Aging: a barrier to renutrition? Nutritional and immunologic evidence in rats[1–3]

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ABSTRACT

Background: Previous reports suggest that correcting the malnourished state is more difficult in elderly people than in younger ones and that protein requirements may be higher in elderly than in younger adults.

Objective: The aim of this study was to establish whether malnourished old rats respond to protein-supplemented nutritional repletion as do young adult rats.

Design: Adult (3 mo old) and old (22 mo old) rats were submitted to dietary restriction programs that induced similar metabolic and nutritional alterations. Malnourished adult and old rats were then killed (R groups) or refed for 1 wk with a high-protein diet (HPD; 23% protein) or a very-high-protein diet (VHPD; 27% protein). Control groups at both ages were fed ad libitum throughout the experiment. Effects of food repletion were evaluated in terms of protein metabolism, intestinal histomorphometry, and nonspecific immune status.

Results: In adult rats, HPD sufficed to increase body weight and restore basal values of liver weight and protein content ($P < 0.01$ compared with the R adult group), nitrogen balance ($P < 0.01$ compared with the R adult group), and hydrogen peroxide production by polymorphonuclear neutrophils and monocytes ($P < 0.01$ compared with the R group); VHPD had no supplementary effect except on nitrogen balance. In old rats, HPD was less effective and greater benefit was observed with VHPD in terms of body weight gain (10%; $P < 0.01$ compared with the old group fed HPD), albuminemia, muscle weight and protein content, plasma arginine concentration, and hydrogen peroxide production by stimulated polymorphonuclear neutrophils and monocytes compared with the old R group ($P < 0.01$).

Conclusion: Aging is a significant variable affecting the response to nutritional support. *Am J Clin Nutr* 2000;72:816–24.

KEY WORDS Aging, dietary restriction, acute renutrition, protein supplementation, protein metabolism, intestinal morphometry, nonspecific immune response, elderly, aged, old, rats

INTRODUCTION

Protein-energy malnutrition is common in the elderly, especially in hospitalized patients (1, 2). The malnutrition arises from a combination of factors including poor diet, social isolation, poverty, physical and psychological illness, and, in some cases, malabsorption syndrome (3). Sullivan et al (1) showed a strong independent correlation between severity of protein-energy malnutrition and 1-y mortality risk. The capacity of elderly persons to respond successfully to renutrition, especially during malnutrition or illness, is less than that of younger persons, and their nutritional requirements are different. Hébuterne et al (4, 5) showed that cyclic enteral renutrition was more effective in middle-aged undernourished patients than in elderly ones in terms of body weight, nutritional variables, fat-free mass, and body cell mass. In addition, in a 15-d course of parenteral nutrition, Shizgal et al (6) showed that the body cell mass improved only in patients aged <65 y. Thus, the response to standard nutritional support seems less effective in elderly malnourished individuals than in younger ones. These observations highlight the need for careful definition of a renutrition strategy suitable for malnourished elderly patients.

The aim of this study was to establish whether malnourished old rats could respond to nutritional repletion as do younger rats. Because supplements may be required to act as a starter of renutrition in old subjects, and because protein requirements in elderly persons are reportedly greater than in healthy adult subjects (7), we tested 2 diets supplemented with high (23%) and very high (27%) amounts of protein. We investigated whether malnutrition-induced alterations, in terms of protein metabolism, gut structure, and nonspecific immune status, were reversible in old rats receiving renutrition via a protein-enriched diet. For this purpose, adult and old rats that were in the same state of dietary restriction at the start of renutrition were refed.

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TABLE 1
Compositions of the experimental diets

	Standard[1]	Low protein[2]	High protein[3]	Very high protein[3]
Protein (% by wt)				
From standard diet	17	5	17	17
From casein	0	0	6	10
Fat (% by wt)	6	6	6	6
Starch (% by wt)	39	30	41	39
Sugar (% by wt)	28	50	20	19
Vitamin and mineral mix (% by wt)	6	6	6	6
Cellulose (% by wt)	4	4	4	4
Total energy (kJ/100 g)	1630	1647	1630	1647

[1] Used for both adult and old rats during acclimatization, throughout the experiment for the control groups, and during the dietary restriction period for the old rats.
[2] Used for the adult rats during the dietary restriction period.
[3] Used for both adult and old rats during refeeding.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Iffa Credo, L'Arbresle, France) were used in all the experiments. Adult and old rats were 3 and 22 mo old, respectively, on arrival at our facility. They were caged individually and kept at 20–23°C in alternate 12-h light-dark cycles. They had free access to water. During acclimatization, all the rats were fed a standard diet (UAR, Villemoisson-sur-Orge, France) ad libitum for 2 wk. Daily spontaneous intakes were determined; adult and old rats consumed 28.8 ± 0.8 and 25.5 ± 0.9 g/d, respectively. Two of us (M-PV and LC) are authorized by the French Ministry of Agriculture and Forestry to perform animal experiments and the recommendations of the US National Research Council for the care and use of laboratory animals were followed (8).

Experimental procedures

The compositions of the experimental diets are given in **Table 1**.

Dietary restriction

Both adult and old rats were allowed to consume 50% (14.4 and 12.8 g/d, respectively) of their spontaneous intakes measured during the second week of the acclimatization period. During dietary restriction, the old rats were fed a standard diet (17% protein) for 12 wk, according to a protocol established in our laboratory (9). However, this dietary restriction program does not produce an equivalent malnourished state in adult rats (10). Because we needed 2 models of dietary restriction that induced similar metabolic and nutritional alterations in both adult and old rats, the adult rats were submitted, in a preliminary experiment, to dietary restriction regimens that differed in duration (1, 3, or 6 wk) and protein intake (0%, 5%, or 10%).

Refeeding

At the end of their dietary restriction program, the adult rats were 5 mo old and the old rats were 25 mo old, which corresponds to 93% of the mean life span of this species. All the animals were refed for 1 wk with 90% of their spontaneous food intakes (25.9 and 22.9 g/d, respectively). In a preliminary experiment, we found that refeeding old rats for 2 or 3 wk with a high-protein diet had no clear effect on nutritional status compared with rats refed for 1 wk only (data not shown). Rats were refed with a diet supplemented with 1.6 g casein hydrolysate/d, ie, 23% protein for the high-protein diet (HPD; n = 6 and n = 7 adult and old rats, respectively) or 3.2 g casein hydrolysate/d, ie, 27% protein for the very-high-protein diet (VHPD; n = 6 and n = 7 adult and old rats, respectively). The casein hydrolysate (UAR) contained free amino acids (69%) and small peptides (31%) of average molecular weight 234 Da. The casein hydrolysate supplement was mixed thoroughly each day with the standard diet in amounts adequate to obtain a homogeneous powder. Food was given in conical porcelain cups to avoid spillage and was limited to 90% of the spontaneous food intake measured during the acclimatization period to make sure all the rats ate all of their ration and hence received the same amounts of food (11). All rats in every group ate all of their ration.

Control groups

Control groups (n = 6 and n = 11 adult and old rats, respectively) for each age were fed the standard diet ad libitum throughout the experiment. Food intakes and body weights were recorded daily for every group. During the last 2 wk of experimentation the rats were placed in individual metabolic cages. Urine was collected in an antiseptic solution (Gifrer Barbezat, Décines, France) on the last 2 d of experimentation for determination of nitrogen balance.

Tissue removal

At the end of the experimental period, the adult and old rats were killed by beheading after ether anesthesia. Blood was taken into sodium heparin (Biochrom, Berlin) to assay plasma albumin and amino acids, and into calcium heparin (Leo, Saint-Quentin-Yvelines, France) to isolate leukocytes.

Small intestine

The abdominal cavity was opened and the small intestine extending from the ligament of Treitz to the cecum was resected. Its total length was determined by vertical suspension with an attached 3-g weight; the intestine was cut at its midpoint into 2 portions, the jejunum and the ileum. The proximal part of the jejunum and the ileum was removed and washed with saline buffer. A 1-cm length of each segment was removed for intestinal morphometry.

Muscles

The right hind limbs were removed and skinned. The soleus and tibialis anterior muscles were rapidly dissected, weighed, and frozen in liquid nitrogen for determination of protein content.

Analytic methods

Intestinal morphometry

Intestinal samples were promptly fixed in Bouin solution and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin. Crypt depth and villus height in the jejunum and the ileum were measured by using a semiautomatic image analyzer (Biocom, Lyon, France). Total villus height is the sum of villus height and crypt depth.

Variables related to protein metabolism

Albuminemia. Plasma albumin concentrations were quantified by immunonephelemetry (Array protein system; Beckman, Gagny, France) using rabbit antibodies (Dako, Trappes, France).

Nitrogen balance. Urine samples were centrifuged at 4500 × g for 10 min at 4°C. An aliquot of the supernate was diluted in water (1:400) before analysis for total nitrogen by chemoluminescence using an automatic apparatus (model 7000N; Antek Instruments, Houston). Nitrogen balance, expressed as mg N/24 h, was calculated as the difference between daily nitrogen intake and daily urinary nitrogen excretion. Nitrogen in stools was considered to be negligible (<5%) and stable during starvation and refeeding (12) and at different protein intakes in mature and old rats (13), and so was ignored here.

Plasma amino acid concentrations. After deproteinization of plasma with sulfosalicylic acid (50 g/L) and centrifugation (4500 × *g* for 10 min at 4°C), supernates were analyzed to determine amino acid concentrations by ion-exchange chromatography as described above. We focused on glutamate + glutamine and arginine concentrations because of their ability to control protein turnover (14, 15) and modulate immune status (16–18).

Tissue protein contents. Tissue protein contents were evaluated as described previously (11). Briefly, frozen tissues were pulverized in ice-cold 10% trichloroacetic acid containing 0.5 mmol EDTA/L (1 mL/100 g tissue). Precipitated proteins were separated by centrifugation at 4500 × *g* for 10 min at 4°C. Fat was then eliminated by using alcohol:ether (1:1 by vol). The protein precipitate was dissolved in 1 mol NaOH/L (4 mL/100 mg tissue) at 40°C for 12 h and total tissue protein content was assayed by using Gornall's method (19).

Variables related to nonspecific immune status

Hydrogen peroxide production by stimulated granulocytes. Granulocytes were isolated from blood by gravity sedimentation using an isotonic gelatin matrix (Plasmagel; Belon, Neuilly-sur-Seine, France) at room temperature for 30 min. Erythrocytes settled at the bottom of the tube and the supernate was drawn off. Residual erythrocytes were destroyed by a hemolytic solution (0.15 mol NH_4Cl/L, 12 mmol $NaHCO_3$/L, 0.1 mmol EDTA/L). Leukocytes were then washed twice with phosphate-buffered saline (PBS) and adjusted to 10^9 cells/L with RPMI-1640 medium (Sigma Chemical Co, Saint-Quentin-Fallavier, France). Cell viability was monitored by using the trypan blue (Sigma Chemical Co) exclusion test.

Leukocytes (10^9/L) were preincubated for 15 min with 5 μmol 2′7′-dichlorofluorescein diacetate/L (DCFH-DA; Acros, Noisy-Le-Grand, France) in a water bath with horizontal agitation at 37°C. Under these conditions, DCFH-DA diffuses into the cells and is hydrolyzed intracellularly into 2′7′-DCFH. White blood cells were then stimulated with 10^{-6} mol phorbol-myristate-acetate/L (PMA; Sigma Chemical Co). This results in a leukocyte oxidative burst, during which nonfluorescent intracellular DCFH is oxidized to highly fluorescent dichlorofluorescein (DCF) by hydrogen peroxide. Individual granulocytes were discerned and DCF fluorescence was measured by using flow cytometry analysis (Epics XL; Beckman Coulter, Villepinte, France). Results were expressed as the ratio of hydrogen peroxide produced by PMA-stimulated leukocytes to that produced by nonstimulated cells.

Tumor necrosis factor α production by stimulated peritoneal macrophages. Macrophages were obtained by 2 successive washings of the peritoneal cavity with 10 mL RPMI-1640 medium. Cells were sedimented at 1200 × *g* for 10 min at 4°C. Cell viability was monitored by using a 0.4% trypan blue exclusion test, and cells were counted and adjusted to a final concentration of 10^9 cells/L in RPMI-1640 medium supplemented with fresh L-glutamine (2 mmol/L; Sigma Chemical Co), an antibiotic solution containing penicillin (100 000 U/L) and streptomycin (100 mg/L; Sigma Chemical Co), and 10% fetal calf serum (Sigma Chemical Co). The macrophage suspension from each rat was distributed in duplicate in multiwell tissue culture plates (Falcon, Lincoln Park, MI). Macrophages were selected for their ability to adhere to the well surface after incubation at 37°C for 2 h in a humidified 5% CO_2 atmosphere. Adherent cells were then washed 3 times with 1 mL RPMI-1640 medium. For each macrophage preparation, 2 wells were used and incubated with either 1 mL RPMI-1640 medium alone or 1 mL RPMI-1640 supplemented with lipopolysaccharide at 40 mg/L (LPS from *Escherichia coli* serotype 0127:B8; Sigma Chemical Co), respectively. Multiwells were incubated again for 3 h under the same conditions. Culture media was collected, and tumor necrosis factor α (TNF-α) released by macrophages was assayed by enzyme-linked immunosorbent assay (Factor test X rat TNFα ELISA kit; Genzyme, Cergy-Saint-Christophe, France). Results were expressed as the ratio of TNF-α produced by LPS-stimulated macrophages to that produced by nonstimulated ones.

Statistical analysis

Data are expressed as means ± SEMs. In the preliminary experiment, to compare restricted adult and old rat models, we used a one-way analysis of variance (ANOVA) and a principal component analysis to confirm the nonsignificant difference between the 2 restricted groups.

In the main study, the experimental design comprised 2 crossed fixed factors with the factor "aging" as 2 classes ("adult" and "old") and the factor "diet" as 4 classes ("control rats," "restricted rats," "HPD-fed rats," and "VHPD-fed rats"). This design allowed statistical analysis by two-way ANOVA. This two-way ANOVA was performed to discriminate between the effects of aging, diet, and their interaction. The level of significance was set at $P < 0.05$ for this test. When the ANOVA indicated significant interactions ($P < 0.05$), differences between individual means were identified by using the Newman-Keuls test. When no significant interaction was found, the marginal means were calculated and compared. Because the Newman-Keuls procedure does not test for type 1 error, we used a Bonferroni correction, for which the significance level was set at $P < 0.01$.

RESULTS

Preliminary experiment: similarity of nutritional status of adult and old rats at the end of the dietary restriction period

Of all the combinations tested during this preliminary experiment (*see* Materials and Methods), a 5%-protein diet for 6 wk

TABLE 2
Main variables of comparison between restricted adult and old rat models before refeeding[1]

	Restricted/control	
	5 mo (n = 6)	25 mo (n = 6)
	%	
Carcass weight	56 ± 2	53 ± 2
Nitrogen balance[2]	31 ± 2	21 ± 7
3-Methylhistidine	54 ± 6	65 ± 9
Soleus protein content	100 ± 3	96 ± 1
Tibialis protein content	96 ± 1	94 ± 2
Liver protein content	93 ± 2	96 ± 5
Jejunal protein content	61 ± 2	74 ± 5
Ileal protein content	52 ± 2	61 ± 7
Jejunal crypt depth	76 ± 3	81 ± 2
Ileal crypt depth	84 ± 2	75 ± 3

[1] Results are expressed as percentage values for restricted rats relative to the mean values obtained for ad libitum fed rats of the same age. Rats were restricted at 50% of ad libitum intake for 6 wk with a 5%-protein diet in adult rats, and for 12 wk with a 17%-protein diet in old rats. There were no significant differences between 5- and 25-mo-old rats.

[2] Nitrogen balance had large variations in old animals and the absence of a significant difference for this variable may be due to type II error.

was found to induce metabolic and nutritional disorders in adult rats similar to those noted in old ones (**Table 2**). This diet was therefore selected for the main study.

Mortality

There was no mortality in adult rats, but 8% of the control old rats and 25% of the diet-restricted old rats died during the experiments. No mortality was observed during the repletion period.

Body weight

At the beginning of the study, the mean body weights were 404 ± 3 and 719 ± 10 g for adult and old rats, respectively (P < 0.01). Food restriction induced a body weight loss of 89 ± 6 g (2.12 g/d) and 317 ± 16 g (3.77 g/d) for adult and old rats, respectively (P < 0.05 compared with corresponding groups at the beginning of the study). After 1 wk of refeeding, the adult animals showed a similar weight gain whatever the regimen (44 and 51 g for adult rats fed HPD and VHPD, respectively). In contrast, little change in body weight occurred in the old rat group consuming the HPD (12 g; P < 0.01 compared with the HPD-fed adult group). A higher weight gain was observed when using the VHPD in the old rats (37 g; P < 0.01 compared with the HPD-fed old group), but it was still significantly lower than that in the adult group fed VHPD (P < 0.05). None of the refed animals lost weight, with one exception in the old rat group fed the HPD.

Intestinal histomorphometry

In the jejunum, dietary restriction–induced alterations were significantly related to a decrease in crypt depth (**Figure 1**). In the ileum, the total villus height modifications were related to a decrease in both crypt depth and villus height (P < 0.01 for both compared with control groups). During refeeding with the HPD, jejunal crypt depths were corrected in both the adult and the old rats (Figure 1), resulting in a restoration of total height (data not shown). Ileal morphometry was not significantly modified by refeeding in the adult group. In refed old rats, ileal crypt depths were significantly improved in the group fed HPD compared with the restricted old group.

Albuminemia

Plasma albumin concentrations were significantly lower in the diet-restricted old rats than in the control old rats (P < 0.01; **Table 3**). During refeeding with the HPD, hypoalbuminemia was not corrected. When the protein intake was raised (ie, by using the VHPD), the old rats showed a normalization in plasma

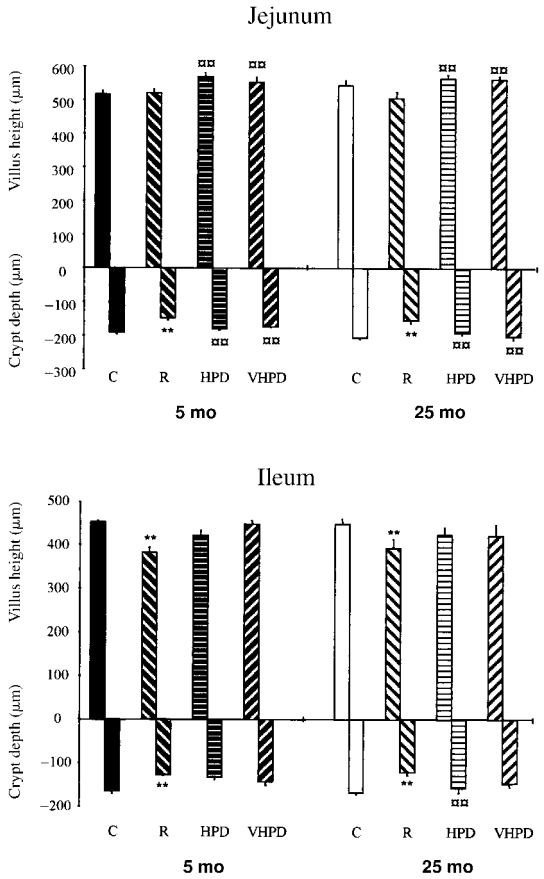

FIGURE 1. Intestinal morphometry after dietary restriction and refeeding in adult (5 mo old) and old (25 mo old) rats. The rats were fed ad libitum (C groups), were diet restricted and then killed (R groups), or were diet restricted and then refed with high-protein (HPD) or very-high-protein (VHPD groups) diets. Villus height is the distance from crypt-villus junction to villus tip, and crypt depth is the distance from crypt-villus junction to the bottom of the crypt. $\bar{x}$ ± SEM. Two-way ANOVA was performed to discriminate among effects of aging, diets, and their interaction. A significant effect of diet (P < 0.0001) was observed in all intestinal segments. A significant effect of aging was shown in jejunal crypt depths (P = 0.01), and a significant interaction between aging and diet effects was found in ileal crypt depths (P < 0.05). When no significant interaction was noted (jejunal villus height and crypt depth and ileal villus height), comparison of marginal means was carried out by using the Newman-Keuls test: $^{**}P$ < 0.01 compared with the C group, P < 0.01 compared with the R group. Because a significant interaction was found for ileal crypt depths, comparison of individual means was valid and was carried out by using the Newman-Keuls test: $^{**}P$ < 0.01 compared with the C group of the same age, P < 0.01 compared with the R group of the same age.

TABLE 3
Plasma albumin concentrations after dietary restriction and refeeding in adult and old rats[1]

	Adult (5 mo old)	Old (25 mo old)
	g/L	
Control	25.2 ± 0.4	24.2 ± 1.3
Restricted	24.4 ± 0.5	18.1 ± 1.0[2,3]
HPD	26.2 ± 0.7	19.9 ± 1.2[3]
VHPD	27.3 ± 0.5	22.1 ± 0.8[3,4]

[1] $\bar{x}$ ± SEM; n = 6–11 rats per group. Control rats were fed ad libitum, restricted rats were diet restricted then killed, HPD rats were diet restricted then refed a high-protein diet (1.6 g casein hydrolysate) for 1 wk, VHPD rats were diet restricted then refed a very-high-protein diet (3.2 g casein hydrolysate) for 1 wk. There were significant effects of aging, diet, and aging × diet, $P < 0.05$ (two-way ANOVA).
[2] Significantly different from the control group of the same age, $P < 0.01$ (Newman-Keuls test).
[3] Significantly different from the restricted group of the same age, $P < 0.01$ (Newman-Keuls test).
[4] Significantly different from adults, $P < 0.01$ (Newman-Keuls test).

albumin concentrations, but values were lower than in adult rats receiving the same diet.

Nitrogen balance

Nitrogen balance was significantly reduced in the diet-restricted groups at both ages compared with the respective control groups (**Figure 2**). Refeeding induced a significant increase in nitrogen balance for all rats, but nitrogen balance tended to increase with protein intake in adult rats whereas old rats received no greater benefit from the VHPD than from the HPD.

Tissue weights and protein contents

No significant change was observed in the soleus muscle in terms of weight or protein content for any treatment or age (**Table 4**). The weight of the tibialis anterior muscle was significantly reduced after HPD refeeding in the old rats compared with the corresponding control rats (Table 4). Tibialis anterior protein content was significantly affected by age and diet in the old rats.

In the liver, dietary restriction induced a severe loss of weight and protein content at both ages (Table 4). Refeeding with either diet induced significant recovery of liver weight and protein content in the adult rats compared with the diet-restricted group, whereas neither variable was fully restored in the old animals. Liver weight and protein content in the old group fed VHPD were higher than in the group fed HPD but remained significantly lower than in the control group.

Plasma amino acid concentrations

Dietary restriction and refeeding had no significant effect on plasma glutamine + glutamate concentrations in adult rats (**Figure 3**), but concentrations of these amino acids were reduced in old diet-restricted rats. In addition, refeeding failed to restore these concentrations in old rats. Plasma arginine concentration was significantly reduced in adult and old rats fed the HPD compared with controls.

Hydrogen peroxide production by granulocytes

Dietary restriction induced a decrease in oxidative activity in polymorphonuclear neutrophils and monocytes from adult and old animals ($P < 0.01$ compared with controls; **Figure 4**). Refeeding induced a significant increase in hydrogen peroxide production in adult rats whatever the diet. Higher hydrogen peroxide production by polymorphonuclear neutrophils and monocytes was observed after the VHPD in adult and old rats compared with the diet-restricted groups.

TNF-α production by peritoneal macrophages

Food restriction was associated with a reduction in TNF-α production by peritoneal macrophages in adult and old rats, although these variations were not significant because of the wide variability of values (**Figure 5**). Nevertheless, adult and old groups fed HPD and VHPD showed an increase in TNF-α secretion in stimulated macrophages compared with diet-restricted adult and old rats.

DISCUSSION

In a previous study (10), we showed the effect of a 6-wk, 50% dietary restriction in adult and old rats: adult rats adapted to a chronic 50% dietary restriction by a rapid stabilization of body weight, preservation of muscle weight and protein content, and moderate deterioration of nitrogen balance. Unlike adult rats, old rats had a defect in adaptation to dietary restriction that resulted in severe weight loss, depletion of visceral protein content, and deterioration of nitrogen balance (9, 10). This difference in the kinetics of response to dietary restriction between adult and old rats makes it difficult to compare young and old rats receiving renutrition. Hence, models of dietary restriction had to be defined that would induce fairly similar nutritional alterations in both adult and old rats. The results of preliminary work (Table 2), in which the duration of dietary restriction and protein intake was varied, indicated that a similar malnourished state could be obtained by using a 50% dietary restriction regimen with less protein (5% compared with 17%) associated with a shorter duration of dietary restriction (6 compared with 12 wk) in adult rats than in old rats (Table 2). However, because the duration of dietary restriction was different between adult and old rats, we cannot be

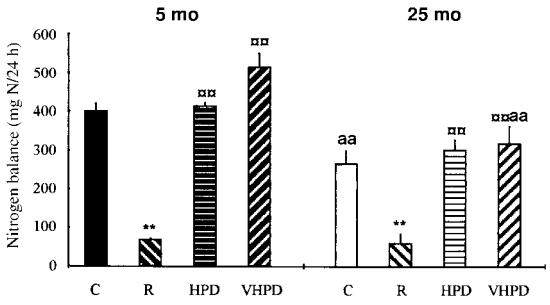

FIGURE 2. Mean (±SEM) nitrogen balance after dietary restriction and refeeding in adult (5 mo old) and old (25 mo old) rats. Groups are as defined in Materials and Methods and in Figure 1: C, control group; R, restricted group; HPD, group fed high-protein diet; VHPD, group fed very-high-protein diet. Results are the difference between daily nitrogen intake and daily nitrogen excretion. Two-way ANOVA was performed to discriminate among effects of aging, diets, and their interaction. Significant effects of aging ($P < 0.0001$) and diet ($P < 0.0001$) with a significant interaction ($P < 0.05$) were observed. Comparison of individual means was carried out by using the Newman-Keuls test: $^{**}P < 0.01$ compared with the C group of the same age, $P < 0.01$ compared with the R group of the same age, $^{aa}P < 0.01$ compared with 5-mo-old rats consuming the same diet.

TABLE 4
Tissue weight and protein content after dietary restriction and refeeding in adult and old rats[1]

	Adult (5 mo old)			Old (25 mo old)		
	Soleus	Tibialis anterior	Liver[2]	Soleus	Tibialis anterior	Liver[2]
Weight (g)						
Control	0.37 ± 0.01	1.76 ± 0.08	12.9 ± 0.2	0.37 ± 0.02	1.37 ± 0.07	18.1 ± 0.7[3]
Restricted	0.29 ± 0.01	1.42 ± 0.02	7.7 ± 0.2[4]	0.37 ± 0.03	1.17 ± 0.11	8.5 ± 0.6[4]
HPD	0.33 ± 0.01	1.56 ± 0.03	11.8 ± 0.3[5]	0.34 ± 0.05	0.93 ± 0.15[3,4]	10.6 ± 0.7[4]
VHPD	0.34 ± 0.01	1.50 ± 0.04	12.9 ± 0.5[5]	0.46 ± 0.04	1.64 ± 0.17[6]	14.6 ± 0.6[4,5,6]
Protein content (mg)						
Control	35.7 ± 2.1	165.0 ± 8.7	2.9 ± 0.1	30.8 ± 1.6	121.9 ± 8.7	3.9 ± 0.2[3]
Restricted	24.5 ± 0.9	141.2 ± 3.1	1.5 ± 0.1[4]	29.1 ± 2.4	110.6 ± 15.0	1.8 ± 0.1[4]
HPD	29.5 ± 1.7	148.6 ± 2.5	2.4 ± 0.1[5]	26.6 ± 3.9	90.0 ± 8.8	2.1 ± 0.1[4]
VHPD	28.8 ± 1.2	145.8 ± 4.1	2.6 ± 0.1[5]	38.3 ± 3.8	156.6 ± 17.0	2.9 ± 0.1[4,5,6]

[1] $\bar{x}$ ± SEM; n = 6–11 rats per group. Control rats were fed ad libitum, restricted rats were diet restricted then killed, HPD rats were diet restricted then refed a high-protein diet (1.6 g casein hydrolysate) for 1 wk, VHPD rats were diet restricted then refed a very-high-protein diet (3.2 g casein hydrolysate) for 1 wk. There were significant effects of aging and diet and an interaction of aging and diet on tibialis anterior weight, liver weight, and liver protein content; and there were significant effects of aging and diet on tibialis anterior protein content ($P < 0.05$, two-way ANOVA). A comparison of marginal means with the Newman-Keuls test indicated a significant effect of aging on tibialis anterior protein content, $P < 0.01$.

[2] Protein content of liver is also in grams.

[3] Significantly different from adults consuming the same diet, $P < 0.01$ (Newman-Keuls test).

[4] Significantly different from the control group of the same age, $P < 0.01$ (Newman-Keuls test).

[5] Significantly different from the restricted group of the same age, $P < 0.01$ (Newman-Keuls test).

[6] Significantly different from the HPD group of the same age, $P < 0.01$ (Newman-Keuls test).

certain that the status of other nutrients, especially micronutrients, was equivalent between groups at the end.

During the refeeding period in this study, 2 diets containing different amounts of casein hydrolysate as the source of protein supplementation were studied. It was shown in other studies that a period of undernutrition induces a decrease in brush border peptidase activity, which in turn may lead to abnormal protein digestion (20, 21). Hence, providing the protein supplement as whole proteins may have introduced bias, especially for the groups fed a very-high-protein diet. Providing a protein supplement as a hydrolysate circumvents this difficulty.

The choice of the amount of protein supplementation was also difficult. We supplied 23% (HPD) and 27% protein in the diet (VHPD) to adult and old malnourished rats. This choice was based primarily on the literature: Chyun and Griminger (22) showed that old rats cannot maintain a positive nitrogen balance with diets containing 5% or 10% protein, even in the absence of stress. Poullain et al (12, 23) described weight gain, improvement of nitrogen retention, and increase in intestinal morphometry and enzyme activities in adult rats after 72 h of food deprivation followed by 24–96 h of ad libitum refeeding with a 27% protein diet in the form of casein hydrolysate. Last, studies in clinical patients (4, 5) indicated that malnourished elderly persons are insensitive to a diet containing a standard amount of protein. Also, because early efficiency of renutrition is mandatory in elderly people to avoid complications (especially infections), it would be most useful to supply supplements that could act as a starter of renutrition.

When considering the high cost of old rats, we decided it was not worthwhile to study rats refed with the standard 17% protein diet and focused our efforts on the HPD and VHPD. The results obtained with these 2 diets vindicate that choice.

Like Reville et al (24), we showed that refeeding food-restricted rats with a high-protein diet induced better body weight recovery in young than in old rats. This age-related difference did not result from changes in food intake because all the rats ingested between 23 and 26 g diet during refeeding. However, because the old rats weighed more than the adults (402 g compared with 315 g), the intake of energy per gram body weight supplied to the old rats was lower than that in their adult counterparts. On the other hand, the difference in weight corresponds roughly to fat mass. Expressing food intake according to fat-free mass would probably indicate very little or no difference between the age groups. In any case, a very-high-protein diet was essential to improve body weight in the old rats.

At the intestinal level, adaptive potential seemed well preserved in the old rats, as evidenced by the rapid improvement of jejunal and ileal morphometry with refeeding. In adult rats, many studies have shown that starvation or malnutrition results in hypofunction and morphologic atrophy of the small intestine, which are reversed in the jejunum (12) and the ileum (25) after 48–96 h of refeeding. However, we observed that adult rats were unable to adapt to ileal modifications induced by dietary restriction. This difference between old and adult rats in refeeding was related to crypt depth variations, suggesting impaired cell renewal. Holt et al (26) showed that the proximal and distal small intestines of old rodents responded to starvation by rapidly reducing crypt cell proliferation rate, and to resumption of food intake by an abnormal increase in proliferation of epithelial cells. In addition, in old animals the nutritional control of intestinal cell proliferation by polyamines seems to be altered (27). Hence, the intrinsic age-related changes in intestinal structure are shown by an incapacity to respond correctly to nutritional variations, ie, undernutrition and refeeding. In addition, a study in elderly men (28) showed that splanchnic extraction of dietary leucine increases with aging. This high splanchnic extraction of amino acids might also explain the higher intestinal response to refeeding, in particular the increase in cell renewal, and the lower plasma amino acid concentrations observed in the present study in old rats refed HPD compared with younger ones.

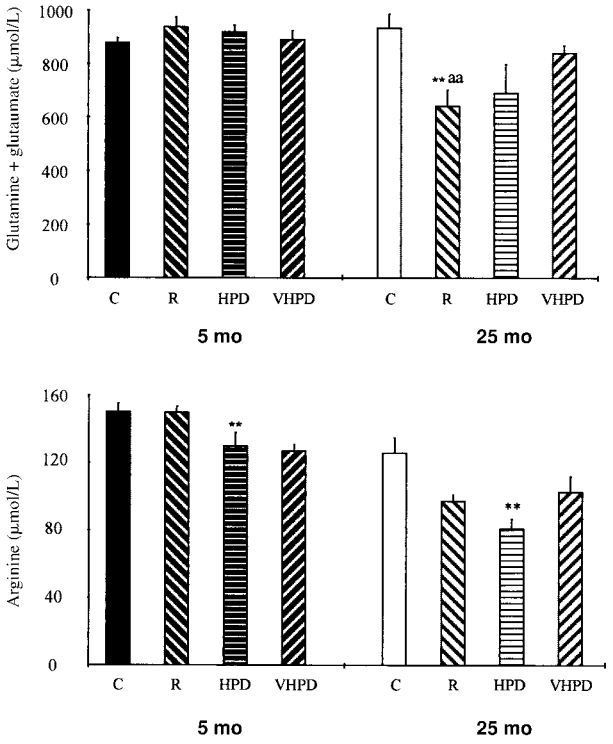

FIGURE 3. Mean (±SEM) plasma glutamine + glutamate and arginine concentrations after dietary restriction and refeeding in adult and old rats. Groups are as defined in Materials and Methods and in Figure 1: C, control group; R, restricted group; HPD, group fed high-protein diet; VHPD, group fed very-high-protein diet. Two-way ANOVA was performed to discriminate among effects of aging, diets, and their interaction. A significant effect of aging ($P < 0.05$) and a significant interaction between aging and diet effects ($P < 0.01$) was observed for glutamine + glutamate. In addition, significant effects of aging ($P < 0.05$) and diet ($P = 0.002$) without interaction were shown for arginine. We used the Newman-Keuls test to compare individual means of plasma glutamine + glutamate concentration: $^{**}P < 0.01$ compared with the C group of the same age, $^{aa}P < 0.01$ compared with the 5-mo-old rats consuming the same diet; when marginal means of plasma arginine concentration were compared, $^{**}P < 0.01$ compared with the C groups.

Finally, it may be that supplying a VHPD to malnourished old rats saturates amino acid utilization in visceral tissues and therefore allows replenishment of the plasma pool of amino acids. A VHPD could thereby allow a higher amino acid impregnation of peripheral tissues, improving muscle weight in old animals fed such a diet.

Refeeding restored nitrogen balance irrespective of the diet used or the age of the rats. However, nitrogen balance was lower in refed old rats than in similar groups of adult rats. These results, consistent with the literature (13, 29, 30), indicate that a protein-enriched diet led to a greater nitrogen retention in adult animals than in old ones. Whether the reduced positive nitrogen balance in the old animals is due only to a delay in achieving normal positive nitrogen balance remains to be determined. The main determinants of nitrogen balance are the net protein balance in the liver, where protein turnover is rapid, and in the muscles, which contain the largest stores of proteins. A decrease in liver weight and protein content was observed in response to dietary restriction, as was a reduction of plasma albumin concentration in old rats, with no improvement when the rats were refed with the HPD, illustrating the frail nutritional status of this group. Munro et al (31) found that albumin synthesis was not stimulated by a normal-protein diet after a low-protein regimen in elderly people. In the present study, the VHPD corrected hypoalbuminemia in old diet-restricted rats, indicating that supplying a higher-protein diet may allow increased hepatic protein synthesis in malnourished old rats.

Immunocompetent cells rely on amino acids as energy substrates (16–18). In agreement with the literature (32–34), we showed here that protein-energy malnutrition caused immunologic abnormalities that were especially marked in the old rats. In our model, we showed that age associated with protein-energy malnutrition induces a drop in hydrogen peroxide production by stimulated polymorphonuclear neutrophils and monocytes, ie, a reduction of microbicidal molecule generation (35). In addition,

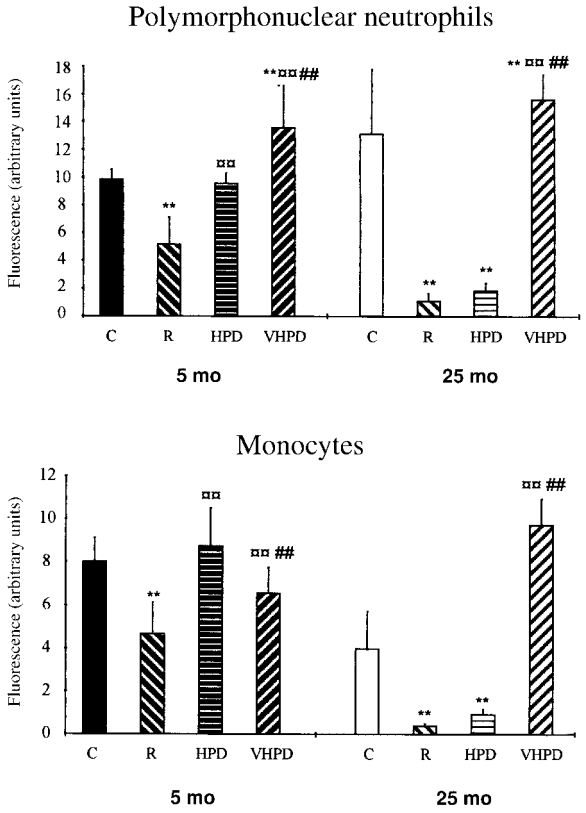

FIGURE 4. Mean (±SEM) hydrogen peroxide production by polymorphonuclear neutrophils and monocytes after dietary restriction and refeeding in adult (5 mo old) and old (25 mo old) rats. Groups are as defined in Materials and Methods and in Figure 1: C, control group; R, restricted group; HPD, group fed high-protein diet; VHPD, group fed very-high-protein diet. Results are expressed as the ratio of stimulated-leukocyte fluorescence to nonstimulated-cell fluorescence. Two-way ANOVA was performed to discriminate among effects of aging, diets, and their interaction. A significant effect of diet ($P < 0.05$) was observed in polymorphonuclear neutrophils and monocytes. Comparison of marginal means was carried out by using the Newman-Keuls test: $^{**}P < 0.01$ compared with the C group, $^{¤¤}P < 0.01$ compared with the R groups, $^{\#\#}P < 0.01$ compared with the group fed the HPD.

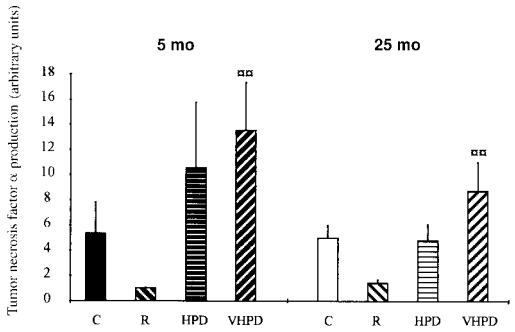

FIGURE 5. Mean (±SEM) tumor necrosis factor α production by peritoneal macrophages after dietary restriction and refeeding in adult (5 mo old) and old (25 mo old) rats. Groups are as defined in Materials and Methods and in Figure 1: C, control group; R, restricted group; HPD, group fed high-protein diet; VHPD, group fed very-high-protein diet. Results are expressed as the ratio of stimulated-macrophage production to nonstimulated macrophage production. Two-way ANOVA was performed to discriminate among effects of aging, diets, and their interaction. A significant effect of diet was observed ($P < 0.01$). Comparison of marginal means was carried out by using the Newman-Keuls test: $P < 0.01$ compared with the R groups.

we observed a reduction in TNF-α secretion by stimulated peritoneal macrophages, which could induce metabolic and immune impairment (36, 37). The depleted immunologic status was not improved by the HPD in the old rats, whereas the VHPD improved both plasma amino acid concentrations and nonspecific immune functions in them. Given that some amino acids have biological properties for immunologic cells, in particular glutamine (17, 38) and arginine (18), which are considered to be modulators of immunity, it may be that a greater peripheral availability of nutrients improves the nonspecific immune response.

In conclusion, as shown in humans (4, 5), the response of rats to renutrition is affected by aging. The results reported here clearly show that nutritional and immunologic variables can be improved when a very high amount of protein is added to the diet of old malnourished rats. Elderly malnourished people may therefore require higher amounts of protein during refeeding, possibly because of increased protein utilization by splanchnic tissues. This higher use may result in a lower availability of nutrients to the peripheral tissues and may contribute to the low anabolic responses during refeeding in the elderly. Thus, studies need to be conducted in malnourished patients with higher-protein diets providing adequate energy intakes to determine the potential nutritional and immunologic roles of such regimens in elderly malnourished people.

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