 h, the remaining pellets were collected, weighed, and compared to the total quantity given and

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control mean daily food intake was recorded. Mice in the restricted groups received either 70 or 55% of the mean weight of control food intake, constituting the 70% (R70%) or the 55% (R55%) total dietary-restricted mice; the food intake of these animals was restricted between the ages of 21 and 61 days. All mice were fed each day at 0900. Body weight was determined every 2 days with an electronic scale adapted to live animals (PM 4600 Mettler, Greifensee, Switzerland) before the food was given. Weight was measured to the nearest 0.01 g. Body length was evaluated without the tail five times during the study.

Sets of five mice from each group, selected at random as early as the 1st day of experimentation, were selected when 21, 26, 31, 41, 51, and 61 days old. The mice were anesthetized (ether) and exsanguinated by cardiac puncture. The spleen and thymus were dissected and rapidly weighed, and the spleen was placed in 15 ml of ice-cold sterile DMEM (Gibco, Grand Island, NY). Single-cell suspensions of splenocytes were prepared by perfusing the spleen with 10 ml of cold DMEM from a syringe. The spleen cells were washed twice by centrifugation at 800 x *g* for 5 min at 4°C, and the pellet was treated with 5 ml of cold 0.30 M ammonium chloride to lyse contaminating erythrocytes. The cells were again washed and resuspended in 8 ml of cold DMEM. Cell recovery and viability was determined by trypan blue exclusion.

The specific surface antigenic determinant of a cell population was characterized in a suspension of living cells by immunofluorescence (IF). The B-lymphocyte population was characterized by fluorescein-isothiocyanate (FITC)-coupled sheep anti-mouse immunoglobulin (Ig) antibody (Ab) (Amersham, Buckinghamshire, UK). The mature T-lymphocyte population was studied with mouse FITC-conjugated anti-Thy-1.2 monoclonal Ab (MoAb) (a gift from P. Carayon, Sanofi Recherche, Montpellier, France), and the suppressor T-lymphocyte population was studied with a mouse FITC-coupled anti-Lyt-2 MoAb (Becton Dickinson, Mountain View, CA). The helper T-lymphocyte subset was studied with a rat anti-L3T4 MoAb (donated by A. Dupuy d'Anjeac, INSERM U236, Montpellier). The FITC-conjugated goat anti-rat Ig Ab (Nordic, Tilburg, Netherlands) was used as a second Ab. The B-, T-, and suppressor T-lymphocyte populations were labeled with a one-step direct IF technique because of the FITC-labeled specific Ab, whereas the helper T-lymphocyte subset was studied with the two-step IF method. The IF medium used was Hanks' balanced salt solution without Ca and Mg or phenol red (Gibco), containing 0.405 mM $MgSO_4$, 0.491 mM $MgCl_2$, 1 mM EDTA, 20 mM sodium azide, and 3% fetal calf serum (FCS) inactivated by heating at 56°C for 30 min. Cells (10^6) were distributed in a 200-µl volume in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark). The plates were centrifugated at 500 x *g* for 5 min at 4°C, and the supernatant was discarded. Ab (100 µl) was added to the pellet of resuspended cells. After 45 min incubation at 4°C in the dark, Ab was removed by centrifugation. Cells were washed with 200 µl IF medium by centrifugation. The second step was carried out in the same way for FITC labeling of cells binding the anti-L3T4 Ab. A control cell suspension without the first anti-L3T4 Ab was used to verify that the second Ab did not react with splenocytes. One hundred microliters of 1.5% paraformaldehyde was added to cells stored in the dark at 4°C. A drop of cell suspension was placed on a slide and examined microscopically. A comparison between the FITC-labeled cells, counted under ultraviolet light, and the total number of lymphocytes counted under visible light gave the percentage of cells recognized by the Ab, i.e., belonging to a defined population.

Spleen cells were used to assess the in vitro proliferative response to lymphocyte mitogens tested with a microculture method to assay mitogen-induced DNA synthesis through thymidine incorporation. The mitogens concanavalin A (ConA, Sigma, St. Louis, MO) at 2 µg/ml and *Escherichia coli* lipopolysaccharide (LPS 0111:B4, Sigma) at 50 µg/ml were used for the stimulation of T and B lymphocytes, respectively. Cells were cultured in DMEM containing 10% heated FCS, 2 mM L-glutamine, 25 mM Hepes, 50 µM 2-mercaptoethanol, and 1/100 Gibco nonessential amino acids. Triplicate splenocyte suspensions of 2.5×10^5 cells from individual mice were placed in 96-well flat-bottom microtiter plates. The suspensions were incubated for 48 h with or without mitogen at 37°C in a humidified atmosphere of air with 5% CO_2. All wells were pulsed with 1 µCi of tritiated thymidine (CEA, Saclay, France) for 18 h before harvesting. The suspensions were collected on filters with a cell microharvester and washed free of soluble tritiated thymidine. The filters were placed in a 2.5-ml nonaqueous liquid scintillation cocktail for the measurement of radioactivity. Mean disintegrations per minute (dpm) were calculated for each set of triplicate cultures. The mean background dpm was given by unstimulated cells. Recorded dpms were converted to stimulation index (SI) values by dividing the response of mitogen-stimulated cultures by nonstimulated-media control response.

All results are expressed as means ± SE. Multiple variance analysis and 2 x 2 adjusted comparisons were used for statistical description of the data to determine the effects of treatment. All results were assessed at the 95% confidence level.

Results

From age 22 to 30 days, the daily consumption of control mice increased from 2.36 to 4.93 $g \cdot mouse^{-1} \cdot day^{-1}$. Then mean consumption of control mice was 5.25 ± 0.08 $g \cdot mouse^{-1} \cdot day^{-1}$ between 31 and 40 days of age, 4.79 ± 0.08 $g \cdot mouse^{-1} \cdot day^{-1}$ between 41 and 50 days, and 4.62 ± 0.09 $g \cdot mouse^{-1} \cdot day^{-1}$ between 51 and 60 days. Restricted mice always ate all the pellets given them daily. In the first 8 days of restriction, mice of the R55% group and even some of the R70% group were greatly affected by the restriction so that they had difficulty in moving and holding the pellets. Subsequently, they seemed to adapt to the restriction, because their general fitness and apparent physical capacities improved.

The weight of R55% mice first decreased until age 27 days then increased in the same manner as the body weight of R70% mice (Fig. 1). At age 61 days, the mean body weight of R70% mice was 69.8% of control mice, and the mean body weight of R55% mice was 51.2 and 73.2% of the weight of age-matched control and

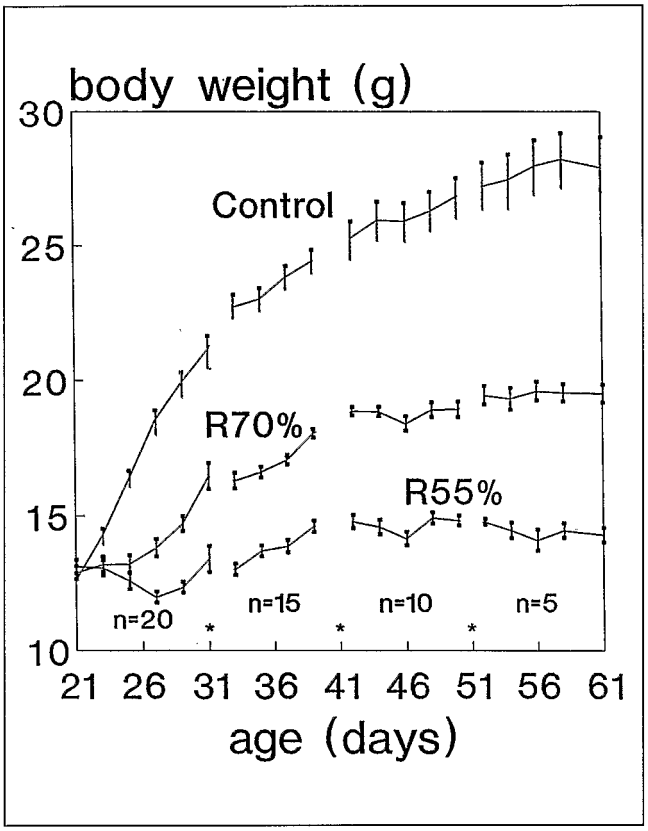

*Fig. 1. Mean ± SE body weight of mice from each group during normal development and (R)70 and 55% undernutrition. At 21 days of age, mean body weight was not significantly (p > 0.05) different between groups. At age 23 days, mean body weight of mice from both restricted groups was not different but was significantly (p < 0.01) lower than control mice. From age 25 days, difference was significant (p < 0.05) between groups. *Days where 5 mice from each group were killed for immunological study, resulting in decreasing group number (n) on which mean body weight was measured.*

R70% mice, respectively. Between 21 and 61 days of age, the body weight of the control mice rose by 15.29 g and that of R70% and R55% mice by only 6.62 and 1.17 g, respectively. From age 29 days on, body length was significantly different between the three groups (Table I). The ratio of body weight to body length in control mice increased with age mainly when the growth was noticeable. This ratio increased in R70% mice but did not increase in R55% mice. The more severe the restriction, the greater the decrease in this ratio compared with the control mice, the effect being more marked in older mice.

Thymus weight in control mice first increased until age 31 days then decreased by 51.5% between 31 and 61 days of age (Fig. 2*A*). Thymus weight in the R70% and R55% mice first decreased dramatically then increased between 31 and 41 days of age and remained quasi stable. The more severe the restriction, the lower the weight of the thymus in restricted mice. The ratio of thymus weight to body weight in control mice decreased from 21 to 61 days of age (Fig. 2*B*). Changes in the ratio of thymus weight to body weight in restricted mice followed the time-course changes in their thymus weight. The more severe the restriction, the more impaired the ratio.

Mean spleen weight was significantly reduced at all ages in the two restricted groups, the effect being more marked in the R55% group (Fig. 3*A*). The ratio of spleen weight to body weight in control mice decreased by 59.5% between 21 and 41 days of age, when growth was noticeable, then remained relatively constant (Fig. 3*B*). The ratio of spleen weight to body weight in R70% mice decreased from 26 to 61 days of age, during which body weight increased but spleen weight varied little. The ratio of spleen weight to body weight in R55% mice decreased between 21 and 31 days of age and was subsequently almost constant over the remainder of the study period, because body weight and spleen weight hardly did not vary. The more severe the restriction, the more diminished the ratio of spleen weight to body weight. Spleen atrophy resulted in a smaller mean value for total count of collected splenocytes (Table II). At 31 days of age, the more severe the restriction, the lower the ratio of splenocyte count to spleen weight (Fig. 4). The difference between the three groups decreased with age. From 41 days of age on, no difference between the two restricted groups was observed, and at 61 days of age, the ratio of splenocyte count to spleen weight of both restricted groups returned to a normal value compared with control mice.

Restricted mice showed diminished B-lymphocyte percentage and increased T-lymphocyte percentage at the four ages studied (Table III). The sum of spleen B- and T-lymphocyte percentages calculated for each mouse showed an almost constant mean value between 69.15 ± 2.20 and 80.33 ± 4.15%, whatever the diet group or age, with the only significant ($p < 0.05$) difference being

Table I. Body Length and Ratio of Body Weight to Body Length of Mice During Normal Development and Undernutrition (R) at 70 and 55%

		Length (cm)			Ratio of Weight to Length (g/cm)		
Age (days)	*n*/group	Control	R70%	R55%	Control	R70%	R55%
23*	20	6.65 ± 0.06	6.69 ± 0.08	6.53 ± 0.06	2.16 ± 0.03	1.97 ± 0.04†	1.99 ± 0.03†
29‡	20	7.60 ± 0.07	7.08 ± 0.06	6.77 ± 0.03	2.60 ± 0.03	2.08 ± 0.03	1.82 ± 0.03
39‡	15	8.29 ± 0.08	7.69 ± 0.06	7.20 ± 0.06	2.95 ± 0.06	2.33 ± 0.02	2.02 ± 0.03
48‡	10	8.52 ± 0.07	7.84 ± 0.08	7.43 ± 0.07	3.09 ± 0.08	2.41 ± 0.04	1.99 ± 0.03
61‡	5	8.90 ± 0.10	7.94 ± 0.12	7.44 ± 0.06	3.13 ± 0.13	2.45 ± 0.02	2.01 ± 0.05

Values are means ± SE.
**p > 0.05, ‡p < 0.01, between groups, †p < 0.01 vs. control group.*

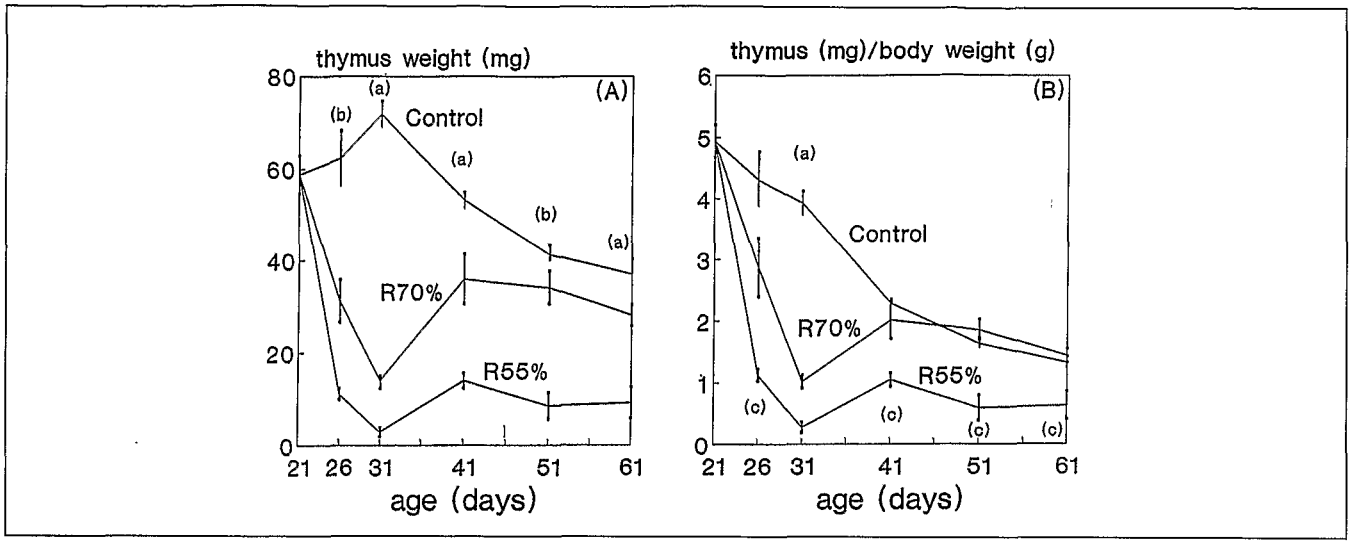

Fig. 2. Mean ± SE thymus weight (A) and ratio of thymus to body weight (B) of 5 mice from each group during normal development and (R) 70 and 55% undernutrition. [a]*p < 0.01,* [b]*p < 0.05, between groups.* [c]*p < 0.01 vs. control and R70% groups.*

between each restricted group and the control mice at 51 days of age. Restricted-mouse suppressor T-lymphocyte percentages were higher than those of control mice except for the R70% group at age 31 days. The difference between the three groups decreased with age, being significant at 31 days of age and not significant at 61 days of age. There was an increase in the percentage of helper T lymphocytes in the spleens of R55% mice with time, which was more noticeable than the increase observed in R70% mice, whereas this percentage was almost constant in control mice. The difference between the three groups increased with age, being not significant at 31 days of age and significant at 61 days of age.

No difference was observed in regard to the ConA-stimulated dpm of the three groups at the three ages (Table IV). LPS-stimulated dpm showed a difference between the control group and the R55% group only, at 41 and 61 days of age. Differences also appeared between groups in regard to unstimulated-culture dpm. Diet-restricted mice had no evidence of infection compared to control mice as this might explain the increased spontaneous tritiated thymidine incorporation. Mainly because of unstimulated-culture dpm, ConA and LPS SI in control mice was much higher at 41 days of age than at both other ages, and SI in R70% mice was lower than in control or R55% mice for both ConA and LPS mitogens at all ages considered. Because of the results of unstimulated culture, SI was difficult to calculate, so another mode of interpreting the results had to be used. When dpm of nonmitogen splenocyte cultures from each mouse was subtracted from dpm of associated stimulated cultures, mean results did not really modify the comparison performed with dpm from stimu-

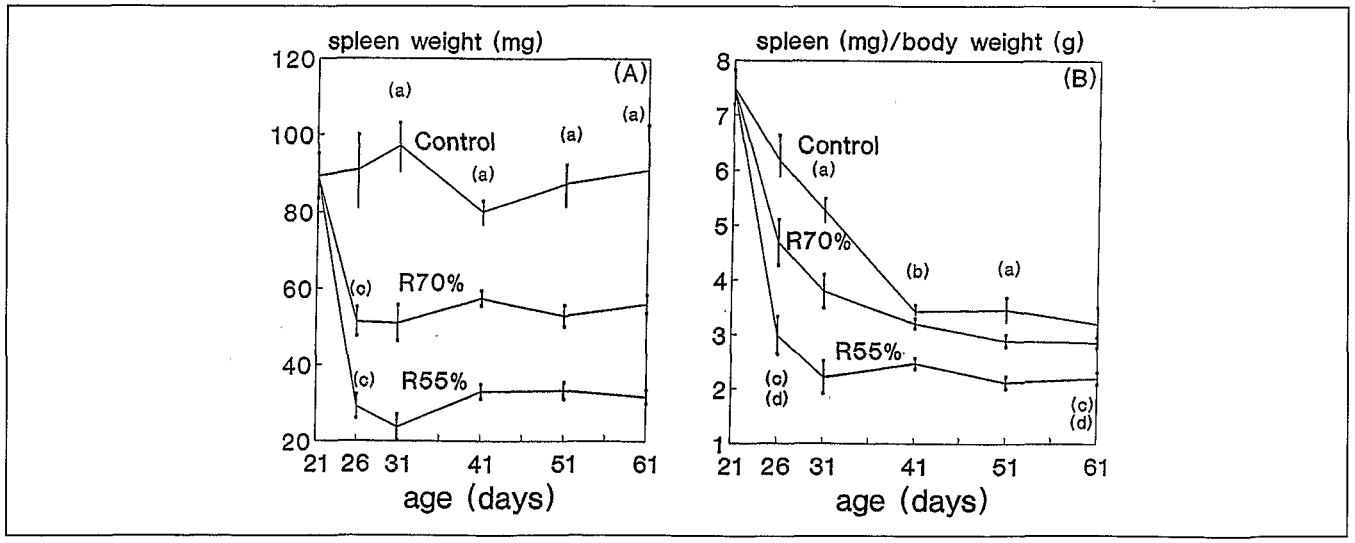

Fig. 3. Mean ± SE spleen weight (A) and ratio of spleen to body weight (B) of 5 mice from each group during normal development and (R)70 and 55% undernutrition. [a]*p < 0.01,* [b]*p < 0.05, between groups.* [c]*p < 0.01 vs. control group.* [d]*p < 0.01 vs. R70% group.*

Table II. Total Lymphocyte Count Per Spleen of 5 Mice/Group During Normal Development and Undernutrition (R) at 70 and 55%

	Splenocyte Count ($\times 10^6$)		
Age (days)	Control	R70%	R55%
21	53.0 ± 4.1		
31*	54.2 ± 9.1	23.7 ± 3.6	5.0 ± 1.4
41*	56.8 ± 5.4	25.4 ± 2.4	12.5 ± 1.7
51*	61.0 ± 2.7	28.1 ± 2.6	17.0 ± 3.3
61†	62.8 ± 8.8	33.2 ± 2.4	21.2 ± 1.8

Values are means ± SE.
**p < 0.01, †p < 0.05, between groups.*

lated cultures alone, except that three differences appeared for the results of LPS stimulation: both restricted groups were the same at age 61 days ($p > 0.05$), the R70% and control groups were significantly ($p < 0.01$) different at age 61 days and there was no significant ($p > 0.05$) difference between the three groups at age 51 days. The results obtained by this technique were divided, for each stimulated culture, by the number of cultured T- or B-splenocytes, obtained for each mouse from the phenotypic study. Mean results of this ratio are given in Fig. 5. For ConA-stimulated T lymphocytes, the more severe the restriction, the more diminished the ratio. For LPS-stimulated B lymphocytes, computation of this ratio showed that it increased with the restriction.

Discussion

Our study was undertaken to determine the effect on mouse immunity of two total dietary-restricted diets resulting in reduced or practically no increase in body weight. Both diets had considerable effects on the growth and general fitness of the mice in the first 8 days. Restricted animals then seemed to adapt to the diet, but weight, length, and ratio of weight to length remained lower than normal values at all ages. The 70% restriction allowed for some growth, although reduced, whereas severe restriction at 55% led first to a decrease in body weight, which recovered only at 30 days of age to the value at weaning and was then maintained without increase. A similar result was obtained in weanling BALB/c mice restricted at 75 and 62% of normal food consumption.[17]

Changes in thymus weight in control mice during the study period reflected the physiological modifications of the organ from weaning to adulthood. In restricted mice, thymus weight decreased considerably just after weaning, when normal thymus was still growing. Thymus of restricted mice then grew between age 31 and 41 days and did not really lose mass when control thymus underwent the normal physiological involution. It is therefore important to compare the effect of a restriction on the thymus over a period. In fact, our results showed that thymus weights in control and R70% mice were similar at age 51 days but that, at that moment, the thymus of control mice underwent normal involution, whereas the thy-

Table III. Percentage of B Lymphocytes Labeled With Anti-Mouse Ig-Specific Antibody, of T Lymphocytes Labeled With Anti-Thy-1.2 Monoclonal Antibody, of Suppressor T Lymphocytes Labeled With Anti-Lyt-2 Monoclonal Antibody, and of Helper T Lymphocytes Labeled With Anti-L3T4 Monoclonal Antibody of 5 Mice/Group During Normal Development and Undernutrition (R) at 70 and 55%

	Age (days)			
Group	31	41	51	61
B lymphocytes				
Control	31.16 ± 1.12*	35.89 ± 1.69†	44.46 ± 2.91	39.51 ± 1.12†
R70%	28.03 ± 3.14	30.42 ± 1.34	30.15 ± 1.54‡	32.44 ± 1.73
R55%	27.88 ± 4.38	19.18 ± 2.73	26.65 ± 2.61‡	21.28 ± 1.47
T lymphocytes				
Control	39.10 ± 3.41	40.54 ± 2.54	33.66 ± 1.14†	31.48 ± 4.04
R70%	45.96 ± 2.89§	43.71 ± 3.90	39.50 ± 1.47	36.71 ± 2.33
R55%	48.89 ± 3.27§	61.15 ± 3.64‡¶	45.10 ± 2.89	49.22 ± 3.94‡¶
Suppressor/cytotoxic T lymphocytes				
Control	23.31 ± 2.15 ‖	17.13 ± 1.72	13.98 ± 0.88	18.94 ± 2.22*
R70%	17.63 ± 0.99	18.84 ± 1.69	15.01 ± 1.47	21.58 ± 2.19
R55%	28.55 ± 2.53	23.48 ± 2.01‡¶	19.26 ± 3.18‡¶	21.70 ± 2.90
Helper/inducer T lymphocytes				
Control	29.00 ± 3.64*	28.94 ± 1.74	32.51 ± 1.89*	31.34 ± 1.21†
R70%	28.80 ± 2.87	27.21 ± 2.67	37.80 ± 2.15	38.42 ± 3.20
R55%	26.66 ± 5.45	37.26 ± 5.12‡¶	35.97 ± 4.50	49.13 ± 2.46

**p > 0.05, †p < 0.01, ‖p < 0.05, between groups.*
‡p < 0.01, §p < 0.05, vs. control group.
¶p < 0.01 vs. R70% group.

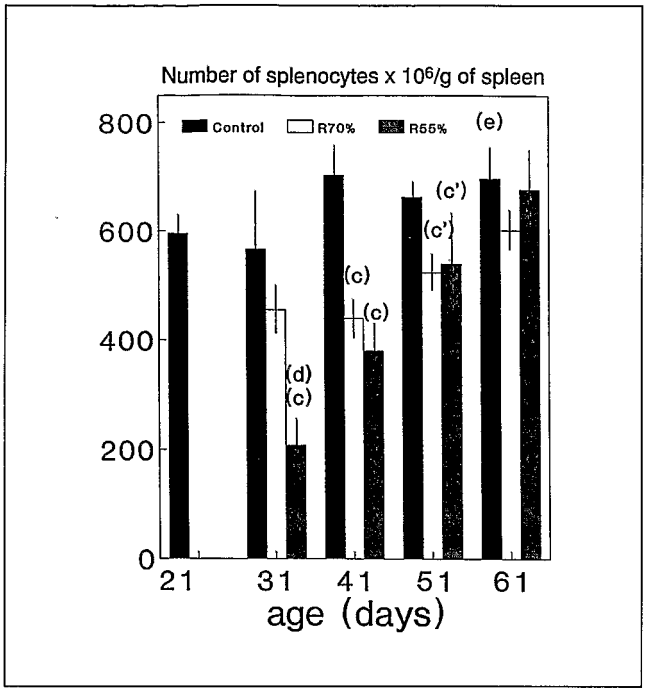

Fig. 4. Mean ± SE ratio of total splenocyte count (x 10^6) to spleen weight of 5 mice from each group during normal development and (R) 70 and 55% undernutrition. [c]*p < 0.01,* [c]*p < 0.05, vs. control group.* [d]*p < 0.01 vs. R70% group.* [e]*p > 0.05 between groups.*

mus of restricted mice reached a plateau after having increased. In addition, thymus weight in R70% mice at 41 days of age was close to that in control mice at age 61 days. This shows how carefully an isolated comparison must be considered between the thymus of growing mice undergoing different treatments over a long period.

From age 41 days on, the R70% mice appeared to be maintaining thymus weight in proportion to their body weight regardless of age, whereas 55% restriction was too severe to allow for such a possibility. Franklin et al.[18] observed the same phenomenon in undernourished rats. McAnulty and Dickerson[7] observed that, after undernutrition, the thymus had a remarkable capacity for recovery and that body weight was more important than age in influencing the rate of thymus recovery. Underfeeding started at weaning results in a slower rate of growth and development; immune function matures relatively slowly in moderately restricted animals so that, in early life, they could have a delayed and slower pattern of natural thymic involution than control animals.[19] It is important, because of its natural variation, that the thymus can adapt to body weight because thymic atrophy may have significant implications with respect to immune function.

The decrease in the percentage of B lymphocytes in the spleen of restricted mice, which was parallel to an increase in the percentage of T lymphocytes, was enhanced with the severity of restriction. However, at the same time, the sum of B- and T-lymphocyte percentages was maintained at an almost constant level at all ages in all groups. Our study stopped when mice were 60 days old, but Hishinuma et al.[20] showed that the percentage of spleen T lymphocytes was dramatically increased in diet-restricted adult mice. Parallel to the increase in total T-lymphocyte percentage, an increase in the percentage of both subsets was observed. The difference in the percentage of suppressor T lymphocytes between the three groups decreased with age, whereas an opposite

Table IV. Disintegrations per Minute (dpm) and Stimulation Indices (SI) (Ratio of Stimulated-Culture dpm to Unstimulated-Culture dpm) in Response to Mitogen Stimulation of Splenocytes of 5 Mice/Group During Normal Development and Undernutrition (R) at 70 and 55%

Age (days) Group	dpm			SI	
	ConA	LPS	Unstimulated Culture	ConA	LPS
41					
Control	513,719 ± 33,328*	131,929 ± 7142	10,635 ± 4155	73.6 ± 17.2	17.6 ± 3.4
R70%	516,564 ± 16,608	144,564 ± 6192	28,742 ± 6105†	20.6 ± 3.2†	5.6 ± 0.9†
R55%	513,630 ± 24,922	105,052 ± 18,924†‡	20,514 ± 8391	58.6 ± 29.7	8.2 ± 2.2†
51					
Control	511,175 ± 20,363*	143,144 ± 28,461	17,874 ± 5794	43.2 ± 13.6	9.4 ± 2.2
R70%	531,126 ± 23,437	164,449 ± 4654	37,708 ± 10,177¶	17.0 ± 3.1	5.4 ± 1.1
R55%	516,364 ± 35,237	108,180 ± 40,004§	11,754 ± 5333‡	106.2 ± 54.4‡¶	10.2 ± 3.3§
61					
Control	481,202 ± 56,795*	182,571 ± 11,046	18,369 ± 2374	27.4 ± 3.9	10.0 ± 1.0
R70%	434,480 ± 37,011	166,650 ± 7913	33,156 ± 5364†	14.4 ± 2.5	5.4 ± 1.1†
R55%	427,985 ± 40,764	139,159 ± 14,605†‡	12,600 ± 2991‡	46.0 ± 13.2‡¶	12.8 ± 2.0‡

Values are means ± SE. ConA, Concanavalin A; LPS, lipopolysaccharide.
p > 0.05 between groups.
†p < 0.01, ¶p < 0.05, vs. control group.
‡p < 0.01, §p < 0.05, vs. R70% group.

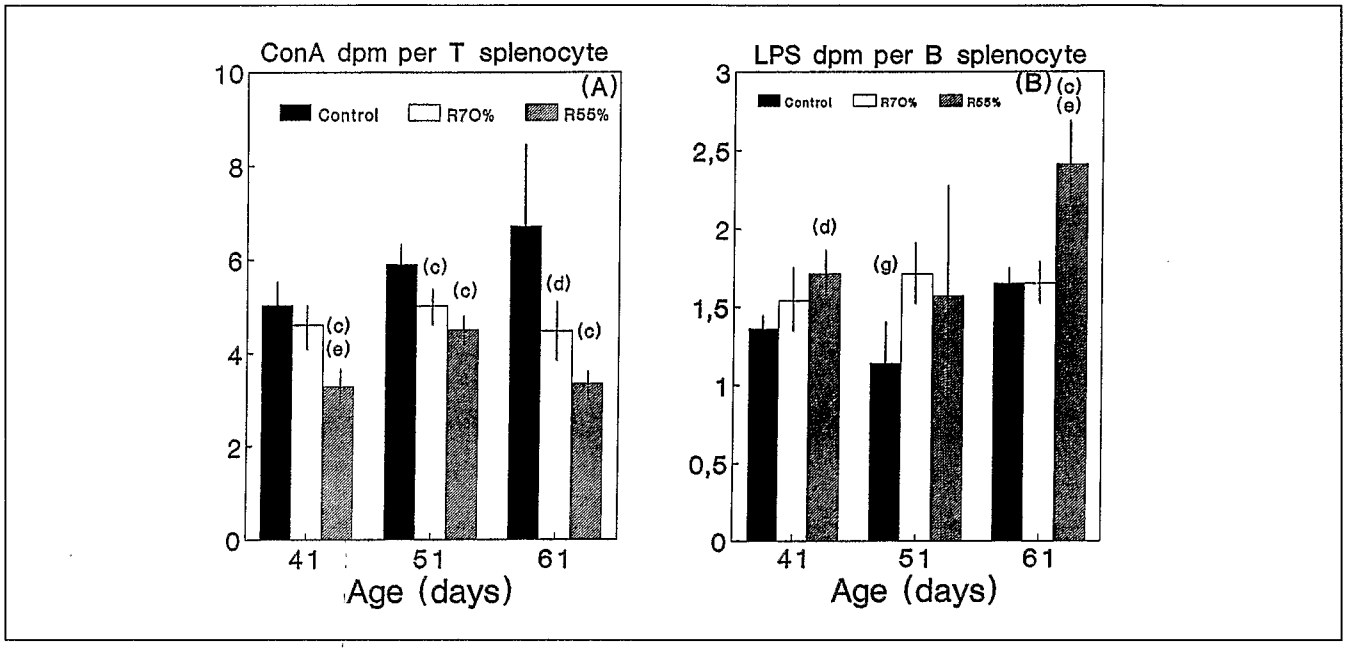

Fig. 5. Mean ± SE ratio of stimulated-culture concanavalin A disintegrations per minute (ConA dpm) minus unstimulated-culture dpm to cultured T-lymphocyte number (A) and ratio of stimulated-culture lipopolysaccharide (LPS) dpm minus unstimulated-culture dpm to cultured B-lymphocyte number (B) of 5 mice from each group during normal development and (R) 70 and 55% undernutrition. $^{c}p < 0.01$, $^{d}p < 0.05$, *vs. control group.* $^{e}p < 0.01$ *vs. R70% group.* $^{g}p > 0.05$ *between groups.*

tendency was noted for the percentage of helper T lymphocytes. In adult mice fed a restricted diet, Hishinuma et al.[20] showed that $L3T4^+$ but not $Lyt\text{-}2^+$ lymphocytes increased, which could confirm the tendency we noted with age. There were some discrepancies in total numbers of helper and suppressor T lymphocytes, which exceeded total T lymphocytes in some cases. This could be the result of changes in levels of natural killer cells, which can express CD8, or antigen-presenting cells, which can express CD4.

Regardless of whether unstimulated-culture dpm values were subtracted, results of ConA stimulation of the three groups were not different, whereas the membrane characterization of antigen showed a higher T-splenocyte percentage in restricted mice. We calculated the ratio of stimulated-culture dpm minus unstimulated-culture dpm to cultured T-splenocyte number for each mouse. It appeared that the T splenocytes of restricted mice had a lower proliferative capacity, especially in R55% mice, under the conditions of this in vitro test. Regardless of whether unstimulated-culture dpm values were subtracted, the results of LPS stimulation of splenocyte culture from R55% mice were lower than those of control and R70% groups, but this could be because the percentage of B splenocytes in the culture was lower in R55% mice, as shown by the phenotypic study. Calculation of the ratio of stimulated-culture dpm minus unstimulated-culture dpm to cultured B-lymphocyte number showed the increased capacity of restricted-mouse B lymphocytes to proliferate in response to LPS stimulation, and this was more marked in the R55% group. It is therefore desirable to evaluate B- and T-lymphocyte population percentages concurrently with the test of proliferative response to mitogen stimulation. This interpretation led us to different conclusions compared with taking only dpm into consideration.

Dpm results from the mitogen-stimulation tests are usually used to calculate SIs. However, our data showed that we must also closely consider unstimulated cultures of cells from restricted animals before coming to a conclusion. Dpms of unstimulated cultures were significantly higher in splenoctyes from R70% mice at the ages studied. Increased incorporation of tritiated thymidine in unstimulated cultures of splenocytes from calorie-restricted rodents was reported previously,[21] as in copper-deficient rats.[22,23] Here, differences in SI between groups were due to differences among unstimulated cultures rather than among stimulated cultures.

In many areas of nutrition-immunity interactions, apparently conflicting data prevent definitive conclusions.[3] An explanation lies in the inherent differences between the test system and the laboratory methods. The duration of cell culture, amount of mitogen, composition of culture medium, and correction of the results for the number of responding cells in vitro are some of the variables that may influence the results of lymphocyte proliferation responses.[8]

Our study, considering the time factor in growing animals, showed that, during adaptation to the restricted diet, some immunological parameters returned to normal values. The results allow us to conclude that both levels of restriction tested yielded different immunologi-

cal effects and may represent effects observed in malnourished children, i.e., moderate and severe malnutrition. Our results showed the importance of evaluating B- and T-lymphocyte percentages in parallel to mitogen stimulation. Here, the proportion of B lymphocytes was decreased and the proportion of T lymphocytes increased in the spleen, but the response capacity of B lymphocytes seemed to be enhanced, whereas that of T lymphocytes seemed to be diminished in growing mice. This could reflect effects observed in malnourished children, i.e., a decrease in T-lymphocyte function and an apparently maintained or enhanced humoral function. The choice of restriction model and the interpretation of its results could provide a correct animal model.

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