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Thymic Epithelial Cells of Severely Undernourished Mice: Accumulation of Cholesteryl Esters and Absence of Cytoplasmic Vacuoles[1] (42021)

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Abstract. The thymic epithelium was compared in weanling male and female CBA/J mice when fed *ad libitum* and when subjected to severe food intake restriction for 14 days. The restriction protocol elicited predominantly a metabolic response to caloric deficit rather than to protein deficiency. Electron microscopy revealed intracytoplasmic accumulations of large, circular, homogeneously electron-dense profiles (with no limiting membrane) in a high proportion of cortical and medullary epithelial cells of thymuses from restricted mice, but not from controls. The electron-dense material was not preserved in the absence of osmium. Thin-layer chromatography (TLC) indicated elevated levels of free and esterified cholesterol, particularly the latter, in whole thymus extracts of restricted mice. Measurements of total cholesterol levels in the thymic extracts were consistent with the results obtained by TLC. In addition, cryostat sections of thymuses from restricted mice, but not from controls, exhibited numerous stained foci throughout the cortex and medulla when treated with oil red O (a general neutral lipid stain) or by the Schultz procedure which is specific for cholesterol. Collectively the results suggest accumulations of cholesteryl esters, together with some free cholesterol, as non-membrane-bound droplets in the cytoplasm of thymic epithelial cells of undernourished mice. It is also of interest that the lipid-laden epithelial cells exhibited none of the cytoplasmic vacuoles observed in controls and believed to be important in thymic hormone secretion. This work provides the first direct evidence of thymus epithelial abnormalities in severe protein-energy malnutrition.

Thymus (T)-dependent immune responses appear more sensitive to protein–energy malnutrition than T-independent responses (1). Associated with this general finding is a selective depletion of mature T lymphocytes from the blood and secondary lymphoid organs in malnutrition (1). Thymic epithelial cells synthesize hormones (2, 3) which are required for T-lymphocyte maturation (4), and the serum level of one such hormone, facteur thymique serique (FTS), is low in malnourished children (5) and rats (6). Brief incubation with thymic hormones induced a normal proportion of mature T lymphocytes to appear in samples of peripheral blood cells from malnourished children (7). Moreover, injection of thymic hormones improved cell-mediated disease resistance in protein-deficient mice (8). The foregoing discoveries suggest that a primary effect of malnutrition on the immune system may lie at the level of the thymus epithelium. To date, however, direct evidence of thymic epithelial pathology in malnutrition appears lacking. The main objective of the present study was to determine the influence of severe food intake restriction on the morphology of thymic epithelial cells in the mouse.

Materials and Methods. *Animals.* Male and female weanling (21 day old) CBA/J mice were individually housed in plastic cages. Room temperature was about 24°C, and a photoperiod of 14-hr light and 10-hr darkness was maintained. All animals were acclimated for 2 days on the purified diet shown in Table I. The mice had free access at all times to clean tap water. In each experiment the feeding period was 14 days, i.e., from 23 to 37 days of age.

Experiment 1. To determine the *ad libitum* food intake of weanling CBA/J mice, 10

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female and 11 male mice were fed the purified diet shown in Table I. Food intake and live weight were recorded daily for each animal.

Experiment 2. The purpose of this experiment was to obtain basic information on the response of the mice to severe food intake restriction. Preliminary results revealed a high mortality rate (about 60%) among mice fed, for 14 days, 50% of the average *ad libitum* intake (g feed/g live weight/day) determined for our conditions in Experiment 1. Severe restriction with a moderate mortality rate (about 19%), however, was achieved by feeding the mice 60% of *ad libitum* intake (live weight basis) for the last 5 days. The purified diet shown in Table I was used. At the end of the experiment serum protein levels, thymic and splenic indices (organ weight per unit of live weight), and maximal liver alanine aminotransferase activities were each determined on separate subgroups from the controls and the restricted mice. In addition, thymuses from 5 controls and 10 restricted animals were embedded in wax for histological examination.

TABLE I. COMPOSITION OF PURIFIED DIET[a]

Ingredient	Amount (g/kg)
Spray-dried egg white[b]	245
Cornstarch[c]	290
Glucose[d]	290
Cellulose[e]	50
Corn oil[f]	75
Vitamin–mineral supplement[g]	50

[a] Proximate analysis (% as-fed basis): dry matter, 92.3; crude protein (% N × 6.25), 19.2; ether extract, 7.9; crude fiber, 3.8; ash, 4.5; gross energy, 4.1 kcal/g. Methods according to the AOAC (9).

[b] U.S. Biochemical Corp., Cleveland, Ohio.

[c] St. Lawrence Starch Company, Ltd., Port Credit, Ontario.

[d] Cerelose, 2001, CPC International, Englewood Cliffs, N.J.

[e] Celufil, nonnutritive bulk, U.S. Biochemical Corp.

[f] Contained sufficient supplemented fat-soluble vitamins to provide per kilogram diet: 1000 IU cholecalciferol; 50 IU all-rac-α-tocopheryl acetate.

[g] The supplement supplied the following levels of nutrients per kilogram diet: 4000 IU retinyl acetate; 0.8 mg menadione sodium bisulfite; 4 mg biotin; 1 mg folic acid; 15 mg niacin; 12 mg D-calcium pantothenate; 10 mg riboflavin; 8 mg thiamin HCl; 9 mg pyridoxine HCl; 0.05 mg cyanocobalamin; 1.2 mg choline chloride; 2.5 g NaCl; 27.8 g dicalcium phosphate (20% Ca, 18% P); 0.9 g magnesium oxide (60% Mg); 10.7 g potassium sulfate (45% K); 21.6 mg cupric sulfate (25% Cu); 174 mg ferrous sulfate (21% Fe); 154 mg manganous sulfate (25% Mn); 0.3 mg potassium iodide (75% I); 0.4 mg sodium selenite (30% Se); 14 mg zinc carbonate (52% Zn). The supplement was brought to appropriate weight with glucose.

Experiment 3. Control mice were fed *ad libitum* the purified diet shown in Table I whereas restricted animals were fed the same diet according to the protocol used in Experiment 2. After the 14-day feeding period thymuses of 9 controls and 16 restricted mice were examined by electron microscopy. Subsequent to this morphological study, the lipid compositions of whole thymus glands from the two groups of mice were compared by thin-layer chromatography (TLC). A total of 5 samples from controls (6 mice in total) and 5 samples from restricted animals (18 mice in total, 3–4 mice pooled per sample) were examined. The total cholesterol content of the thymuses was also determined from measurements made on aliquots of the thymus lipid extracts used for TLC. Finally, total neutral lipids as well as cholesterol, specifically, were visualized histochemically in thymuses from 6 controls and 7 restricted mice.

Assay of serum protein level. Serum was stored at −20°C and assayed by the biuret method (10).

Assay of maximal liver alanine aminotransferase activity. Livers were stored at −80°C and the assay of Segal and Matsuzawa (11) was followed in modified form. In particular, 1.0 ml of 1.3 *M* L-alanine was used in an assay volume of 3.0 ml containing 10 μl of supernatant from a liver homogenate made with 10 vol of glass-distilled water. Assays were conducted at room temperature, and a linear decrease in optical density (340 nm) was maintained for at least 10 min in all test samples.

Light microscopy. Thymuses were fixed at room temperature for several days in a commercially prepared mixture (Perfix, Fisher Scientific Co., Don Mills, Ontario) of isopropyl alcohol (16.8% w/v), paraformaldehyde (4.1% w/v), and trichloroacetic acid (2.0% w/v). The tissue was embedded in Paraplast (Fisher Scientific Co., Don Mills, Ontario), and sections about 6 μm in thickness were stained with Mayer's hematoxylin and eosin (12). At least five sections per animal were examined.

Electron microscopy. Thymus blocks were fixed by immersion for 2 hr in 2% (w/v) glutaraldehyde. All steps in the tissue processing procedure were conducted at room temperature. Some tissue (5 controls and 13 restricted mice) was postfixed for 2 hr in 1% (w/v) OsO_4, while other tissue (4 controls and 3 restricted mice) was not osmicated. The fixative vehicle which also served as the wash buffer was 0.12 *M* Sorensen's phosphate buffer (300 mOsm, pH 7.4). Dilution dehydration (13) was performed in ethanol and the tissue was embedded in Epon 812. At least four sections per animal were viewed.

Thin-layer chromatography. Thymuses were weighed, pooled, and homogenized at room temperature in 2-ml glass-distilled water. Each thymus homogenate from control mice contained about 40 mg of tissue (wet wt), while each homogenate from restricted animals contained about 5 mg of tissue. The protein content of 0.2-ml aliquots from each homogenate was measured by the Lowry method (14). Lipids were extracted from the remaining 1.8 ml of each homogenate by the method of Bligh and Dyer (15). Quantities of the chloroform layer corresponding to the same amount of thymus protein (about 780 μg) were evaporated to dryness under a stream of nitrogen, dissolved in 25 μl chloroform: methanol (2:1, v/v), and subjected to TLC by the method of Mercer and Holub (16). Lipid standards by which the fractions could be identified were generously supplied by Dr. B. J. Holub.

Assays of thymus cholesterol level. The total cholesterol concentration was determined by the method of Bhandaru *et al.* (17) in the chloroform extracts used, also, for TLC.

Histochemical visualization of lipids. Thymuses were frozen in liquid nitrogen and cryostat sections 8 μm in thickness were postfixed in formol calcium for 3 hr at 4°C. For each mouse at least four sections were stained with the nonspecific neutral lipid stain oil red O (18) and at least two sections were subjected to the Schultz procedure which specifically visualizes cholesterol present in either free or esterified form (12). Cryostat sections from thymuses of both groups of mice were extracted in chloroform:methanol (2:1, v/v) for 1 hr at room temperature to serve as negative controls for both staining procedures. Sections from livers and adrenal glands of well-nourished, healthy mice were used as positive controls for the oil red O and Schultz methods, respectively.

Results and Discussion. Linear regression of food intake against time (Experiment 1) produced the following equations for male and female mice, respectively: $Y = -0.0041X + 0.23$ ($r^2 = 0.83$, $P < 0.01$) and $Y = -0.0047X + 0.26$ ($r^2 = 0.81$, $P < 0.01$), where Y is food intake (g food/g live weight/day) and X is time in days. Because good growth rates were achieved (males: initial and final live weights 11.6 ± 1.8 g and 20.6 ± 1.6 g, respectively; females: initial and final live weights 10.5 ± 1.6 g and 17.0 ± 0.9 g, respectively; means ± SD), the food intake

TABLE II. EXPERIMENT 2: PERFORMANCE PARAMETERS OF CONTROL (*AD LIBITUM*-FED) AND FOOD INTAKE-RESTRICTED MICE[a]

Parameter	Group of mice		*P* value[b]
	Control	Restricted	
Initial live weight (g)	11.6 ± 1.8 (21)	12.3 ± 1.8 (19)	0.439
Final live weight (g)	18.7 ± 2.3 (21)	8.5 ± 1.1 (19)	<0.001
Thymic index (mg/g live weight): (a) Males	3.0 ± 0.7 (6)	0.3 ± 0.1 (16)	<0.001
(b) Females	4.4 ± 0.7 (5)	0.4 ± 0.1 (14)	<0.001
Splenic index (mg/g live weight)	3.2 ± 0.4 (11)	1.0 ± 0.3 (26)	<0.001
Serum protein level (g/100 ml)	5.3 ± 0.2 (9)	3.3 ± 0.4 (14)	<0.001
Maximal liver ALAT[c] activity (μmole NADPH oxidized/g wet wt min^{-1})	42.3 ± 12.6 (9)	63.9 ± 20.4 (18)	0.009

[a] Means ± SD (number of mice).
[b] Two-tailed Student's *t* test.
[c] ALAT: alanine aminotransferase.

prediction equations were considered suitable for use throughout this work in setting the daily intakes of restricted mice.

The results shown in Table II indicate the severity of the food intake restriction protocol which resulted in a 30% loss of initial live weight during the 14-day period both by males and females (Experiment 2). Reduced thymic and splenic indices (Table II) are widely recognized responses to severe protein–energy malnutrition (1). Histologically, the thymuses of all restricted mice exhibited cortical lymphocytic depletion. This is a common feature of protein–energy malnutrition in experimental animals and human beings (1). Low serum protein levels were observed in the restricted mice (Table II) and are also a common feature of severe protein–energy malnutrition (1). It appears that the predominant metabolic response of the restricted mice in this study was to caloric deficit. Liver alanine aminotransferase is a key gluconeogenic enzyme (19) which exhibits increased maximal activity (wet weight basis) when energy is the primary limiting factor (20) but decreased maximal activity when protein is

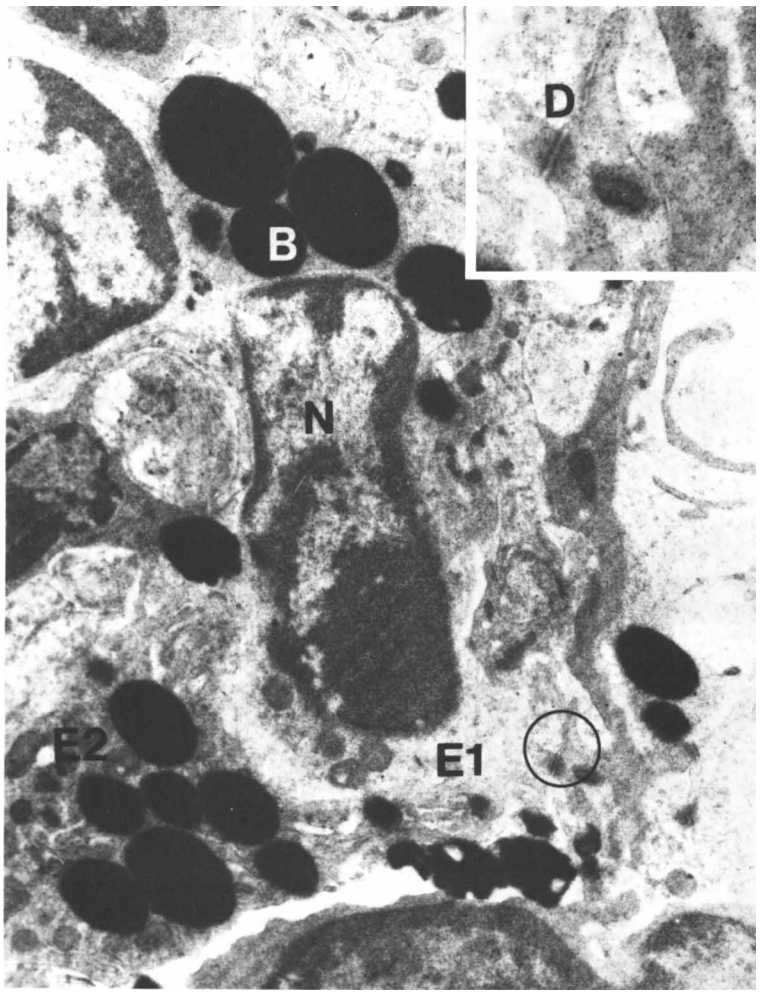

FIG. 1. Electron micrograph of thymic epithelial cells from restricted mouse. Labeled structures include nucleus (N), desmosome (D), and electron-dense bodies (B). Two adjacent thymic epithelial cells (E1 and E2). Circled structure, a desmosome, shown within the inset. Glutaraldehyde and osmium fixation; unstained. ×11880; inset ×17820.

the main limiting factor (20). An increase in the activity of this enzyme was induced by the food restriction protocol adopted in this work (Table II).

Thymic epithelial cells were identified ultrastructurally by the presence of desmosomes or tonofilaments which are characteristic features of these cells (21–23). A high proportion of cortical and medullary epithelial cells in thymuses of restricted mice displayed intracytoplasmic accumulations of large, circular, homogeneously electron-dense profiles when fixed in glutaraldehyde and osmium (Fig. 1). No similar accumulations of electron-dense structures were observed in the thymus epithelial cells of well-nourished mice. The electron-opaque material appeared to be mainly lipid in nature, presumably with abundant carbon-to-carbon double bonds (24), because omission of osmium fixation resulted in replacement of the electron-dense structures by electron-lucent regions of similar size, shape, and distribution. It was also apparent from examination of nonosmicated tissue that the majority of lipid droplets were not surrounded by a limiting membrane. In addition, the lipid-laden epithelial cells failed to exhibit cytoplasmic vacuoles (Fig. 1) which were abundant in the controls and which are characteristic of epithelial cells both in the cortex and in the medulla of well-nourished animals (21–23). This phenomenon merits further study since the numerous cytoplasmic vacuoles of normal thymus epithelial cells are considered to participate in secretion of thymic hormones (4).

Oil red O staining revealed abundant foci of dye uptake throughout the cortex and medulla of thymuses from restricted mice, while no uptake of stain was discernible in thymuses of well-nourished mice. Chloroform:methanol extraction abolished oil red O uptake by the thymuses of restricted mice. The thymus epithelium could not be distinctly visualized in cryostat sections, but it is difficult to assign the oil red O-positive sites to structures other than the lipid droplets observed ultrastructurally. The osmiophilic droplets, therefore, appear to include significant quantities of neutral lipid.

A typical TLC separation of whole thymus lipid extracts is shown in Fig. 2. Extracts from restricted mice consistently exhibited a large, intensely fluorescent band of cholesteryl ester(s), while controls displayed only a trace of this material. Moreover, restricted mice exhibited a more intense band of free cholesterol than controls as well as a diffuse band, not visible in extracts of controls, within the free fatty acid zone. By contrast, extracts from restricted mice contained only a trace of triglycerides, an observation consistent with the extreme caloric deprivation of these animals. The results with regard to the cholesterol-containing bands are supported by the total cholesterol analyses (Table III) which revealed a several-fold greater cholesterol concentration (wet weight basis) in thymuses of restricted mice than in controls. The same conclusion was apparent when the results were expressed on a thymus protein basis (values not shown). The Schultz histochemical test supported and extended these biochemical results. Thymuses of restricted mice exhibited numerous foci of reaction product

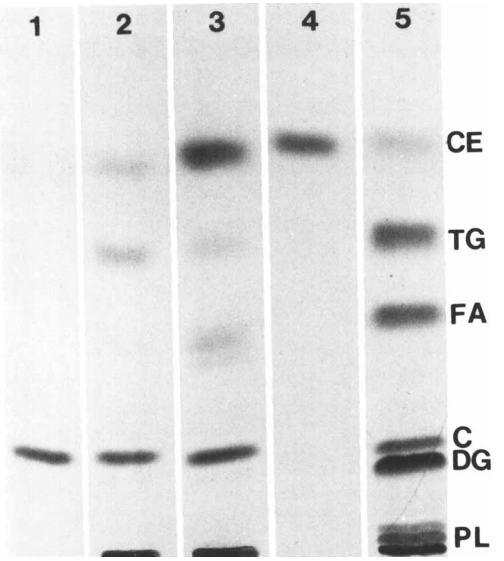

FIG. 2. Thin-layer chromatography of chloroform: methanol extracts of whole thymuses from mice fed *ad libitum* (2) and from restricted mice (3). Lipid standards are shown in columns 1, 4, and 5. Phospholipids (PL), diglycerides (DG), free cholesterol (C), free fatty acids (FA), triglycerides (TG), and cholesteryl esters (CE). For the purpose of photography thymus extracts were spotted corresponding to 1.7 mg protein, and the plate was sprayed with 50% (v/v) aqueous sulfuric acid and heated at 160°C for 30 min.

(abolished by extraction with chloroform: methanol) throughout the cortex and medulla (Fig. 3), but thymuses of control animals displayed no Schultz positivity except for a faint reaction with cellular limiting membranes. The Schultz-positive foci clearly correspond to the neutral lipid-containing sites identified with oil red O and, in turn, correspond to the lipid droplets observed ultrastructurally within thymic epithelial cells of restricted mice. Similarly, the TLC study (although conducted with whole thymus extracts) may be interpreted, on the basis of the present morphological results, to show that the lipid accumulations of restricted mice are composed primarily of cholesteryl ester(s) together with some free cholesterol. The intense osmiophilia of the lipid droplets suggests a high proportion of unsaturated fatty acids within the cholesteryl ester fraction, but the identity of the fatty acids awaits future investigation.

The results of this investigation provide the first direct evidence of thymic epithelial cell abnormalities in protein–energy malnutrition. The phenomenon of epithelial lipid accumulation does not resemble cholesteryl ester storage disease in which lysosomal lipase deficiency results in accumulations of lipid surrounded by a limiting membrane (26). It is relevant to consider the findings of the present investigation in the light of the cytoplasmic cholesteryl ester cycle proposed for macrophages by Brown *et al.* (27). The model focuses on the enzyme acyl-CoA: cholesterol acyltransferase (ACAT). Increase in ACAT activity (e.g., resulting from free cholesterol influx into the cytoplasm) results in cholesteryl ester accumulation within non-membrane-bound cytoplasmic droplets (27). By contrast, a decrease in ACAT activity (e.g., resulting from receptor-mediated free cholesterol efflux) has the opposite effect (27). In this regard the high free-cholesterol level apparent by TLC in thymuses of restricted mice (presumably in the epithelial cells) is of interest as are reports of elevated ACAT activity in the liver (28) of starved rats. Both the mechanism of cholesteryl ester accumulation within thymic epithelial cells and the influence (if any) of this phenomenon on epithelial functions remain to be determined. Similarly, the mechanism underlying the absence of cytoplasmic vacuoles from lipid-laden epithelial cells as well as the functional significance of this abnormality are presently unknown.

TABLE III. EXPERIMENT 3: TOTAL CHOLESTEROL LEVELS OF THYMUSES[a]

	Group of mice		*P* value[b]
	Control	Restricted	
Total cholesterol level (μg/mg wet wt)	1.8 ± 0.2 (5)	6.9 ± 2.6 (5)	0.009

[a] Means ± SD (number of samples).
[b] Two-tailed Student's *t* test conducted for means with unequal variances, i.e., $df = 4$ (25).

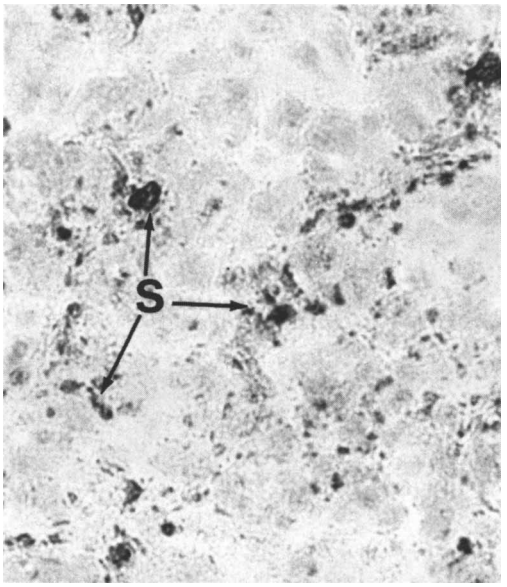

FIG. 3. Cryostat section from thymus of a restricted mouse. Schultz reaction product is labeled "S." Liquid nitrogen fixation. ×760.

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