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Triiodothyronine Improves the Primary Antibody Response to Sheep Red Blood Cells in Severely Undernourished Weanling Mice[1] (42565)

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Abstract. Three experiments were conducted in which weanling mice were fed a nutritionally complete diet either *ad libitum* or in restricted quantities such that they lost about 30% of their initial weight over a 14-day period. In Experiments 1 and 2, half the animals from each group received dietary triiodothyronine (T_3) supplements. In Experiment 3, food-intake-restricted mice were fed graded levels of potassium iodide. Malnutrition reduced the number of nucleated cells per spleen, the number of splenic IgG plaque-forming cells (PFC) per 10^6 cells, and the serum antibody titers against sheep red blood cells. T_3 supplements increased antibody titers, the number of nucleated cells per spleen, and both IgM and IgG PFC per 10^6 spleen cells in malnourished mice, but had no effect on well-nourished mice. The beneficial effect of T_3 was not a result of improved protein, energy, or iodine status in the malnourished mice.

Impairments of thymus-dependent immune functions are widely recognized in severely protein–energy malnourished humans and animals (1). Little consideration, however, has been given to the possibility that the observed immunological impairments result, not directly from the nutritional deficiency, but indirectly from the altered hormonal profile (2) which occurs in malnutrition. A corollary to this hypothesis is that immune functions of malnourished humans and animals might be improved by manipulation of hormone levels without the necessity of improving nutritional status. In the present work this hypothesis was tested using triiodothyronine (T_3).

Serum levels of total and free T_3 are depressed in severely protein–energy malnourished humans (2), rats (3), and mice (4). In well-nourished animals serum thyroid hormone levels correlate with thymus-dependent immune functions (5–8) and serum thymulin levels (9, 10). Moreover, unpublished preliminary results from the authors' laboratory revealed a several-fold increase in primary serum hemagglutinin titer to sheep red blood cells (SRBC) when severely food-intake-restricted weanling mice were fed diets containing various T_3 levels between 0.1 and 0.3 mg T_3/kg diet ($n = 35$ per group, $P < 0.05$ by two-tailed Student's t test). The present experiments were conducted to investigate more fully this apparent effect of T_3 supplements on a thymus-dependent immune function, the primary antibody response to SRBC, of severely undernourished weanling mice.

Materials and Methods. *Animals and dietary treatments.* Three experiments were conducted using 21-day-old weanling male CBA/J mice. Animals were individually housed in a windowless room maintained at 28°C. Lights were on from 7:00 AM to 9:00 PM. Mice were acclimated for 2 days to a nutritionally complete diet described previously (11). They were then fed this diet for 14 days either *ad libitum* (C mice) or in restricted daily amounts such that the animals lost about 30% of their initial body weight in an approximately linear manner during the 14-day feeding period (R mice). In Experiments 1 and 2 half the animals from each of the *ad libitum* and restricted groups received dietary T_3 (Sigma Chemical Co., St. Louis, MO) at a level of 0.2 mg/kg diet (groups designated CT_3 and RT_3). T_3 was mixed into the diet at the required level by first preparing a premix of oil-free diet containing 0.1 mg T_3/g. In Experiment 3 restricted mice were fed either the basal

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diet (0.25 mg/kg iodine) or the basal diet supplemented with potassium iodide to contain 2.5 or 25 mg/kg iodine (groups designated RI1 and RI2, respectively). The latter supplementation levels were chosen to ensure adequate iodine absorption but to avoid iodine toxicity (12). Body weights and feed intakes of the mice in all experiments were determined daily.

Experiment 1. After 9 days on the experimental diets, mice were immunized intraperitoneally with 4×10^8 washed SRBC. Five days later mice were anesthetized with Metofane (Pitman-Moore, Inc., Washington Crossing, NJ) and orbital blood samples were taken for measurement of hemagglutinin titers and serum free T_3 levels. Spleens were removed for measurement of the plaque-forming cell (PFC) response to SRBC.

Experiment 2. This experiment was conducted in order to assess the nutritional status of mice treated as in Experiment 1. Diets and handling of mice were as described in Experiment 1 except that animals were not immunized. At the end of the 14-day feeding period orbital blood samples were taken for measurement of serum protein levels by the biuret method (13) and serum free T_3. Spleens were removed and weighed. Carcasses were analyzed for protein content by the Kjeldahl procedure (14) and for lipid content by the method of Bligh and Dyer (15).

Experiment 3. This experiment was conducted to determine whether the effect of added dietary T_3 in Experiment 1 was due to the increased iodine intake of the T_3-supplemented animals. Mice were fed for 14 days according to one of four dietary protocols—C, R, RI1, or RI2—and were handled as in Experiment 1.

Serum free T_3 level. Serum free T_3 levels were measured by radioimmunoassay using a kit from Amersham (Oakville, Ontario). Reagents and serum were used at half the volumes suggested by the manufacturer. Preliminary work showed that this modification, which permitted analyses of 50 μl of serum, did not affect results.

Plaque-forming cell assay. Preliminary work indicated that the number of both direct and indirect plaques in well-nourished mice (CBA/J males at 5 weeks of age) was maximal 5 days after immunization. Splenic plaque-forming cells (PFC) were measured essentially according to the method of Cunningham and Szenberg (16). Cell suspensions were prepared by passing spleens through 100-mesh wire screens into medium (RPMI 1640 containing 10% fetal calf serum; Flow Laboratories, Inc., Mississauga, Ontario). Cell viability, as measured using trypan blue, was always greater than 95%. The reaction mixture consisted of 100 μl of spleen cell suspension containing between 5×10^4 and 10^6 nucleated cells, 40 μl of 25% washed SRBC, 50 μl of a 1:4 dilution of guinea pig serum in medium and (for direct plaques) 10 μl of medium or (for indirect plaques) 10 μl of a 1:50 dilution, in medium, of goat anti-mouse IgG Fc fragment (Daymar Laboratories, Inc., Toronto, Ontario). Slides were incubated for 2 hr at 37°C before plaques were counted. Unimmunized, well-nourished mice produced 1–3 IgM PFC/10^6 cells and no IgG PFC.

Hemagglutinin titers. Serum hemagglutinin titers were measured by a standard method (17). Plates were allowed to settle for 2 hr at 37°C. Titer was determined as the base 2 logarithm of the reciprocal of the highest serum dilution at which spreading of SRBC was observable. The minimum titer detectable by this method was 2. Unimmunized, well-nourished mice produced no detectable response.

Statistics. Statistical analyses were performed according to the SAS User's Guide (18). For Experiments 1 and 2, factorial (2×2) analyses of variance were performed and orthogonal contrasts were used to compare least-squares means. For Experiment 3 a one-way analysis of variance was used and means were compared by least significant difference (LSD). Where necessary, data were normalized by log transformation before analyses were performed. In these cases, antilogs of the log means are quoted in the tables. If data could not be normalized by transformation, comparisons were made with the Kruskal–Wallis nonparametric test. For all data, only a priori comparisons were made, i.e., in Experiments 1 and 2 between the groups C and R, C and CT_3, and R and RT_3, and in Experiment 3 between groups C and R, R and RI1, and R and RI2. This approach was chosen mainly so that T_3- or iodine-related effects would be compared only between groups of animals exhibiting similar weight changes. Moreover the procedure serves to emphasize that the main

TABLE I. EXPERIMENT 1: INITIAL WEIGHTS, WEIGHT CHANGES, FEED INTAKES, AND SERUM FREE T_3 LEVELS

Group	Initial weight (g)	Weight change (% of initial)	Feed intake (g/14 days)	Serum free T_3 (pg/ml)
C	10.8	93	40.3	0.55
CT_3	9.5	100	40.1	4.88
R	10.8	−25[a]	14.9[a]	0.06[a,b]
RT_3	11.3	−28	18.6[c]	0.91[c]
Error mean square	2.477	276.54	5.059	0.0275[d]

[a] R value significantly different from C.
[b] All values below detection limit of assay.
[c] Significantly different from unsupplemented of same dietary group.
[d] From ANOVA using log-transformed data. Mean values are antilogs of log means.

comparisons relevant to the present hypothesis were between group R and restricted groups with T_3 or iodine supplement. A significance level of 5% was used.

Results. *Experiment 1.* Two of eleven RT_3 mice died during the feeding period but there were no mortalities in any other group. All results are given for surviving mice only, of which there were nine in each restricted group and eight in each *ad libitum* group.

Initial weights, weight changes expressed as percentage of initial weight, 14-day feed intakes, and serum free T_3 levels are shown in Table I. Feed intakes of RT_3 mice were greater than those of R mice although the degree of weight loss was not different in the two groups. T_3 supplementation had no effect on either weight gain or feed intake of the *ad libitum*-fed mice. The R protocol decreased serum free T_3 level as reported previously from this laboratory (4). Dietary T_3 supplements increased free T_3 levels in both restricted and *ad libitum*-fed mice.

Splenic nucleated cell numbers and antibody responses to SRBC are shown in Table II. Although spleens were small in the malnourished mice, the number of IgM-secreting cells on a per 10^6 cell basis in R mice was not different from that in C mice. The number of IgG PFC per 10^6 cells and of both IgM and IgG PFC per spleen was decreased in the R group. T_3 supplements increased the number of nucleated cells recovered per spleen and the number of both IgM- and IgG-secreting cells expressed both per spleen or per 10^6 cells in the malnourished mice, but had no significant effect on any of these parameters in the well-nourished mice. Furthermore, only one of nine R mice had any detectable IgG, but six of nine RT_3 mice had detectable IgG PFC.

TABLE II. EXPERIMENT 1: SPLENIC NUCLEATED CELL NUMBERS, PLAQUE-FORMING CELL RESPONSES AND HEMAGGLUTININ TITERS TO SRBC

Group	No. of cells/spleen ($\times 10^{-6}$)	PFC/10^6 cells		PFC/spleen ($\times 10^{-3}$)		Hemagglutinin titer[a]
		IgM	IgG	IgM	IgG	
C	79	246	182	17.9	14.2	7.0
CT_3	104	269	255	22.6	26.4	6.7
R	7[b]	640	2[b]	4.0[b]	0.01[b]	4.6[b]
RT_3	11[c]	1414[c]	27[c]	11.6[c]	0.3[c]	6.3[c]
EMS[d]	0.0187[e]	443952	0.6474[e]	0.1055[e]	0.6446[e]	1.041

[a] Reciprocal of base 2 log of maximum dilution which produced spreading of SRBC.
[b] R value significantly different from C.
[c] Different from unsupplemented of same food intake level.
[d] Error mean square.
[e] From ANOVA using log transformed data. Mean values are antilogs of log means.

These proportions were different by the Chi-square test for association ($\chi^2 = 8.40$, $P < 0.005$). Finally, the R protocol decreased serum hemagglutinin titers, and T_3 increased the mean titer of the food-intake-restricted mice, but had no effect on the mean titer of the well-nourished mice.

Experiment 2. One of 10 R mice and three of 10 RT_3 mice died during the experiment, but there were no mortalities in the *ad libitum*-fed groups. Physical parameters and carcass composition data are shown in Table III. Weight changes, feed intakes, and serum free T_3 levels were similar to those found in Experiment 1. Splenic index (mg spleen/g body weight) showed the same pattern as spleen cell number in Experiment 1, i.e., this parameter was decreased by the R protocol but was larger in RT_3 mice than in R mice. Serum protein level and percentage carcass protein and lipid were decreased in the malnourished mice and were not increased by T_3 supplementation. T_3 decreased serum protein level in both well-nourished and malnourished mice.

Experiment 3. Two of 16 RI1 and one of 14 RI2 mice died during the feeding period but there were no mortalities in groups R or C. The restriction protocols decreased weight gain, feed intake, serum free T_3 level, spleen cell number, IgM PFC/spleen, and IgG PFC expressed per spleen and per 10^6 splenocytes and hemagglutinin titers, but had no effect on IgM PFC per 10^6 cells compared to control (Tables IV and V). Neither level of iodine supplementation affected any of the parameters measured when compared to group R.

Discussion. The present results support the hypothesis that the T-dependent antibody response, at least to a strong immunogen such as SRBC, can be improved in severely malnourished individuals independently of their nutritional status. It will be of interest to determine whether the beneficial effect of T_3 extends to other immune responses in malnourished animals. The objective of this work is of significance because, regardless of medical care, severely malnourished individuals frequently die of infectious disease before they can be nutritionally rehabilitated. It must be emphasized, however, that low T_3 levels in malnutrition represent an adaptive response to this condition, in particular for the conservation of peripheral tissues as protein and energy reserves (19). Presumably the low serum protein level in the RT_3 group reflects a degree of deadaptation to malnutrition imposed by an elevation in metabolic rate. Any clinical application of the present findings with T_3 will therefore be limited to short-term therapies, perhaps in conjunction with nutritional support. It would be of great interest to determine the influence, in the present experimental sys-

TABLE III. PHYSICAL PARAMETERS OF MICE IN EXPERIMENT 2[a]

Group	Initial weight (g)	Weight change (% of initial)	Splenic index (mg/g)	Feed intake (g/14 days)	Serum free T_3 (pg/ml)	Serum protein (g/dl)	Carcass analysis (% wet wt)	
							Protein	Lipid
C	12.0	83	3.7	43.5	0.39	6.8	16	6.2
CT_3	12.2	71	4.5	43.7	3.29[b]	5.2[b]	15	6.3
R	11.0	−26[c]	1.1[c]	16.3[c]	0.06[d]	6.1[c]	13[c]	3.0[c]
RT_3	10.8	−28	1.3[b]	18.3[b]	0.89[b]	5.2[b]	14	2.5
Error mean square	3.288	225.55	0.00641[e]	0.00192[e]	—[f]	0.00181[e]	—[f]	—[f]

[a] *N* values for all data but serum protein levels are C, 8; CT_3, 8; R, 9; RT_3, 7; for serum protein: C, 8; CT_3, 8; R, 8; RT_3, 6.

[b] Significantly different from unsupplemented of same dietary group.

[c] R value significantly different from C.

[d] Seven of nine values below detection limit of assay.

[e] From ANOVA using log-transformed data. Mean values are antilogs of log means.

[f] Kruskal–Wallis test. Rank sums for free T_3 levels were C, 116; CT_3, 225; R, 45; RT_3, 142; for carcass protein: C, 204; CT_3, 177; R, 56; RT_3, 91; for carcass lipid: C, 189; CT_3, 191; R, 90; RT_3, 58.

TABLE IV. EXPERIMENT 3: INITIAL WEIGHTS, WEIGHT CHANGES, FEED INTAKES, AND SERUM FREE T_3 LEVELS[a]

Group	Initial weight (g)	Weight change (% of initial)	Feed intake (g/14 days)	Serum free T_3 (pg/ml)[b]
C	12.5	70	37.3	0.47
R	12.7	−30[c]	16.1[c]	0.05[c]
RI1	12.8	−29	16.4	0.08
RI2	11.9	−30	15.6	0.06
Error mean square	1.570	29.306	5.963	—[d]

[a] *N* values for initial weight, weight change, and feed intake were C, 8; R, 12; RI1, 14; RI2, 13; for free T_3: C, 8; R, 11; RI1, 13; RI2, 11.

[b] Ten of 11 R, 11 of 13 RI1, and nine of 11 RI2 values were below detection limit of assay.

[c] R value significantly different from C.

[d] Kruskal–Wallis test. Rank sums were C, 307; R, 186.5; RI1, 248.5; RI2, 204.

tem, of a thyromimetic drug such as SK & F L-94901. This newly developed compound appears to exert some of the effects of T_3 but with minimal influence on metabolic rate (20).

Despite the relatively short feeding period used in the present investigation, the R protocol produces animals which exhibit many features of nonedematous protein–energy malnutrition (PEM) rather than simply acute starvation. With regard to thyroid hormones, acute starvation reduces the serum T_4/T_3 ratio (21) whereas the present protocol increases this ratio many-fold (4). Moreover free and total T_3 and total T_4 levels in the serum are reduced in our model to an extent seen only in chronic human and rodent malnutrition (4). In addition, acute starvation causes elevation in serum zinc (22) and blood hemoglobin (23) levels, whereas these are normal or reduced in human PEM (24, 25) and in mice subjected to the present R protocol (unpublished results). The severity of malnutrition imposed by the present restriction protocols is apparent particularly from the weight loss and mortality results. The hypoproteinemia in R mice is unlikely to be of biological significance except that if may reflect incipient liver failure. Reduced serum albumin and total protein levels occur for this reason in the terminal stages of nonedematous PEM in humans (25).

The carcass protein and lipid analyses (Experiment 2) reflect mobilization of these components by the R and RT_3 mice. The RT_3 group required a greater food intake than the R mice in order to maintain the same rate of weight loss. The carcass composition and

TABLE V. EXPERIMENT 3: SPLENIC NUCLEATED CELL NUMBERS, PLAQUE-FORMING CELL RESPONSES AND HEMAGGLUTININ TITERS TO SRBC[a]

Group	No. of cells/spleen ($\times 10^{-6}$)	PFC/10^6 cells		PFC/spleen ($\times 10^{-3}$)		Hemagglutinin titer[b]
		IgM	IgG	IgM	IgG	
C	51	225	85	9.52	4.04	6.2
R	5[c]	360	4[c]	1.21[c]	0.02[c]	3.8[c]
RI1	5	150	3	0.57	0.01	2.8
RI2	6	377	10	1.11	0.04	3.6
EMS[d]	66.039	66219	0.9240[e]	0.2090[e]	0.8938[e]	0.0322[e]

[a] *N* values for cell number, IgM and IgG PFC were C, 7; R, 10; RI1, 11; RI2, 11; for titers: C, 8; R, 11; RI1, 14; RI2, 13.

[b] Reciprocal of base 2 log of maximum dilution which produced spreading of SRBC.

[c] Value significantly different from C.

[d] Error mean square.

[e] From ANOVA using log transformed data. Mean values are antilogs of log means.

serum protein analyses, however, permit the conclusion that, in spite of the increased flux of nutrients and energy through the RT_3 mice, the protein and energy status of these animals was not improved over that of R mice. Part of the increased nutrient and energy flow may have been directed toward some components of the immune system. Thus T_3 may have exerted its effect by improving the nutritional status of a single, high-priority physiological system. Alternatively, T_3 supplementation might have increased the availability of the trace nutrient, iodine, since 0.2 mg T_3 per kg of diet supplies 0.12 mg iodine/kg. Intestinal iodine absorption efficiency is modestly impaired by malnutrition (26) and T_3 could simply have served as an intestinal carrier for iodine. The ineffectiveness of iodine at increasing the antibody response of restricted mice, however, indicates that iodine per se was not the limiting factor but that the hormonal form of this nutrient was required.

The results suggest that the effect of T_3 on IgM production by RT_3 mice could, in part, be due to the measured numerical increase in nucleated spleen cells, many of which may be lymphocytes. T_3 may also improve the responsiveness to antigens of individual immunocompetent splenocytes. The influence of T_3 on IgG-producing plasma cells of malnourished mice is also of interest. It is tempting to speculate that T_3 may play a role in immunoglobulin class switching and that the food intake restriction model of the present study may be of use in investigating the factors involved in the regulation of this phenomenon.

Others have reported improved cell-mediated disease resistance (to Listeria monocytogenes) in severely protein-deficient young adult rats (27) and in moderately protein-deficient young adult mice (28) injected, respectively, with the polysaccharide lentinan and the thymus extract thymosin fraction V (TF5). The primary action of both lentinan (29) and TF5 (30) is generally considered to be immunoenhancement through direct stimulation of the immune system. It is unknown whether the effect of T_3 in the present system is by direct action on immune cells or is mediated indirectly. Further, it is unclear whether the present results represent a physiological or a pharmacological effect of T_3. The trend toward elevation in spleen cell number and measures of antibody response in CT_3 mice, however, suggests a pharmacological effect. Furthermore, T_3 improved even those aspects of the anti-SRBC antibody response which were not diminished by a protocol of undernutrition longer in duration (6 weeks) but of less severity (weight gain equal to 20% of initial weight) than the present procedure (31). Moreover T_3 produced results similar to the present findings in a low-protein model of wasting malnutrition (B. Woodward, K. Perry, and S. M. Filteau, Canad Fed Biol Soc **30,** 1987, Abstract) in which serum total and free T_3 levels are not depressed (4). Additional work is required to identify the target immune cells in the present system, to determine T_3 dose–response relationships for the various aspects of the anti-SRBC response, and to determine the influence of undernutrition on T_3 receptor numbers and affinity in the target immune cells. Moreover, the present results should stimulate experimentation with other immunopharmacological agents in severe malnutrition.

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