

ORIGINAL ARTICLE

Immunosuppression in undernourished rats: the effect of glutamine supplementation

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Abstract—Objective: The aim of our study is to determine the effect of a 30-day-period caloric restriction (CR) upon the immune response of rats and the influence of glutamine upon mononuclear cells proliferation and cytokine production.

Methods: Male albino Wistar rats were submitted to CR receiving an amount of food equivalent to 50% of the mean amount consumed by the control animals. We measured the incorporation of [2-¹⁴C]-thymidine by lymphocytes obtained from the spleen and mesenteric lymph nodes, plasma glucose and glutamine concentration, as well as cytokine production by cultivated cells, in the presence of glutamine.

Results: Rats submitted to CR presented reduced body weight (49%) and decreased splenic leukocyte number. CR led to a reduction in the proliferative response of lymphocyte. Spleenocytes from CR animals produced less γ -interferon and interleukins 1, 4 and 10 in 48 h culture than did those from control rats. The same pattern is observed in cells obtained from the mesenteric lymph nodes. The addition of glutamine 2 mM to the culture medium restored spleen and mesenteric lymph node cells' proliferative response and the production of interleukin 2 by cells obtained from the spleen and from the mesenteric lymph nodes.

Conclusions: The present data reinforce that undernutrition decreases in vitro immune cell function and indicates that, in such circumstances, glutamine supplementation could reverse some of the changes observed in the functionality of cultured immune cells. The presence of the amino acid at physiological concentration, however, reinforces the diversion of the immune response towards a Th₁-like response.

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Introduction

Undernutrition is still a noticeable cause of impaired immunocompetence, and has been shown to be an important causal factor in the increased susceptibility to infectious diseases in normal and hospitalized patients (1, 2). Rapidly proliferating tissues are very sensitive to changes in nutrient availability and hormonal status. For this reason, it may be expected that subtle changes in the immune system, which presents a rapid turnover of cells and enormous protein synthesis, may be observed in malnourished animals (3).

Undernutrition depresses both cell-mediated and humoral immunity, resulting in thymus, spleen and lymph node atrophy (4–6), significantly impairs macrophage activation (7), decreases the absolute number of T cells (8), decreases thymic hormone production (9) and delayed-type hypersensitivity (10, 11), and impairs development of cytotoxic T cells (12). The changes observed, however, depend on the severity and time of exposure to caloric restriction (CR) (5, 13, 14), as well as on the type of dietary protein consumed (15–17).

The lack of appropriate food intake is a decisive factor in predisposing to infections and death (15, 18, 19), as happens frequently to low-income individuals. In such cases, changes in the immune system, as that observed in thymus, could be considered as part of a process of closing-down functions which can be best sacrificed in the short term to ensure long-term survival (15). Calorie restriction, on the other hand, appears to preserve immune function in autoimmune-susceptible mice (20, 21) and to delay cellular events involved in the ageing process, prolonging the life span of small animals (20–22). There is, however, relatively little amount of research directed toward the effects of dietary restriction in young animals. The aim of our study is, therefore, to determine the effect of a 30-day-period CR restriction upon the immune response of rats and the influence of glutamine upon mononuclear cells proliferation and cytokine production.

Materials and methods

Animals

Male albino Wistar rats ($n=10$) weighing 150 g were obtained from the Institute of Biomedical Sciences,

University of São Paulo. The rats were kept at 23°C and a light/dark cycle of 12/12 h, lights on at 7:00 am.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Mannheim, GmbH, Lewes, East Sussex, UK, Sigma–Aldrich, WI, USA, and Life Technologies, SP, Brazil.

Caloric restriction protocol

The animals were housed individually and weighed two times a week, during 1 month. The group submitted to CR received the amount of food equivalent to 50% of the mean amount consumed by the control animals. After 30 days of CR, the animals presented reduced body weight (Fig. 1). The protocol was approved by the Ethics Committee for Animal Research from the Institute of Biomedical Sciences, University of São Paulo.

Incorporation of [2-¹⁴C]-thymidine by lymphocytes obtained from the spleen and mesenteric lymph nodes (MLNs)

After spleen and MLNs disruption in an inox mesh, as previously described (23), cell suspensions were centrifuged at 500 g for 15 min, 37°C, and cell viability determined by using Trypan blue exclusion test. The cells were cultured in RPMI-1640 medium without glutamine, with 10% homologous serum, for 24 h at 37°C in an artificially humidified atmosphere of 5% CO₂ in air, under sterile conditions. The cells were cultured in a LAB-LINE Microprocessor CO₂ incubator (LAB LINE, USA) in 96-well plates (Corning, NY, USA), at a density of 2×10^5 cells/well (total volume, 200 µl), stimulated with concanavalina A, 20 µg/well. After 24 h

in culture, more than 98% of mononuclear cells were still viable, as measured by Trypan blue exclusion test. To address the possible effect of glutamine upon cell proliferation, the cells were cultured in the presence of exogenous glutamine (0.75 mM, CR + G) or alanine (0.75 mM, CR + Al), while those from the CR group were cultured in an RPMI medium with 0.45 mM glutamine, as these are plasma glutamine concentration described for control and CR groups, respectively (Table 3).

The cells were then pulsed with 20 µl (0.02 µCi) [2-¹⁴C]-thymidine (sp. Act. 56.0 mCi nM⁻¹) diluted in sterile PBS. Cells were then maintained under these conditions for an additional 16 h and harvested automatically by a multiple cell harvester onto a filter paper (cat n° 11731 Skatron Combi, Suffolk, UK). The paper discs containing the labelled cells were added to vials containing 5 ml of Ecolume (ICN, CA, USA scintillation cocktail), and counted in a Beckman-LS 5000TD liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

Plasma metabolite measurements

Plasma glucose concentration was determined as previously described by Trinder (24) and plasma glutamine concentration was determined using the method described by Windmueller and Spaeth (25).

Determination of cytokine production

Mononuclear cells obtained from the spleen and MLNs ($\pm 79\%$ lymphocytes) were plated (1.0×10^6 cells/ml) in plastic petri dishes in RPMI 1640 medium enriched with glutamine 2 mM and 10% homologous serum, in the presence of phytohemagglutinin (PHA) (10 µg/ml) to stimulate interleukins 1, 4 and 10 (IL-2, IL-4, IL-10), γ -interferon (INF γ) and tumour necrosis factor- α (TNF α) production or lipopolysaccharide (LPS) (10 µg/ml) to stimulate IL-1 α production. After 48 h, the concentration of the cytokines was measured in the supernatant using commercially available ELISA-kits (Amersham-Life Science, R&D System, Inc., Minneapolis, MN, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

A semi-quantitative RT-PCR method was used for the estimation of the concentration of glutamine synthetase mRNA. The red portion of the gastrocnemius muscle (100 mg) was homogenized according to Chomczynski and Sacchi (26). RNA concentration was determined spectrophotometrically (Beckman DU 640, Fullerton, CA, USA). A 33 µl pre-mix containing 3 µg RNA, 10 U of placental RNase inhibitor, 2 µl oligo(dt), 2 µl dNTP (10 nmol), 2 µl DTT, 10 U of Moloney-murine leukaemia virus reverse transcriptase, and 4 µl $10 \times$ reaction

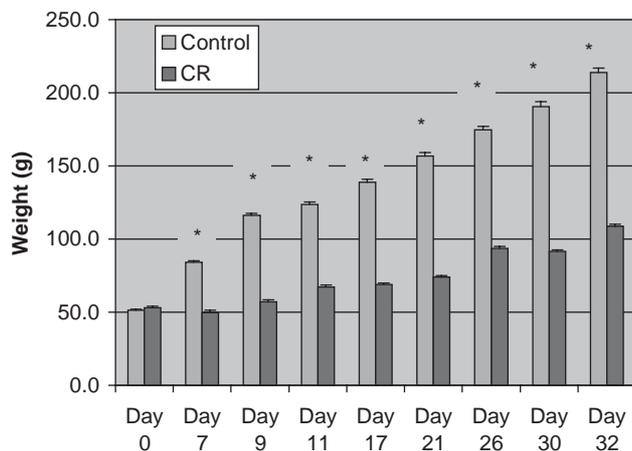


Fig. 1 Body weight gain (g) in control and undernourished rats. The results represent the mean of 10 rats \pm SEM. * $P < 0.05$ for comparison with the caloric restriction group; CR—rats submitted to caloric restriction.

buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 150 mM MgCl₂ in nuclease free water) was used to reverse transcribe RNA into cDNA. The RT-mixture was incubated at 80°C for 3 min, followed by 21°C for 10 min, 42°C for 30 min and then 99°C for 10 min. Two microlitres of the product obtained was subjected to 1% agarose and ethidium bromide electrophoresis. The sequences of glutamine synthetase primers were derived from published sequences (27) and that for RPL19 used as internal control. Each 3 µl PCR mixture contained 40 ng cDNA, 0.5 U AmpliTaq Gold Polymerase (Perkin-Elmer, Foster City, CA, USA), 2.5 nmol each dNTP, and 10 µM of glutamine synthetase primers in reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂). The reaction proceeded at 94°C, 28 cycles of 1 min, for denaturation, annealing at 60°C for 35 s and extension at 72°C for 1 min. Five microlitres of the PCR mixture was then subjected to polyacrylamide gel electrophoresis (agarose 1.2% and ethidium bromide) and the semi-quantitative analysis was performed with a Typhoon (Molecular Dynamics) and the program Image QuANT TM (4, 27).

Statistical analysis

The data obtained were compared using ANOVA and the post-hoc test of Tukey and the level of significance of at least $P < 0.05$ was chosen for all statistical comparisons. The data are presented as mean \pm SEM.

Results

To verify whether the CR imposed on the animals led to undernutrition, body weight and splenic leukocyte number were determined. Rats submitted to CR presented reduced body weight (49%, Fig. 1) and decreased splenic leukocyte number (58% fewer cells, data not shown).

The influence of CR upon the immune system was addressed by evaluating MLNs and spleen lymphocytes

proliferation and their ability to produce cytokines after 48 h in culture. CR led to a reduction in the proliferative response of lymphocytes from the MLNs and in those from the spleen (52% and 61%, respectively, Table 1). Undernutrition also provoked a marked reduction in the response of such cells to concanavalin A, a mitogen for T cell, 49% and a 21% decrease in the proliferative response of cells from the MLN and spleen, respectively (Table 1), was observed.

Splenocytes from CR animals produced less INF γ and IL-1, IL-4 and IL-10 in 48 h culture (reduction of 49%, 43%, 45% and 40.5%, respectively, Table 2) than did those from control rats. The same pattern is observed in cells obtained from the MLNs which showed a reduction of 57.8%, 39%, 29% and 33.2% in INF γ and IL-1, IL-4 and IL-10 production, respectively (Table 2). Cells obtained from the spleen of CR rats showed, however, increased production of IL-2 (19%, Table 2).

The animals submitted to CR presented, also, reduced plasma glutamine concentration (36.3%) but increased concentration of glutamine synthetase mRNA, as measured by a semi-quantitative RT-PCR (Table 3).

The addition of glutamine 0.75 mM to the culture medium restored spleen and MLN cells proliferative response as shown in Table 1, as well as the production of INF γ and IL-1 (Table 2). The presence of glutamine in concentrations similar to those found for the control group, in the culture medium also increased the production of IL-2, 23.7% and 17.9% by cells obtained from the spleen and from the MLNs, respectively (Table 2), and that of TNF, 14% and 29% (Table 2). The addition of alanine, however, did not change the proliferative response, nor cytokine production by cells obtained from the MLNs or spleen (Tables 1 and 2). In fact, these experiments with the addition of alanine to the culture medium were made with another set of undernourished rats, in which we found the same pattern of changes observed previously, and it is important to clarify if the results obtained with glutamine supplementation were specific or not.

Table 1 Proliferative response of lymphocytes obtained from the MLN (LFN) and splenocytes (spleen) of control and undernourished rats (CR) in the presence or not of glutamine (CR + G) or alanine (CR + Al) in the culture medium

	No addition	SEM	ConA	SEM
Spleen C	2598	156.7	7684.6	429.6
Spleen CR	989.6*	81.7	2292F*	167.9
Spleen CR + G	2250.3 [†]	187.6	7139.6 [†]	332.8
Spleen CR + Al	1067.4*	98.5	2673.4*	223.6
LFN C	10859	673.8	18509	1072.3
LFN CR	5182.7*	342.1	4444.3*	766.4
LFN CR + G	8737.4* [†]	549.6	17099 [†]	983.7
LFN CR + Al	5401.5*	287.5	4608.5*	659.7

* $P < 0.05$ for comparison with the values obtained for cells obtained from the control animals.

[†] $P < 0.05$ for comparison with the values obtained for cells obtained from CR group.

C—control group, CR—cells from rats submitted to caloric restriction; CR + G—cells from rats submitted to caloric restriction cultivated in the presence of glutamine, CR + Al—cells from rats submitted to caloric restriction cultivated in the presence of alanine.

The results are presented as decay per minute, as mean \pm SEM of 10 animals.

Table 2 Cytokine production by lymphocytes obtained from the MLNs (LFN) and spleen after 48 h of culture in the presence or not of glutamine or alanine added to the medium

Cytokines	Spleen C		Spleen CR		Spleen CR+G		Spleen CR+Al	
TNF	1879.5	47.8	1762.1	65.1	2139.6	78	1851.5	102
INF	32.4	1.3	16.5*	0.8	30.9	1.1	15.1*	1.1
IL-1	769.5	32.1	438.5*	45.8	771.3	67.4	397.5*	35.7
IL-2	1473.8	85.3	1759.2*	70.4	1823.5*	65.1	1902.2*	81.4
IL-4	370.6	21.7	201.4*	26.7	223.7*	23	196.5*	31
IL-10	128.5	9.7	76.4*	6.9	81.6*	5.9	74.3*	4.7

Cytokines	LFN C		LFN CR		LFN CR+G		LFN CR+Al	
TNF	1528.4	142.4	1662.9	89.4	1972.8	99.2	1712.8	86.4
INF	27.5	1.1	11.6*	0.3	29.4	1.2	12.6*	0.9
IL-1	659.3	33.8	399.4*	38.5	602.6	29.4	351.2*	32.7
IL-2	1903.6	98.4	2128.4	76.9	2245.7*	88.6	2103.6	102.7
IL-4	427.4	31.4	301.3*	19.5	276.4*	29.6	286.5*	17.6
IL-10	165.3	10.5	110.4*	10.9	128.4*	8.6	102.7*	14.1

* $P < 0.05$ for comparison with the values obtained from the control (C) group.

C—control group, CR—cells from rats submitted to caloric restriction; CR+G—cells from rats submitted to caloric restriction cultivated in the presence of glutamine, CR+Al—cells from rats submitted to caloric restriction cultivated in the presence of alanine.

The results are expressed as pg/ml and represent the mean \pm SEM of 10 animals.

Table 3 Plasma glucose (mg/dL) and glutamine (mM) concentration and that of glutamine synthetase (GS) mRNA in soleus muscle of control and undernourished rats (CR)

	Control	SEM	CR	SEM
Glucose	117.4*	8.7*	115.2	6.3
Glutamine	721.7*	56.8*	459.2*	38.5
GS-mRNA	1.61*	0.34*	6.85*	1.14

* $P < 0.05$ for comparison with the values obtained from the control group.

CR—cells from rats submitted to caloric restriction.

The results represents the mean \pm SEM of 10 animals.

Discussion

As previously described, undernutrition leads to immunosuppression (4, 14, 28), associated with a reduced body weight gain. CR also led to thymus atrophy, with important changes in thymus structure, as observed in our animals (data not shown) indicating a severe loss of thymus function, since the differences between the cortical and medullar zone of the lobules was lost (6). Despite the changes observed in the thymus, a moderate increase in splenic T cells responsiveness to polyclonal mitogens stimulated by increased IL-2 production by cells from undernourished rats has been reported (4). In our animals, we also observed an increased production of IL-2 after CR, but not only by cells from the spleen, but also by cultured cells obtained from the MLNs (LFN). These results, however, are not a consensus (3). Our data demonstrated, as well, that the production of TNF was not altered by CR, while that of INF, IL-1, IL-4 and IL-10 was reduced, indicating that CR modifies specific aspects of the immune response, leading to a diversion towards a Th₁-like response, with a decreased production of specific antibodies and mucosal IgA levels (29).

These changes in cytokine production by cultured mononuclear cells from the spleen and MLNs were

accompanied by a reduction in the proliferative response of such cells to concanavalin A, a mitogen for T-cell. Considering that IL-2 is a very important cytokine in stimulating T-cell proliferation and that its production is increased in CR rats, we can hypothesize an impairment in the function of antigen presenting cells as previously described by Zhang and Petro (4).

To better address the mechanism involved in the effects of CR upon the immune response of the animals we determined plasma glutamine concentration, since glutamine is an essential amino acid for the immune cells, including lymphocytes and antigen presenting cells such as B-lymphocytes and macrophages (23, 30).

In fact, a 24 h fast induced an increase in plasma glutamine concentration (27), abolished by an additional stressful stimulus and, as demonstrated by our data, after a chronic CR regime. Considering that glutamine is a non-essential amino acid, produced in large scale by the skeletal muscles, we evaluated glutamine synthetase expression in such muscles. It is interesting to note, however, that the expression of glutamine synthetase mRNA is increased in the skeletal muscle of undernourished rats. The increase in glutamine synthetase activity and expression could be related to the reduced plasma glutamine concentration, as previously demonstrated by Labow and colleagues (31), who showed a 3.5-fold increase in glutamine synthetase activity in skeletal muscle after glutamine starvation. The increase in enzyme expression, however, was not matched by an increase in plasma glutamine concentration, probably due to an increased demand of glutamine from cells such as immune cells and enterocytes, and possibly by other organs, in excess of the individual's capability for its synthesis (32). In this way, it is possible that during such starvation regime glutamine would be utilized as a neoglycogenic substrate in liver, helping in keeping glycaemia in normal values. In such circumstances, an increase in enzyme expression

would be taken as an adaptative response to hypoglutaminemia during starvation, open the possibility that glutamine supplementation to the culture medium of cells obtained from CR rats reversed part of the changes in the immune response first observed.

The addition of glutamine, mimicking physiological concentrations, recovered partially the changes observed in cytokine production by cells obtained from the spleen and the MLNs of undernourished rats, except that of IL-4 and IL-10, indicating a more pronounced diversion towards a Th₁-like response. It is interesting to mention, however, that glutamine addition to the culture medium restored the proliferative response of mononuclear cells to concanavalin-A, suggesting that the antigen presenting cells recovered their ability to stimulate cell proliferation in the presence of adequate plasma glutamine concentration. It is interesting to note that alanine added to the culture medium, instead of glutamine, did not change any of the parameters studied. The set of experiments with alanine supplementation was performed in a second group of undernourished rats, that presents however, the same pattern of changes in lymphocytes proliferation as those observed in the first experiment.

The present data reinforce that undernutrition decreases *in vitro* immune cell function and indicate that, in such circumstances, glutamine supplementation could reverse some of the changes observed in the functionality of cultured immune cells. The presence of the amino acid at physiological concentration, however, reinforces the diversion of the immune response towards a Th₁-like response.

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