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Localisation of islet amyloid peptide in lipofuscin bodies and secretory granules of human B-cells and in islets of type-2 diabetic subjects

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Summary. Islet amyloid peptide (or diabetes-associated peptide), the major component of pancreatic islet amyloid found in type-2 diabetes, has been identified by electron-microscopic immunocytochemistry in pancreatic B-cells from five non-diabetic human subjects, and in islets from five type-2 diabetic patients. The greatest density of immunoreactivity for islet amyloid peptide was found in electron-dense regions of some lysosomal or lipofuscin bodies. The peptide was also localised by quantification of immunogold in the secretory granules of B-cells, and was present in cytoplasmic lamellar bodies. Acid phosphatase activity was also demonstrated in these organelles. Immunoreactivity for insulin was found in some lysosomes. These results suggest that islet amyloid peptide is a constituent of normal pancreatic B-cells, and accumulates in lipofuscin bodies where it is presumably partially degraded. In islets from type-2 diabetic subjects, amyloid fibrils and lipofuscin bodies in B-cells showed immunoreactivity for the amyloid peptide. Abnormal processing of the peptide within B-cells could lead to the formation of islet amyloid in type-2 diabetes.

Key words: Islet amyloid peptide – Pancreatic islets – Type-2 diabetes – Insulin – Lysosomes – Secretory granules – Man

Hyaline material in the islets of Langerhans was recognised as a possible pathological factor in the development of diabetes (Opie 1901). The amorphous deposits were subsequently shown to be amyloid (Ehrlich and Ratner 1961) and were unrelated to systemic amyloid disease. Islet amyloid has been found in up to 72% of type-2 diabetic subjects over the age of 40 (Schneider et al. 1980). Although there is a strong association with diabetes (Bell 1959) islet amyloid has been found at post-mortem in elderly non-diabetic subjects with a prevalence of between 8–55% (Bell 1959; Westermark et al. 1987a) and, hence, the deposits have been considered by some to be a non-specific feature of ageing. However, studies in a group of spontaneously diabetic monkeys have shown that amyloid development precedes intolerance to glucose (Howard 1986), suggesting that it could be a causative or predisposing factor to diabetes. Appearance of amyloid in older subjects at post-mortem could therefore represent subclinical prediabetic conditions.

The peptide forming the major component of the insoluble islet deposits has recently been isolated and characterised from pancreatic tissue of type-2 diabetic subjects (Cooper et al. 1987; Westermark et al. 1987b). It consists of 37 amino acids and shows significant structural homology, particularly at its C-terminal region, with calcitonin gene-related peptide, a product of alternative gene processing in thyroid C-cells (Fig. 1) (Rosenfeld et al. 1983). There is also a distant homology with the A-chain of insulin (Cooper et al. 1987). The peptide has been termed either Diabetes Associated Peptide, DAP (Clark et al. 1987; Cooper et al. 1987) since it was extracted from pancreatic tissue of diabetic subjects or Insulinoma Amyloid Polypeptide/Islet Amyloid Polypeptide, IAPP, since it was extracted from a human insulinoma and from pancreatic islets (Westermark et al. 1987a, b). In this report it is called islet amyloid peptide (IAP).

The amyloid deposits can occupy up to 80% of the islet space and disrupt the normal islet architecture (Clark et al. 1986). The fibrils lie between the capillaries and the endocrine cells, closely apposed to the hormone-producing cells and within deep invaginations of the B-cell plasma membrane (Westermark 1973; Clark et al. 1987). These anatomical changes could affect the normal passage of glucose and hormones within the islets and thereby lead to the characteristic abnormalities of insulin secretion of type-2 diabetes. The origin of IAP is not known, but there is an

1 10
Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-
20
Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-
30 37
Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr

Fig. 1. The amino acid sequence for IAP

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association of amyloid with B-cells: IAPP has been shown to be the component peptide of amyloid from an insulinoma (Westermark et al. 1987a), and in diabetic subjects, amyloid is not found in the islets of the pancreatic head which contain few B-cells (Clark et al. 1984).

Immunoreactivity for $IAPP_{7-17}$ and IAP_{19-37} has been demonstrated with light microscopy in islet B-cells of non-diabetic subjects (Clark et al. 1987; Westermark et al. 1987a, c). Immunoreactivity for $IAPP_{7-17}$ in feline islet cells has been found in the halo of insulin granules (Johnson et al. 1988) but the peptide has not been localised in human B-cells. To establish the origin of the peptide in cells not associated with amyloid, IAP has been identified within B-cells from non-diabetic subjects by use of quantitative immunogold cytochemistry and observations made on tissue from five type-2 diabetic subjects. Some of the results have been published in abstract form (Clark et al. 1988a).

Materials and methods

Pancreatic tissue

Non-diabetic subjects. Pancreatic tissue was obtained from 4 kidney donors, aged 17, 17, 17 and 44 (the pancreas being removed at the time of organ donation) and from a surgical partial pancreatectomy specimen resected from a man aged 65 because of a pancreatic carcinoma (normal pancreatic tissue was obtained proximal to the localised tumour). Since the operation the patient has been shown to have normal glucose tolerance.

Type-2 diabetic subjects. A type-2 diabetic subject aged 67 years had a partial pancreatectomy for a suspected tumour. Pancreas from the resected specimen was fixed in 4% glutaraldehyde in phosphate buffer, pH 7.4. No tumour was detected in this patient or in the specimen.

Pancreatic tissue resected at post-mortem (2.5–20 h following death) from 4 type-2 diabetic subjects, aged 48–84 years was fixed in normal saline containing 10% formaldehyde (formol-saline). Small specimens were removed from the body region of the pancreas and processed for electron microscopy.

Specimen preparation

Pancreatic tissue from the non-diabetic subjects was dissected free from adipose tissue, trimmed into 2 mm cubes and fixed in 2% paraformaldehyde, 0.5% glutaraldehyde in cacodylate buffer, pH 7.4. Half the specimens were post-fixed in buffered 1% osmium tetroxide. All specimens were dehydrated through ethanol and embedded in Spurr resin (Taab Laboratories, Reading, UK).

Immunocytochemistry

Ultrathin sections (~60 nm thick) of pancreatic islets were cut from the tissue blocks and supported on nickel grids. A rabbit polyclonal antiserum to synthetic IAP_{19-37} raised in our laboratory was used for immunohistochemistry. Osmium was removed from the sections with saturated sodium periodate (10–30 min) and the sections incubated for 2 h with antisera, 1:500 dilution (IAP) or 1:1000 dilution (insulin), and then for 1 h with protein A-gold (Janssen Pharmaceuticals, Olen, Belgium) diluted 1:50. Tissue contrast was enhanced by staining with uranyl acetate and lead citrate and sections examined with a Jeol-JEM 100S electron microscope.

Specificity of the antiserum to IAP was demonstrated by preabsorbtion tests, in which immunoreactivity was not inhibited by preabsorption overnight at 4° C with human calcitonin gene-related peptide (10^{-8} M) or with human insulin (Novo Laboratories, Denmark) (10^{-8} M), but was blocked by preabsorption with the immunogen IAP_{19-37} (5×10^{-7} M). Immunoreactivity to insulin was demonstrated by use of a polyclonal antiserum to insulin (ICN Immunobiologicals, High Wycombe, UK) raised in a guinea-pig. Preabsorption of this antiserum with human insulin (10^{-8}) blocked immunoreactivity.

Histochemistry for acid phosphatase

Thin slices of one non-diabetic pancreas were prepared for examination of acid phosphatase acitivity (Gomori 1941); aldehyde-fixed tissue was washed in acetate buffer, pH 5.0, and reacted with beta-glycerophosphate in lead nitrate for 10–20 min at 37° C. Electron-dense lead phosphate deposits were formed at the site of enzyme activity. Specimens were rinsed in acetate buffer and cacodylate buffer, pH 7.4, and then processed by the usual method for embedding in resin.

Qantification of immunoreactivity

Photomicrographs (printed at a final magnification of 30000) were prepared of systematic randomly selected adjacent regions of the B-cells in a pancreatic islet (total area, 100–500 μm^2). For analysis of immunogold density, the B-cell was subdivided into granule cores, granule halos, lysosomal or lipofuscin bodies, mitochondria, nuclei and the remainder of the cytoplasm. The numbers of gold grains situated on these compartments and over extracellular spaces were counted on the photomicrographs. The area of each compartment was estimated by point-counting using a 1 cm quadratic lattice and the numerical density of the gold particles was calculated for each cellular compartment. Similar, low densities of immunoreactive gold were found over extracellular spaces, nuclei and the remainder of the cytoplasm. Binding over these regions was taken to represent non-specific, random background deposition of gold particles. The density of immunogold in each compartment was compared with this background by application of the χ^2 test for each individual and the paired t-test for all subjects together.

Results

Tissue preservation was good in all surgical specimens; mitochondria were intact and cellular membranes well preserved. In post-mortem specimens fixed in formol-saline, intact nuclei, insulin granule cores, lipofuscin bodies and amyloid fibrils were easily recognised, even though membranes and endoplasmic reticular stucture were poorly preserved. Despite this, in both the surgical and post-mortem specimens, clear immunoreactivity for IAP was found in the islet amyloid and B-cells of the type-2 diabetic subjects and in the B-cells from the non-diabetic subjects.

Non-diabetic subjects

In B-cells of non-diabetic subjects the highest density of immunoreactivity for IAP was located in electron-dense re-

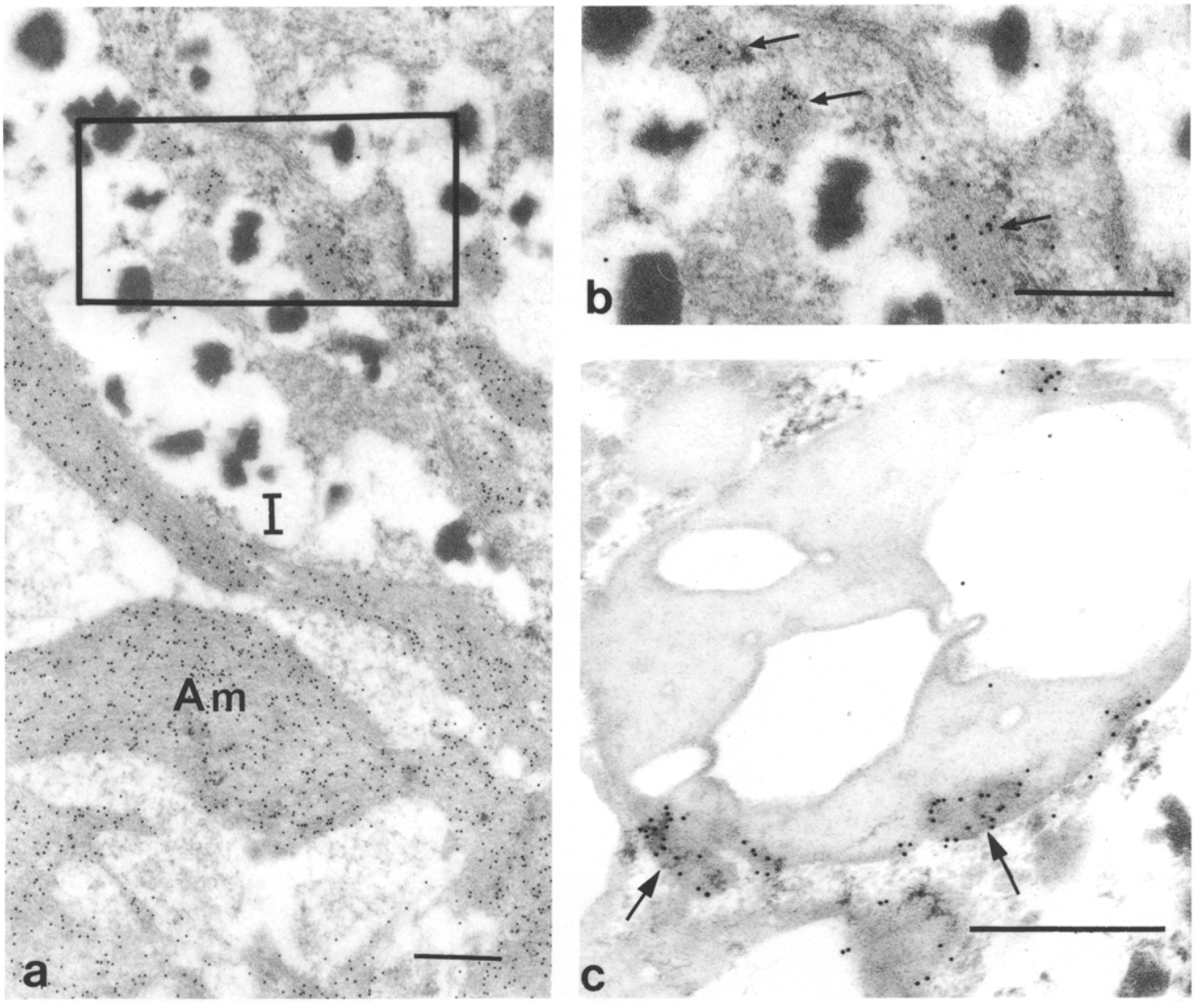

Fig. 2a–c. Islets of type-2 diabetic subjects showing immunoreactivity for DAP. **a** Immunogold deposition on extracellular amyloid (*Am*) enclosing unlabelled areas which possibly represent cellular debris. Adjacent B-cells containing insulin granules (*I*). *Bar:* 0.5 μm; **b** Enlarged region of **a** showing small areas of immunolabelled amyloid (*arrows*) apparently within the B-cell. *Bar:* 0.5 μm; **c** Immunogold deposition on electron-dense regions of a lipofuscin body in a post-mortem specimen fixed in 10% formol-saline. *Bar*: 0.5 μm

Table 1. Clinical details of diabetic subjects

Patient Number	Age	Sex	Therapy	Duration of diabetes	Cause of death
1	67	M	O/I	10 years	Alive
2	84	F	O	12 years	Pneumonia
3	50	M	O	1 year	Stroke
4	76	F	O/I	28 years	MI
5	48	M	I	4 years	Cirrosis

The surgical specimen was obtained from Patient 1. *O* oral sulphonylurea; *I* insulin

gions of lysosomal, lipofuscin bodies (Fig. 2a) (Table 1); in two subjects (4 and 5) the density of immunogold over lipofuscin bodies was 30- and 165-fold greater than over background. However, in 3 other subjects no appreciable IAP could be detected in lysosomes. There was considerable structural heterogeneity among lysosomes and lipofuscin bodies within a single cell. Many did not show immunoreactivity for IAP. Multivacuolar lipofuscin bodies also had acid phosphatase activity restricted to the electron-dense regions. Immunoreactivity for insulin was detected within the electron-dense regions of some lipofuscin bodies.

Multilayered membrane bodies in the cytoplasm showed immunoreactivity for IAP (Fig. 2c) and acid phosphatase activity (Fig. 2b). These structures were distinct from fibrillar amyloid. Immunoreactive membranous bodies were often adjacent to secretory granules (Fig. 2c).

Immunoreactivity for IAP was present within the granules of B-cells; gold particles were located over both the halos and dense core of the granules (Fig. 2d). Quantification in individuals showed significant ($p < 0.001$) immunoreactivity over the granule halo compared with background in each of 3 normal subjects (nos 1, 3 and 5) compared with background as well as an overall significant increase for the group (Table 1). In two individuals (subjects 3 and

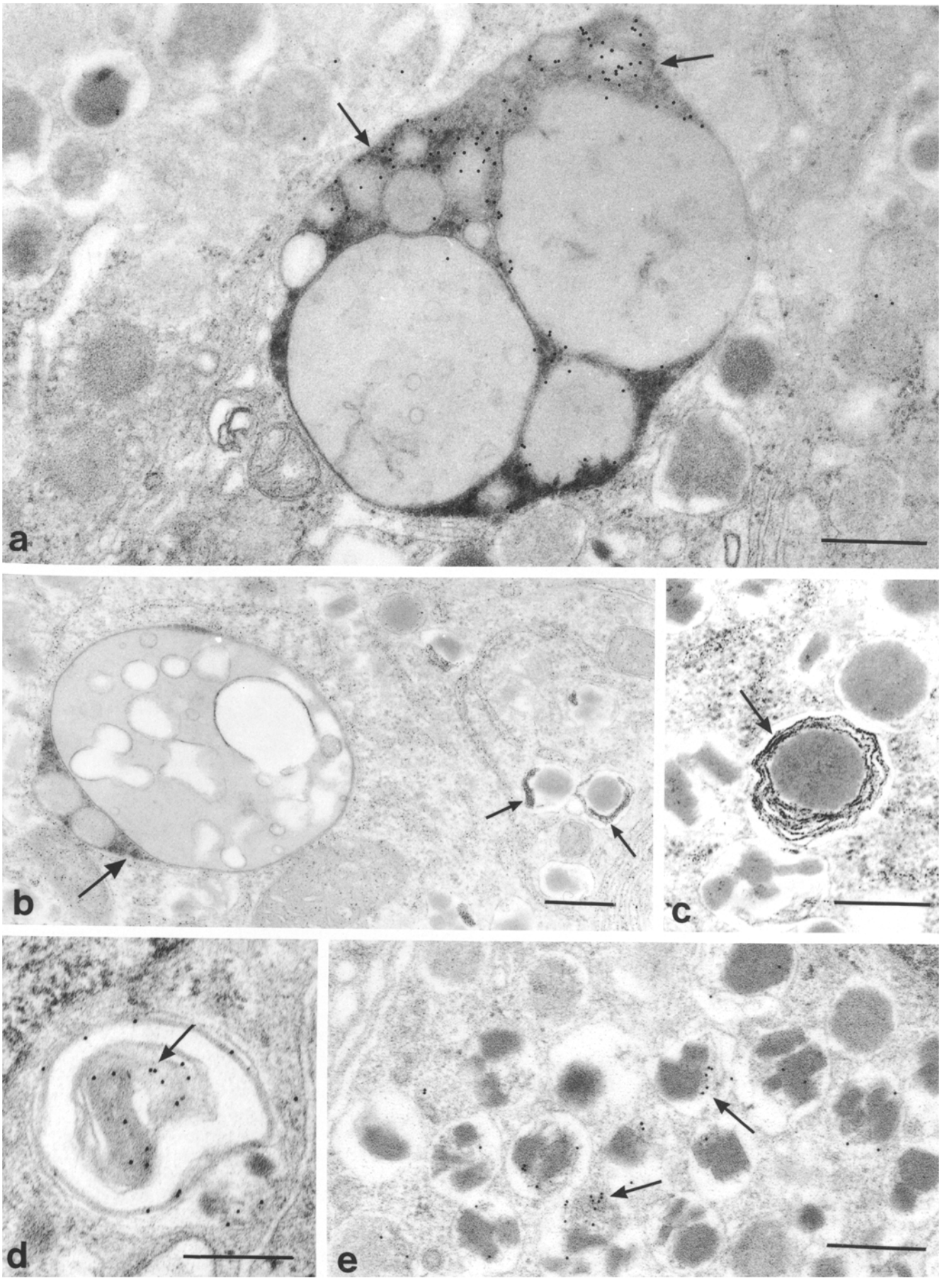
a
b
c
d
e

Table 2. Immunoreactivity for IAP in B-cells

Subject	Age	Immunogold distribution							
		Granule core		Granule halo		Lysosome		Background	
	Years	Density	Area	Density	Area	Density	Area	Density	Area
Human									
1	44	0.7	22	2.5[a]	26	0.4	26	1.1	123
2	17	0.3	15	1.6	21	0	4.4	1.2	135
3	17	2.9[a]	38	4.8[a]	84	0	2.6	1.6	322
4	17	0.7	60	1.4	26	26[a]	16	0.9	175
5	65	3.0[a]	14	3.3[a]	18	102[a]	9	1.6	57
Mean		1.5		2.7				1.3	
±SEM		1.3		1.4				0.4	
Paired *t*-test P = compared with background		0.6		0.05					

Immunoreactivity of IAP in B-cells expressed as the numerical density of immunogold particles over B-cell granule cores, halos, lysosomes and background in 5 subjects. Immunogold density in gold particles/μm^2. Area examined in μm^2

[a] X^2 test, $p < 0.001$, significantly greater than background for that specimen

5) but not the group as a whole, there was also significant ($p < 0.001$) immunoreactivity over the granule core (Table 1). The antiserum to insulin showed, as expected, binding of gold particles over the granule cores. Acid phosphatase activity was noted in the halos of some B-cell granules (Fig. 2b).

Type-2 diabetic subjects

Amyloid fibrils showed a high density of immunogold binding (Fig. 1a), and its location could be easily identified in both the surgical specimen and the post-mortem tissue. The amyloid was situated adjacent to both peripheral and central capillaries in the islets and was bordered by insulin-containing B-cells, glucagon-containing A-cells and somatostatin-containing D-cells. Shafts of amyloid fibrils penetrated the adjacent B-cells but were not found between the islet cells. Irregularly shaped areas resembling fragments of cytoplasm were present within the amyloid deposits and were not immunoreactive for IAP (Fig. 1a). Small regions of immunoreactive amyloid fibrils were present within the B-cell profiles and appeared to be entirely surrounded by cytoplasm in the plane of the section (Fig. 1b). In the surgical specimen fixed in 4% glutaraldehyde little immunogold was present on the B-cells either within the lysosomes or associated with the densely packed insulin granules. However, immunoreactivity for IAP was present within the lipofuscin bodies of the B-cells in the post-mortem specimens fixed in formol-saline (Fig. 1c). The poor cytoplasmic preservation in the post-mortem specimens precluded accurate quantification of immunogold.

Fig. 3a–e. Localisation of IAP by immunogold and acid phosphatase in B-cells of non-diabetic human subjects. **a** Immunogold particles showing IAP in electron-dense areas of a lipofuscin body. *Bar:* 0.5 μm; **b** Acid phosphatase activity shown as electron-dense areas of lead deposition in the lipofuscin body (*arrow*) and within the B-cell granule halo (*small arrows*). *Bar:* 0.5 μm; **c** Acid phosphatase activity in a 'lamellar body' adjacent to a secretory granule. *Bar:* 20 nm; **d** Immunoreactivity for IAP in an intracellular 'lamellar body'. *Bar:* 0.5 μm; **e** IAP localisation in secretory granules. *Bar:* 0.5 μm

Discussion

In B-cells of normal subjects, IAP immunoreactivity was located in lipofuscin bodies, insulin granules, and in multilayered cytoplasmic "lamellar bodies". There was considerable variability of the immunoreactivity between patients and within different cells of the same islet. In the type-2 diabetic subjects, the islet amyloid was intensely immunoreactive for IAP confirming light-microscopic results (Clark et al. 1987), and immunoreactivity to IAP was also found in lipofuscin bodies of islet B-cells.

The finding of immunoreactivity for IAP in lipofuscin bodies of both diabetic and non-diabetic subjects suggests that the peptide may be degraded by an intracellular pathway. Acid phosphatase activity was present in similar regions of the lipofuscin indicating that the peptide is present in the biochemically active areas. Heterogeneity was marked; some large vacuolated lipofuscin bodies showing little enzyme activity and often no immunoreactivity for IAP. Insoluble ceroid and lipofuscin pigments are thought to be formed within the lysosomes from incompletely processed material, including peroxidised lipids crosslinked to peptides (Chio and Tappel 1969). The absence of immunoreactivity for IAP in all lipofuscin bodies could indicate heterogeneity of function of the lysosomes or that degradation of the peptide has been completed or lack of availability of the epitope due to crosslinking of side chains. Lipofuscin bodies are found in neonatal tissues (Mann and Yates 1974) but accumulate with age in many tissues of the body, including the central nervous system (Mann and Yates 1974) and heart (Jayne 1950). The method of formation of amyloid is unknown but it is possible that progressive accumulation of intracellular lipofuscin bodies, containing IAP or a precursor peptide, could lead to polymerisation of IAP into insoluble amyloid fibrils and cell death. This hypothesis is supported by the finding of apparently intra-

cellular amyloid in B-cells of type-2 diabetic subjects and evidence that cellular debris is present in the islet amyloid deposits. Light microscopy has shown that amyloid deposition is associated with destruction of the islet cells in type-2 diabetes (Clark et al. 1988b).

Immunoreactivity for IAP was present within the B-cell granule halos surrounding the dense cores of crystalline insulin. This result is comparable to that found in feline pancreas where immunoreactivity for IAPP was associated with insulin granules (Johnson et al. 1988). The presence of the amyloid peptide and insulin both in granules and in lysosomes suggests that the two peptides are degraded by intracellular lysosomal pathways (Halban and Wollheim 1980; Orci et al. 1984; Borg and Schnell 1986) but insoluble residues of IAP can accumulate in lipofuscin bodies. In the rat, mature insulin granules are incorporated into lysosomes, possibly by crinophagy, leading to degradation of insulin (Meda 1978; Orci et al. 1984). It is probable that C-peptide, betagranin and other compounds located within the granule compartment would be similarly catabolised and some of the products of enzyme activity recycled to the cell. The finding of insulin immunoreactivity in human B-cell lysosomes suggests that a similar mechanism for insulin degradation is present in man.

The mechanisms regulating lysosomal uptake of insulin granules are unknown but may be related to the cellular concentration of mature granules: diazoxide, which inhibits insulin secretion without affecting biosynthesis, increases the activity of islet lysosomal enzymes (Skoglund et al. 1987) and crinophagy is increased at glucose concentrations that are subthreshold for stimulation of insulin release (Skoglund et al. 1987; Halban and Wollheim 1980). If the intracellular pathway for catabolism of IAP is similar to that of insulin, high levels of biosynthesis associated with low plasma glucose concentrations could lead to accumulation of IAP in lysosomes.

Lamellar myeloid bodies have been identified in isolated rat islets in tissue culture (Halban et al. 1979; Zwahlen et al. 1979). These structures resemble the end products of lysosomal activity found in other tissues, which are known as residual bodies. Myeloid bodies are released from rat B-cells by exocytosis and this is reduced by cyproheptadine, an agent that inhibits insulin secretion (Zwahlen et al. 1979). Immunoreactivity for IAP in lamellar bodies suggests a membrane-bound location for the peptide or its precursor but further identification of the nature of these bodies is needed.

Immunoreactivity for IAP in both diabetic and non-diabetic B-cells suggests that the amyloid peptide is derived from a normal cellular constituent. The gene encoding human IAP has been isolated from a genomic library and located on chromosome 12. The peptide is derived from a larger propeptide and the predicted amino acid sequence is identical to that of the extracted peptide with the exception that the peptide may be amidated on the carboxy terminal in the native IAP (Mosselman et al. 1988). This suggests that, unlike amyloid derived from prealbumin (Pras et al. 1983) and cystatin C (Palsdottir et al. 1988), in which a genetically altered protein is produced, islet amyloid is formed from the normally-occurring peptide. Identification of more than one cDNA sequence encoding IAP by screening an insulinoma library suggests that amyloid may develop by inefficient splicing of the prohormone in insulinoma tissue (Sanke T et al. 1988). A similar situation may be present in islet cells of type-2 diabetic subjects; aberrant biochemical processing of IAP could be the result of a primary abnormality in the B-cell or secondary to the effects of hyperglycaemia (e.g. during hypersecretion of insulin). This could lead to its accumulation in the B-cell with subsequent formation of amyloid deposits.

Since the peptide is located in organelles showing acid phosphatase activity it is possible that post-translational changes could take place at acid pH or that IAP has a role in the regulation of enzyme activity. Acid phosphatase activity in granule halos has been reported in rats (Meda 1978) and the activity of the peptidases responsible for cleavage of proinsulin is optimal at low pH (Orci et al. 1986; Davidson and Hutton 1987). Analysis of the cDNA for the A4 protein from amyloid of Alzheimer's disease indicates a much longer precursor peptide which includes a domain with homology with the Kunitz family of serine protease inhibitors (Ponte et al. 1988; Carrell 1988). The peptide of hereditary cerebral amyloidosis, cystatin C, is also a protease inhibitor (Barrett et al. 1984). The precursor of the Alzheimer protein has, like some other proteases, a hydrophobic region suggesting it is membrane bound. Since IAP is found in the halos of insulin granules and lamellar bodies it could be membrane bound.

Decreased immunoreactivity for the islet amyloid peptide (IAPP) has been reported in islets of type-2 diabetics (Westermark et al. 1987c). In our study, immunoreactivity for IAP was noted in lipofuscin deposits in B-cells of five diabetic subjects and the density of the lipofuscin bodies corresponded with the density of immunoreactivity observed with light microscopy of specimens from the same subjects. No apparent reduction in the peptide in tissue from type-2 diabetic subjects was noted. Since the accumulation of amyloid in the pancreas might account for the late onset of a hereditary disease, determination of the role of the formative peptide and its localisation in normal and diabetic pancreas may be pertinent.

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