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Persistent reduction of pancreatic Beta-cell mass after a limited period of protein-energy malnutrition in the young rat*

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Summary. Kwashiorkor, the human disease of protein-energy malnutrition, has been implicated in the aetiology of malnutrition-related diabetes mellitus, a form of diabetes not uncommon in developing countries. We have previously demonstrated that temporary protein-energy malnutrition in young rats causes a persisting impairment of insulin secretion. The present study investigates whether this secretory deficiency is accompanied by structural alterations of the endocrine pancreas. Three-week-old rats were weaned onto semi-synthetic diets containing either 15% or 5% protein and these diets were maintained for 3 weeks. From 6 weeks of age all rats were fed a commercial chow containing 18% protein. The endocrine pancreas was investigated by light and electron microscopic morphometry at 3, 6 and 12 weeks of age. In rats not subjected to protein-energy malnutrition there was a progressive increase, with age, of total pancreatic Beta-cell weight and individual Beta-cell size. In 6-week-old rats fed the low protein diet total pancreatic Beta-cell weight and individual Beta-cell size were diminished. In 12-week-old rats previously fed the low protein diet total Beta-cell weight remained lower compared to control rats. It is concluded that protein-energy malnutrition early in life may result in a diminished reserve for insulin production. This may predispose to glucose intolerance or even diabetes in situations with an increased insulin demand.

Key words: Malnutrition-related diabetes mellitus, kwashiorkor, protein-calorie malnutrition, rat, pancreatic islets, pancreatic Beta cell, insulin, light microscopy, electron microscopy, morphometry.

The relationship between affluent food supply, obesity and diabetes mellitus is well known and has been the subject of numerous studies. The observation made in 1907 [1] that the spectrum of diabetes in tropical regions of the Third World differs from that in western society and that the disease may be related to malnutrition is not as well recognized. However, in recent years the malnutrition-related diabetic syndromes have been delineated and are now regarded as separate entities [2]. The exact role of malnutrition in the aetiology and pathogenesis of these types of diabetes remains obscure [3].

Kwashiorkor, the disease of protein malnutrition in the young, is characterized by, among other symptoms and signs, impaired glucose tolerance and a diminished or even absent insulin secretory response to glucose [4–20]. This appears to be a feature typical of protein deficiency, since balanced malnutrition, as in marasmus, has little or no effect on glucose tolerance and insulin secretion [7, 9, 11, 12, 15, 18]. Following nutritional rehabilitation, patients with kwashiorkor show a rapid improvement of glucose tolerance and insulin secretory response to glucose within weeks but short-term recovery remains incomplete [8–11, 13–16, 20]. Long-term follow-up has been difficult for practical reasons and the outcome influenced by socio-economic factors and diet following the immediate recovery from malnutrition. The studies available are contradictory and suggest either complete normalization [11, 18, 21] or a persisting impairment of glucose tolerance [22, 23].

The rapid initial recovery of glucose homeostasis upon treatment of kwashiorkor suggests a reversible functional impairment of the pancreatic Beta cell. However, if diminution of Beta-cell mass were to be shown, the case for protein deficiency in the aetiology of malnutrition-related diabetes would be strengthened [3]. Histopathological investigations of the endocrine pancreas of children who have died during the acute phase of kwashiorkor are confusing since hypertrophic [24–28], normal [29–33] and atrophic [4, 24, 26, 34, 35] pancreatic islets have been re-

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ported. Studies of the endocrine pancreas in patients who have suffered from kwashiorkor but subsequently recovered are to our knowledge not available. Thus, studies in man do not give a clear indication as to whether protein-energy malnutrition causes structural alterations of the pancreatic islets.

The stunting of growth, the impaired glucose tolerance and blunted insulin secretory response to glucose of kwashiorkor can be reproduced in animal models of protein-energy malnutrition [36–43]. Following nutritional rehabilitation growth is resumed, glucose tolerance normalized but a lowered capacity for insulin secretion persists [41, 42, 44]. Reports on the histopathology of the pancreatic islets during experimental protein-energy malnutrition are again divergent suggesting hypertrophy [45], no change [46, 47] or atrophy [48–53]. Quantitative morphometry at light and electron microscopic levels was used here to study the structural alterations in the pancreatic Beta cell of the rat during experimental protein-energy malnutrition and after nutritional rehabilitation.

Materials and methods

Diets

Pelleted semi-synthetic diets were made by Ewos (Södertälje, Sweden) as previously described in detail [54]. They contained either 15% protein (control or "C" diet) or 5% protein (low protein or "LP" diet). The restriction of protein content in the LP diet was compensated for by increasing the proportion of carbohydrate. Diets were thus isoenergetic and identical except for their protein content. The commercial chow R3 (Ewos) contained (weight/weight [w/w]) 18% protein, 5% fat, 52% carbohydrates and 3.5% fibre. R3 pellets and semi-synthetic C diet support growth of young rats to the same extent [44].

Animals

Sprague-Dawley rats were obtained from a local colony. Animals had free access to R3 pellets and tap water throughout breeding, gestation and lactation. Male rats were caged overnight with females and pregnancy confirmed by the presence of sperm in vaginal smears on the following morning, designated day 0 of pregnancy. Pregnant rats were caged individually and spontaneous delivery took place on day 22 of pregnancy. The day after delivery large litters were reduced to 12 pups. Litters of less than 6 were not used. At 21 days of age, male rats were weaned onto either C diet (C rats) or LP diet (LP rats). These diets were fed ad libitum for 3 weeks when both C and LP rats, at the age of 6 weeks, returned to a diet of R3 pellets up to the end of the experiment at 12 weeks of age. Rats fed the LP diet reduced their food intake to one-third the amount consumed by C rats (C.J. Crace, unpublished observation). The deficiency state induced should therefore be regarded as a combined protein-energy malnutrition rather than as pure protein deficiency [55, 56].

Light microscopy and light microscopic morphometry

Rats were weighed and killed by cervical dislocation at 3, 6 and 12 weeks of age. Pancreatic glands were rapidly dissected free from surrounding tissues, weighed and fixed by immersion in Bouin's fluid. The fixed tissue was embedded in paraffin according to a standardized protocol, cut into 7 μm thick sections, and mounted on glass slides. Insulin was demonstrated with the unlabelled peroxidase-anti-peroxidase technique [57]. The primary antibody was raised in guinea pigs against bovine insulin (ICN ImmunoBiologicals, Lisle, Ill., USA). Secondary antibodies and peroxidase-anti-peroxidase complex were from Dakopatts (Glostrup, Denmark). The immunostained sections were lightly counterstained with haematoxylin. The specificity of the immunostaining was tested according to Goldman [58] and included the application of primary antisera adsorbed with 100 ng/ml insulin, glucagon, somatostatin or pancreatic polypeptide.

To obtain an estimate of the Beta-cell volume fraction of the pancreas, each gland was sectioned throughout. To avoid bias due to regional variations in islet distribution and cell composition [59] sections used for measurements were selected by systematic random sampling. Every 50th section was taken from glands of 3-week-old weanlings and 6-week-old LP rats, every 100th section from glands of 6-week-old C rats and every 150th section from glands of 12-week-old C and LP rats. This sampling procedure yielded 6–12 sections from each pancreas.

The Beta-cell volume fraction was determined by planimetric analysis using a MOP-Videoplan image analysis system (Kontron Bildanalyse, Munich, FRG). The sectional area of total pancreatic and non-pancreatic tissue, mostly lymph nodes and adipose tissue, was measured at a magnification of 156×, and the area of insulin-positive cells at a magnification of 470×. Crude pancreatic weight was corrected for the proportion of non-pancreatic tissue in the sections. In the following "pancreatic weight" refers to this corrected value. On the assumption that endocrine and exocrine pancreas differ only slightly in density [60], Beta-cell weight was calculated from the pancreatic weight and Beta-cell volume fraction. To allow comparison between animals of different sizes, Beta-cell weight was finally expressed per unit body weight.

Electron microscopy and ultrastructural morphometry

Rats, aged 3, 6 or 12 weeks, were killed by retrograde perfusion with 2.5% (volume/volume [v/v]) glutaraldehyde in a phosphate buffer, pH 7.2, through the aorta under sodium pentobarbital anaesthesia. The pancreatic glands were quickly removed and kept immersed in fixation medium. After postfixation in 1% (w/v) osmium tetroxide the tissue was embedded in Epon 812 [61]. The pancreatic islets were identified in semithin sections stained with 1% (w/v) toluidine blue in 25 mmol/l disodium tetraborate. Ultrathin sections were prepared and contrasted with uranyl acetate [62] and lead citrate [63]. Electron microscopy was carried out using a Jeol 100B transmission electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) with the magnification calibrated against a diffraction grating replica with 28800 lines per inch (Ernest F. Fullam, Latham, NY, USA).

Two ultrathin sections, cut at least 20 μm apart, were obtained by random selection from one islet from each of four rats in every experimental group. For each experimental group a total of 32 electron micrographs were taken. The fields were distributed over the sections by systematic random sampling.

In the morphometric analysis of pancreatic Beta cells, endocrine non-Beta-cells and non-endocrine tissue were excluded from the measurements. The volume of Beta cells per unit tissue volume (V_{VC}) was thus by definition equal to unity. The volume of Beta-cell nuclei per unit tissue volume (V_{VN}), the surface area of Beta cells per unit tissue volume (S_{VC}), the number of Beta cells per unit tissue volume (N_{VC}) and secondary morphometric parameters were determined as previously described [64].

Statistical analysis

Results are given as means ± SEM. Differences between N and LP rats were evaluated using Student's two-tailed *t*-test for independent observations.

Table 1. Morphometric parameters of rats fed diets with normal (C) or low (LP) protein content between 3 and 6 weeks of age

Morphometric parameter		Rats				
		3-week-old weanlings	6-week-old C rats	6-week-old LP rats	12-week-old C rats	12-week-old LP rats
Body weight	(g)	42 ± 1	152 ± 7	41 ± 1[b]	387 ± 9	347 ± 8[a]
Pancreatic weight	(mg)	170 ± 4	630 ± 39	190 ± 8[b]	1630 ± 33	1150 ± 36[b]
Pancreatic weight/body weight	$(mg \cdot g^{-1})$	4.1 ± 0.1	4.2 ± 0.1	4.5 ± 0.1	4.2 ± 0.1	3.3 ± 0.1[b]
Beta-cell volume fraction	$(mm^3 \cdot 10^3 \cdot mm^{-3})$	4.8 ± 0.3	5.3 ± 0.2	3.6 ± 0.1[b]	6.1 ± 0.2	4.5 ± 0.3[b]
Beta-cell weight	(mg)	0.83 ± 0.05	3.32 ± 0.14	0.68 ± 0.04[b]	9.98 ± 0.37	5.18 ± 0.36[b]
Beta-cell weight/body weight	$(mg \cdot 10^3 \cdot g^{-1})$	19.6 ± 0.7	22.0 ± 0.8	16.5 ± 0.8[b]	25.8 ± 1.0	15.0 ± 1.1[b]

[a] $p < 0.01$; [b] $p < 0.001$. Differences between C rats and LP rats were evaluated by Student's t-test. The results are given as mean values ± SEM for 7–8 rats

Results

Three-week-old rats weaned onto the C diet rapidly increased in weight and an almost four-fold weight gain was noted up to 6 weeks of age (Table 1). Rats weaned onto the LP diet did not gain weight. Following refeeding of standard chow at 6 weeks of age the LP rats resumed growth but at 12 weeks they still remained smaller than C rats.

Light microscopic investigation of pancreatic sections from C rats showed a well-preserved endocrine and exocrine morphology which changed little with age. At 6 weeks of age the pancreatic islets of LP rats appeared small and with tightly packed cells. There were no signs of leucocyte infiltration, nuclear pycnosis, or cell fragmentation and death. At 12 weeks of age the pancreas of LP rats had recovered and could not be distinguished from that of 12-week-old C rats.

Light microscopic morphometry showed that the volume fraction of Beta cells in the pancreas increased with age in C rats and a more than ten-fold increase of total Beta-cell mass was achieved between 3 and 12 weeks of age (Table 1). When expressed per unit body weight Beta-cell weight increased by 25% between 3 and 12 weeks of age.

At 6 weeks of age, at the end of the period on LP diet, the Beta-cell volume fraction in the pancreas of LP rats had decreased to two-thirds that of C rats (Table 1). Total Beta-cell mass was also reduced and, when expressed per unit of body weight, only 75% of that of 6-week-old C rats. During the recovery period between 6 and 12 weeks of age pancreatic Beta-cell volume fraction increased in LP rats but reached only 75% of that of 12-week-old C rats. Total Beta-cell mass of LP rats also remained lower and was only one half that of C rats at 12 weeks of age, and when expressed per unit body weight, only 60% that of C rats.

At the ultrastructural level Beta cells could be identified by their secretory granule morphology. In 3-week-old weanling rats Beta cells had rounded nuclei with dense heterochromatin distributed along the inside of the nuclear membrane (Fig. 1A). Large nucleoli were seldom observed, the synthetic apparatus, consisting of the endoplasmic reticulum and Golgi complex, was easily identified and secretory granules abundant. At 6 weeks of age the Beta cells of C rats had larger nuclei with little or no condensed chromatin, large nucleoli and the synthetic apparatus was usually prominent (Fig. 1B). There was a considerable variation in secretory granule content and secondary lysosomes of a crinophagic type were common. At 12 weeks of age the ultrastructural morphology of Beta cells of C rats was similar to that at 6 weeks of age (Fig. 1C).

The Beta cells of 6-week-old LP rats were small and their nuclei contained large amounts of condensed chromatin but no nucleoli (Fig. 1D). The cytoplasm contained small numbers of secretory granules, a small Golgi complex but a relatively large endoplasmic reticulum. There were no signs of nuclear pycnosis or cell disintegration and death. At 12 weeks of age the Beta cells of LP rats still retained some nuclear heterochromatin and nucleoli could be seen (Fig. 1E). The secretory apparatus was well developed and the content of secretory granules had increased compared to 6-week-old LP rats.

Ultrastructural morphometry showed that the average volume of individual Beta cells (V_{VC}/N_{VC}) almost doubled between 3 and 12 weeks of age in C rats (Table 2). The average nuclear volume of the Beta cells (V_{VN}/N_{VC}) also increased but only by 24% over the same period.

By contrast, at 6 weeks of age the average Beta-cell volume (V_{VC}/N_{VC}) of LP rats was only two-thirds that of C rats (Table 2). The average nuclear volume (V_{VN}/N_{VC}) was also diminished in comparison with the 6-week-old C rats. Following refeeding both average Beta-cell volume (V_{VC}/N_{VC}) and average nuclear volume (V_{VN}/N_{VC}) of LP rats increased and did not differ significantly from C rats at 12 weeks of age.

Discussion

The increase with age of total Beta-cell mass in C rats confirms previous investigations of Beta-cell growth [65, 66]. The combined data of total Beta-cell mass and individual Beta-cell size indicate that approximately one half of the expansion of Beta-cell mass is due to an increase of Beta-cell number and the rest depends on a doubling of Beta-cell size. Previous functional studies have shown that the insulin secretory response matures after weaning and develops further throughout adult life [44, 67]. The replica-

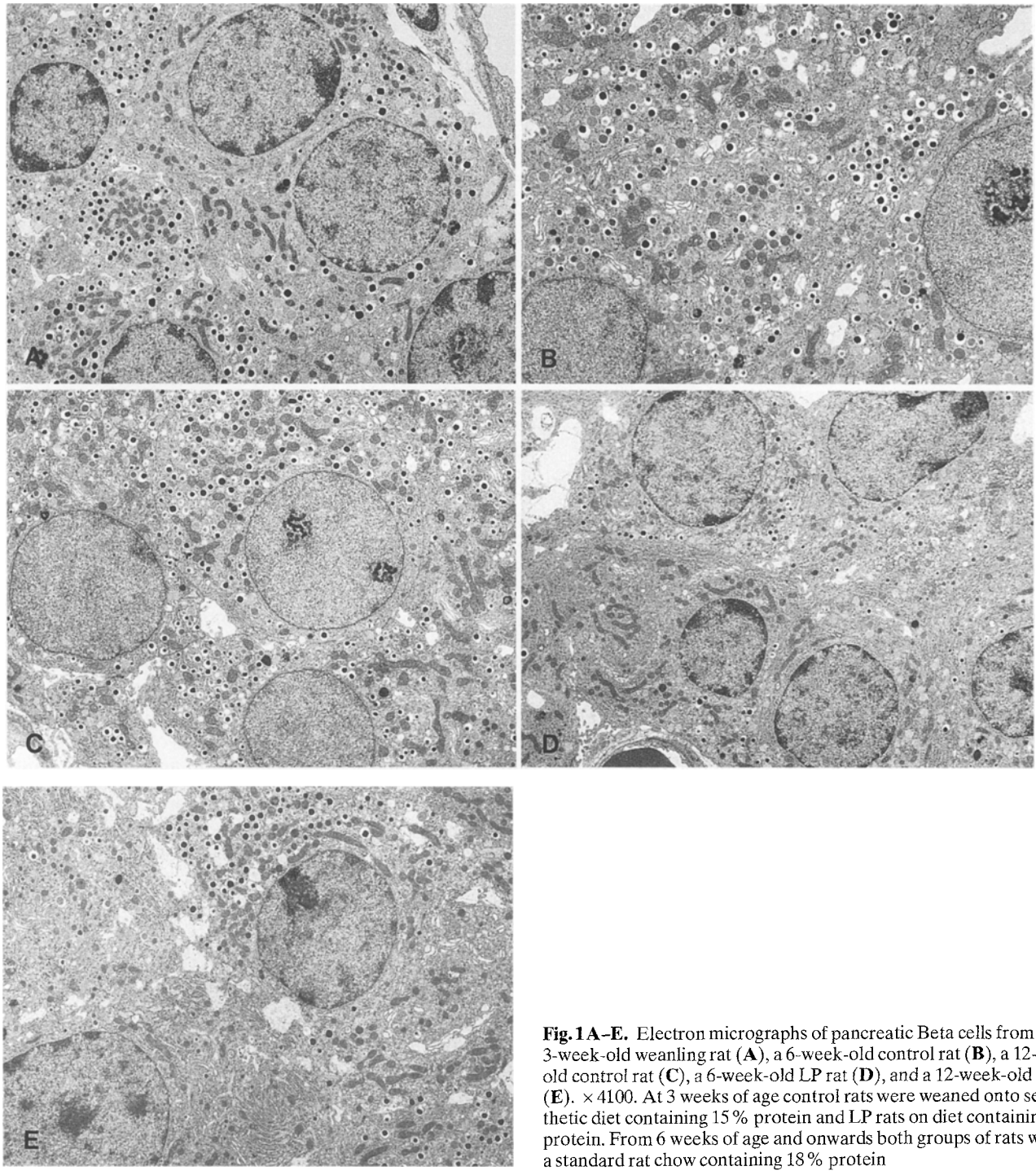

Fig. 1 A–E. Electron micrographs of pancreatic Beta cells from a 3-week-old weanling rat (**A**), a 6-week-old control rat (**B**), a 12-week-old control rat (**C**), a 6-week-old LP rat (**D**), and a 12-week-old LP rat (**E**). × 4100. At 3 weeks of age control rats were weaned onto semi-synthetic diet containing 15% protein and LP rats on diet containing 5% protein. From 6 weeks of age and onwards both groups of rats were fed a standard rat chow containing 18% protein

tion, growth and functional development of the Beta cells of C rats thus shows that the islet organ has considerable plasticity and adapts to variations in functional demand during development [68].

In 6-week-old LP rats total islet volume was diminished and the Beta cells showed ultrastructural signs of atrophy confirming previous observations of small Beta cells with hyperchromatic nuclei but lack of degenerative changes and signs of cell death in experimental protein-energy malnutrition [49, 51–53]. The measurements of total Beta-cells mass and individual Beta-cell size indicate that total Beta-cell number had not increased in LP rats between 3 and 6 weeks of age. It is notable that early experimental studies [45–47] suggesting hypertrophy or no changes of the islets were not quantitative and focussed on exocrine pancreas rather than pancreatic islets. The consequence of experimental protein-energy malnutrition thus appears to be inhibition of replication and atrophy of the Beta cells. In this context it should, however, be noted that the reduction of Beta-cell mass demonstrated is not sufficient to explain the severely blunted insulin secretory response to glucose in vivo and in vitro [42, 44]. Changes in Beta-

Table 2. Morphometric parameters of Beta cells in pancreatic islets of rats fed diets with normal (C) or low (LP) protein content between 3 and 6 weeks of age

Morphometric parameter		Rats				
		3-week-old weanlings	6-week-old C rats	6-week-old LP rats	12-week-old C rats	12-week-old LP rats
V_{VC}	$(\mu m^3 \cdot \mu m^{-3})$	1.000[a]	1.000[a]	1.000[a]	1.000[a]	1.000[a]
S_{VC}	$(\mu m^2 \cdot \mu m^{-3})$	0.515 ± 0.021	0.459 ± 0.018	0.556 ± 0.017[d]	0.433 ± 0.016	0.446 ± 0.013
N_{VC}	$(10^3 \cdot \mu m^{-3})$	1.04 ± 0.56	0.87 ± 0.08	1.32 ± 0.08[d]	0.56 ± 0.11	0.81 ± 0.06[b]
V_{VC}/N_{VC}	(μm^3)	960 ± 512	1150 ± 106	756 ± 47[c]	1790 ± 349	1240 ± 86
V_{VN}	$(\mu m^3 \cdot \mu m^{-3})$	0.18 ± 0.02	0.16 ± 0.01	0.17 ± 0.01	0.12 ± 0.01	0.13 ± 0.01
V_{VN}/N_{VC}	(μm^3)	173 ± 93	185 ± 21	129 ± 11[b]	210 ± 44	165 ± 16

[a] V_{VC} is by definition assigned the value of 1.000 as explained in the Materials and methods section.
[b] $p < 0.05$; [c] $p < 0.01$; [d] $p < 0.001$. Differences between C rats and LP rats were evaluated by Student's *t*-test. The results are given as mean values ± SEM of 32 observations.

The morphometric parameters are:
V_{VC}, volume of Beta-cells per unit tissue volume;
S_{VC}, surface area of Beta-cells per unit tissue volume;
N_{VC}, number of Beta-cells per unit tissue volume;
V_{VC}/N_{VC}, average Beta-cell volume;
V_{VN}, volume of Beta-cell nuclei per unit tissue volume and
V_{VN}/N_{VC}, average Beta-cell nuclear volume

cell function must also be investigated to explain this effect of protein-energy malnutrition.

The major novel observation of the present investigation is that temporary protein-energy malnutrition in young rats causes a dimunition of pancreatic Beta-cell mass which is not compensated for despite apparent adequate nutritional rehabilitation. Although at 12 weeks of age LP rats had increased Beta-cell number almost five-fold and total Beta-cell mass eight-fold they still did not reach the values of C rats. Since the rats grow at a normal rate and have a normal glucose tolerance [42], it could be argued that the Beta cells have only adapted to a new metabolic state. However, perfusion of the pancreas from 12-week-old LP rats has shown loss of first phase insulin release and reduction of the second phase [44]. It would therefore appear that the protein-energy malnutrition has a direct effect on the Beta cells and leaves them with not only a blunted insulin secretory response but also with a diminished capacity for Beta-cell growth and replication.

Beta-cell atrophy appears to be a feature typical of protein deficiency. If rats are energy restricted by feeding them a diet of adequate nutritional composition but in amounts reduced to stunt growth, the proportion of Beta-cell mass to body weight is not decreased [69–71] and the insulin secretory response to glucose is maintained [72]. This parallels the situation seen in human disease, since in marasmus insulin secretion is preserved but in kwashiorkor, insulin secretion is impaired [11, 12, 15, 18]. The pathology of the pancreatic islets in human kwashiorkor is controversial but may depend on the duration of protein-energy deficiency [24]. Taken together, available data from morphological and functional studies in animals and humans suggest that kwashiorkor of some standing causes atrophy of the pancreatic Beta cells. It is not known whether adults who suffered from kwashiorkor in childhood have a reduced Beta-cell mass or other persisting alterations of the endocrine pancreas. The epidemiological evidence for a relationship between protein-energy malnutrition and malnutrition-related diabetes nevertheless makes it tempting to speculate that, if the reserves for insulin production are diminished by malnutrition, impaired glucose tolerance or even diabetes may develop if demands on insulin secretion are increased by intercurrent illness or other, hitherto unknown, environmental factors.

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