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Islet cell antigens and autoantigen(s): Monoclonal antibodies and further characterization

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Abstract. A novel series of murine monoclonal antibodies to islet cells (I-45, I-51, I-52 and I-39) have been generated using human insulinoma homogenate as the immunogen in order to characterize pathogenetically relevant islet cell autoantigen(s). Differentiation antigens recognized by these islet cell monoclonal antibodies displayed varied cytological distribution (pan-islet or peripheral mantle only). Monoclonal antibody I-45 reacted with all endocrine subsets of the pancreatic islet, similar to the reactivity of islet cell autoantibody positive sera from type I diabetes subjects. Preexposure to pH2 abolished the immunoreactivity of the autoantigen; I-45 antigen was also sensitive to low pH. Preexposure to 100° C for 1 h did not significantly alter the immunoreactivity of islet antigens recognized by ICAb positive patient sera and monoclonal antibody 1-39, thus demonstrating the extraordinary heat stability of the corresponding epitopes; those recognized by I-45 were less heat stable. Islet cells were found to share I-45 differentiation antigen(s)/epitope(s) with other neuroendocrine cells, *viz.* amerior pituitary, adrenal medulla and gut endocrine cells.

Keywords. Autoimmunity; type I diabetes; islet cell antigens; islet cell antibodies; monoclonal antibodies; heat stable antigen; neuroendocrine differentiation molecules; immunohistochemistry.

1. Introduction

In order to understand the mechanisms involved in the breakdown of self-tolerance in type I diabetes, it is essential to identify the target antigen(s) involved in the autoimmune *ß*-cell destructive process. The two lines of evidences reported thus far elaborate about (i) a putative 64 kDa islet protein (expression increased by high glucose, glutamic acid decarboxylase enzyme activity and possibly a heat shock protein), and (ii) islet cell glycolipid(s), as the autoantigen(s) (ICAgs) (Colman *et al* 1988; Kampe *et al* 1989; Baekkeskov *et al* 1990; Christie *et al* 1990; Jones *et al* 1990). Detailed characterization (structure/function) of any of these antigens is yet to be established. This has prompted us to look for alternative approaches to study and identify the relevant molecule(s), including the use of islet cell monoclonal antibodies (ICMAbs) as probes for easier isolation, and characterization of ICAgs. It is however pertinent to note that none of the few ICMAbs described to date have been shown to be directed against pathologically relevant islet autoantigens/ epitopes, and the relationship between the human islet cell antibodies (hICAbs) of type I diabetes and these ICMAbs is still unclear. However, analogous monoclonal antibody approach has been successful in thyroid autoimmunity, through the identification, isolation and gene cloning of the thyroid microsomal autoantigen

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(thyroid peroxidase), utilizing corresponding monoclonal antibody probes (Ruf *et al* 1987).

2. Materials and methods

Hybridoma reagents and plasticware were obtained from Flow Laboratories, UK. Immunological reagents were obtained from Sigma Chemical Company, St. Louis, MO, USA (proteinA–FITC and anti-mouse Ig—FITC).

2.1 *Tissues*

Human pancreas and human insulinoma used were discarded surgical specimens obtained from surgery. Other human tissue specimens were obtained during autopsy. Monkey tissues were obtained from animals sacrificed during other research studies at our institution. The tissues obtained were cut into 0·5 cm blocks and snap frozen in liquid nitrogen. These blocks were preserved at –70° C till use. For immunohistochemistry, 5 μm sections were cut at –25° C and the sections placed on gel chrome coated glass slides (Srikanta *et al* 1985).

2.2 *Assay for hICAb*

The unfixed pancreas sections were incubated for 30 min with 20 μl serum, washed thrice and incubated with protein A–FITC conjugate for 30 min. After further washings, sections were mounted in 30% glycerol in PBS and screened under fluorescence microscope.

2.3 *Assay for ICMAb*

The pancreas sections were incubated for 30 min with 20 μ1 culture supernatant, washed thrice and incubated with anti-mouse Ig—FITC conjugate for 30 min. After further washings, sections were mounted in 30% glycerol in PBS and screened under fluorescence microscope.

2.4 *Distribution of antigens in tissues*

This was studied with unfixed sections of pituitary (monkey), pineal (monkey). thyroid (human), parathyroid (human carcinoma), adrenal (human) and duodenum (human). These tissues were procured, preserved and processed as mentioned above. Immunohistochemical assays were carried out using the same protocol as for screening ICMAbs (vide supra).

2.5 *Hybridoma production*

Human insulinoma homogenates were intraperitoneally administered to Balb/c mice (three injections: days 0, 15 and 30) and splenocytes were fused with the mouse

myeloma cell line P3X63 Ag 6·5·3 in 5:1 ratio and plated into 96 well tissue culture plates containing feeder cells. The wells were periodically supplemented with selection media (HAT) and supernatant screened on 14th day post-fusion. Two different screening strategies were used for identifying murine ICMAb secreting hybridomas, based on two different rationales: (i) Frozen unfixed section indirect immunofluorescence assay: This was similar to the hICAb assay for patient serum autoantibodies, and is expected to detect reactivity towards 'anchored' or 'membrane related' antigens, analogous to the antigens pathologically important in human type I diabetes. (ii) Fixed section indirect immunofluroscence assay: This was done using formalin fixed, paraffin embedded pancreatic sections. The sections were deparaffinized and rehydrated prior to the assay. This assay is expected to detect reactivity towards islet hormones and other soluble islet antigens. Hybrid colonies secreting ICMAbs were cloned by limiting dilution method (single cell/clone per well) and expanded in cell culture.

2.6 *Heat stability of antigens*

Unfixed human pancreas sections were heated in a hot air oven for 15 min at 50°, 60^0, 70°, 80°, 90° and 100° C. The sections were treated with four different hICAb positive patient sera and assay was carried out to find whether the islet antigens were still reactive to hICAbs. As the reactivity persisted, unfixed pancreas sections were, subsequently , preheated similarly at 100° C for 30 min, 45 min and 1 h, prior to hICAb assays. Using similar protocols, ICMAb I-45 and I-39 immunoreactivities were tested on pancreatic sections preheated at 100° C for 1 h.

2.7 *Treatment with organic solvents*

Unfixed cryostat sections of the pancreas were incubated for 10 min at room temperature in chloroform : methanol (2:1), chloroform, methanol and acetone. Then these sections were studied for immunoreactivity of islet cell antigens using patient serum hICAbs and ICMAbs I-45 and I-39.

2.8 *pH sensitivity*

Unfixed pancreatic sections were incubated for 10 min at pH 7·5 or 2·0 at 37° C. After preincubation at pH 2·0 sections were washed once at pH 7·5. Immunoreactivity of islet cell antigens was checked on these treated sections using patient serum hICAbs and ICMAbs I-45 and I-39.

2.9 *Enzyme treatment*

Enzymes pronase and trypsin were used as described by Nayak *et al* (1985). The sections were preincubated for 1 min in solutions containing pronase or trypsin before the assay.

2.10 *Periodate oxidation*

Oxidation with sodium metaperiodate was carried out as follows: Slides with pancreas sections were immersed in a solution containing 50 mM sodium metaperiodate (Sigma, USA) for 10 min at 4° C in the dark, washed with PBS containing glycine (0·1 M) and tested for antigen immunoreactivity using patient serum hICAbs and ICMAbs I-45 and I-39.

3. Results

3.1 *Hybridoma production*

Out of the 744 wells plated, 626 wells had hybrid colonies and all these were screened by frozen section assay and 296 wells were screened by fixed section assay. A total of 70 wells, named I-1 to 1-79 were cryospreserved. Supernatants of the hybrids named I-45, -51, -52, -39, -50, -63, -64, -65, -67, -69, -70, -72, -76 were found to be islet reacting. I-45, I-51, I-52 and I-39 were identified by frozen section assay. and selected for further studies. (Other clones were identified on assay with fixed pancreas sections and data with respect to these are not included in this communication.)

3.2 *Islet cell reactivity*

ICMAb I-45 stained all endocrine cell subsets of the islets, with a reactivity pattern similar to hICAbs, showing a uniform "flat" (cytoplasmic) pan-islet staining [figure la (i)]. The isotype of MAB I-45 was IgG 2a. I-51 and -52 reacted only with the islet mantle leaving the islet *β*-cell core unstained [figure 1a (ii), (iii)]. With I-51 and I-52 in unilobular islets, immunostaining appears only in the rim [figure la (ii)]; in multilobular islets, cells at the islet rim as well as those at the periphery of the individual lobules within the islet are stained [figure 1 a (iii)].

3.3 *Effect of heat*

Pretreatment of pancreas sections to 100° C for 1 h did not significantly alter the immunoreactivity of islet antigens recognized by hICAbs and ICMAb I-39; those recognized by ICMAb I-45 were relatively less stable [figure lb (i), (ii)].

3.4 *Effect of organic solvents*

Chloroform or acetone did not abolish the islet antigen immunoreactivity, whereas with methanol or chloroform:methanol pretreatment, the islet antigen immunoreactivity was completely abolished (table 1).

3.5 *Effect of pH*

Preexposure to pH 2 (0·5 M phosphate, 10 min) abolished the immunoreactivity of

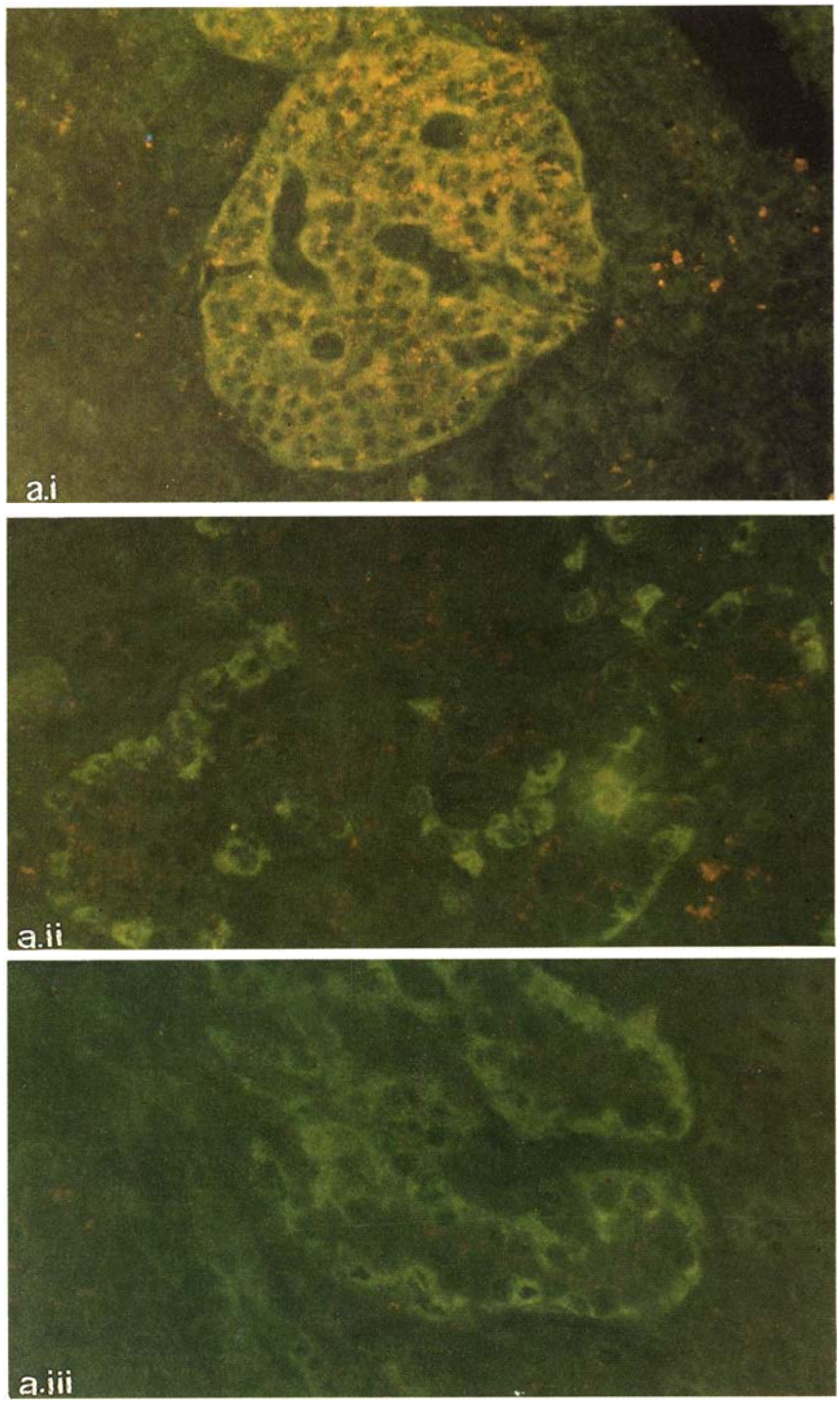

Figure la. Monoclonal islet cell antibodies: Reactivity with unfixed cryostat sections of human pancreas (indirect immunofluorescence, IFL**).** (i) I-45, (ii) I-51 and (iii) I-52. I-45 reacts with all the endocrine cell subsets of the pancreatic islet. I-51 and I-52 react with the peripheral islet mantle cells only.

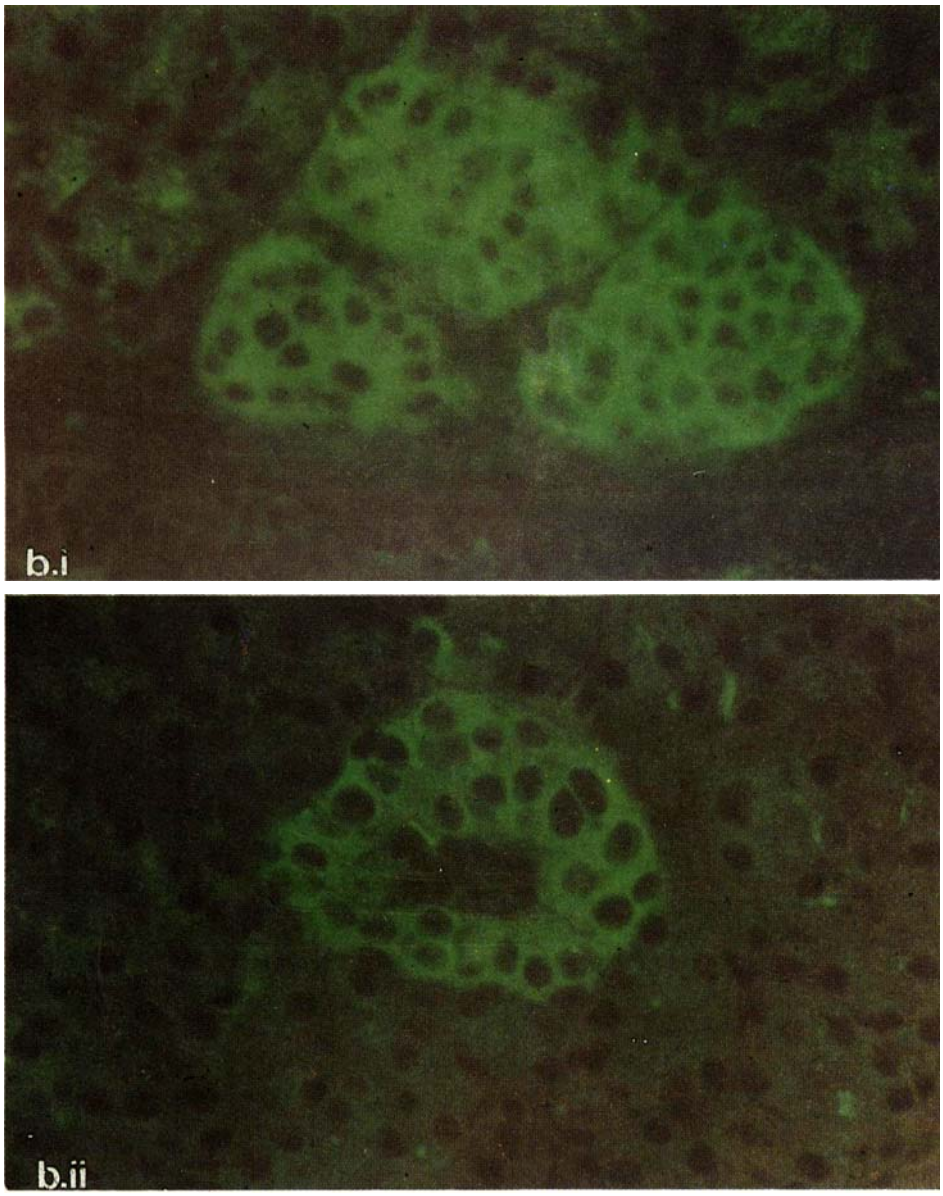

Figure lb. Reactivity of islet cell autoantibody (hICAb) positive patient sera (type I diabetes) with unfixed cryostat sections of human pancreas (IFL). (i) Without preheating of the pancreating sections and (ii) after dry heating for 1 h at 100° C.

the hICAb autoantigen. ICAg recognized by ICMAb I-45 was also sensitive to low pH (table 1).

3.6 *Effect of proteolytic enzymes*

Trypsin or pronase did not alter the immunoreactivity of ICAgs recognized by patient serum hICAbs or ICMAbs I-45 and I-39 [figure lc (i)].

Table 1. Physicochemical characteristics of islet antigens.

Pretreatment	hICAg	I-45 Ag
Periodate oxidation	–	–
Chloroform : methanol (2:1)	–	–
Methanol	–	–
Chloroform	+	+
Heat (dry, 100° C, 1 h)	+	+/–
pH 2	–	+/–
Protease	+	+
Islet distribution	Pan-islet	Pan-islet

–, Immunoreactivity abolished; +, immunoreactivity retained; +/–, immunoreactivity weak.

3.7 *Periodate oxidation*

By this treatment, immunoreactivity of ICAgs recognized by hICAb and ICMAb I-45 were abolished, but not the reactivity of I-39 [figure lc (ii)].

3.8 *Tissue distribution of antigens/epitopes recognized by ICMAb I-45*

The antigen/epitope recognized by ICMAb I-45 showed a neuroendocrine distribution; apart from pancreatic islets, I-45 antigen was demonstrated in adrenal medulla, anterior pituitary, and gut endocrine cells (figure 1d). This monoclonal was reacting with unfixed parathyroid adenoma section also (data not shown). I-45 failed to react with posterior pituitary, adrenal cortex, pineal or thyroid follicular cells. I-39 reacted with thyroid follicular cells, whereas the reactivities of I-51 and I-52 were more restricted.

3.9 *Competition experiments*

In competition experiments based on the indirect immunofluoreseence assay MAbs I-45, I-51, I-52 and I-39 did not compete with hICAb suggesting separate/distinct epitopes.

4. Discussion

Using the hybridoma technology a new library of murine monoclonal antibodies to islet cells has been developed. These ICMAbs showed different cytological immunostaining patterns in the pancreatic islets, depending upon the expression of the corresponding ICAgs by the different endocrine cell subsets (β, α, δ). The more promising of the ICMAbs was I-45, as its reactivity pattern was very similar to hICAbs.

The remarkable "heat stability" of the autoantigens/epitopes recognized by hICAbs of type I diabetes points to the possibility of non-protein epitope(s). This is consonant with the data that these epitopes are preserved on treatment with proteolytic enzymes, whereas treatment with metaperiodate abolishes the ICAg

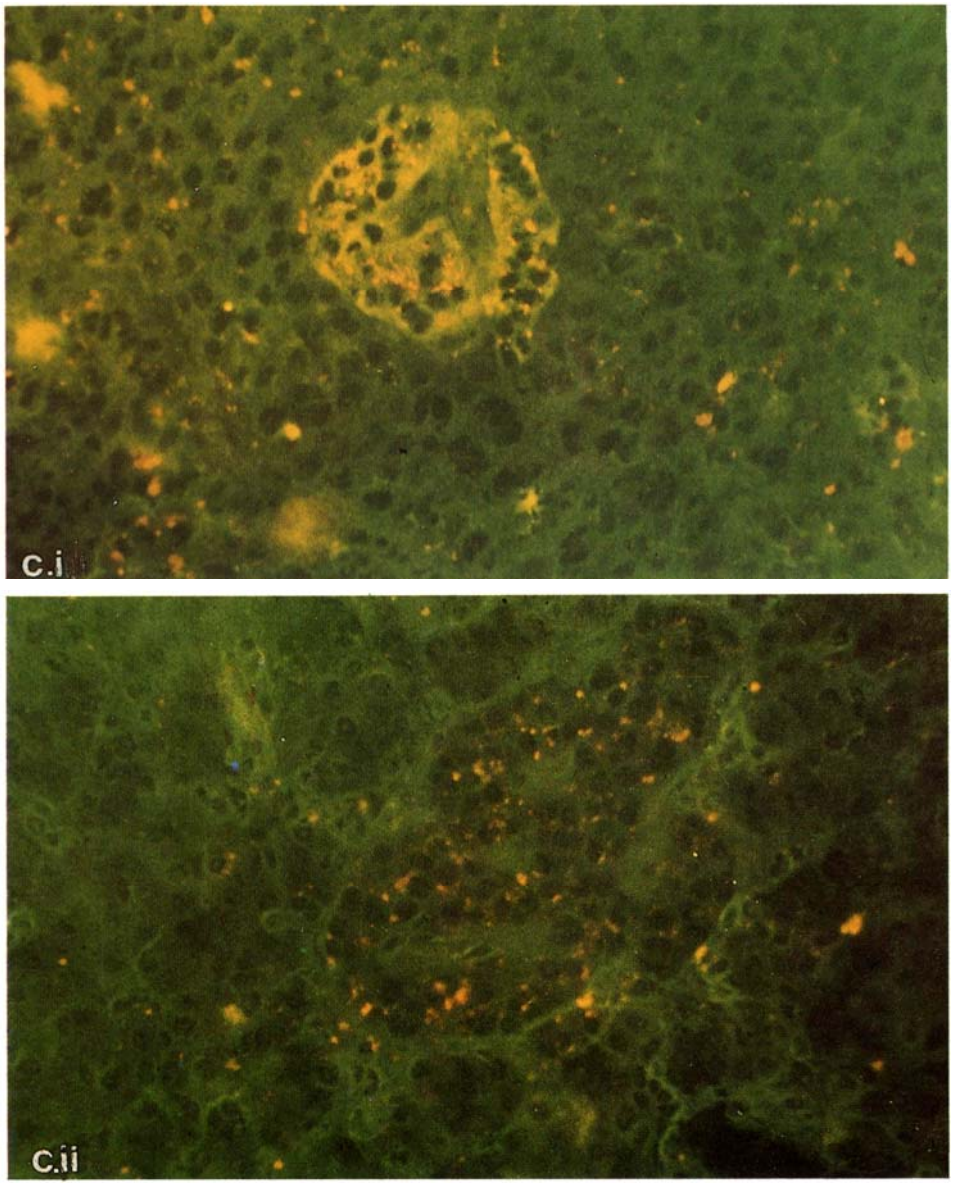

Figure 1c. Monoclonal islet cell antibody I-45: Reactivity with unfixed cryostat sections of human pancreas (IFL) pretreated with (i) pronase or (ii) sodium metaperiodate.

immunoreactivity. Metaperiodate oxidation breaks vicinal diol carbon–crbon linkage suggesting carbohydrate specificity (glycoconjugate ICAgs) (Spiro 1964).

The tissue distribution of I-45 antigen/epitope was neuroendocrine specific,

Figure 1d. I-45 antigen/epitope: Neuroendocrine distribution. Monoclonal antibody immunoreactivity with unfixed cryostat sections (IFL). (i) Adrenal (human), (ii) duodenum (human) and (iii) pituitary (monkey). Monoclonal antibody I-45 reacts with adrenal medullary cells, gut endocrine cells and anterior pituitary cells.

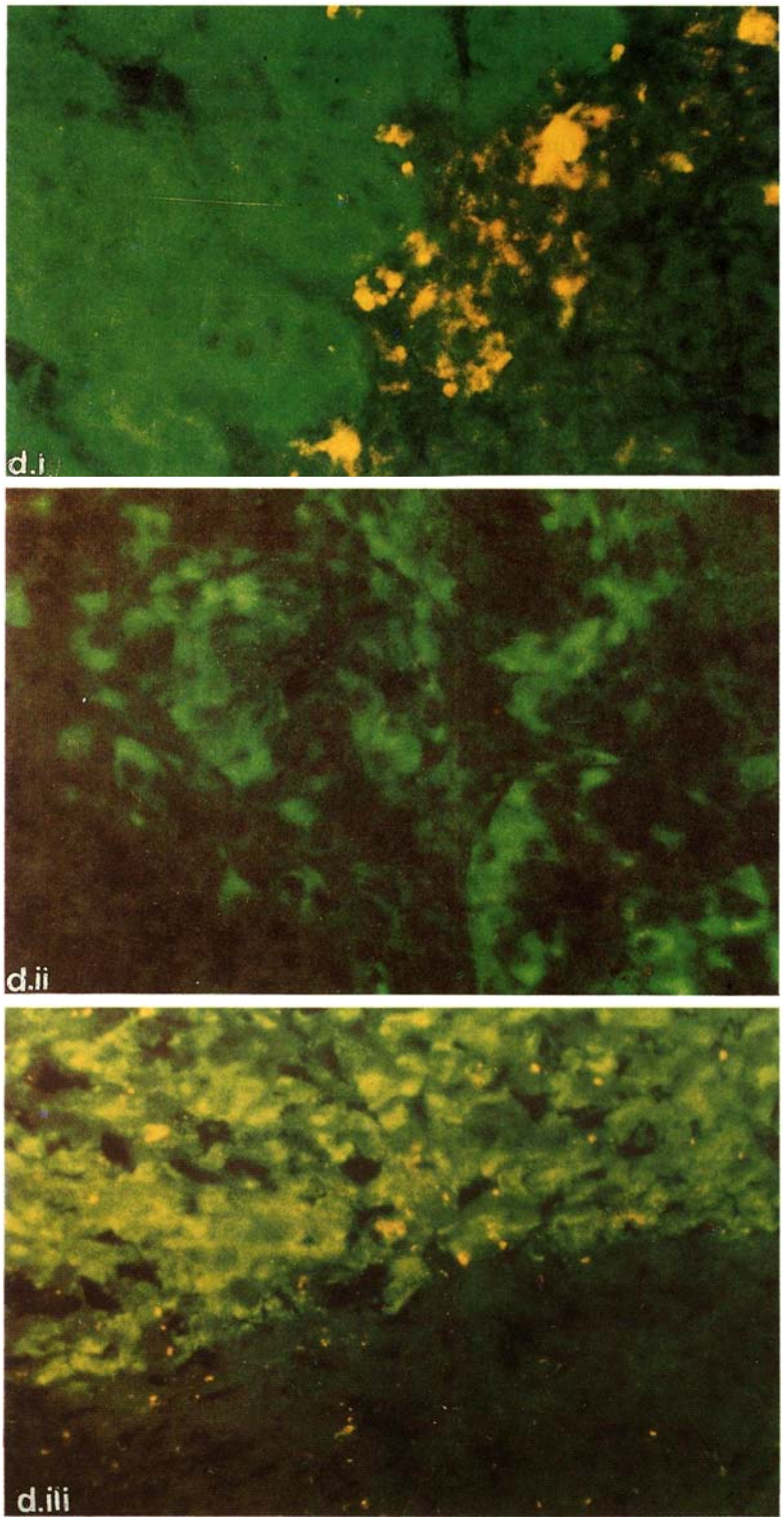

Figure 1 d.

similar to that of chromogranin A and neuron-specific enolase antigens (Krisch *et al* 1988; Lloyd *et al* 1988; Schmechel *et al* 1988). This molecule is different from the HISL-19 antigen described (Krisch *et al* 1988) as the reactivity of HISL-19 MAb on islets was shown to be relatively more with islet peripheral mantle α-cells and this antigen was not detected on parathyroid by immunohistochemistry (Krisch *et al* 1986, 1988). Such sharing of differentiation antigens/epitopes is speculated to have interesting physiological and pathological relevance: it may indicate common embryological origin, *e.g.* from neural crest or neurectoderm; or it may reflect common modes of functional differentiation and specialization, *e.g.* polypeptide and amine hormone biosynthesis and secretion (steroid secreting endocrine cells negative) (Douarin 1988). This may also have relevance in the "clustering" of multiple organ specific autoimmune diseases and multiple endocrine neoplasia (MEN) syndromes.

Also, it is possible that there is no direct induction of antibodies by islet cell antigens. Rather there may simply be a cross reaction of antibodies directed against an environmental factor (*e.g.* infectious agents/viruses) which cross react with molecules of islet cells with common epitopes (molecular mimicry).

Despite many physicochemical similarities, the epitopes recognized by the hICAb of type I diabetes and the ICMAbs studied, appear to be disparate (in preliminary competition experiments/immunohistochemistry — prior binding of polyclonal hICAb to pancreatic islets did not inhibit the subsequent binding of ICMAb I-45 or I-39). While the autoantigenic, hICAb-reactive epitope is evolutionarily well conserved and is islet restricted, I-45 epitope is neuroendocrine differentiation related. These findings do not rule out the possibility that both hICAb and ICMAb I-45 epitopes can be harboured on the same antigen molecule (*e.g.* multiple carbohydrate determinants on an identical protein backbone). The reasons why ICMAbs recognizing the autoantigenic hICAb epitope(s) have not been generated so far are intriguing; the autoantigen(s)/epitope(s) being evolutionarily conserved may not be able to generate an antibody response in the immunized mice used for hybridoma generation, or the repertoire of all possible islet cell antigen(s)/epitopes may not yet been fully explored.

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