Hematological and immunological effects of excess dietary leucine in the young rat[1]

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ABSTRACT Young rats were subjected to a dietary leucine overload for several weeks. Although no significant changes in growth, food consumption, and hematological and immune responsiveness occurred when the basic diet was balanced (18% casein), rats which were both overloaded with leucine and subjected to a protein-poor diet (4% casein) displayed a strong impairment of immunological reactions to sheep red cells (rosette and plaque-forming cells in the spleen, serum hemagglutinins, and hemolysins). The effect was significantly more pronounced than in rats on a protein-poor nonsupplemented diet or in pair-fed controls on a balanced diet. The immunodepression was as profound as after complete protein deprivation. It is suggested that a secondary defect in valine and isoleucine utilization may play a role in the harmful effects of excess leucine, because isoleucine and especially valine have been shown to be highly deleterious to lymphopoiesis. *Am. J. Clin. Nutr.* 30: 1645–1654, 1977.

A protein-deprived diet which is protracted for several weeks provokes an important reduction of certain immunological responses in the rat, especially of those responses directed toward heterologous red blood cells. This effect concerns the titers of serum hemagglutinins and hemolysins (1–6) as well as the number of hemolytic plaque-forming cells (PFC) (4–9) and rosette-forming cells (RFC) (3–6, 9), although the latter are much more resistant than PFC in protein-deprived adult rats (3–6).

On the other hand, it has been reported that dietary overload with leucine induced a secondary deficiency in valine and isoleucine (10–12) which may be due to an excessive activation of enzymes of the catabolic pathway of branched chain amino acids (13, 14).

A lack of isoleucine and especially of valine leads to an inhibition of leukopoiesis (granulocytes as well as lymphocytes), with involution of the lymphoid organs, especially the thymus, and a dramatic drop in the blood lymphocyte levels (15–18). Therefore, it was of interest to know whether an overload of leucine also induces lymphopenia and atrophy of the thymus, and whether there is a depression of the immunological potential, which is known to depend primarily on lymphocyte activity.

The experiments reported in this paper indeed revealed an immunodepressive effect of excess intake of leucine, but only in rats sensitized by a protein-poor diet and not in well-nourished animals.

Materials and methods

Animals and diets

Sixty-one young pathogen-free male Charles River rats which initially weighed about 100 g each were divided into seven groups fed the following diets: I—a balanced 18% casein diet[4] (eight rats); II—the same diet with L-leucine (Ajinomoto, Tokyo) (nine rats); III—a diet containing only 4% casein[4] (nine rats); IV—

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[4] This diet contained (in g/100 g of dry weight): vitamin-free casein, 18; sucrose, 55.4; white dextrin, 10; peanut oil, 10; salt mixture, 5.7, choline chloride, 0.5; inositol, 0.06. It also contained the following vitamins (in milligrams): thiamin, 1.0; riboflavin, 2.0; niacin, 20.0; calcium pantothenate, 3.0; pyridoxine, 2.0; folic acid, 0.1; vitamin B_{12}, 0.005; biotin, 0.02; ascorbic acid, 25.0; DL-α-tocopheryl acetate, 1.0; and retinyl palmitate, 5,000 IU, and ergocalciferol, 1,000 IU. The salt mixture (Orent-Keiles mixture no. 22) (19) consisted of (in percentages): $CaCO_3$, 18.90; $CaHPO_4 \cdot 2H_2O$, 12.60; KCl, 14.90; $MgSO_4$, 8.77; $NaH_2PO_4 \cdot H_2O$, 20.10; NaCl, 22.20; NaI, 0.0026; $Na_2SiO_3 \cdot 9H_2O$, 0.61; $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 1.22; $CuSO_4 \cdot 5H_2O$, 0.44; $MnSO_4 \cdot 4H_2O$, 0.087; $ZnCl_2$, 0.18. In the protein-deficient diets casein was partly or completely replaced by sucrose; all the other components were the same.

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the same diet supplemented with L-leucine (nine rats); V—an 18% casein diet given in amounts not exceeding those ingested in group IV (eight rats); and VI and VII—diets completely deprived of protein[4] administered for 4 weeks (10 rats) or 5 weeks (eight rats). The other diets were maintained for 6 weeks (group I) or 8 weeks (Groups II to V). Drinking water was allowed ad libitum. Leucine was introduced into the diet at a concentration of 3% during the first 18 days and at 7% during the following period in order to accustom the animals to this compound.

The rats were housed in individual compartments at 22 C. Body weights were recorded weekly and the dietary intake was measured daily.

The duration of the diet in groups VI and VII was shortened because of the poor resistance of young rats (30 days of age at the start of the experiments) to complete protein deprivation.

Hematological and immunological examinations

On day −8 before killing, the rats of all groups were immunized with an intraperitoneal injection of sheep red blood cells (SRBC) (2.4×10^9 cells/100 g of body weight) without adjuvant added. The animals were killed by cardiac exsanguination. The blood was used for titration of serum antibodies. Thymus, spleen, and cervical nodes were excised free from any adhering connective tissue and weighed. Cell density in the spleen (number of lymphocytes per milligram of tissue) and the total lymphocytic population of this organ was determined (20), as was the percentage of viable lymphocytes. Viability was measured using a dye (erythrosin) exclusion test, which was performed immediately after addition of the dye to the cell suspension (21).

On the day the animals were killed, a leukocyte count was performed on tail blood samples, with counting of granulocytes and lymphocytes per cubic millimeter, as calculated from their percentage distribution.

The immunological tests consisted of the enumeration of RFC and PFC in the spleen according to the methods of Zaalberg (22) and Cunningham and Szenberg (23), respectively, and of the titration of serum antibodies (hemagglutinins and hemolysins) according to methods used previously (4).

Separation of immunoglobulins IgG was accomplished by treating sera with 2-mercaptoethanol (2-ME) (4, 24). The two antibody titers were expressed as decimal logarithms of geometrical means of the highest dilutions giving an agglutination visible with the naked eye or giving complete hemolysis, respectively. In the protein-deficient groups the quantities of serum were too small to permit the separate titration of IgG hemolysins in each rat.

Results

Body weight and food intake (Tables 1, 2, and 3)

The body weight increased almost 4-fold in 6 weeks in group I (18% casein control rats), and growth was not at all delayed after addition of leucine (group II).

In the protein-deficient (groups III and IV) (4% casein) the body weight remained at the initial level. Administration of leucine was without any effect. In control rats of group V pair-fed with those of group IV (4% casein + leucine) the increase in body weight was much lower (only +50% of the initial values) than in groups I and II fed the same balanced diet ad libitum without or with extra leucine, but it was significantly higher than in the 4% casein groups III and IV.

The rats which were completely deprived of protein (Groups VI and VII) lost 35% of their initial weight after 4 (VI) or 5 (VII) weeks. Their aspect and behavior (weakness, inactivity, and thinning of the hair, which became dull, ruffled and dirty) indicated a state of cachexia. Indeed, two rats of the group VII died before the end of the experimental period.

The food intake was also the same in the absence and the presence of extra leucine when the diet was well provided with protein (I and II). In group III (4% casein) the food intake was closer to that of protein-free groups (VI and VII) than to that of the 18% casein groups. Contrary to what had been noticed with the latter groups, a 7% leucine supplement still significantly reduced food ingestion in rats on a 4% casein diet (compare group IV to group III, Table 3). In rats on a protein-free diet, food consumption did not exceed 50% of the initial consumption of nondeficient animals (I and II).

Hematological results

Lymphoid organs (Tables 1 and 4). Expressed per 100 g of body weight (which was necessary because of the unequal duration of certain diets and the difference of final body weights), the weights of the spleen and cervical lymph nodes did not differ significantly in rats whose diets were or were not supplemented with leucine, when the protein intake was adequate. Although the thymus was somewhat smaller in supplemented animals, the lack of significant thymic involution militated against the existence of a nonspecific stress effect of excess leucine when given with a balanced diet (Table 1).

Neither were there any changes in the cellular density and total level of splenic lymphocytes, nor in the viability of these cells after overcharge of leucine (Table 4).

TABLE 1
Body weights and weights of lymphoid organs in rats nourished with balanced or protein-deficient diets—effect of overload with leucine*

Group (no. of rats)	Diet	Duration (days)	Body weight (g)		Weight of the lymphoid organs (mg)					
					Thymus		Cervical lymph node		Spleen	
			Initial	Terminal	Absolute	Relative	Absolute	Relative	Absolute	Relative
I (8)	18% casein ad libitum	42	105.4 ± 3.0	376.5 ± 5.6 b_2; c_{3-7}	790.6 ± 70.3 c_{3-7}	210.6 ± 20.2 b_3; $c_{4,6,7}$	21.8 ± 5.8 $a_{3,4}$; $b_{6,7}$	5.9 ± 1.6	812.9 ± 32.1 c_{3-7}	216.0 ± 8.1 $b_{3,4}$; $c_{6,7}$
II (9)	18% casein + leucine	56	105.3 ± 1.6	426.9 ± 13.4 b_1; c_{3-7}	695.3 ± 47.8 c_{3-7}	164.7 ± 13.5 $c_{4,6,7}$	21.1 ± 3.8 a_5; $b_{3,4}$; $c_{6,7}$	5.0 ± 0.9	829.6 ± 55.7 c_{3-7}	195.6 ± 14.3 a_6; b_7
III (9)	4% casein	56	105.9 ± 3.1	98.8 ± 9.0 $c_{1,2}$	129.7 ± 32.9 b_{5-7}; $c_{1,2}$	114.6 ± 24.5 $b_{1,6,7}$	6.3 ± 0.8 a_1; $b_{2,7}$	6.4 ± 0.5	163.1 ± 19.0 $b_{6,7}$; $c_{1,2,5}$	164.2 ± 11.5 a_7; b_1
IV (9)	4% casein + leucine	56	107.8 ± 3.9	92.4 ± 2.8 $c_{1,2,5-7}$	70.6 ± 5.5 $c_{1,2,5-7}$	76.9 ± 5.9 $c_{1,2,5-7}$	7.0 ± 1.2 $a_{1,7}$; b_2	7.6 ± 1.3	166.7 ± 9.0 $c_{1,2,5-7}$	179.9 ± 5.9 $b_{1,6,7}$
V (8)	18% casein pair-fed with IV	56	108.4 ± 1.3	154.4 ± 4.7 $c_{1-4,6,7}$	255.8 ± 9.1 b_3; $c_{1,2,4-7}$	166.8 ± 7.8 $c_{4,6,7}$	8.2 ± 3.3	5.1 ± 1.8	317.1 ± 32.1 $c_{1-4,6,7}$	202.8 ± 14.6 $b_{6,7}$

In group III (4% casein, nonsupplemented) the absolute weights of the lymphoid organs were obviously much lower than in the 18% casein control group I. That was also the case for the relative weights of the spleens and the thymuses as well as for the lymphocyte number and viability in the spleen.

The addition of leucine to a 4% casein diet (group IV) did not significantly change the weight of the thymuses compared to group III. This was due mainly to the high variability in the latter group, with an equal number of low and high values. An important difference was noticed when the weights of the lymphoid organs ot the 4% casein groups (III and IV) were compared to those of pair-fed group V. The absolute weights of the spleens and thymuses and the total number of splenic lymphocytes were all much higher in the pair-fed animals. These differences were greater between IV and V than between III and V for the relative weights of the thymuses, and for the viability of splenic cells. They were significant only for the ratio IV/V. Thus the changes in the lymphoid organs exhibited by the group III and especially the group IV could not be ascribed exclusively to the decrease in the food intake observed in these animals.

In rats completely deprived of protein (VI and VII) the absolute and relative weights of thymuses and spleens were much lower than in rats of group V (Table 1), and after 35 days of protein deprivation the lymphocyte population of the spleen and the viability of these cells were also below those of rats maintained for 56 days on a 4% casein diet with or without added leucine (Table 4).

Blood leukocytes (Table 5). Although important individual variations were observed even in nondeficient rats, a highly significant leukopenia occurred in the five malnourished groups as compared to the controls, with a selective drop in lymphocyte levels. No differences were noticed between rats that were or were not supplemented with leucine.

Immunological results (Tables 6 and 7)

The RFC and PFC levels and the serum antibody titers were not influenced by the addition of leucine to a balanced diet. A 4% casein diet not overloaded with this amino

TABLE 2
Body weight changes after 4 weeks of diet*

Group	Diet	Body weight changes (g) after 28 days
I	18% casein ad lib.	+208.7 ± 7.6 c_{3-7}
II	18% casein + leucine	+205.5 ± 9.3 c_{3-7}
III	4% casein	−10.4 ± 5.4 b_5; $c_{1,2,6/7}$
IV	4% casein + leucine	−21.8 ± 3.9 $c_{1,2,5,6/7}$
V	18% casein pair-fed with IV	+10.4 ± 2.1 b_3; $c_{1,2,4,6/7}$
VI + VII	Protein-free	−41.5 ± 1.3 c_{1-5}

* Values given are grams ± SEM. The significant differences are presented as in Table 1.

TABLE 3
Mean daily intake per rat and per week in the different dietary groups[a]

Time periods (weeks)	Group I (18% casein)	Group II (18% casein + leucine)	Group III (4% casein)	Group IV (4% casein + leucine)	Group VI (protein-free, 4 weeks)	Group VII (protein-free, 5 weeks)
1st	16.9 ± 0.6	16.8 ± 0.5	10.9 ± 0.4	10.4 ± 0.3	8.0 ± 0.2	7.5 ± 0.5
2nd	21.7 ± 0.8	21.1 ± 0.4	10.9 ± 0.6	10.2 ± 0.3	7.7 ± 0.4	8.5 ± 0.8
3rd	23.7 ± 0.9	24.2 ± 0.4	10.7 ± 0.5	8.3 ± 0.3	8.0 ± 1.0	7.3 ± 0.6
4th	25.6 ± 0.8	25.9 ± 0.7	10.8 ± 1.0	6.9 ± 0.3	6.4 ± 0.4	7.1 ± 1.2
5th	25.4 ± 0.6	25.8 ± 0.4	10.3 ± 0.6	7.7 ± 0.3		6.8 ± 1.2
6th	25.7 ± 0.6	26.0 ± 0.6	10.7 ± 0.6	7.4 ± 0.5		
7th		25.9 ± 0.6	10.3 ± 0.8	7.3 ± 0.4		
8th		24.1 ± 1.0	9.9 ± 0.8	7.7 ± 0.2		

[a] Values given are grams of dry weight ± SEM. The rats of group V pair-fed with group IV consumed completely their daily ration.

TABLE 4
Number and viability of lymphocytes in the spleen as function of the protein levels in diet—effect of overload with leucine*

Group (no. of rats)	Diet	Lymphocytes in the spleen			
		No./mg of splenic tissue	No. in the entire organ ($\times 10^3$)		% viable splenic lymphocytes
			Absolute no.	No./100 g of body weight	
I (8)	18% casein ad lib. (42 days)	1,220,860 ± 62,780 c_{3-7}	990,789 ± 59,876 c_{3-7}	263,168 ± 15,139 b_5; $c_{3,4,6,7}$	62.45 ± 1.99 a_5; $c_{3,4,6,7}$
II (9)	18% casein + leucine (56 days)	1,131,020 ± 57,800 b_{3-5}; $c_{4,6,7}$	944,050 ± 88,180 c_{3-7}	223,908 ± 23,120 b_3; $c_{4,6,7}$	58.26 ± 1.38 $b_{3,6}$; $c_{4,7}$
III (9)	4% casein (56 days)	712,578 ± 101,797 a_7; b_2; c_1	127,684 ± 26,950 a_6; b_7; $c_{1,2,5}$	119,616 ± 19,533 $a_{5,7}$; b_2; c_1	47.51 ± 2.50 a_7; b_2; c_1
IV (9)	4% casein + leucine (56	623,750 ± 58,308	106,226 ± 10,496	113,739 ± 12,387	44.92 ± 2.57

TABLE 5
Numbers of total leukocytes, neutrophils and lymphocytes per cubic millimeter of blood after administration of various diets*

Group	Diet (duration)	Total leukocytes	Neutrophils	Lymphocytes
I	18% casein ad lib. (42 days)	19,562 ± 1,607 c_{3-7}	2,498 ± 1,178	16,963 ± 839 c_{3-7}
II	18% casein + leucine (56 days)	19,747 ± 2,176 $b_{3,6}$; $c_{4,5,7}$	3,747 ± 1,261	15,827 ± 1,352 c_{3-7}
III	4% casein (56 days)	9,433 ± 1,461 b_2; c_1	1,949 ± 528	7,444 ± 1,165 $c_{1,2}$
IV	4% casein + leucine (56 days)	8,400 ± 979 $c_{1,2}$	1,644 ± 241	6,942 ± 537 $c_{1,2}$
V	18% casein pair-fed (56 days)	6,000 ± 1,015 $c_{1,2}$	818 ± 166	5,090 ± 853 $c_{1,2}$
VI	Protein-free (28 days)	10,415 ± 1,198 b_2; c_1	2,408 ± 641	7,947 ± 696 $c_{1,2}$
VII	Protein-free (35 days)	6,975 ± 950 $c_{1,2}$	1,435 ± 241	5,540 ± 785 $c_{1,2}$

* Values given are ± SEM. The significant differences are presented as in Table 1.

acid (group III) did not induce by itself any significant decrease in the immunological responses studied except for a drop in the total number of RFC and PFC in the spleen as a whole, undoubtedly because of the atrophy of this organ.

On the contrary, the same protein-poor diet supplemented with leucine (group IV) provoked a dramatic fall of the RFC and PFC number, not only per entire spleen but also per 10^6 splenic lymphocytes. Significant decreases in serum antibody titers were also observed as compared to those of 4% casein nonsupplemented rats. This anomaly concerned primarily 2-ME-resistant immunoglobulins (IgG). These Ig's were completely absent 1 week after immunization in all of the rats of group IV (4% casein with extra leucine) which were examined. Separation of IgG could be carried out in seven rats of this group for hemagglutinins but only in three rats for hemolysins because of the low amounts of serum available.

In pair-fed controls (group V) the levels of RFC and PFC were significantly higher than in group IV (4% casein with extra leucine) although they were much lower than in the controls fed ad libitum (group I). However, the serum antibody titers did not differ from those of the latter group except for IgG hemolysins.

Rats completely deprived of protein (groups VI and VII) displayed a highly significant drop in RFC/10^6 lymphocytes as well as per entire spleen when compared to rats of the 18% casein groups (I and II) and to those of the 4% casein group (III) nonsupplemented with leucine. There was, however, no significant difference between protein-deprived rats and 4% casein rats supplemented with leucine (group IV). The number of PFC was also sharply decreased in almost all protein deprived animals (groups VI and VII).

Total titers of serum hemagglutinins and hemolysins were significantly decreased as compared to 18% casein controls only in group VII (35 days of protein deprivation), but hemagglutinin levels of the IgG class were low in both protein-deprived groups. IgG hemolysins were completely absent in the three rats of each of the groups VI and VII for which their separation could be performed.

Thus the tests employed in the present research revealed that a protein-poor diet (4% casein) overcharged with leucine given for 56 days was as immunosuppressive as a diet which was completely deprived of protein for 4 or 5 weeks. A protein-poor diet without extra leucine resulted only in a decrease in the total number of splenic immunocytes without any change in their concentration or in the titers of serum antibodies.

Discussion

1) The relatively good tolerance of excess leucine, when given to rats with a diet bal-

PFC	per entire spleen
	93,500 ± 9,160
	c_{3-6}
	118,550 ± 26,030
	c_{3-6}
	9,000 ± 3,680
	a_4; $c_{1,2}$
	817 ± 279
	a_4; $c_{1,2}$
	8,260 ± 2,110
	$b_{4,6}$; $c_{1,2}$
	932 ± 385
	b_5; $c_{1,2}$
	1,299
	(32; 536; 0; 4628)

anced in its protein content, has already been reported by Harper et al. using 1.5 or 3.0% of this amino acid/100 g of dry food (25), and only temporary retardation of growth was observed with 5 or 7% of leucine when it was added to a low-protein (9% casein) diet (26, 27). As in our experiments, food intake was depressed after a low-pro-

TABLE 7
*Effect of excess leucine on serum antibody titers in rats fed a balanced or protein-deficient diet and immunized to SRBC**

Group	Diet (no. of rats)	Hemagglutinins (log 10)		Hemolysins (log 10)	
		Total	IgG	Total	IgG
I	18% casein ad libitum (8)	3.424 ± 0.097 b_7; c_4	2.634 ± 0.136 $a_{6,7}$	2.634 ± 0.075 $c_{4,7}$	1.731 ± 0.136 a_5; $c_{6,7}$
II	18% casein + leucine (9)	3.712 ± 0.071 $b_{3,5}$; $c_{4,7}$	3.010 ± 0.194 $b_{6,7}$	2.776 ± 0.110 b_6; $c_{4,7}$	1.919 ± 0.170 b_5; $c_{6,7}$
III	4% casein (9)	2.876 ± 0.261 $b_{2,4}$	2.236 ± 0.399 (7 rats)	2.408 ± 0.399 (7 rats) b_7; c_4	0.903 ± 0.535 (4 rats)
IV	4% casein + leucine	1.940 ± 0.134	0	1.003 ± 0.174	0

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