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The haemochromatotic human pancreas: a quantitative immunohistochemical and ultrastructural study

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Summary. Insulin, glucagon, somatostatin and pancreatic polypeptide cells were quantified after immunoperoxidase staining in sections of pancreases obtained from nine control subjects and seven diabetic patients with primary or secondary iron overload. One was normoglycaemic, two had glucose intolerance and four presented insulin-requiring diabetes. The whole pancreas was studied, taking into account the heterogeneous distribution of the endocrine cells. In the diabetic patients, the weight of the pancreas tended to be lower. Iron overload predominated in the exocrine tissue, whereas in islets iron concentration was quite variable from case to case. At the Haemalun-Eosine staining the histological appearance of the islets was normal, their shape and size being unchanged; amyloid deposits were absent, as were atrophic islets. Immunoperoxidase staining revealed a severe reduction in the number of immunoreactive B cells in the four diabetic patients. The mass of immunoreactive B cells was calculated from their volume density and from the weight of each lobe of the pancreas. It averaged 950 mg in control subjects, 1580 mg in the normoglycaemic patient, 1010 mg in patients with glucose intolerance and 180 mg in insulin-requiring diabetic patients. The electron microscopic examination, performed in four cases, revealed that the iron deposits were restricted to B cells and associated with progressive loss of their endocrine granules. The study shows that the pancreatic islet abnormalities in iron overloaded diabetic patients are completely different from those of Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetic patients. This constitutes a further argument for a specific role of iron in the pathogeny of diabetes in haemochromatotic patients.

Key words: Endocrine pancreas, haemochromatosis, immunocytochemistry, ultrastructure, diabetes.

Though the frequent association of iron overload and diabetes has long been recognized [1, 2], the exact nature of this relationship remains controversial. It is still unclear whether the diabetic disease is inherited or at least related to familial factors, or if the iron overload has a direct diabetogenic role. Epidemiological studies have led to opposite conclusions [3–7]. However, large prospective studies by Simon at al. [8, 9] indicate that the frequency of diabetes in relatives of haemochromatotic patients bears no relation to the carbohydrate regulation of these patients. These results suggest that the tendency to develop diabetes is not genetically inherited in these patients, but that iron overload is diabetogenic per se. The frequency of diabetes observed in secondary iron overload, in experimental animal models [10], in the Bantu population [11] and in patients suffering from sidero-achrestic anaemia or from thalassaemia [12, 13] reinforces this hypothesis. In the latter group, glucose intolerance correlates significantly with the number of received transfusions. However, a higher prevalence of diabetes in relatives of thalassaemic diabetic patients [13] suggests that associated ground factors contribute to precipitation of the disease by iron overload.

Only a few recently reviewed studies [14] have analysed the morphological consequences of iron overload on the endocrine pancreas. Early reports [15, 16] indicated that the iron deposits are located in B cells, but suggested [17] that the destruction of B cells does not result from these iron deposits, since diabetes could be observed in cases with only minor iron overload in the islets.

The aim of the present study was to (1) investigate, by quantitative immunochemical methods and electron microscopy, the specific characteristics of the islets of Langerhans in diabetes associated with iron surcharge; and (2) determine whether islet morphology of this secondary diabetes is closer to that of diabetes of Type 1 or Type 2, or whether it corresponds to that of a third type.

Materials and methods

Pancreases were obtained within 6 h after death from seven male diabetic patients with hepatic and pancreatic iron surcharge and from nine age-matched male control subjects. These latter died from diseases not known to affect the pancreas and did not exhibit glucose intolerance. Clinical data of patients with iron overload are given in Table 1.

Familial history of diabetes was reported in none of the seven cases and could even be ruled out by systematic interrogation.

After careful dissection, the whole pancreas was cut in parallel slices 2–3 mm thick. All slices were numbered, weighed, fixed in Bouin-Allen's fluid and embedded in paraffin. The limits of the lobe rich in pancreatic polypeptide (PP) cells [18, 19] were delineated and the weight of each lobe was calculated as previously reported [20]. Specimens were then taken in the posterior part of the head (lobe rich in PP-cells), in the anterior part of the head, in the body and in the tail.

Table 1.

Patient no.	Age	Diagnosis	Cause of death	Plasma ferritin level ($n < 300$ mg/ml)	Glucose homeostasis	Treatment		Liver iron overload
						Diabetes (duration)	Iron overload	
1	56	Micronodular cirrhosis Secondary haemochromatosis Unknown etiology	Diffuse gastrointestinal bleeding	-	Normal	-	-	Moderate
2	57	Micronodular cirrhosis Primary haemochromatosis	Hepatoma	4100	Fasting blood glucose 9 mmol/l	-	-	Massive
3	60	Macronodular cirrhosis Secondary haemochromatosis Hepatitis B	Hepatic insufficiency	705	Fasting blood glucose 15 mmol/l	-	-	Massive
4	43	Micronodular cirrhosis Secondary haemochromatosis Alcoholism	Sepsis	-	Insulin requiring diabetes	Insulin (4 m)	-	Moderate
5	50	Micronodular cirrhosis Secondary haemochromatosis Alcoholism	Hepatic insufficiency	1490	Insulin requiring diabetes	Insulin (5 m)		Massive
6	70	Micronodular cirrhosis secondary haemochromatosis Sideroachrestic anemia	Oesophageal varices bleeding	1520	Insulin requiring diabetes	Sulfonylurea (6 y) Insulin (2 y)	-	Massive
7	62	Micronodular cirrhosis Primary haemochromatosis	Hepatoma	490 (under iron deprivative treatment)	Insulin requiring diabetes	Insulin (14 y)	Desferoxamine Phlebotomy	Massive

Table 2.

Patient no.	Pancreatic weight (g)	PP-rich lobe relative weight (%)	Mesenchymal fraction (%)
1	120	11.2	28.8
2	138	13.2	33.8
3	46	16.2	28.2
4	76	13.5	22.9
5	39	12.2	43.4
6	34	20.3	47.3
7	34	15.7	34.8
Control subjects ($n=9$)	87 ± 32	9.7 ± 2.7	19.2 ± 6.1

From each of them, six consecutive sections were stained by Haemalun Eosine, Grimelius [21] and the PAP technique of Sternberger [22] with guinea-pig anti-insulin serum (Dr. P. H. Wright, Indianapolis, Ind, USA), rabbit anti-glucagon serum (Dr. A. Like, Worcester, Mass, USA), rabbit anti-somatostatin serum (Dr. W. Gepts, Brussels, Belgium) and rabbit anti-pancreatic polypeptide serum (Dr. R. E. Chance, Indianapolis, Ind, USA) at dilutions of 1/3000, 1/4000, 1/10 000 and 1/40 000 respectively. Iron deposits were simultaneously demonstrated by the Prussian blue reaction [23]. The specificity of each reaction was assessed by absorption of the antiserum with the corresponding antigen. The volume density of each endocrine cell type and that of mesenchymal tissue was determined by the point counting method of Chalkley [24] as previously reported [20]. From the volume density of each endocrine cell type measured in the two lobes of the pancreas, the proportion of mesenchymal tissue and the absolute weight of this two lobes, the total mass of the different endocrine cells was calculated.

Small specimens were also taken in the corpus from cases 3, 5, 6 and 7, fixed in glutaraldehyde solution, postfixed in osmium and embedded in Epon 812 for electron microscopy.

Statistical analysis

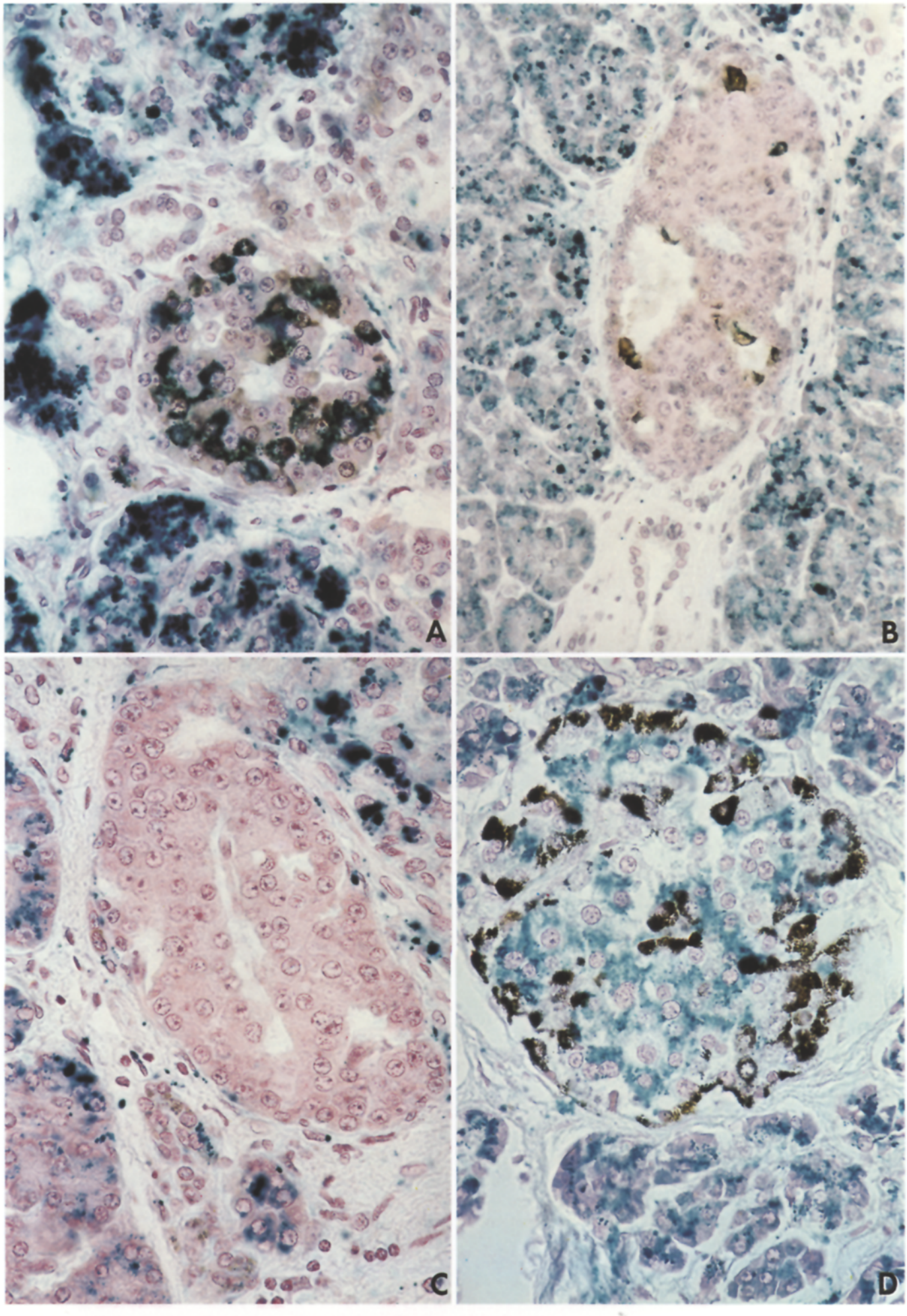
Results are presented as means ± SD. The significance of differences between means was assessed by the Wilcoxon rank-sum test [25]. A p value of < 0.05 was considered statistically significant.

Results

Macroscopy

Its firm consistency and its chamois-brown colour, after removal of the peripancreatic fat, give the haemochro-

Fig. 1 A–D. Double staining technique of pancreatic tissue: Prussian blue method for iron detection (in blue) and peroxidase antiperoxidase technique (in brown) with anti-insulin serum **(A–C)** or Grimelius technique (dark brown) (D). **A** Numerous B cells containing iron (Prussian blue and anti-insulin immunoperoxidase). (Case 2) **B–C** Marked decrease (case 4) or disappearance (case 7) of both insulin immunoreactivity and iron deposits in the islets. **D** Iron deposits predominate in endocrine cells from the central part of the islets. Grimelius positive cells (A and PP cells) are devoid of iron (Case 2)


A
B
C
D

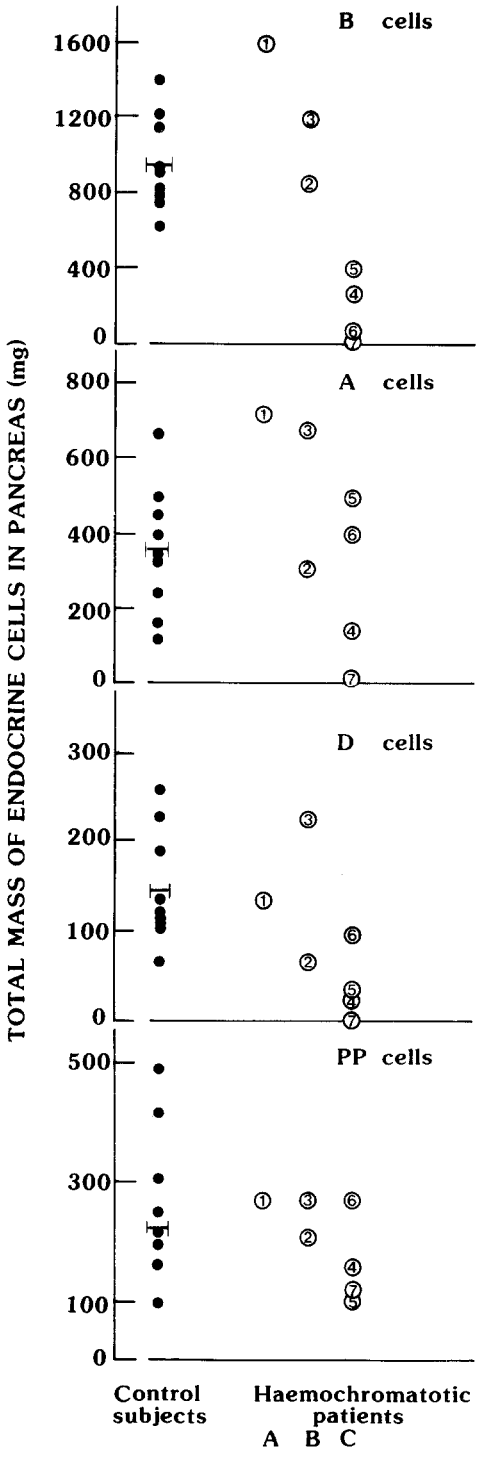


Fig. 2 A–C. Estimated total mass of each endocrine cell type in the pancreas of control and normoglycaemic **(A)**, gluco-intolerant **(B)** and insulin-requiring diabetic haemochromatotic patients **(C)**

matotic pancreas a particular and typical aspect. As shown in Table 2, the weight of the pancreas was clearly lower in insulin-requiring diabetic haemochromatotic patients (nos. 4–7) than in the normoglycaemic haemochromatotic patient (no. 1), in whom it remained within the normal values. The relative weight of the lobe rich in PP cells was increased in these diabetic patients ($p < 0.05$), as reported in classical Type 1 and 2 diabetic patients [26].

Conventional microscopy

The mesenchymal component of the gland, consisting of fat or fibrous tissue, was much more abundant than in controls (Table 2). As classically described [27], iron deposits predominated in exocrine tissue and in macrophages of the connective tissue and was less frequent in islets, whatever the etiology of iron overload. It is noteworthy, however, that iron overload was more important in the islets of normoglycaemic and non-insulin-dependent diabetic haemochromatotic patients (nos. 1–3) than in those of insulin-dependent diabetic haemochromatotic patients (nos. 4–7). The shape and size of the islets were unchanged in haemochromatotic patients; their number seemed to be normal in all cases but one (no. 7), in whom they were less numerous. However, even in this case atrophic irregular islets, typical of insulin-dependent diabetic patients, were not observed. Moreover, amyloid deposits, frequently detected in islets of non-insulin-dependent diabetic patients, were absent from islets of haemochromatotic diabetic patients.

Quantitative immunocytochemistry

Immunocytochemical techniques disclosed several peculiarities of the islets of haemochromatotic patients which had escaped detection after haemalun-eosin staining. Iron pigments were found almost only in immunoreactive B cells; A, D and PP cells were devoid of iron deposits, as were most of the non-immunoreactive cells of the islets. It is immediately observed that marked differences may exist in the number of immunoreactive B cells from case to case (Fig. 1). Although not quantified by morphometry, a positive correlation seemed to exist between the iron overload and the number of immunoreactive B cells. The total mass of endocrine tissue average 1667 mg (range: 1064–2426) in control subjects, 2696 mg in the normoglycaemic haemochromatotic patient (no. 1), and 2347 and 1428 in the two haemochromatotic patients with glucose intolerance (no. 2 and 3). On the other hand, it was markedly decreased (1018–138) in the four insulin-requiring diabetic haemochromatotic patients (nos. 4–7). The calculated mass of each endocrine cell type in the pancreases of controls and haemochromatotic patients is shown by Fig. 2. In insulin-requiring diabetic haemochromatotic patients the mass of immunoreactive B cells was markedly lowered. The mass of A cells and that of D cells was either decreased or normal, and that of PP cells was unchanged. In the two haemochromatotic patients with glucose intolerance the mass of B, A, D and PP cells remained within the normal values. In the normoglycaemic patient, the mass of B and A cells was higher than in controls, whereas that of D and PP cells was within normal values. The volume density of all immunodetected

cells in the islets (sum of A, B, D and PP) was clearly lower than the volume density of islet cells measured after haemalun-eosin staining in insulin-requiring haemochromatotic patients (Fig. 3). Such was not the case in control subjects or in other haemochromatotic patients, in whom the ratio was close to unity. This indicates that certain islet cells are not detected by immunocytochemistry in the group of diabetic haemochromatotic patients.

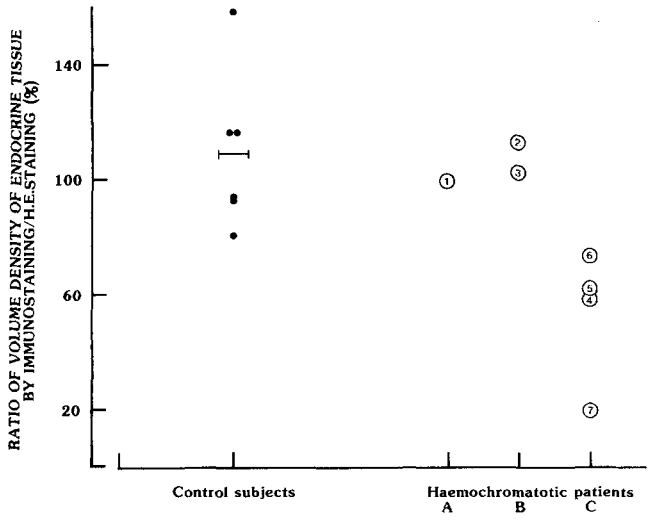


Fig. 3. Ratio of the volume density of endocrine tissue estimated on immunostained slices on Haemalun Eosin staining in the pancreas of control and normoglycaemic **(A)**, gluco-intolerant **(B)** and insulin-requiring diabetic haemochromatotic patients **(C)**

Ultrastructural analysis

Ultrastructural analysis of the islets was performed in one patient with glucose intolerance (no. 3) and in three insulin-requiring haemochromatotic patients (nos. 5–7). In patient 3, a high content in typical β-granules allowed easy recognition of B cells (Fig. 4a), the proportion of which appears normal. Iron overload was evident from the presence of numerous lysosomes containing haemosiderin deposits. The amount of haemosiderin pigments was quite variable from cell to cell. The rough endoplasmic reticulum (RER) was well preserved, its sheets sometimes being undoubled. No degenerative changes occurred, and the rate of granulation remained similar to that of controls, with most of the granules being of the mature crystalline type. A, D and PP cells did not show ultrastructural abnormalities and, in particular, were devoid of haemosiderin deposits. In patient 5, B cells no longer constituted the predominant population of the islets, non-B cells being the most numerous. Lipofuchsin inclusions and degenerative vacuoles were observed in B cells. Haemosiderin deposits were restricted to B cells. As iron overload, the rate of granulation was quite variable from cell to cell but was, in general, lower than in normal B cells

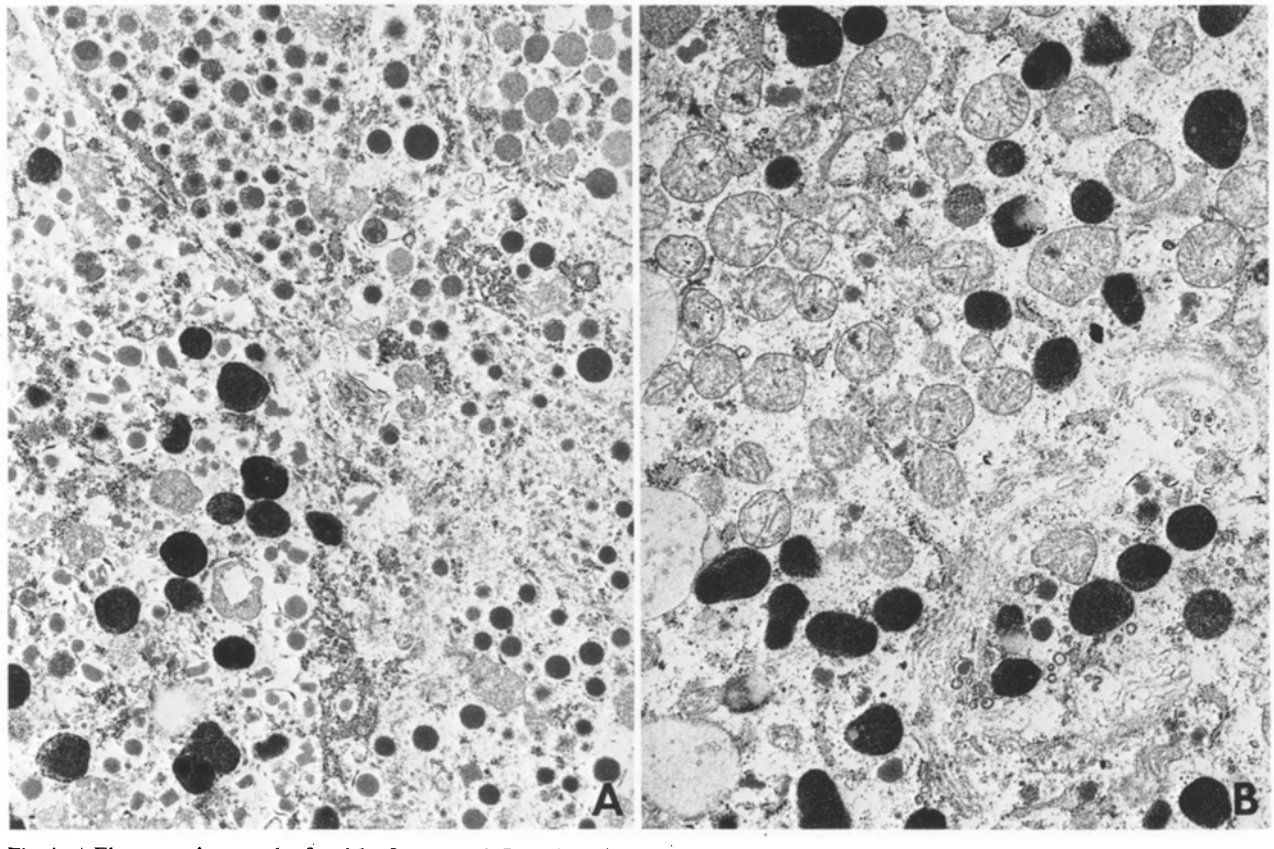


Fig. 4. **A** Electron micrograph of an islet from case 3. Iron deposits are restricted to B cells. Numerous typical β-granules are still present, but their electron density is weaker than in normal B cells. **B** Electron micrograph of a B cell from case 5. Only few granules are detected in the cytoplasm

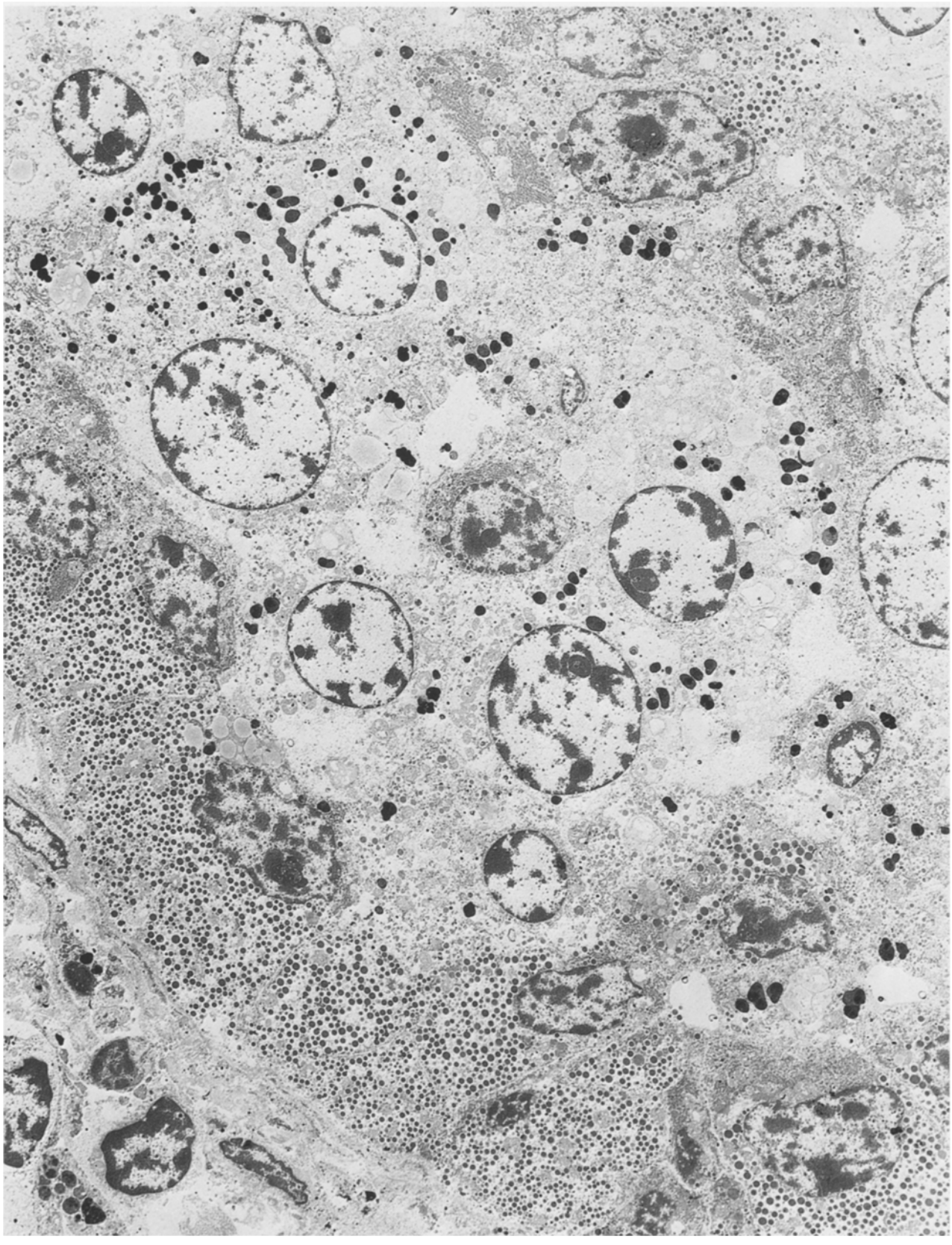


Fig. 5. Low magnification of an islet from case 5. A and D cells remain normally granulated and devoid of haemosiderin. B cells show a major iron surcharge and a clear decrease in the number of insulin granules

(Fig. 4b). Pictures suggest that the decrease in β-granules number correlates with the importance of the iron overload. However, haemosiderin pigments were exclusively observed in still granulated B cells. Non-granulated or poorly granulated cells with non-typical granules were seen in islets. These scarce granules were of small size and electron dense. Most often, these cells were devoid of iron or contained only a small amount. The ultrastructural aspect of A, D and PP cells was not particular. Islets were also observed to be formed in close contact with exocrine ducts, sometimes incorporating ductular cells within the endocrine structure. In patients 6 and particularly 7, islets were rare and B cells infrequent. These cells were difficult to recognize, from the low number and weak density of their granules, in comparison with non-β-granules (Fig. 5). Their cytoplasms were clear, with vacuoles or white spaces. As in the other cases, iron was restricted to B cells and its amount was quite variable. Particular cells were also encountered with empty granules or granules containing only finely granular material. They were devoid of iron. These cells were located in islets, but no formal morphological argument allows their endocrine nature to be ascertained. Multivesicular bodies and undoubling of the RER sheets were observed, not only in some B cells, but also in other endocrine cells.

Discussion

The seven patients included in this series were selected on the basis of iron deposits in the liver, mostly in parenchymal and ductular cells, confirmed in most of the cases by a clinical diagnosis of iron overload (high seric ferritin level and Iron Binding Capacity). The etiology of the iron overload (primary or secondary) was not taken into account.

Several arguments support the hypothesis of a specific role of iron overload in the development of diabetes in haemochromatotic patients. First, the high frequency of diabetes (or glucose intolerance) associated with haemochromatosis (% in this series) cannot be fortuitous. Second, none of the patients became diabetic before an iron overload in the liver was detected. Third, the morphological aspect of the pancreas was similar in the primary and secondary types of the disease. Fourth, our study clearly shows that the islet morphology in diabetes associated with haemochromatosis differs from that of the other types of diabetes.

The normal size and round shape of the islets in haemochromatotic patients make them indeed totally different from the pseudoatrophic irregular islets consistently observed in chronic Type 1 diabetic patients [20, 28]. Moreover, the mass of immunoreactive B cells, though very low in some insulin-requiring haemochromatotic patients, is still much higher than in Type 1 diabetic patients. Several features also make these islets distinguishable from those of Type 2 diabetic patients. First, they never contain amyloid deposits. Second, the mass of immunoreactive B cells in the haemochromatotic insulin-requiring diabetic patients is markedly lower than in Type 2 diabetic patients [20], even when these latter require insulin treatment. Third, apparently normal islets, where only three or four B cells can be detected by immunostaining, have never been observed in pancreases of Type 2 diabetic patients.

A very good correlation exists between the morphometrical estimation of the B-cell mass by immunochemistry and the clinical data. The patient with a B-cell mass higher than in control subjects does not show any glucose intolerance, in spite of an important iron overload. The persistence of normoglycaemia in this case could be explained by an adaptative hyperplasia of the cells, balancing a possible functional deficiency. The two patients with glucose intolerance display a relatively normal mass of B cells, suggesting that this glucose intolerance has to be related to the existence of a functional B-cell deficiency. Lastly, in the four insulin-requiring diabetic patients, the mass of immunoreactive B cells is markedly decreased, explaining the clinical symptomatology. The exact evaluation of the decrease in the B-cell mass in the four insulin-requiring diabetic patients is, however, difficult to ascertain from immunohistochemical quantitative study alone since, as proposed previously [31], a marked decrease or a lack of granulation may lead to an underestimation by these techniques. This is the reason to compare VV of insular cells on HES stained sections to the summation of the VV for each immunodetected endocrine cell type. From this comparison, it becomes evident that not only a decrease in the real mass of the endocrine cells explains the low endocrine cell mass measured, at least in cases with a long duration of diabetes, but that an underestimation of endocrine cells also is responsible for it, probably due to a low hormonal content. Electron microscopy reinforces this hypothesis, since numerous poorly granulated B cells are present in these islets; the observation that A and D cells are still well granulated allows us to conclude that the underestimation related to degranulation is restricted to B cells. Non-granulated cells are also encountered in the islets of insulin-requiring haemochromatotic patients. The real significance of these cells is not clear; they may correspond to old B cells in which insulin synthesis has been interrupted and iron deposit excluded. They could also be new immature endocrine cells of the B- or non-B-cell line, not yet differentiated or unable to differentiate in this particular context. The presence of very small amounts of iron in a few of these cells may, however, constitute an argument to think that they belong to the B-cell line.

It remains difficult to ascertain whether the iron overload is the only factor responsible for the development of diabetes or whether other elements may interfere with the importance of the glucose intolerance, with its insulin-dependent character, or with the rapidity of its appearance. Thus, no correlation seems to exist between the suspected duration of iron overload and the advent of diabetes. However, the importance of iron

overload in the liver, as in the pancreas, does also not correlate with the duration of iron overload. This apparent discrepancy can be explained by a different velocity of iron overloading [29, 30].

Electron microscopic analysis and iron detection by the Prussian blue method combined with immunocytochemistry, formally demonstrate that iron overload is restricted to B cells. The reason for this restriction remains unknown. The persistence of a normal or high glucagon secretion [32, 33] in these patients is in agreement with our observation, but does not allow us to conclude, as previously [34], that the islets of Langerhans are normal in iron overloaded patients.

In conclusion, immunocytochemical quantitative studies and electron microscopic analysis demonstrate that the islets of Langerhans in iron overloaded patients do not share the morphological characteristics of islets of Type 1 nor Type 2 diabetic patients, but show a particular aspect related to the existence of iron surcharge. It is evident that haemochromatotic diabetes and Type 1 diabetes are completely different entities. Our study strongly suggests that such is also the case for haemochromatotic diabetes and Type 2 diabetes. However, the formal proof of this proposal requires elucidation of the pathogenesis of Type 2 diabetes.

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