DEVELOPMENT OF ENDOCRINE CELLS IN THE HUMAN FETAL PANCREAS

Ronald R. de Krijger

DEVELOPMENT OF ENDOCRINE CELLS IN THE HUMAN FETAL PANCREAS

DE ONTWIKKELING VAN ENDOCRIENE CELLEN IN HET HUMANE FOETALE PANCREAS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR

AAN DE ERASMUS UNIVERSITEIT ROTTERDAM

OP GEZAG VAN DE RECTOR MAGNIFICUS

PROF. DR. P.W.C. AKKERMANS M.A.

EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES.

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP

WOENSDAG 9 NOVEMBER 1994 OM 11.45 UUR

DOOR

RONALD RENE DE KRIJGER

GEBOREN TE ROTTERDAM

Offsetdrukkerij Haveka B.V.,
Alblasserdam

PROMOTIECOMMISSIE

PROMOTORES:	Prof. Dr. H. Galjaard
	Prof. Dr. H.K.A. Visser
CO-PROMOTOR:	Dr. G.J. Bruining
OVERIGE LEDEN:	Prof. Dr. F.T. Bosman
	Prof. Dr. S.W.J. Lamberts

The work presented in this thesis was generously supported by the "Diabetes Fonds Nederland". Printing of this thesis was possible by the generous support from Novo-Nordisk Nederland BV, and from the "Diabetes Fonds Nederland".

Voor Annet,

Voor mijn ouders,

Voor mijn schoonmoeder.

CONTENTS

Introduction.

The development of tissues can be studied by observing the proliferation and differentiation of the composing cell types. In this thesis the development is studied of the endocrine human fetal pancreas throughout the second trimester of pregnancy. There are two main reasons to study the fetal development of islet cells. The first concerns the detection of islet stem cells, which have remained unidentified so far. It is conceivable that proliferating endocrine cells or their precursors are detectable in the rapidly growing human fetal pancreas, whereas human adult ß cells have limited proliferative capacity (De Vroede et al., 1990). Identification of islet precursor cells and knowledge of the stimulation of islet cell proliferation allows the in vitro replication of islet cells. This may eventually result in therapeutic strategies in diseases in which insulin-producing ß cells are underrepresented, most notably diabetes mellitus.
The second reason is the link that has emerged from the literature between fetal islet (ß) cell development and adult pathologic states as type-I (insulin-dependent) and type-II (non-insulin-dependent) diabetes mellitus. Clinical studies have indicated that the endocrine cell mass depends on events during pregnancy. Small for gestational age (SGA) infants, as a result of intrauterine nutritional deficits, have pancreata containing less ß cells (Van Assche and Aerts, 1979). Hales et al. (1991) find a relationship between low birth weight and glucose intolerance later in life. Experimental studies suggest that aberrant tolerance induction during fetal development may play an important role in the evolvement of autoimmune diseases (e.g. type-I diabetes mellitus). The timing of fetal antigen expression may determine whether the immune system will be tolerant to these antigens. In this thesis potential autoantigens involved in type-I diabetes mellitus have been studied, using type-I diabetic patient antisera.
Antigen expression patterns, related to proliferation and differentiation of fetal islet cells, may be important for the pathogenesis of type-I (insulin dependent) diabetes mellitus, which is reviewed in Chapter 2. The human fetal pancreas was studied because rodent fetal development and rodent models for type-I diabetes mellitus are essentially different.
Apart from morphological and morphometrical studies (Stefan et al., 1983, Clark and Grant, 1983), little was known about the human fetal pancreas when the studies presented in this thesis were started. The aim of the thesis was to increase the knowledge of endocrine cell development in the human fetal pancreas, investigating:

1. Developmental islet cell interrelationships of the four major endocrine pancreatic cell types: insulin- (ß), glucagon- (α), somatostatin- (δ), and pancreatic polypeptide- (PP) containing cells.
2. Identification of potential endocrine stem cells.
3. Separation of the islet cells from the other pancreatic cells for in vitro studies.
4. The reactivity of type-I diabetic patient sera to fetal islet cells and the development of the fetal thymic T-cell repertoire, both of which relate to tolerance development.

The actual experimental work is described in Chapters 3-7. In the general discussion (Chapter 8), the experimental work is discussed, proposing future experiments.

REFERENCES

Clark A, and Grant AM. (1983). Quantitative morphology of endocrine cells in human fetal pancreas. Diabetologia 25:31-35.

De Vroede MA, In 't Veld PA, and Pipeleers DG. (1990). Deoxyribonucleic acid synthesis in cultured adult rat pancreatic B cells. Endocrinology 127:1510-1516.

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, and Winter PD. (1991). Fetal and infant growth and impaired glucose tolerance at age 64 years. Br Med J 303:1019-1022.

Stefan Y, Grasso S, Perrelet A, and Orci L. (1983). A quantitative immunofluorescent study of the endocrine cell populations in the developing human pancreas. Diabetes 32:293-301.

Van Assche FA, and Aerts L. (1979). The fetal endocrine pancreas. Contrib Gynaecol Obstet 5:44-57.

Chapter 1. Islets of Langerhans and their development.

* Adult pancreatic islet anatomy.

In the experimental work of this thesis, hormone and protein expression patterns of human adult pancreatic islets were used for comparison with those in human fetal pancreata. In this section, the anatomy of adult islets of Langerhans is briefly reviewed.

The pancreas consists mainly of digestive enzyme producing exocrine cells, arranged in acini. In 1869 the islets of Langerhans, now known to consist of endocrine cells, were discovered. Human adult pancreata contain over 1 million islets of Langerhans, interspersed throughout the exocrine parenchyma. The evolutionary advantage of having many small islets instead of one large islet organ, as fish have (Endo et al., 1991), remains unclear. The islets vary considerably in size; a typical islet consists of 2,500 cells and measures 140 μm in diameter (Hellman and Hellerstrom, 1969). The islets comprise 1% of the adult pancreatic mass (Weir and Bonner-Weir, 1990). Four major endocrine cell types have been characterized in islets: insulin-producing ß cells, glucagon-producing α cells, somatostatin-producing δ cells, and pancreatic polypeptide-producing PP cells. In the adult islet of Langerhans the central ß cell core constitutes 80% of the islet volume and is surrounded by a mantle of the other three cell types. In the caudal part of the pancreatic head, which stems from the ventral pancreatic primordium, the islet mantle consists mainly of PP cells and δ cells. The remaining part of the pancreas stems from the dorsal pancreatic primordium and contains islet mantles made up of α and δ cells. In the human adult pancreas, ß cells are the predominant islet cell type (65-80% of the endocrine cells), followed by α cells (15-20%), δ cells (5-10%), and PP cells (5-10%) (Stefan et al., 1982, Rahier et al., 1981). Electronmicroscopic analysis shows that each endocrine pancreatic cell type contains characteristic secretory granules. Granules of α cells are black, electrondense, sometimes with a dark grey outer rim; ß cell granules have crystalline black cores surrounded by a white halo or, when immature, are uniformly black to grey; δ cell granules are the largest in size, relatively electronlucent, light grey. These granule characteristics were found in both human adult and human fetal pancreas (Like and Orci, 1972, Dudek and Boyne, 1986) **(FIGURE 1A,B)**. The granule morphology of PP cells has not been described clearly in the literature. This cell type has not been studied in this thesis, because its frequency in the human fetal pancreas (except the caudal pancreatic head) is very low.

Communication between islet cells occurs through gap junctions, allowing the passage of molecules up to 1.2 kD, and through desmosomes and tight junctions (Pipeleers et al., 1992).

Islets are richly vascularized, receiving 10% of the pancreatic blood flow, whereas they make up only 1% of the adult pancreatic mass (Lifson et al., 1980). The afferent arteriole enters straight into the ß cell core. Within the islet, a glomerular-like capillary network ensures a vascular order of perfusion from ß to α to δ cells (Stagner and Samols, 1992, Weir and Bonner-Weir, 1990). The order of vascularization of the islet cells indicates that intra-islet regulation of insulin secretion through intravascular release of somatostatin and/or glucagon is unlikely. Individual ß cells have been shown to have a distinct orientation, facing an arteriolar capillary with its basolateral portion and a venous capillary with its apical portion. This latter portion contains relatively more insulin secretory granules (Bonner-Weir, 1988). Between the lateral surfaces of ß cells, canaliculi span the distance between arterial and venous domains, providing an interstitial flow (Bonner-Weir, 1989). The microvilli in these canaliculi are enriched in glucose transporters allowing glucose sensing (Orci et al., 1989).

Innervation of the islets occurs through sympathetic (adrenergic) nerves, parasympathetic (cholinergic) nerves, as well as peptidergic nerves (Larsson, 1979, Ahrén et al., 1986). The functional significance and the target cells of these nerves are largely unknown.

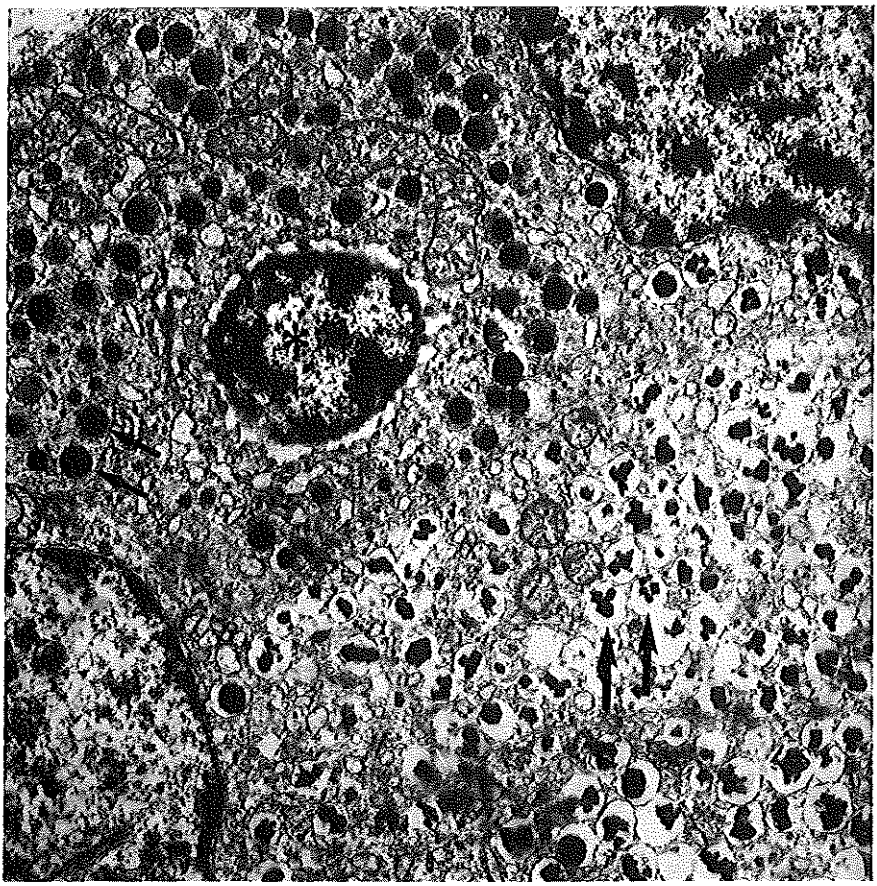


Figure 1A. Electronmicroscopic analysis of a 15 week human fetal pancreas, embedded in Epon. A ß cell, of which part of the nucleus can be observed in the lower left corner, can be recognized by characteristic secretory granules with a crystalline black core and a white surrounding halo (large arrows). The cell in the left upper part of the picture (nucleus marked by asterisk) is a characteristic α cell, with round electrondense secretory granules, sometimes with a slightly less electrondense outer rim (small arrows). Final magnification is 16440x.

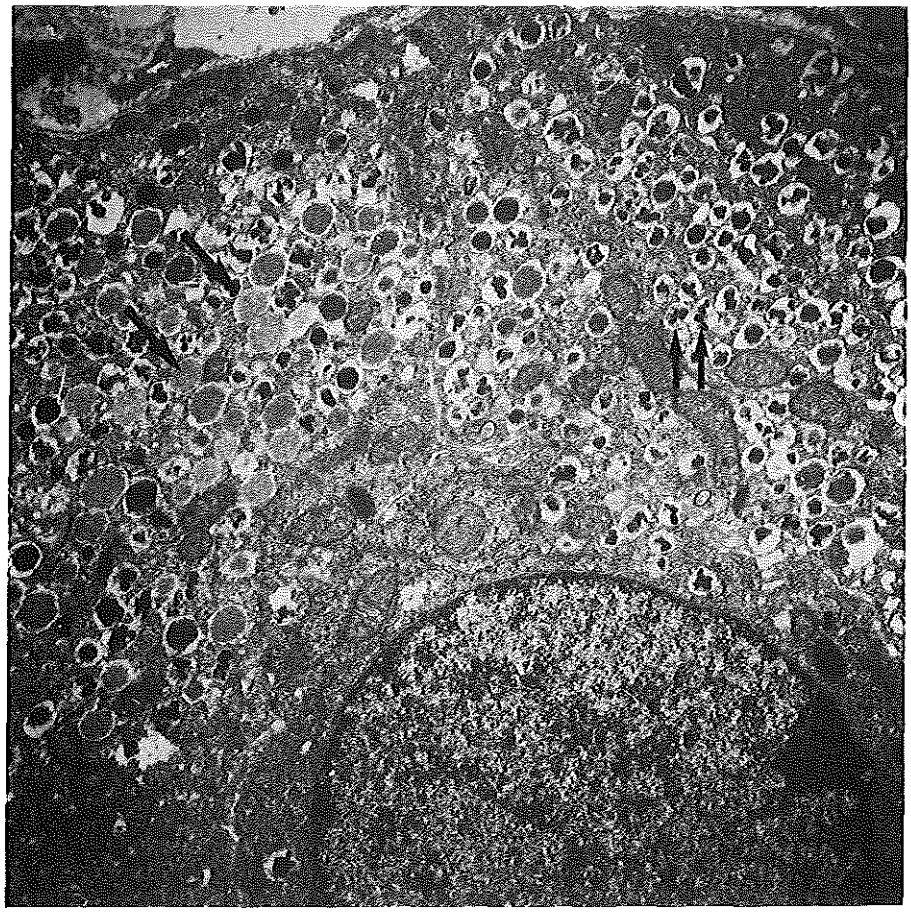


Figure 1B. Electron microscopic analysis of a 15 week human fetal pancreas, embedded in Epon. A fetal ß cell, containing two ultrastructurally different granule types, can be observed. The characteristic ß cell secretory granule type, shown in figure 1A can be seen in right part of this picture (small arrows). In addition, granules filled with less electrondense, non-crystallized material are mainly found in the left part (large arrows). Later immuno-electronmicroscopic experiments revealed that these granules also contain insulin. The latter granule type has been suggested to represent a fetal type of ß cell granule. Final magnification is 16440x.

*** Fetal anatomical pancreatic islet development.**

The initiation of pancreatic development may be observed as early as 4 weeks gestation in the human embryo **(FIGURE 2A,B)**. A dorsal and ventral outpocketing of foregut endoderm, at the site of the future duodenum, contain cells that proliferate into the adjacent mesenchyme. Dudek et al. (1991) showed that adult ductal epithelium without hormone producing cells, co-transplanted with fetal mesenchyme, resulted in the formation of islets with insulin and glucagon containing cells. They postulated that the fetal mesenchyme may have a differentiating effect on ductal epithelium. At 7 weeks the ventral and dorsal primordia fuse to become the pancreas. Proliferating cellular cords of the pancreatic diverticula form branching ductules. Both exocrine and islet cells are thought to differentiate from ductal epithelial precursor cells. The first sign of hormone production detectable by immunohistochemistry is at 7 weeks gestation, when glucagon producing cells appear (Assan and Boillot, 1973, Dubois, 1989). At 8 weeks gestation, ß cells, δ cells, and PP cells can be found (Stefan et al., 1983). Initially, single endocrine cells are found in the pancreatic parenchyma. With advancing fetal age, endocrine cell clusters of increasing size can be observed, leading to the formation of mature islets of Langerhans. Studies with mouse chimaeras showed that islets derive from several independent ancestor cells (Deltour et al., 1991). Chimaeric embryos resulted from a cross between transgenic mice carrying the human insulin gene with a normal mouse strain. Species-specific monoclonal anti-insulin antibodies discriminated the origin of the ß cells. Several small clusters of ß cells, derived from both embryos, suggested multiple ancestor cells with limited intra-islet proliferative capacity. In the human fetal pancreas the first mantle islets, which are thought to represent the mature islet type, appear at 15 weeks (Dubois, 1989) **(FIGURE 3A,B)**. It remains unknown however to what extent the organogenesis of islets in the fetal pancreas is a continuous process during fetal development, and whether each fetal pancreas presents islets at different stages of development.

It is known that the proportion of all endocrine cells in the fetal pancreas (8-40 weeks gestation) and in the neonatal pancreas is higher than in adults (Stefan et al., 1983, Rahier et al., 1981) and that the frequency of endocrine cell types in islets in the caudal part of the head differs from that in the rest of the pancreas. The proportion of each of the major hormone containing cell types in the human fetal pancreas differs from that in the adult pancreas.

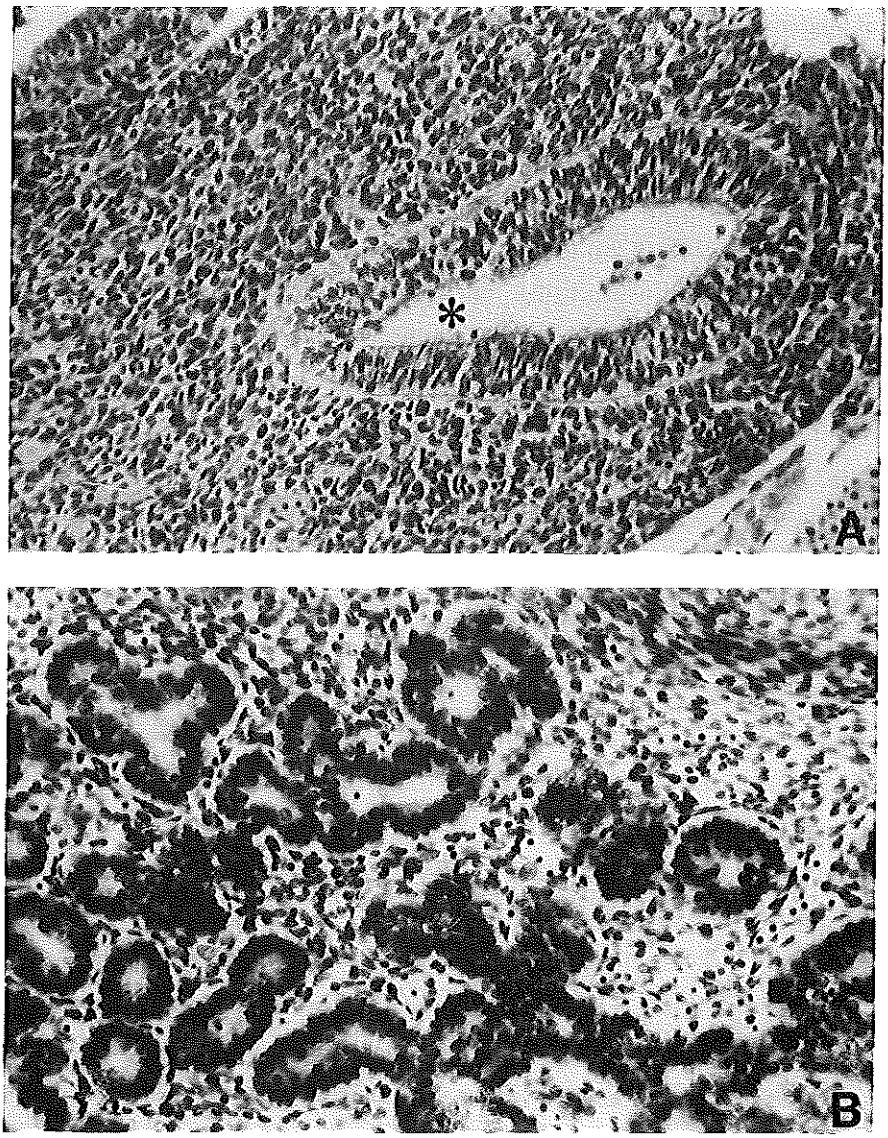


Figure 2. Haematoxylin/eosin staining of a 4 week old embryo (A) and a 6 week old embryo (B). In A a notch in the duodenum can be seen, indicating the initiation of pancreatic development (asterisk). In B primitive ductular structures can be observed.

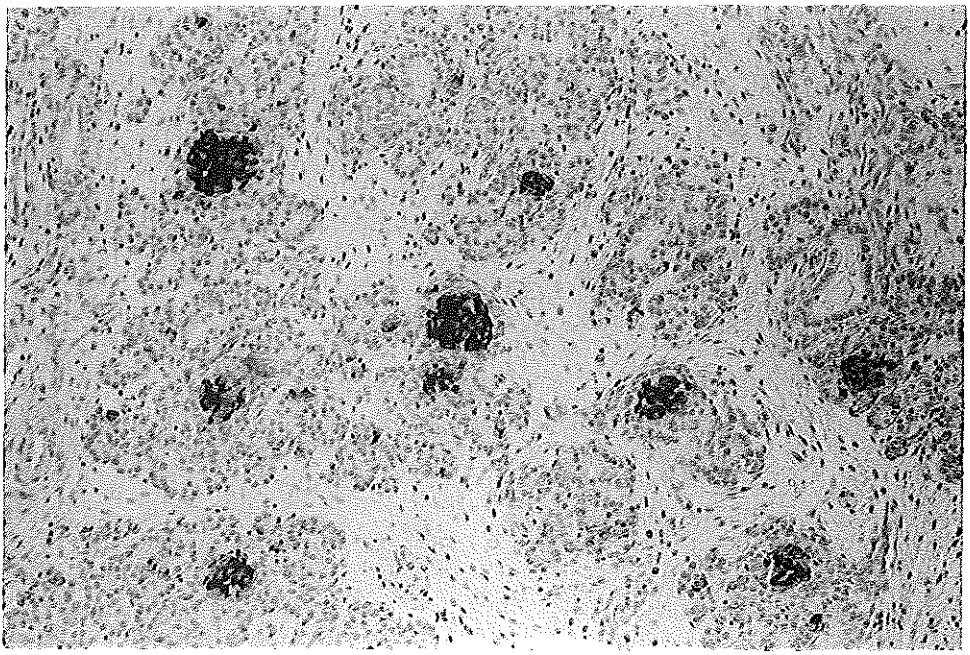


Figure 3A. Light microscopic picture of a section of a 16 week human fetal pancreas, stained by the indirect peroxidase technique. Figure 3A and 3B represent consecutive sections using antibodies to insulin and to somatostatin respectively. In A the insulin positive core of the islets is evident, as well as several smaller endocrine cell clusters and single insulin positive cells.

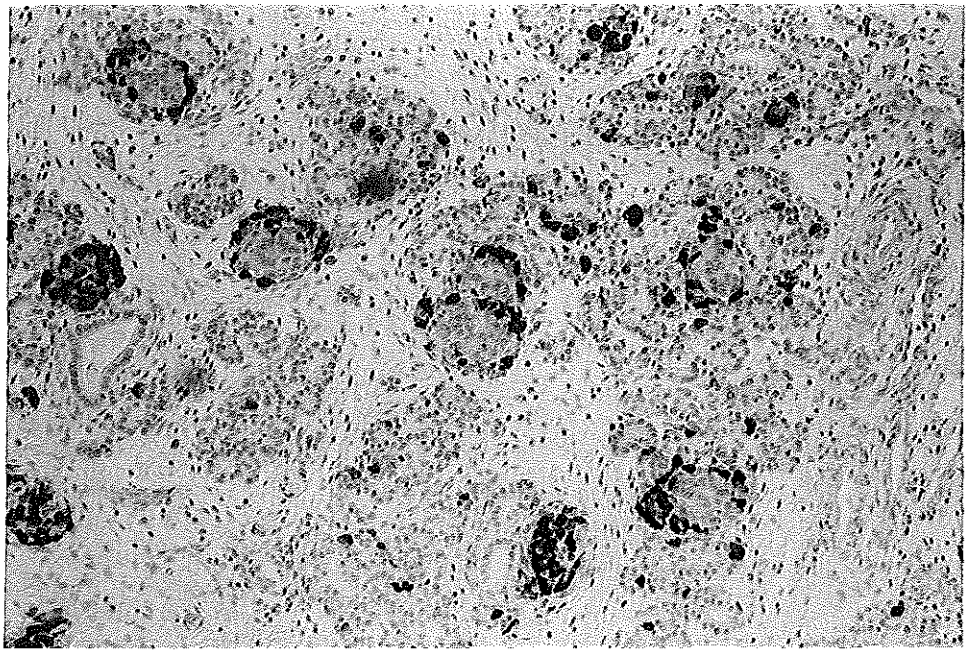


Figure 3B. Light microscopic pictures of sections of a 16 week human fetal pancreas, stained by the indirect peroxidase technique. Figure 3A and 3B represent consecutive sections using antibodies to insulin and to somatostatin respectively. In B, somatostatin positive cells are located peripheral to the ß cell core in the three central islets. Abundant (single) δ cells can be observed throughout this section, examplifying the higher relative amount of δ cells in the fetal pancreas compared with the adult pancreas.

Beta cells constitute 50-55% of the endocrine cells, α cells 15-20%, δ cells 25-30%, and PP cells 5-10%. Thus ß cells are less frequent, and δ cells are more abundant than in the human adult pancreas.

Could this difference be associated with a differing developmental timing of individual endocrine cell types? Experiments described in Chapters 3-5 address the anatomical islet development in the human fetal pancreas, taking into account the difference in distribution of endocrine cells between the caudal pancreatic head and the remaining part of the pancreas. The first aim was to study the presence and coexpression of islet hormones to gain insight in islet cell interrelationships. Second, ß cell specific proteins are of interest, because in type-I diabetes ß cells are selectively destructed by an autoimmune mechanism (see Chapter 2). In the next section, expression of (ß cell specific) proteins related to the insulin machinery is reviewed, with special reference to expression during fetal development.

*** Functional activity of islet ß cells.**

In this section the key function of adult ß cells, the controlled production, storage, and release of insulin is reviewed, with a special focuss on the expression of ß cell (specific) proteins. The precursor molecule of insulin, preproinsulin, is formed by the ribosomes on the rough endoplasmic reticulum. Its signal peptide is cleaved off and proinsulin is transported to the Golgi apparatus. Molecule-specific "sorting" domains are suggested in the trans-Golgi region, which direct proteins released through the so-called regulated pathway to secretory granules. Alternatively, constitutively released proteins are packed in other vesicles (Orci et al., 1987a, Halban, 1991). Proinsulin to insulin conversion takes place in clathrin-coated secretory granules (Orci et al., 1987b), by the endopeptidases I and II, also called prohormone convertases (PC), and carboxypeptidase H (Smeekens et al., 1991, Guest et al., 1989). This conversion yields insulin and C-peptide in equimolar concentrations. However, none of the enzymes involved in proinsulin conversion is specific for the pancreatic ß cell (Smeekens et al., 1991, Guest et al., 1991). In preliminary experiments, no reproducible staining pattern could be obtained in human fetal islet cells, using antibodies to prohormone convertases 2 and 3.

In addition to the abovementioned secretory granule membrane (clathrin) and matrix proteins (conversion enzymes, proinsulin, insulin, and C-peptide) two more ß cell secretory granule proteins, chromogranin-A and islet amyloid polypeptide (IAPP) were considered. In this thesis chromogranin-A and IAPP were selected as a neuroendocrine marker and a ß cell marker respectively (Chapter 5) **(FIGURE 4)**.

Chromogranin-A is a protein of secretory granules in many neuroendocrine cell types (O'Connor et al., 1983; Wilson and Lloyd, 1984), and is probably a precursor protein for regulatory peptides (Hutton et al., 1987; Iacangelo et al., 1988). It is processed by proteolytic cleavage to betagranin (Hutton et al., 1985) or pancreastatin. Betagranin is expressed in a subpopulation of α and ß cells, pancreastatin is found in the majority of ß and δ cells, but not in α cells (Jensen et al., 1991). Pancreastatin is involved in the suppression of glucose-stimulated insulin release (Tatemoto et al., 1986). The function of betagranin is yet unknown.

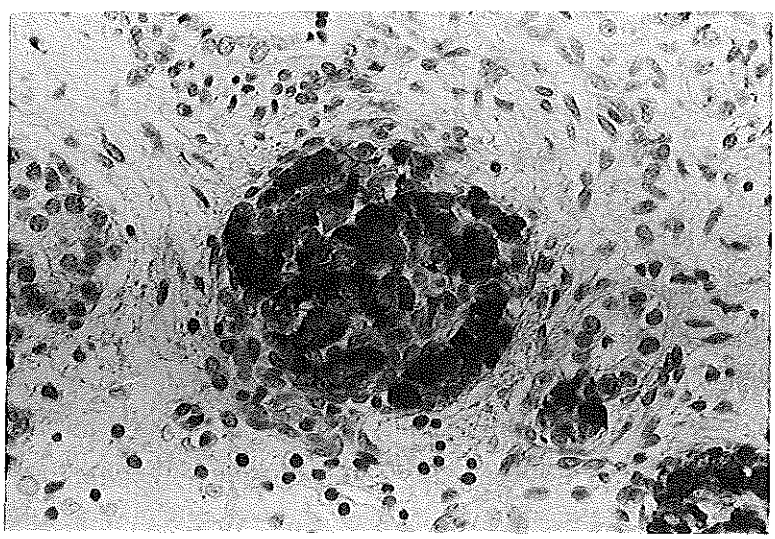


Figure 4. Light microscopy of a 14 week old section of human fetal pancreas stained with an antibody to chromogranin-A, using the indirect peroxidase technique. A mixture of chromogranin-A positive and negative cells is observed in this islet.

IAPP is a 37 amino acid protein, colocalized with insulin in human adult and fetal ß cell secretory granules, not in other human islet cells (Lukinius et al., 1989). It can be isolated from amyloid deposits in type II (non-insulin dependent) diabetes mellitus patients (Cooper et al., 1987, Westermark et al., 1987). During human fetal development IAPP expression starts at 12 weeks gestation in a proportion of the ß cells, mainly those located in endocrine cell clusters (Rindi et al., 1991, In 't Veld et al., 1992). The proportion of IAPP expressing ß cells increases to almost 100% in human adult islets. Studies in transformed mouse and rat cell lines suggest differential regulation of gene expression for IAPP and insulin, because IAPP is also found in glucagon or somatostatin expressing cells (Madsen et al., 1991). Using other cell lines, however, similar promotor elements have been found in both genes (German et al., 1992). Thus, IAPP seems a ß cell specific marker, which is expressed relatively late during development. It is unknown whether the IAPP positive subpopulation of ß cells differs functionally or developmentally from IAPP negative ß cells.

Insulin is discharged from the ß cell by exocytosis of secretory granules in response to an appropriate stimulus. More than 99% of all proinsulin is directed through this regulated secretory pathway in normal ß cells (Halban, 1991). The most important physiologic stimulus for secretory granule release in adult ß cells is glucose. A biphasic insulin response occurs after in vitro or in vivo stimulation of adult human or rodent islets. Many other factors are known to influence insulin granule release. Glucagon, leucine, arginine and the pharmacologic drug theophylline all stimulate insulin release (Samols et al., 1965, Pipeleers et al., 1985). Endocrine cell-to-cell contacts have a positive effect on secretory granule release (Halban et al., 1982, Pipeleers et al., 1982, Pipeleers, 1984). By contrast, somatostatin inhibits insulin release (Alberti et al., 1973).

Human fetal ß cells do not show an adult insulin release pattern in response to glucose stimulation (Otonkoski et al., 1991). However, glucagon, arginine, and theophylline cause an increase of fetal insulin release above basal levels regardless of glucose concentration (Otonkoski, 1988a). It seems necessary to culture fetal islets or islet-like cell clusters (Otonkoski et al., 1988b) of 12 to 20 weeks gestation for several weeks to months or to transplant them into an intermediate host before they acquire an adult insulin release pattern (Tuch et al., 1985, Korsgren et al., 1991). Addition of nicotinamide to the culture medium has resulted in biphasic insulin release from islet-like cell clusters after 7 days of culture (Otonkoski et al., 1993). Apparently, further

maturation of fetal ß cells is needed to obtain glucose responsiveness. At present, it is unknown which molecules in the ß cells are induced in these in vivo and in vitro experiments. One or both of the molecules described underneath may be involved.

The coupling of glucose stimulation to secretory granule exocytosis is currently investigated (by others). Two isoforms (GLUT1 and GLUT2) of a family of structurally related glucose transporters have been identified on the lateral surfaces of pancreatic ß cells (Orci et al., 1989, Yasuda et al., 1992). This may serve as the initial interface for glucose sensing by ß cells (Weir and Bonner-Weir, 1990). The K_m of GLUT2 is 15-20 mM, resulting in a variable glucose effect in the physiologic range (3-9 mM).

Glucokinase is a ß cell specific rate limiting enzyme for the high K_m conversion of glucose to glucose 6-phosphate (Meglasson and Matschinsky, 1986, Jetton and Magnuson, 1992). Increases of intracellular glucose, caused by GLUT2 mediated glucose uptake, are translated by glucokinase into an increased glucose usage. The resultant increase in intracellular $[Ca^{2+}]$ stimulates the release of insulin secretory granules. Heterogeneous glucokinase expression has been found in rat ß cells, implicating functionally different ß cell subpopulations (Jetton and Magnuson, 1992). It is conceivable that the expression of either glucose transporter isoforms or glucokinase is lacking in human fetal ß cells. Preliminary experiments with an antibody to glucokinase did not show its presence in human fetal ß cells. However, this finding awaits further confirmation.

In summary, many factors exert influence on the insulin molecule on its pathway from gene transcription to exocytosis into the bloodstream. The secretory granules for the storage and regulated release of insulin can be regarded as a micro-society of interacting matrix and membrane proteins, only some of which are known yet. Islet amyloid polypeptide is, in addition to insulin, one of few ß cell specific proteins. The heterogeneity of its expression is also observed for molecules involved in stimulus-secretion coupling of insulin release: glucokinase and glucose transporters. The functional and immunological consequences of such heterogeneous expressions in fetal islet cells during midgestation may be important.

The protein expression pattern of (fetal) islet (ß) cells is also largely determined by the embryological origin of these cells. What is presently known about the embryological origin of islet cells?

*** embryological origin of endocrine pancreatic cells and islet cell interrelationships.**

Two theories exist regarding the origin of endocrine pancreatic cells: the neuroectodermal hypothesis and the endodermal hypothesis. The first hypothesis, by Pearse (1977), was based upon his amine precursor uptake and decarboxylation concept (APUD concept). Cells sharing these characteristics would all be derived from the neural crest and migrate into the respective "target" organs at an early stage of differentiation. In his study Pearse showed that islet cells belong to the APUD cells. In support, numerous neuronal molecules have been detected in pancreatic islet cells (see table 1). Pancreatic ß cells possess synaptic-like microvesicles (SLMV's), the endocrine cell counterpart of small synaptic vesicles (SSV's) in neurons (Navone et al., 1986). These recycling microvesicles share several membrane and matrix molecules with neuronal SSV's. Synaptophysin is found in the membrane of SLMV's and SSV's. It is present in all islet cell types, suggesting that α and δ cells also contain SLMV's (Redecker et al., 1991). In addition, synaptophysin has been found in a variety of neuroendocrine neoplasms, and is superior to chromogranin A as a marker of neuroendocrine tumors (Wiedenmann et al., 1986, Chejfec et al., 1987). GABA is found in SLMV's of ß cells, not of other islet cells (Garry et al., 1987, Reetz et al, 1991). GABA is the major nonpeptide inhibitory neurotransmitter in the central nervous system. A paracrine role of GABA in the islets is suggested (Rorsman et al., 1989, Reetz et al., 1991). Production of GABA from glutamate in the central nervous system involves the enzyme glutamic acid decarboxylase (GAD), of which two isoforms, GAD65 and GAD67, exist. In the human adult pancreas, GAD65, identified as a target of autoimmune destruction in type-I diabetes mellitus (see Chapter 2), is detectable in ß cells (Vincent et al., 1983, Gilon et al., 1988) and in few other islet cells (Petersen et al., 1993). The functional and immunological significance of GAD expression in non-ß islet cells is yet unclear. In the human fetal pancreas, SLMV proteins have not been studied systematically.

Neuronal cell adhesion molecules are also found in islet cells. These molecules are thought to be involved in (fetal) islet development, because of the non-random distribution of the different islet cell types within an islet. The 135 kD form of neural cell adhesion molecule (N-CAM) is expressed in neonatal and adult rat islet cells, with higher expression on non-ß-cells than on ß cells (Möller et al., 1992). An essential role for N-CAM has been proposed in the calcium-independent aggregation of islet cells. In addition, three cadherins, E-CAD, N-CAD, and R-CAD, have been detected on pancreatic islet cells (see general discussion).

Other neuronal proteins expressed in endocrine pancreatic cells of mouse, rat, and human include neuron-specific enolase (Polak et al., 1984), HNK-1 (Shioda et al., 1984), neuropeptide Y (Teitelman et al., 1993), and tyrosine hydroxylase (Teitelman and Lee, 1987, Teitelman et al., 1988). In Chapter 4 and 5 we describe the use of synaptophysin and HNK-1 as neuroendocrine markers in the analysis of islet cell interrelationships and in the detection of islet stem cells. Islet cells do not only express neuronal proteins, but also display functional neuronal characteristics (see table 1). Single ß cells kept in vitro form neurite-like processes, containing neurofilament, but they do not when cultured as larger cell aggregates (Teitelman, 1990). An insulin secreting ß cell line (RINm5F) can be induced to form neuritic extensions (Polak et al., 1993).

The second hypothesis, the endodermal hypothesis, disagrees with Pearse's neuroectodermal hypothesis. Le Douarin (1978), using quail neural crest transplanted in chick embryos to produce chimeras, showed that quail cells could not be found in the endocrine pancreas. In agreement with this, Pictet et al. (1976) removed the ectoderm from rat embryos at E9, and found undisturbed development of endocrine pancreatic cells in vitro. These experiments show that, unless migration occurs at a very early developmental stage, it seems unlikely that the neuroectoderm gives rise to endocrine pancreatic cells. Because hormone containing cells are frequently found near the pancreatic ducts (Pictet and Rutter, 1972), they probably derive from precursors in these ducts. Indeed, adult ductal epithelium can differentiate into insulin- and glucagon-producing cells if induced appropriately by fetal mesenchyme (Dudek et al., 1991). In a 90% pancreatectomy rat model, sequential proliferation in ducts of decreasing size is found (Bonner-Weir et al., 1993). Near the smallest ductules new pancreatic islets and endocrine cells appeared. It must be noted that the latter model is one of forced regeneration and the mechanisms may not be comparable to those in the physiological proliferation/differentiation occurring during fetal development.

TABLE 1

Common features (proteins and functional characteristics) of pancreatic islet cells and neurons.

- glutamic acid decarboxylase (GAD65/GAD67)*
- gamma-amino butyric acid (GABA)
- synaptophysin*
- tyrosine hydroxylase (TH)
- DOPA decarboxylase
- neuronal cell adhesion molecule (N-CAM)*
- neuron specific enolase (NSE)
- HNK-1*
- electric excitability
- neurite extention

Proteins marker with an asterisk (*) are discussed in this thesis.

In conclusion, despite a remarkably similar protein expression pattern in endocrine pancreatic cells and neuronal cells, no direct evidence in support of a neuroectodermal origin of endocrine pancreatic cells has been found. Islet cells probably derive from endodermal precursor cells in the pancreatic duct. The observed protein similarity may result from similar gene regulation in both cell types.

The cell lineage relationship of the four major endocrine pancreatic cell types has been a matter of extensive investigation. Presently, α, ß, δ, and PP cells are thought to derive from a common pluripotent precursor cell.

Evidence has come from different experiments. Firstly, hormone coexpression is found in fetal mouse, pig, and human pancreas (Alpert et al., 1988, Lukinius et al., 1992, De Krijger et al., 1992). In the mouse embryo glucagon is the first hormone to appear at day E10. At E12, insulin positive cells appear, all coexpressing glucagon. The percentage of insulin containing

cells that coexpress glucagon decreases to negligable levels after birth, at E20. Similarly, somatostatin containing cells coexpress insulin when they first appear at E17. By immuno-electronmicroscopy the subcellular distribution of coexpression has been studied. In the human fetal pancreas one study found granules to contain more than one hormone (Lukinius et al., 1992), whereas another study observed two or more morphologically and immunohistochemically distinct granule types in the same cell without intragranular coexpression (De Krijger et al., 1992). A prominent role for pancreatic polypeptide is suggested by Herrera et al. (1991). They detected PP expression by immunohistochemistry and by reverse transcriptase (RT)-PCR at day E10.5 in the mouse embryo, concurrent and coexpressed with glucagon. Recently, it has been shown that the early detection of PP may have resulted from crossreactivity of the PP antibody with neuropeptide Y (Teitelman et al., 1993).

A second line of evidence for a common endocrine precursor cell comes from the observation that islet hormones are coexpressed in neuroendocrine tumors and in cell lines. The RIN cell line, stemming from a radiation induced islet cell tumor in NEDH rats, and several sublines coexpress insulin, glucagon and somatostatin (Chick et al., 1977, Philippe et al., 1987). The MSL cell line and its subclones, stemming from a liver metastasis of the same rat islet cell tumor, also have a heterogeneous and multihormonal phenotype. Successive subcutaneous transplantation of this pluripotent rat islet tumor cell line allowed segregation of an insulinoma causing hypoglycaemia from a glucagonoma producing anorexia (Madsen et al., 1993). It was concluded that both tumors can derive from the same pluripotent MSL cell, which supports the idea of a common origin for at least α and ß cells.

Finally, neuroendocrine markers have been used to study islet cell interrelationships. Tyrosin hydroxylase (TH) was detected in mouse embryos in proliferating cells and in cells expressing either glucagon or insulin (Teitelman and Lee, 1987). In adult mouse islets, TH can still be found, but only in ß cells, which do not proliferate. Thus, TH cells seem to give rise to α and ß cells in the mouse embryo. In a further study, using models for ß cell hyperplasia, the adult TH containing ß cells are suggested to be on a pathway to senescence.

In contrast to insulin-only cells, the TH-insulin cells do not proliferate (Teitelman et al., 1988). Recently, neuropeptide Y (NPY) has been reported to colocalize with insulin and glucagon from day E9.5 of mouse embryogenesis (Teitelman et al., 1993). This peptide may play a role in the paracrine regulation of insulin secretion (Jamal et al., 1991, Waeber et al., 1993). TH and NPY are expressed not only in α and ß cells, but in δ and PP cells as well. They can

be taken as an indication of the neural character of pancreatic islet cells, and suggest derivation from a common progenitor cell of the four major islet cell types. As mentioned above, other neuroendocrine marker proteins have also been used succesfully to study islet cell interrelationships (Chapter 4).

In summary, pluripotent stem cells probably give rise to all islet cell types. Islet cells may initially express multiple hormones. In such cells, a set of islet cell hormone and neural genes seems to be expressed simultaneously, although there may be a delay between transcription and translation (Herrera et al., 1991, Gittes and Rutter, 1992, Teitelman, 1993, Teitelman et al., 1993). Neuroendocrine marker proteins, such as TH and NPY, may aid to detect a common ancestry for islet cells.

Multiple hormone expressing precursor cells, or precursor cells identified by other marker proteins do not necessarily represent pluripotent endocrine stem cells. What is currently known about replication of islet cells or islet precursor cells?

*** endocrine stem cells.**

Stem cells can be subdivided in pluripotent stem cells and committed stem cells. Pluripotent stem cells have the capability of self-renewal and can give rise to a broader range of differentiated cell types than committed stem cells. All stem cells have the capability to proliferate. Under basal conditions hormone expressing islet cells appear to have little or no proliferative capacity (De Vroede et al., 1990; De Krijger et al., 1992). However, if stimulated by growth factors, after pancreatectomy, or during pregnancy, hormone containing islet cells, including ß cells, can be observed to proliferate, either by bromodeoxyuridine (BrdU) uptake studies or by ^{3}H-thymidine autoradiography (Billestrup and Nielsen, 1991, Parsons et al., 1992, Bonner-Weir et al., 1993). In the fetal rat pancreas the proliferative compartment (PC) comprises 3% of the islet cells (Swenne, 1982), and can be increased to maximally 10% of the islet cells by an increase in the glucose concentration. The majority of the islet cells seem to be in an irreversible G_0 phase. This assumption has been questioned recently in a quantitative analysis of proliferating cells after prolactin stimulation (Brelje et al., 1994). At any rate, the proliferative fraction of islet cells, with an estimated cell cycle time of 14.9 hours (Swenne, 1982), cannot account for the increase in islet cells during fetal development. Neoformation of endocrine cells from unidentified

endocrine stem cells must therefore occur. So far no pluripotent stem cell markers are available, which indicates the importance of finding such markers (see Chapter 4 and general discussion). The factors involved in ß cell replication and their mechanisms of action have been studied mainly in fetal rat islets, during pregnancy and in cell lines. The stimulatory effect of growth hormone (GH) on insulin production and ß cell replication is well documented (Swenne et al., 1987, Swenne and Hill, 1989, Nielsen et al., 1989, Billestrup and Nielsen, 1991). In many tissues, GH mediates its effects through insulin-like growth factors (IGF's), which are produced locally and exert their effect via autocrine or paracrine mechanisms (D'Ercole et al., 1984). IGF-I has been detected by immunohistochemistry in ß cells of the human fetus and adult rat (Han et al., 1987a, Hansson et al., 1988, Hill et al., 1987), but mRNA could not be found (Han et al., 1987b, Beck et al., 1988). In another study adult human and rat α and δ cells, but not ß cells, contained IGF-I (Maake and Reinecke, 1993). These contradictory findings may be explained by uptake of IGF-I in ß cells through IGF-I receptors, which have been shown on rat α and ß cells (Van Schravendijk et al., 1987). IGF-I mRNA is found to be increased in epithelial and connective tissue cells in focal areas of regeneration in a 90% rat pancreatectomy model, suggesting a role in growth and/or differentiation (Smith et el., 1991). IGF-II in adult rat and human ß cells and in human fetal ß cells may also be involved in the regulation of islet growth and differentiation (Maake and Reinecke, 1993, Miettinen et al., 1993). It seems conceivable that GH acts at least partly trough IGF-I in the islet, because GH stimulates fetal and adult rat islet IGF-I release and an IGF-I antibody blocks GH stimulated islet cell replication. By contrast, other studies do not find an IGF-I mediated GH effect (Billestrup and Nielsen, 1991). This may be explained by binding of IGF-I to IGF binding proteins (IGFBP's). GH, IGF's, and IGFBP's may form a complex network of interacting substances which influence islet cell replication.

Other members of the growth hormone family, placental lactogen (PL), and prolactin (PRL), also stimulate the proliferation of fetal and newborn rat ß cells (Nielsen, 1982). The physiological action of PL and PRL occurs during pregnancy, which is highlighted by an increase of pancreatic ß cells (Parsons et al., 1992). GH and PRL receptors show both species and tissue differences and have been shown in human fetal and rat islets and islet cell lines (Polak et al., 1990, Hill et al., 1992, Möldrup et al., 1990, 1993). Species and receptor interactivity of members of the growth hormone family interferes with clear analysis of their effects.

Both the high- and low-affinity nerve growth factor receptor (NFGr) have been identified in fetal rat islets and ß-cell lines (RINm5F and INS-1) (Scharfmann et al., 1993). In RINm5F cells NGF can induce neurite formation, and NGF also increases c-fos mRNA expression, indicating that NGF receptors are functional. Recent information about the importance of NGF for the proliferation and/or differentiation of islet cells, in particular ß cells, will be presented in the general discussion.

Transforming growth factor-alpha (TGF-α) can be detected in the human fetal pancreas at 15-20 weeks gestation in ducts and islet ß cells (Miettinen et al., 1992). A further suggestion for a role of TGF-α comes from a study by Wang et al. (1993). They observed metaplastic ductules in transgenic mice overexpressing TGF-α, but no increase in islet mass. Overexpression of gastrin, transiently expressed in islets during fetal development (Brand and Fuller, 1988), in double transgenic mice decreased the TGF-α-stimulated ductular metaplasia. Moreover, islet mass was significantly increased over control mice. Thus, the combination of TGF-α and gastrin is suggested in islet neogenesis from ductular epithelium in the adult mouse pancreas.

In summary, pluripotent islet stem cells have remained elusive, due to the lack of marker proteins. A series of growth factors appear to be involved in proliferation and differentiation of islet cells and islet precursor cells.

Ahrén B, Taborsky GJ Jr, Porte D Jr. (1986). Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. Diabetologia 29:827-836.

Alberti KGM, Christensen NJ, Christensen SE, and Hansen AP. (1973). Inhibition of insulin secretion by somatostatin. Lancet ii:1299-1301.

Alpert S, Hanahan D, and Teitelman G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 53:295-308.

Assan R, and Boillot J. (1973). Pancreatic glucagon and glucagon-like material in tissues and plasma from human fetuses 6-26 weeks old. Pathol Biol 21:149-155.

Beck F, Samani NJ, Byrne S, Morgan K, Gebhard R, and Brammar WJ. (1988). Histochemical localization of IGF-I and IGF-II mRNA in the rat between birth and adulthood. Development 104:29-39.

Billestrup N, and Nielsen JH. (1991). The stimulatory effect of growth hormone, prolactin, and placental lactogen on ß-cell proliferation is not mediated by insulin-like growth factor-I. Endocrinology 129:883-888.

Bonner-Weir S. (1988). Morphological evidence for pancreatic polarity of beta-cell within islets of Langerhans. Diabetes 37:616-621.

Bonner-Weir S. (1989). Potential "sensing" and "secreting" domains of the pancreatic B-cell. Diabetes 38 (suppl.2):98A

Bonner-Weir S, Baxter LA, Schuppin GT, and Smith FE. (1993). A second pathway for regeneration of adult exocrine and endocrine pancreas. Diabetes 42:1715-1720.

Brand SJ, and Fuller PJ. (1988). Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. J Biol Chem 263:5341-5347.

Brelje TC, Parsons JA, and Sorenson RL. (1994). Regulation of islet ß-cell proliferation by prolactin in rat islets. Diabetes 43:263-273.

Chejfec G, Falkmer S, Grimelius L, Jacobsson B, Rodensjö M, Wiedenmann B, Franke WW, Lee I, and Gould VE. (1987). Synaptophysin, a new marker for pancreatic neuroendocrine tumors. Am J Surg Pathol 11:241-247.

Chick WL, Warren S, Chute RN, Like AA, Lauris V, and Kitchen KC. (1977). A transplantable insulinoma in the rat. Proc Natl Acad Sci USA 74:628-632.

Cooper GJS, Willis AC, Clark A, Turner RC, Sim SB, and Reid KBM. (1987). Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. Proc Natl Acad Sci USA 84:8628-8632.

De Krijger RR, Aanstoot HJ, Kranenburg G, Reinhard M, Visser WJ, and Bruining GJ. (1992). The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev Biol 153:368-375.

Deltour L, Leduque P, Paldi A, Ripoche MA, Dubois P, and Jami J. (1991). Polyclonal origin of pancreatic islets in aggregation mouse chimaeras. Development 112:1115-1121.

D'Ercole AJ, Stiles AD, and Underwood LE. (1984). Tissue concentration of somatomedin C: further evidence for multiple sites of biosynthesis and paracrine or autocrine mechanisms of action. Proc Natl Acad Sci USA 81:935-939.

De Vroede MA, In 't Veld PA, and Pipeleers DG. (1990). Dexoyribonucleic acid synthesis in cultured adult rat pancreatic B cells. Endocrinology 127:1510-1516.

Dubois PM. (1989). Ontogeny of the endocrine pancreas. Horm Res 32:53-60.

Dudek RW, and Boyne AF. (1986). An excursion through the ultrastructural world of quick-frozen pancreatic islets. Am J Anat 175:217-243.

Dudek RW, Lawrence IE, Hill RS, and Johnson RC. (1991). Induction of islet cytodifferentiation by fetal mesenchyme in adult pancreatic ductal epithelium. Diabetes 40:1041-1048.

Endo Y, Chiba A, and Honma Y. (1991). Exocytotic release of neurotransmitters from the nerve fibers in the endocrine pancreas (Brockman body) of the teleost Takifugu niphobles. Neurosc Letters 126:60-62.

Garry DJ, Sorenson RL, and Coulter HD. (1987). Ultrastructural localization of gamma amino butyric acid immunoreactivity in B cells of the rat pancreas. Diabetologia 30:115-119.

German MS, Moss LG, Wang J, and Rutter WJ. (1992). The insulin and islet amyloid polypeptide genes contain similar cell-specific promotor elements that bind identical ß-cell nuclear complexes. Mol Cell Biol 12:1777-1788.

Gilon P, Campistron G, Geffard M, and Remacle C. (1988). Immunocytochemical localization of GABA in endocrine cells of the rat entero-pancreatic system. Biol Cell 62:265-273.

Gittes GK, and Rutter WJ. (1992). Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci USA 89:1128-1132.

Guest PC, Pipeleers D, Rossier J, Rhodes CJ, and Hutton JC. (1989). Co-secretion of carboxypeptidase H and insulin from isolated rat islets of Langerhans. Biochem J 264:503-508.

Guest PC, Ravazzola M, Davidson HW, Orci L, and Hutton JC. (1991). Molecular heterogeneity and cellular localization of carboxypeptidase H in the islets of Langerhans. Endocrinology 129:734-740.

Halban PA, Wollheim CB, Blondel B, Meda P, Niesor EN, and Mintz DH. (1982). The possible importance of contact between pancreatic islet cells for the control of insulin release. Endocrinology 111:86-94.

Halban PA. (1991). Structural domains and molecular lifestyles of insulin and its precursors in the pancreatic beta cell. Diabetologia 34:767-778.

Han VKM, Hill DJ, Strain AJ, Towle AC, Lauder JM, Underwood LE, and D'Ercole AJ. (1987a). Identification of somatomedin C/insulin-like growth factor immunoreactive cells in the human fetus. Pediatr Res 22:245-249.

Han VKM, D'Ercole AJ, and Lund PK. (1987b). Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 236:193-197.

Hansson HA, Nillson A, Isgaard J, Billig H, Isaksson O, Skottner A, Andersson IK, and Rozell B. (1988). Immunohistochemical localization of insulin-like growth factor I in the pancreas of normal and diabetic adult rats. Histochemistry 89:403-410.

Hellman B, and Hellerström C. (1969). Histology and histophysiology of the islets of Langerhans in man. In: Pfeiffer EF, ed. Handbook of diabetes mellitus. Munich: Lehmanns Verlag, pp 89-118.

Herrera PL, Huarte J, Sanvito F, Meda P, Orci L, and Vassalli JD. (1991). Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development 113:1257-1265.

Hill DJ, Frazer A, Swenne I, Wirdnam PK, and Milner RDG. (1987). Somatomedin-C in human fetal pancreas. Cellular localization and release during culture. Diabetes 36:465-471.

Hill DJ, Riley SC, Bassett NS, and Waters MJ. (1992). Localization of the growth hormone receptor, identified by immunocytochemistry, in second trimester human fetal tissues and in placenta throughout gestation. J Clin Endocrinol Metab 75:646-650.

Hutton JC, Hansen F, and Peshavaria M. (1985). Betagranins: 21kDa co-secreted peptides of the insulin granule closely related to adrenal medullary chromogranin A. FEBS Lett 188:336-340.

Hutton JC, Davidson HW, and Peshavaria M. (1987). The mechanisms of chromogranin A processing. Nature 325, 766.

Iacangelo AL, Fischer-Colbrie R, Koller KJ, Brownstein MJ, and Eiden LE. (1988). The sequence of porcine chromogranin A messenger RNA demonstrates chromogranin A can serve as the precursor for the biologically active hormone, pancreastatin. Endocrinology 122:2339-2341.

In 't Veld PA, Zhang F, Madsen OD, and Klöppel G. (1992). Islet amyloid polypeptide immunoreactivity in the human fetal pancreas. Diabetologia 35:272-276.

Jamal H, Jones PM, Byrne J, Suda K, Ghatei MA, Kanse SM, and Bloom SR. (1990). Peptide contents of neuropeptide Y, vasoactive intestinal polypeptide, and ß-calcitonin gene-related peptide and their messenger ribonucleic acids after dexamethasone treatment in the isolated rat islets of Langerhans. Endocrinology 129:3372-3380.

Jensen TB, Fahrenkrug J, and Sundler F. (1991). Immunocytochemical localisation of pancreastatin and chromogranin A in porcine neuroendocrine tissues. Reg Pept 36:283-297.

Jetton TL, and Magnuson MA. (1992). Heterogeneous expression of glucokinase among pancreatic ß cells. Proc Natl Acad Sci USA 89:2619-2623.

Korsgren O, Jansson L, Eizirik D, and Andersson A. (1991). Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. Diabetologia 34: 379-386.

Larsson L. (1979). Innervation of the pancreas by substance P, enkephalin, vasoactive intestinal polypeptide and gastrin/CCK immunoreactive nerves. J Histochem Cytochem 27:1283-1284.

Le Douarin N. (1978). The embryological origin of the endocrine cells associated with the digestive tract: experimental analysis based on the use of a stable cell marking technique. In: Bloom SR, ed. Gut hormones. London: Churchill Livingstone, pp. 49-56.

Lifson N, Kramlinger KG, Mayrand RR, and Lender EJ. (1980). Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. Gastroenterology 79:466-473.

Like AA, and Orci L. (1972). Embryogenesis of the human pancreatic islets: a light and electron microscopic study. Diabetes 21 (suppl.2):511-534.

Lukinius A, Wilander E, Westermark GT, Engström U, and Westermark P. (1989). Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets. Diabetologia 32:240-244.

Lukinius A, Ericsson LE, Grimelius L, and Korsgren O. (1992). Ultrastructural studies of the ontogeny of fetal human and porcine endocrine pancreas, with special reference to colocalization of the four major islet hormones. Dev Biol 153:376-385.

Maake C, and Reinecke M. (1993). Immunohistochemical localization of insulin-like growth factor 1 and 2 in the endocrine pancreas of rat, dog, and man, and their coexistence with classical islet hormones. Cell Tissue Res 273:249-259.

Madsen OD, Nielsen JH, Michelsen B, Westermark P, Betsholz C, Nishi M, and Steiner DF. (1991). Islet amyloid polypeptide and insulin expression are controlled differently in primary and transformed islet cells. Mol Endocrinol 5:143-148.

Madsen OD, Karlsen C, Nielsen E, Lund K, Kofod H, Welinder B, Rehfeld JF, Larsson LI, Steiner DF, Holst JJ, and Michelsen BK. (1993). The dissociation of tumor-induced weight loss from hypoglycemia in a transplantable pluripotent rat islet tumor results in the segregation of stable α- and ß-cell tumor phenotypes. Endocrinology 133:2022-2030.

Meglasson MD, and Matschinsky FM. (1986). Pancreatic islet glucose metabolism and regulation of insulin secretion. Diabetes Metab Rev 2:163-214.

Miettinen PJ, and Heikinheimo K. (1992). Transforming growth factor-alpha (TGF-α) and insulin gene expression in the human fetal pancreas. Development 114:833-840.

Miettinen PJ, Otonkoski T, and Voutilainen R. (1993). Insulin-like growth factor-II and transforming growth factor-α in developing human fetal pancreatic islets. J Endocrinol 138:127-136.

Möldrup A, Billestrup N, and Nielsen JH. (1990). Rat insulinoma cells express both a 115kDa growth hormone receptor and a 95-kDa prolactin receptor, structurally related to the hepatic receptors. J Biol Chem 265:8686-8690.

Möldrup A, Douglas Petersen E, and Nielsen JH. (1993). Effects of sex and pregnancy hormones on growth hormone and prolactin receptor gene expression in insulin-producing cells. Endocrinology 133:1165-1172.

Möller CJ, Christgau S, Williamson MR, Madsen OD, Zhan-Po N, Bock E, and Baekkeskov S. (1992). Differential expression of NCAM and cadherins in pancreatic islets, glucagonomas and insulinomas. Mol Endocrinol 6:1332-1342.

Navone F, Jahn R, Di Giola G, Stukenbrok H, Greengard P, and De Camilli P. (1986). Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J Cell Biol 103:2511-2527.

Nielsen JH. (1982). Effects of growth hormone, prolactin, and placental lactogen on insulin content and release and deoxyribonucleic acid synthesis in cultured pancreatic islets. Endocrinology 110:600-606.

Nielsen JH, Linde S, Welinder BS, Billestrup N, and Madsen OD. (1989). Growth homrone is a growth factor for the differentiated pancreatic beta cell. Mol Endocrinol 3:165-173.

O'Connor DT, Burton D, and Deftos LJ. (1983). Immunoreactive human chromogranin A in diverse polypeptide producing human tumors and normal endocrine tissues. J Clin Endocrinol Metab 57:1984-1986.

Orci L, Ravazzola M, Storch MJ, Anderson RGW, Vassalli JD, and Perrelet A. (1987a). Proteolytic maturation of insulin is a post-golgi event which occurs in acidifying clathrin-coated secretory vesicles. Cell 49:865-868.

Orci L, Ravazzola M, Amherdt M, Perrelet A, Powell SK, Quinn DL, and Moore HPH. (1987b). The transmost cisternae of the golgi complex: a compartment for sorting of secretory and plasma membrane proteins. Cell 51:1039-1051.

Orci L, Thorens B, Ravazzola M, and Lodish HF. (1989). Localization of the pancreatic beta cell glucose transporter to specific plasma membrane domains. Science 245:295-297.

Otonkoski T. (1988a). Insulin and glucagon secretory responses to arginine, glucagon, and theophylline during perifusion of human fetal islet-like cell clusters. J Clin Endocrinol Metab 67:734-740.

Otonkoski T, Andersson S, Knip M, and Simell O. (1988b). Maturation of insulin response to glucose during human fetal and neonatal development. Studies with perifusion of pancreatic isletlike cell clusters. Diabetes 37:286-291.

Otonkoski T, Knip M, Wong I, and Simell O. (1991). Lack of glucose-induced functional maturation during long-term culture of human fetal islet cells. Life Sciences 48:2157-2163.

Otonkoski T, Beattie GM, Mally MI, Ricordi C, and Hayek A. (1993). Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. J Clin Invest 92:1459-1466.

Parsons JA, Brelje TC, and Sorenson RL. (1992). Adaptation of islets of Langerhans to pregnancy: increased islet cell proliferation and insulin secretion correlates with the onset of placental lactorgen secretion. Endocrinology 130:1459-1466.

Pearse AGE. (1977). The APUD concept and its implications: related endocrine peptides in brain, intestine, pituitary, placenta, and anuran cutaneous glands. Med Biol 55:115-125.

Petersen JS, Russel S, Marshall MO, Kofod H, Buschard K, Cambon N, Karlsen AE, Boel E, Hagopian WA, Hejnæs K, Moody A, Dyrberg T, Lernmark Å, Madsen OD, and Michelsen BK. (1993). Differential expression of glutamic acid decarboxylase in rat and human islets. Diabetes 42:484-495.

Philippe J, Chick WL, and Habener JF. (1987). Multipotential phenotypic expression of genes encoding peptide hormones in rat insulinoma cell lines. J Clin Invest 79:351-358.

Pictet R, and Rutter WJ. (1972). Development of the embryonic endocrine pancreas. In: Steiner DF, and Frenkel M, eds. Handbook of physiology, sec. 7. Washington DC: American Physiology Society, pp. 25-66.

Pictet R, Rall LB, Phelps P, and Rutter WJ. (1976). The neural crest and origin of the insulin-producing and other gastrointestinal hormone-producing cells. Science 191:191-193.

Pipeleers D, In 't Veld P, Maes E, and Van de Winkel M. (1982). Glucose-induced insulin release depends on functional cooperation between islet cells. Proc Nat Acad Sci USA 79:7322-7325.

Pipeleers D. (1984). Islet cell interactions with pancreatic B-cells. Experientia 40:1114-1126.

Pipeleers DG, Schuit FC, In 't Veld PA, Maes E, Hooghe-Peeters EL, Van de Winkel M, and Gepts W. (1985). Interplay of nutrients and hormones in the regulation of insulin release. Endocrinology 117:824-833.

Pipeleers D, Kiekens R, In 't Veld P. (1992). Morphology of the pancreatic ß-cell. In: Ashcroft FM, and Ashcroft SJH, eds. Insulin. Molecular biology to pathology. Oxford: IRL Press, pp 5-32.

Polak JM, Bloom SR, and Marango PJ. (1984). Neuron-specific enolase, a marker for neuroendocrine cells. In: Falkmer S, Hakanson R, and Sundler F, eds. Evolution and tumor pathology of the neuroendocrine system. Amsterdam: Elsevier, pp.433.

Polak M, Scharfmann R, Ban E, Haour F, Postel-Vinay MC, and Czernichow P. (1990). Demonstration of lactogenic receptors in the rat endocirne pancreas by quantitative autoradiography. Diabetes 39:1045-1049.

Polak M, Scharfmann R, Seilheimer B, Eisenbarth G, Dressler D, Verma IM, and Potter H. (1993). Nerve growth factor induces neuron-like differentiation of an insulin-secreting pancreatic beta cell line. Proc Natl Acad Sci USA 90:5781-5785.

Rahier J, Wallon J, and Henquin JC. (1981). Cell populations in the endocrine pancreas of human neonates and infants. Diabetologia 20:540-546.

Redecker P, Jörns A, Jahn R, and Grube D. (1991). Synaptophysin immunoreactivity in mammalian endocrine pancreas. Cell Tissue Res 264:461-467.

Reetz A, Solimena M, Matteoli M, Folli F, Takei K, and De Camilli P. (1991). GABA and pancreatic ß cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. EMBO J 10:1275-1284.

Rindi G, Terenghi G, Westermark G, Westermark P, Moscoso G, and Polak JM. (1991). Islet amyloid polypeptide in poliferating pancreatic B cells during development, hyperplasia, and neoplasia in humans and mice. Am J Pathol 138:1321-1334.

Rorsman P, Berggren PO, Bokvist K, Ericson H, Möhler H, Östenson CG, and Smith PA. (1989). Glucose-inhibition of glucagon secretion involves activation of $GABA_A$-receptor chloride channels. Nature 341:233-236.

Samols E, Marri G, and Mars V. (1965). Promotion of insulin secretion by glucagon. Lancet ii:415-416.

Scharfmann R, Tazi A, Polak M, Kanaka C, and Czernichow P. (1993). Expression of functiional nerve growth factor receptors in pancreatic ß-cell lines and fetal rat islets in primary culture. Diabetes 42:1829-1836.

Shioda Y, Nagura H, Tsutsumi Y, Shimamura K, and Tamaoki N. (1984). Distribution of Leu 7 (HNK-1) antigen in human digestive organs: an immunohistochemical study with monoclonal antibody. Histochem J 16:843-854.

Smeekens SP, Avruch AS, LaMendola J, Chan SJ, and Steiner DF. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc Natl Acad Sci USA 88:340-344.

Smith FE, Rosen KM, Villa-Komaroff L, Weir GC, and Bonner-Weir S. (1991). Enhanced insulin-like growth factor I gene expression in regenerating rat pancreas. Proc Natl Acad Sci USA 88:6152-6156.

Stagner JI, and Samols E. (1992). The vascular order of islet cellular perfusion in the human pancreas. Diabetes 41:93-97.

Stefan Y, Orci L, Malaisse-Lagae F, Perrelet A, Patel Y, and Unger RH. (1982). Quantitation of endocrine cell content in the pancreas of non-diabetic and diabetic humans. Diabetes 31:694-700.

Stefan Y, Grasso S, Perrelet A, and Orci L. (1983). A quantitative immunofluorescent study of the endocrine cell populations in the developing human pancreas. Diabetes 32:293-301.

Swenne I. (1982). The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. Diabetes 31:754-760.

Swenne I, Hill DJ, Strain AJ, and Milner RDG. (1987). Growth hormone regulation of somatomedin C/insulin-like growth factor I production and DNA replication in fetal rat islets in tissue culture. Diabetes 36:288-294.

Swenne I, and Hill DJ. (1989). Growth hormone regulation of DNA replication, but not insulin production, is partly mediated by somatomedin C/insulin-like growth factor I in isolated pancreatic islets from adult rats. Diabetologia 32:191-197.

Tatemoto K, Efendic S, Mutt V, Makk G, Feistner GJ, and Barchas JD. (1986). Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. Nature 324:476-478.

Teitelman G, and Lee JK. (1987). Cell lineage analysis of pancreatic islet cell development: glucagon and insulin cells arise from catecholaminergic precursors present in the pancreatic duct. Dev Biol 121:454-466.

Teitelman G, Alpert S, and Hanahan D. (1988). Proliferation, senescence, and neoplastic progression of beta cells in hyperplastic pancreatic islets. Cell 52:97-105.

Teitelman G. (1990). Insulin cells of pancreas extend neurites but do not arise from the neuroectoderm. Dev Biol 142:368-379.

Teitelman G. (1993). On the origin of pancreatic endocrine cells, proliferation and neoplastic transformation. Tumor Biol 14:167-173.

Teitelman G, Alpert S, Polak JM, Martinez A, and Hanahan D. (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 118:1031-1039.

Tuch BE, Jones A, and Turtle JR. (1985). Maturation of the response of human fetal pancreatic explants to glucose. Diabetologia 28:28-31.

Van Schravendijk CFH, Foriers A, Van den Brande JL, and Pipeleers DG. (1987). Evidence for the presence of type I insulin-like growth factor receptors on rat pancreatic A and B cells. Endocrinology 121:1784-1788.

Vincent SR, Hökfelt T, Wu JY, Elde RP, Morgan LM, and Kimmel JR. (1983). Immunohistochemical studies of the GABA system in the pancreas. Neuroendocrinology 36:197-204.

Waeber G, Thompson N, Waeber B, Brunner H, Nicod P, and Grouzmann E. (1993). Neuropeptide Y expression and regulation in a differentiated rat insulin-secreting cell line. Endocrinology 133:1061-1067.

Wang TC, Bonner-Weir S, Oates PS, Chulak M, Simon B, Merlino GT, Schmidt EV, and Brand SJ. (1993). Pancreatic gastrin stimulates islet differentiation of transforming growth factor α-induced ductular precursor cells. J Clin Invest 92:1349-1356.

Weir GC, and Bonner-Weir S. (1990). Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest 85:983-987.

Westermark P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, and Johnson KD. (1987). Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. Proc Natl Acad Sci USA 84:3881-3885.

Wiedenmann B, Franke WW, Kuhn C, Moll R, and Gould VE. (1986). Synaptophysin: a marker protein for neuroendocrine cells and neoplasms. Proc Natl Acad Sci USA 83:3500-3504.

Wilson BS, and Lloyd RV. (1984). Detection of chromogranin in neuroendocrine cells with a monoclonal antibody. Am J Pathol 115:458-468.

Yasuda K, Yamada Y, Inagaki N, Yano H, Okamoto Y, Tsuji K, Fukumoto H, Imura H, Seino S, and Seino Y. (1992). Expression of GLUT1 and GLUT2 glucose transporter isoforms in rat islets of Langerhans and their regulation by glucose. Diabetes 41:76-81.

Chapter 2. Type I (insulin-dependent) diabetes mellitus.

* pathogenesis.

Diabetes mellitus is a common chronic disorder, affecting 2-4% of the population in Western countries. Two main types of diabetes mellitus are distinguished: type-I (insulin-dependent) diabetes (prevalence 0.2-0.4%), and type-II (non-insulin-dependent) diabetes (prevalence 2-4%). Both forms of diabetes mellitus are characterized by a disturbed carbohydrate metabolism. Type-I diabetes develops mainly in childhood, with an incidence peak around the onset of puberty. More than 65% of the patients present with symptomps before the age of 18 (Vaandrager et al., 1984).

When islets of Langerhans of recent onset type-I diabetic patients are studied, massive inflammatory infiltration is found (Foulis et al., 1991). The inflammatory process, called insulitis, selectively destroys the islet ß cells. Extensive evidence has been collected to support the notion that type-I diabetes is an autoimmune disorder. Macrophages and T-lymphocytes are known to be present in the cellular infiltrates in the islets of Langerhans (Botazzo et al., 1985). In NOD mice and BB rats, animal models of type-I diabetes, disease can be prevented by the administration of silica particles which are known to be selectively toxic to macrophages (Oschilewski et al., 1985). This indicates that macrophages are required for the immunological destruction of ß cells. The exact role of macrophages remains unknown. Evidence has accumulated for an essential role of cellular autoimmunity (both of CD4+ and CD8+ T-lymphocytes) in ß cell destruction (Bendelac et al., 1987, Miller et al., 1988, Birk and Cohen, 1993, for review). First, immunosuppressive drugs, such as cyclosporin A, have a modulating effect on the development of type-I diabetes mellitus in NOD mice and BB rats as well as in patients (Laupacis et al., 1983, Stiller et al., 1984). Second, diabetes may be prevented by treatment with antibodies to specific T-cell subsets (Boitard et al., 1982). Third, type-I diabetes can be transferred from one animal to another (= adoptive transfer) by using splenocytes, purified T-lymphocytes, and T-cell clones (Bendelac et al., 1987). T-lymphocyte-clones, reactive to ß cell preparations can be isolated from the blood of recent-onset type-I diabetic patients. Features of cellular immunity also precede the onset of type-I diabetes (Harrison et al., 1992). This indicates that type-I diabetes results from a longer period of immune destruction (**FIGURE 5**). The variation in age of onset of type I diabetes suggests that this period may have a variable length. The

factors influencing the destruction rate are unknown. Recently, the relevance of pre-existing ß cell mass and ß cell repair mechanisms on the evolvement of type-I diabetes have attracted increasing attention (Hales et al., 1992, Eizirik et al., 1993). In table 2 targets of cellular autoimmunity in type-I diabetes mellitus are indicated.

Concurrent with cellular immunity, autoantibodies have been detected in the serum of recent onset diabetic patients (targets indicated in table 2). Autoantibodies found in type-I diabetes do not appear to have a pathogenic effect. Adoptive transfer experiments and in vitro culture of islets of Langerhans or ß cells in the presence of diabetic sera does not result in diabetes or ß cell dysfunction respectively (Koevary et al., 1983, Wicker et al., 1986, Mandrup-Poulsen et al., 1990). After transplantation of pancreata between discordant identical twins the islets in the transplanted pancreas are destructed without appearance of autoantibodies in the serum (Sibley et al., 1985).

Insulin autoantibodies (IAA) are found in sera of untreated patients, excluding the possibility that they are formed after insulin administration (Palmer et al., 1983, Wilkin et al., 1985). Islet cell cytoplasmic antibodies (ICA) are present in up to 80% of patient sera at diagnosis (Botazzo et al., 1974, Doniach et al., 1985), and can be found up to 10 years before the clinical onset of type-I diabetes. They are detected by immunohistochemistry of pancreatic sections. ICA-positive sera stain all islet cell types ("whole islet staining pattern"). The assay has been standardized through the use of reference sera (Greenbaum et al., 1992). Thus, ICA have been used for screening of first degree relatives or populations for potential prediabetic patients and for prediction (Bingley et al., 1989, 1993, Bruining et al., 1989). Within the follow-up time of these studies not all ICA positive persons go on to clinical type-I diabetes. This may depend on the age of the individual, ICA levels, complement-fixing ability of the ICA, and other unidentified parameters. There is probably more than one ICA target molecule. A sialoglycoconjugate has been suggested as a target epitope (Nayak et al., 1985). Absorption of ICA sera with glutamic acid decarboxylase (GAD) partially but not completely blocked ICA reactivity (Atkinson et al., 1993), indicating that GAD is also an ICA target. Apart from the whole islet staining pattern of ICA-positive sera, a restricted pattern, in which only ß cells stain, has been observed (Genovese et al., 1992). The different patterns of ICA reactivity may have implications for the risk to develop type-I diabetes (Gianani et al., 1992). Information of ICA reactivity in the human fetal pancreas is limited (Sundqvist et al., 1991).

Autoantibodies to a 64kD protein were first described in 1982 by Baekkeskov et al. They are present in 80-90% of patients at the onset of disease, and can be found several years before onset (Atkinson et al., 1990). The 64kD protein has been identified as glutamic acid decarboxylase (GAD), a neuronal enzyme synthesizing GABA (Baekkeskov et al., 1990). Two forms, GAD65 and GAD67, exist, coded for by two different genes. The two forms of GAD are present in neurons and rat ß cells. Human ß cells only contain GAD65 (Aanstoot, 1993).
Similarly, antibodies to a 38kD protein were found in 20-30% of type-I diabetic patients at onset of disease (Baekkeskov et al., 1982), and in 15% of prediabetic individuals.
In summary, type-I diabetes is considered an autoimmune disease in which both humoral and cellular factors are involved. Cellular factors are presently thought to initiate and propagate the selective destruction of ß cells. Autoantibodies may be used for screening and prediction. It is speculated that ß cell mass and repair mechanisms may play a role in the outcome of autoimmune ß cell destruction, which takes place over an extended period of time. In the human fetal pancreas, few immunologically relevant molecules have been studied. For the understanding of the pathogenesis of type-I diabetes it is important to know whether, and if so, where and when, such relevant molecules are expressed during human fetal islet development. In Chapter 6, the findings on ICA reactivity in the human fetal pancreas are presented, and in the general discussion the preliminary results of GAD65 expression are mentioned.

TABLE 2

Targets of humoral and cellular autoimmunity in type-I diabetes mellitus

HUMORAL	CELLULAR
- insulin	- heat shock protein 65 (hsp65)
- islet cell antibodies (ICA)	- 38 kD protein
- GAD65	- GAD65
- 38 kD protein	
- carboxypeptidase H	
- islet cell autoantigen 69 (ICA69)	

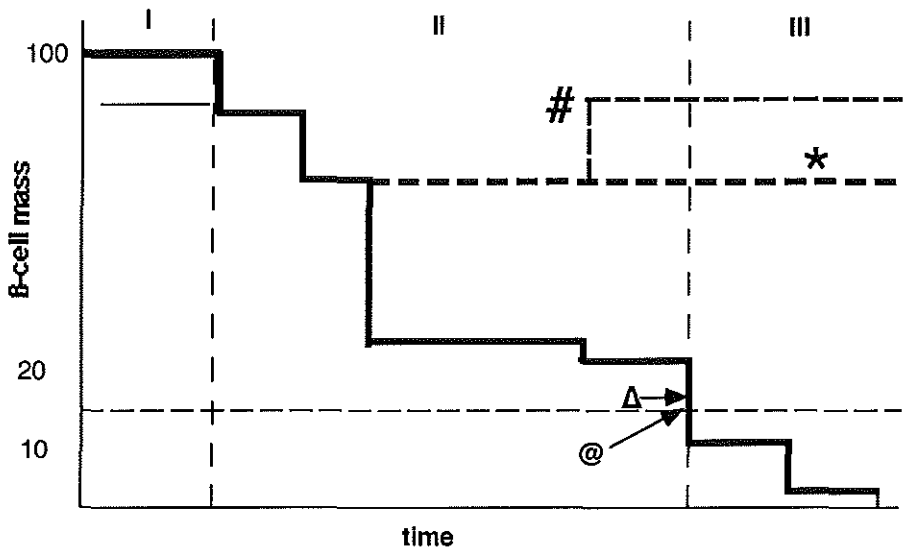


Figure 5. Schematic representation of the decrease in ß cell mass in time, during the different phases of autoimmune destruction leading to type-I diabetes mellitus. This model was proposed by Bruining (1984). In Phase I no ß cell destruction is evident; genetic predisposition, however, may be present. In Phase II, autoimmune destruction starts, triggered by environmental or other (unknown) factors. Phase II has a variable length and may last many years. During this phase, autoimmune destruction may stop (*) or ß cell regeneration may occur (#). When ß cell destruction continues, and cannot be compensated for by ß cell repair, clinical symptoms of type-I diabetes will become evident (@). This last decline in ß cell mass may be relatively rapid, caused by a period of stress or by some viral or bacterial infection (△).

*** etiology.**

In the etiology of type-I diabetes both genetic and environmental factors are important, demonstrated by a concordance rate for monozygotic twins of 35-50% (Olmos et al., 1988). The discordance has been explained in immunological studies, revealing a difference in T-cell receptor specificity in monozygotic twins. Identical twins differ in immunological response patterns, as a result of intrauterine germline rearrangement of T-cell receptor genes. HLA identical siblings of type-I diabetic patients have a 15-25% risk of developing type-I diabetes themselves (Deschamps et al., 1984). This illustrates the importance of HLA genes in determining genetic predisposition. However, the exact gene(s) in the HLA complex which are involved in type-I diabetes are still a matter of investigation.

The HLA complex is a large multigene complex, located on the short arm of chromosome 6. It contains two clusters of highly polymorphic, multiallelic genes, named class I (HLA-A, B and C) and class II (HLA-DR, DQ and DP). In the class I and II gene regions, other, non-HLA genes are also found. Interspaced between the class I and II regions is a cluster of unrelated genes, referred to as the class III region (Trowsdale and Campbell, 1992). All HLA molecules are heterodimers composed of an α and a ß chain. Class I molecules are expressed on most cells. Class II molecules are mainly expressed on macrophages, B-lymphocytes, activated T-lymphocytes, and dendritic cells.

Susceptibility to type-I diabetes was first associated with the class I molecules B8 and B15, later with the class II molecules DR3 and DR4 which are in linkage disequilibrium with B8 and B15, respectively (Tiwari and Terasaki, 1985). Then, HLA-DR3/4 heterozygotes were found to be at high risk to develop type-I diabetes, leading to idea that haplotypes could interact to confer susceptibility or protection (Nerup et al., 1987). Subsequently, a strong association between type-I diabetes and HLA-DQ has been found (Todd et al., 1987). The absence of aspartic acid at position 57 of the HLA-DQ ß chain and the presence of arginine at position 52 of the HLA-DQ α chain was suggested to confer strong susceptibility for type-I diabetes (Todd et al., 1987, Khalil et al., 1990, Trucco, 1992, for review). Other studies have shown that the relative risk associated with different DQ molecules varies considerably, particularly in Japanese subjects (Thomson et al., 1988, Lundin et al., 1989).

The functional link between class I and II MHC molecules and type-I diabetes mellitus may be understood from their function. Class I molecules bind and present peptide fragments to the T-cell receptors of $CD8^+$ (cytotoxic) T-cells, while class II molecules do the same to $CD4^+$ (helper) T-cells (Rothbard and Gefter, 1991). The polymorphism of HLA molecules is mainly localized in the peptide binding clefts (Bjorkman et al., 1987). The shape of the cleft determines which peptides can be bound. DQ molecules could exert their protective or susceptibility effects in each of two phases. Firstly, during tolerance induction, described below, DQ molecules are involved in the intra- or extrathymic presentation of ß-cell peptides (Möller et al., 1990). Second, they may present ß-cell peptides in adult life, after ß cell damage by environmental factors.

Virus infections are the best studied environmental factor in relation to type-I diabetes. Serological studies and case histories have indicated that rubella, mumps, Coxsackie B4, and cytomegalovirus may be involved in the development of type-I diabetes (Banatvala et al., 1985, Yoon et al., 1976). Viruses can produce type-I diabetes after an interval of many years from the time of infection, exemplified by the congenital rubella syndrome (Menser et al., 1978). Alternatively, repeated attacks by ß-cell tropic viruses, in combination with a susceptible genotype, may also lead to type I diabetes. Molecular mimicry is one of the mechanisms by which viruses may trigger autoimmunity against ß cells (Bae et al., 1990). Homology of viral antigens and host determinants has been suggested for the P2C protein of Coxsackie B4 virus and GAD65 (Kaufman et al., 1992, Bu et al., 1992). Another model for virus-induced autoimmunity comes from a transgenic mouse study by Oldstone et al. (1991). Expression of lymphocytic choriomeningitis virus (LCMV) proteins under the control of the insulin promotor resulted in insulitis and diabetes after a challenge with LCMV.

In summary, type-I diabetes etiology involves genetic and environmental determinants. Of the genetic determinants, the HLA region has been studied extensively. HLA-DQ gene polymorphisms seem to play an important role in type-I diabetes susceptibility. Other genetic factors remain to be determined. Environmental factors, especially viruses, are suspected in the etiology of type-I diabetes, but need further investigation.

*** tolerance induction in relation to type-I diabetes mellitus.**

The function of the immune system is to mount a protective response against foreign invaders. At the same time it is crucial not to mount responses against antigens that belong to the own body. Thus, components of the immune system (T- and B-lymphocytes) must learn to discriminate self from non-self antigens. This process occurs during fetal development and is called tolerance induction. Loss of tolerance or failure of tolerance induction leads to autoimmune disease, such as type-I diabetes mellitus. Several causes of lack of tolerance are discussed below and summarized in table 3.

The repertoire of T- and B-lymphocyte activity is formed and modulated in the thymus, early in development (Kappler et al., 1987, Pullen et al., 1988, Von Boehmer et al., 1989). For tolerance induction to occur antigens must be processed and presented to thymic T-lymphocyte receptors in the context of MHC class I and II molecules. A protective or susceptibility effect of HLA-DQ genes may influence tolerance induction. The cells involved in this processing and presentation may be thymic: macrophages, dendritic cells, and cortical epithelial cells, as well as extrathymic: splenic antigen presenting cells (Lorenz et al., 1989, Swat et al., 1991). A major focus of study has been the question how the body could discharge potentially harmful (= autoreactive) cells and at the same time retain potentially beneficial (= alloreactive) cells, to provide an adequate immune defence against foreign antigens. During development, immature T-cells arrive in the thymic cortex. Here, positive selection occurs when T-cells possess receptors to self peptide/MHC complexes. These complexes are presented by thymic epidermal stromal cells. T-cells that fail to recognize MHC become subject to apoptosis, or programmed cell death (Sha et al., 1988). Negative selection against T-cells with high affinity receptors to self antigen/MHC complexes occurs in the thymic medulla. Presentation of self-antigens occurs by dendritic cells. Again, T-cells may be deleted physically by apoptosis (Jenkinson et al., 1989), or through functional deletion (clonal anergy) (Ramsdell et al., 1989, 1990). A recent study describes that fetal mouse thymocytes are protected from deletion early in ontogeny. Thus, negative selection is a late ontogenic event (Finkel et al., 1992).

Despite thymic selection autoreactive T-cells have been detected in many normal individuals to many self proteins, suggesting that additional mechanisms prevent autoimmunity in those cases. It is conceivable that not all antigens are presented in the thymus. The time frame of presentation may be essential (see below).

Peripheral tolerance induction was considered (Rocha and Von Boehmer, 1991). Tolerance to the vast number of peripheral antigens may occur through down-regulation of T-cell receptors (Schönrich et al., 1991). Due to the inability of non-immune cells, such as islet ß cells, to provide co-stimulatory signals necessary for T-cell activation, most T-cells are anergized or become ignorant (Schwartz, 1990, Zinkernagel et al., 1991). The situation changes when ß cell peptides are presented in sufficient quantities by antigen presenting cells, which deliver the co-stimulatory signals. This may occur through molecular mimicry after viral infection. A number of studies indicates the importance of fetal tolerance development, in relation to type-I diabetes.

First, in transgenic mice expressing the SV40 large T-antigen under control of the rat insulin II gene promotor, the occurrence of tolerance to large T-antigen depends on the timing of expression of the transgene (Adams et al., 1987). When this ß-cell specific protein is expressed during late embryological or early neonatal development, mice are normal. However, when expression appears later, autoantibodies are found and the islets are destroyed by lymphocytic infiltrates. T-cell reactivity to large-T antigen is also reported (Jolicoeur, personal communication).

Second, myelin basic protein-specific (MBP-specific) autoreactive T-cells, playing a role in multiple sclerosis, have features in common with fetal T-cells (Zhang and Heber-Katz, 1992). The paucity of N-region additives of the T-cell receptors of MBP-specific T-cells is also found in fetal T-lymphocytes. The authors suggest that the autoreactive T-cells have an early ontogenic origin and have left the thymus at an early developmental stage. Thus, they escape negative selection in the fetal thymus.

Third, defective macrophage-like antigen presenting cells have been demonstrated in the thymus of diabetes prone BB/Wor rats (Georgiou et al., 1988). These cells are normally involved in tolerance development, which may be disturbed in these animals, resulting in type-I diabetes. Other experiments in the BB rat show that intrathymic transplantation of islets may induce unresponsiveness and thus prevention of diabetes (Posselt et al., 1990, Koevary et al, 1992).

Finally, it was recently proposed that a mutation in one of the peptide transporter genes RING4 or RING11, essential for proper assembly of class I MHC molecules may have an important effect on tolerance induction. Without class I MHC surface expression, no presentation of endogenous molecules to the immune system (T-lymphocytes) can occur. This could result in an increased susceptibility for the development of autoimmune disorders. (Faustman et al., 1991).

In summary, tolerance induction is an incompletely understood process, in which potential autoreactive T-cells are deleted. Failure of deletion or a disturbance in the regulation of remaining autoreactive T-cells may result in autoimmune diseases such as type-I diabetes mellitus. Antigen presentation seems an essential component of tolerance induction. Therefore, antigens which may be relevant in type-I diabetes mellitus have been studied (Chapter 6). The results of collaborative work on the development of the T-cell repertoire in human fetal thymocytes is presented in Chapter 7. These experiments suggest that human fetal pancreas, spleen, and thymus should be studied together for the analysis of tolerance induction (see general discussion).

TABLE 3

Causes of lack of tolerance

Lymphocyte related:

- failure of positive selection
- failure of negative selection
- failure of suppression mechanisms
- failure of peripheral tolerance (anergy/ignorance)

Antigen related:

- sequestration in place or time
- molecular mimicry
- alterations of self antigens (different processing or different post-translational modification)
- combination of self antigens with a retroviral particle

Antigen presenting cell related:

- aberrant MHC class II expression
- aberrant processing and presentation of antigen

Aanstoot HJ. (1993). Identification, characterization and application of autoantigens in type I diabetes mellitus. Thesis, Erasmus University.

Adams TE, Alpert S, and Hanahan D. (1987). Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic beta cells. Nature 325:223-228.

Atkinson MA, MacLaren NK, Scharp DW, Lacy PE, and Riley WL. (1990). 64000 M, autoantibodies as predictors of insulin-dependent diabetes. Lancet 335:1357-1360.

Atkinson MA, Kaufman DL, Newman D, Tobin AJ, and Maclaren NK. (1993). Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. J Clin Invest 91:350-356.

Bae YS, Eun HM, Pon RT, Giron GW, and Yoon JW. (1990). Two amino acids, Phe-16 and Ala-776, on the polyprotein are most likely to be responsible for the diabetogenicity of EMC virus. J Gen Virol 71:639-645.

Baekkeskov S, Nielson JH, Marner B, Bilde T, Ludvigsson J, and Lernmark Å. (1982). Autoantibodies in newly diagnosed diabetic children immunoprecipitate specific human pancreatic islet cell proteins. Nature 298:167-169.

Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, and DeCamilli P. (1990). Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151-156.

Banatvala JE, Schernthaner G, Schrober E, DaSilva LM, Bryant J, Borckenstein MM, Brown D, and Menser MA. (1985). Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile onset insulin-dependent diabetes mellitus in Britain, Austria and Australia. Lancet i:1409-1412.

Bendelac AC, Carnaud C, Boitard C, and Bach JF. (1987). Syngeneic tranfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both Lyt2+ and L3T4+ T cells. J Exp Med 166:823-832.

Bingley PJ, and Gale EAM. (1989). Prediction of type I diabetes: The Bart's Windsor family study. In: Andreani D, Kolb H, and Pozzilli P, eds. Immunotherapy of type I diabetes. Chichester: John Wiley & Sons, pp. 137-145.

Bingley PJ, Bonifacio E, and Gale EAM. (1993). Can we really predict IDDM? Diabetes 42:213-220.

Birk OS, and Cohen IR. (1993). T-cell autoimmunity in type I diabetes mellitus. Curr Opin Immunol 5:903-909

Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, and Wiley DC. (1987). Structure of the human class I histocompatibility antigen, HLA-A2. Nature 329:506-518.

Boehmer H von, Teh HS, and Kisielow P. (1989). The thymus selects the useful, neglects the useless and destroy the harmful. Immunol Today 10:57-61.

Boitard C, Chatenoud LM, and Debray-Sachs M. (1982). In vitro inhibition of pancreatic beta-cell function l lymphocytes from diabetics with associated autoimmune diseases: a T-cell phenomenon. J Immunol 129:2529-25

Botazzo GF, Florin-Christensen A, and Doniach D. (1974). Islet-cell antibodies in diabetes mellitus with autoimm polyendocrine deficiencies. Lancet ii:1279-1283.

Botazzo GF, Dean BM, McNally JM, MacKay EH, Swift PGF, and Gamble DR. (1985). In situ characterizati of autoimmune phenomena and epxression of HLA class II molecules in the pancreas in diabetic insulitis. Engl J Med 313:353-360.

Bruining GJ, Molenaar JM, Grobbee DE, Hofman A, Scheffer GJ, Bruining HA, De Bruyn AM, and Valkenburg HA. (1989). Ten-year follow-up study of islet cell antibodies and childhood diabetes mellitus. Lancet i:1100-1103.

Bu DF, Erlander MG, Hitz BC, Tillakaratne NJK, Kaufman DL, Wagner-McPherson CB, and Tobin AJ. (1992). Two human glutamate decarboxylases 65-kDa GAD and 67-kDa GAD, are encoded by a single gene. Proc Natl Acad Sci USA 89:2115-2119.

Deschamps I, Lestradet H, Busson M, and Hors J. (1984). Evaluation of recurrence risk in siblings of diabetic children: importance of age and birth order in relatiion to HLA genotypes. Diabetes Res 1:125-135.

Doniach D, Spender KM, and Botazzo GF. (1985). Immunology of insulin-dependent diabetes. In: Rose NR, and Mackay IR, eds. The autoimmune disease. Orlando: Academic Press, pp. 227-242.

Eizirik DL, Sandler S, and Palmer JP. (1993). Repair of pancreatic ß cells. A relevant phenomenon in early IDDM? Diabetes 42:1383-1391.

Faustman D, Li X, Lin HY, Fu Y, Eisenbarth GS, Avruch J, and Guo J. (1991). Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. Science 254:1756-1761.

Finkel TH, Kappler JW, and Marrack PC. (1992). Immature thymocytes are protected from deletion early in ontogeny. Proc Natl Acad Sci USA 89:3372-3374.

Foulis AK, McGill M, and Farquharson MA. (1991). Insulitis in type 1 (insulin-dependent) diabetes mellitus in man; macrophages, lymphocytes, and interferon-gamma containing cells. J Pathol 165:97-103.

Genovese S, Bonifacio E, McNally JM, Bean BM, Wagner R, Bosi E, Gale EAM, and Botazzo GF. (1992). Distinct cytoplasmic islet cell antibodies with different risks for type-I (insulin-dependent) diabetes mellitus. Diabetologia 35:385-388.

Georgiou HM, Lagarde AC, and Bellgrau D. (1988). T cell dysfunction in the diabetes-prone BB rat: a role for thymic migrants that are not T cell precursors. J Exp Med 167:132-148.

Gianani R, Pugliese A, Bonner-Weir S, Shiffrin AJ, Soeldner JS, Erlich H, Awdeh Z, Alper CA, Jackson RA, and Eisenbarth GS. (1992). Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type I diabetes. Diabetes 41:347-353.

Greenbaum C, Palmer J, Nagataki S, Yamaguchi Y, Molenaar JL, Van Beers WAM, MacLaren NK, Lernmark Å, and participating laboratories. (1992). Improved specificity of ICA assays in the fourth international immunology of diabetes serum exchange workshop. Diabetes 41:1570-1574.

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, and Winter PD. (1991). Fetal and infant growth and impaired glucose tolerance at age 64 years. Br Med J 303:1019-1022.

Harrison LC, Chu SX, DeAizpurua HJ, Graham M, Honeyman MC, and Colman PG. (1992). Islet-reactive T cells are a marker of preclinical insulin-dependent diabetes. J Clin Invest 89:1161-1165.

Jenkinson EJ, Kingston R, Smith CA, Williams GT, and Owen JJT. (1989). Antigen-induced apotosis in developing T-cells: a mechanism for negative selection of the T cell receptor repertoire. Eur J Immunol 19:2175-2177.

Kappler JW, Roehm N, and Marrack P. (1987). T cell tolerance by clonal elimination in the thymus. Cell 49:273-280.

Kaufman DL, Erland MG, Clare-Salzer M, Atkinson MA, MacLaren MA, and Tobin AJ. (1992). Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J Clin Invest 89:283-292.

Khalil I, d'Auriol L, Gobet L, Morin L, Lepage V, Deschamps I, Park MS, Degos L, Galibert F, and Hors J. (1990). A combination of HLA-DQß Asp57-negative and HLA-DQα Arg52 confers susceptibility to insulin-dependent diabetes mellitus. J Clin Invest 85:1315-1319.

Koevary S, Rossini A, Stoller W. Chick W, and Williams RM. (1983). Passive transfer of diabetes in the BB/W rat. Science 220:727-728.

Koevary SB, and Blomberg M. (1992). Prevention of diabetes in BB/Wor rats by intrathymic islet injection. J Clin Invest 89:512-516.

Laupacis A, Stiller CR, Gardell C, Keown P, Dupré J, Wallace AC, and Thibert P. (1983). Cyclosporin prevents diabetes in BB Wistar rats. Lancet i:10-12.

Lorenz RG, and Allen PM. (1989). Thymic cortical epithelial cells can present self-antigens in vivo. Nature 337:560-562.

Lundin KEA, Rönningen KS, Aono S, Spurkland A, Gaudernack G, Isshiki G, and Thorsby E. (1989). HLA-DQ antigens in Japanese patients with insulin-dependent diabetes mellitus. Detection of a DRw8 DQw8 haplotype. Tissue Antigens 34:233-241.

Mandrup-Poulsen T, Mölvig J, Andersen HU, Helqvist S, Spinas GA, Munck M, and the Canadian-European randomized control trial group. (1990). Lack of predictive value of islet cell antibodies, insulin antibodies, and HLA-DR phenotype for remission in cyclosporin-treated IDDM patients. Diabetes 39:204-210.

Menser MA, Forrest JM, and Bransby RD. (1978). Rubella infection and diabetes mellitus. Lancet i:57-60.

Miller BJ, Appel MC, O'Neill JJ, and Wicker LS. (1988). Both the Lyt-2+ and L3T4 T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. J Immunol 140:52-58.

Möller E, Böhme J, Valugerdi MA, Ridderstad A, and Olerup O. (1990). Speculation on mechanisms of HLA associations with autoimmune diseases and the specificity of autoreactive T lymphocytes. Immunol Rev 118:5-19.

Nayak RC, Omar MAK, Rabizadeh A, Srikanta S, and Eisenbarth GS. (1985). "Cytoplasmic" islet cell antibodies: evidence that the target antigen is a sialoglycoconjugate. Diabetes 34:617-619.

Nerup J, Mandrup-Poulsen T, and Mölvig J. (1987). The HLA-IDDM association: implications for etiology and pathogenesis of IDDM. Diab Metab Rev 3:779-802.

Oldstone MBA, Nerenberg M, Southern P, Price J, and Lewicki H. (1991). Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell 65:319-331.

Olmos P, A'Hern R, Heaton DA, Millward BA, Risley D, Pyke DA, and Leslie RDG. (1988). the significance of the concordance rate for type I (insulin-dependent) diabetes in identical twins. Diabetologia 31:747-750.

Oschilewski U, Kiessel U, and Kolb H. (1985). Administration of silica prevents diabetes in BB rats. Diabetes 34:197-199.

Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, and Paquette TL. (1983). Insulin antibodies in insulin dependent diabetes before insulin treatment. Science 222:1337-1339.

Posselt AM, Barker CF, Tomaszewkski JE, Markmann JF, Choti MA, and Naji A. (1990). Induction of donor-specific unresponsiveness by intrathymic islet transplantation. Science 249:1293-1295.

Pullen AM, Marrack P, and Kappler JW. (1988). The T-cell receptor repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature 335:796-801.

Ramsdell F, Lantz T, and Fowlkes BJ. (1989). A nondeletional mechanism of self-tolerance. Science 246:1038-1041.

Ramsdell F, and Fowlkes BJ. (1990). Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. Science 248:1342-1348.

Rocha B, and Von Boehmer H. (1991). Peripheral selection of the T cell repertoire. Science 251:1225-1228.

Rothbard JB, and Gefter ML. (1991). Interactions between immunogenic peptides and MHC proteins. Annu Rev Immunol 9:527-565.

Schwartz RH. (1990). A cell culture model for T lymphocyte clonal anergy. Science 248:1349-1355.

Schönrich, G, Kalinke U, Momburg F, Malissen M, Hammerling GJ, and Arnold B. (1991). Down-regulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell 65:293

Sha WC, Nelson CA, Newberry RD, Kranz DM, Russell JH, and Loh DY. (1988). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature 336:73-76.

Sibley RK, Sutherland DER, Goetz F, and Michael AF. (1985). Recurrent diabetes mellitus in the pancreas iso- and allograft. A light and electron microscopic and immunohistochemical analysis of four cases. Lab Invest 53:132-144.

Stiller CR, Dupre J, Gent M, Jenner MR, Keown PA, Laupacis A, Martell R, Rodger NW, Von Graffenried B, and Wolfe BMJ. (1984). Effects of cyclosporin immunosuppression in insulin-dependent diabetes mellitus of recent onset. Science 223:1362-1367.

Sundkvist G, Bergqvist A, Weibull H, Bergqvist D, Fält K, Landin-Olsson M, and Lernmark Ä. (1991). Islet cell antibody reactivity with human fetal pancreatic islets. Diab Res Clin Pract 14:1-8.

Swat W, Ignatowicz L, Von Boehmer H, and Kisielow P. (1991). Clonal deletion of immature CD4+8+ thymocytes in suspension culture by extrathymic antigen-presenting cells. Nature 351:150-153.

Thomson G, Robinson WP, Kuhner MK, et al. (1988). Genetic heterogeneity, models of inheritance, and risk estimates for a joint study of Caucasians with insulin-dependent diabetes mellitus. Am J Hum Genet 43:799-816.

Tiwari JL, and Terasaki PI. (1985). Juvenile diabetes mellitus (insulin-dependent). In: Tiwari JL, Terasaki PI (eds). HLA and disease associations. New York: Springer Verlag, pp. 185-210.

Todd JA, Bell JI, and McDevitt HO. (1987). HLA-DQ beta gene contributes to susceptibility to insulin-dependent diabetes mellitus. Nature 329:599-604.

Trowsdale J, and Campbell RD. (1992). Complexity in the major histocompatibility complex. Eur J Immunogenetics 19:43-55.

Trucco M. (1992). To be or not to be ASP 57, that is the question. Diabetes Care 15:705-715.

Vaandrager GJ, Bruining GJ, Veenhof FJ, and Drayer NM. (1984). Incidence of childhood diabetes in The Netherlands: a decrease from north to south over North-Western Europe. Diabetologia 27:203-206.

Wicker LS, Miller BJ, and Mullen Y. (1986). Transfer of autoimmune diabetes mellitus with splenocytes from non-obese diabetic (NOD) mice. Diabetes 35:855-860.

Wilkin TJ, Hoskins PJ, Armitage M, Rodier M, Casey C, Diaz JL, Pyke DA, and Leslie RDG. (1985). Value of insulin autoantibodies as serum markers for insulin dependent diabetes mellitus. Lancet i:480-482.

Yoon JW, Austin M, Onodera T, and Notkins AL. (1976). Virus-induced diabetes mellitus: isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 300:1173-1179.

Zhang XM, and Heber-Katz E. (1992). T cell receptor sequences from encephalitogenic T cells in adult lewis rats suggest an early ontogenic origin. J Immunol 148:746-752.

Zinkernagel RM, Pircher HP, Ohashi P, Oehen S, Odermatt B, Mak T, Arnheiter H, Bürki K, and Hengartner H. (1991). T and B cell tolerance and responses to viral antigens in transgenic mice: implications for the pathogenesis of autoimmune versus immunopathological disease. Immunol Rev 122:133-171.

Introduction to the experiments.

The aims of the work presented in this thesis are: 1) identification of islet stem cells; and 2) investigating the importance of the fetal pancreas and the fetal immune system for the develoment of type-I diabetes mellitus. For these experiments the human fetal pancreas was chosen because rodent pancreas development and rodent models for type-I diabetes mellitus differ essentially from human development and human type-I diabetes mellitus respectively.

Embryonal mouse studies have shown that the four major islet cell types derive from a common precursor cell. In mouse embryonal pancreas hormone coexpression was found, suggesting that these cells might be precursor cells. As this had not been studied in the human fetal pancreas, double label immunohistochemistry and double label immuno-electronmicroscopy were performed to detect coexpression of insulin, glucagon and somatostatin. We were able to show that different combinations of hormone coexpression also occur in the human fetal pancreas, but such cells do not proliferate, or proliferate at a level below the detection limit of the assay used (Chapter 3).

In a subsequent series of experiments, neuroendocrine marker molecules, which had been used in the detection of potential endocrine precursor cells in mice, were tested for the detection of islet stem cells. Two of these markers, N1 and HNK-1, were found in proliferating cells as well as in hormone containing islet cells, suggesting them as precursor cell markers (Chapter 4). Again, double label immunohistochemistry and BrdU incorporation were used.

These findings, however, did not prove that marker-positive proliferating cells indeed differentiate into marker-positive hormone containing cells. In Chapter 5, the new monoclonal antibody N1 was characterized and used for sorting human fetal pancreatic single cell suspensions by fluorescence activated cell sorting. These sorted cell suspensions should then be studied in vitro, adding growth factors or differentiation inducing factors. However, the culture conditions for these sorted cells were suboptimal, leading to cell death and fibroblast overgrowth.

The pathogenesis of type-I diabetes mellitus has been outlined in Chapter 2 as a selective autoimmune destruction of pancreatic ß cells. Many potential humorally or cellularly defined autoantigens have been found, but the cause of autoimmunity remains unknown. Transgenic mouse studies have suggested that tolerance induction of the immune system against antigens occurs during a critical period of fetal development and failure may lead to autoimmunity. To investigate this hypothesis for type-I diabetes autoimmunity, two questions should be answered: 1. Are

islet (ß) cell antigens, relevant to type-I diabetes mellitus, expressed in fetal pancreatic islet cells at this stage of development? and 2. At what stage of development does tolerance induction occur in the human fetal pancreas?

Using antisera from type-I diabetic patients islet cell antibody (ICA) reactivity was shown in human fetal islet cells (Chapter 6). In preliminary experiments (Chapter 8) GAD65 expression was also shown.

The time course of tolerance development, as many other aspects of immune system development, is unknown in the human fetus. Therefore, as a start and in collaborative experiments, the formation of the T-cell repertoire was studied in human fetal immune system organs. These experiments did not allow any conclusions with regard to the time course of tolerance development but indicated that T-cell receptor rearrangements occur, involving the majority of TCR V gene families (Chapter 7).

The midgestational human fetal pancreas contains cells co-expressing islet hormones

R.R. de Krijger[1], H.J. Aanstoot[1], G. Kranenburg[1], M. Reinhard[1], W.J. Visser[2], and G.J. Bruining[1]

Dept. of Pediatrics[1], and Dept. of Cell Biology and Genetics[2], Erasmus University and Academic Hospital/SophiaChildrens Hospital, Rotterdam, The Netherlands

Developmental Biology 153:368-375 (1992)

SUMMARY

In the fetal development of the mouse pancreas, endocrine cells have been found that express more than one hormone simultaneously. Our objective was to evaluate the existence of such cells in the human fetal pancreas. We found cells coexpressing two of the major pancreatic hormones (insulin, glucagon, and somatostatin) in sections of eight midgestational (12-18 weeks) pancreata and in 0-7% of cells in single-cell suspensions from midgestational pancreata. By electron microscopy, using granule morphology and immunoelectron microscopic techniques, we could confirm these findings and even detect cells containing three hormones. Morphologically different granules contained different immunoreactivities, suggesting parallel regulation of hormone production and packaging. In six newborn pancreata (born after 22-40 weeks of gestation), we could not find any multiple-hormone-containing cells. Subsequently, we evaluated whether multiple-hormone-containing cells proliferate by using pancreatic fragments and single-cell preparations at the light and electron microscopic level (six pancreata). No endocrine hormone-containing cells incorporated bromodeoxyuridine during a 1-hr culture period, indicating that these cells have lost the ability to proliferate under the conditions chosen. We conclude that, as in mice, the human fetal pancreas of 12-18 weeks of gestation contains endocrine cells that express multiple hormones simultaneously. These (multiple) hormone-containing cells do not seem to proliferate under basal conditions.

INTRODUCTION

Mature hormone-producing cells usually synthesize and secrete one type of hormone. In the adult islet of Langerhans, four endocrine cell types have been described: alpha cells produce and secrete glucagon, beta cells insulin, delta cells somatostatin, and PP cells pancreatic polypeptide (Larsson, 1978). Each cell type contains secretory granules with a specific ultrastructure. Recently it was shown that during mouse pancreatic development, endocrine cells could be found that express more than one of these hormones simultaneously (Alpert et al., 1988).

Our objective was to assess whether multiple-hormone-containing cells are present in the development of the human pancreas. Fetal pancreata of 12-40 weeks gestational age were studied. Also single-cell preparations of part of these samples were studied by immunohistochemistry and

immunoelectron microscopy. Finally, we studied the proliferation of multiple-hormone-containing cells under basal conditions, using the bromodeoxyuridine (BrdU) incorporation technique.

MATERIALS AND METHODS

Tissues and cells. Human fetal pancreata between 12 and 18 weeks of gestational age were obtained from abortions, after signed informed consent of the women concerned and with permission of the local ethical committee. Gestational age was assessed by echographic measurement of the biparietal diameter. Abortions were induced mechanically, resulting in a warm ischemia time of 15-25 min. Tail parts of the pancreas were immediately fixed in 4% buffered formalin or in 0.4% glutaraldehyde and 1% acrolein for light or electron microscopical processing respectively. Pancreata between 22 and 40 weeks of gestational age were obtained from deceased patients of a neonatal intensive care unit after parental permission, during routine obductions. The neonates died of causes not related to pancreas pathology. Pancreata were fixed for light and electron microscopy at autopsy, less than 12 hr after death.

From the fetal pancreata between 12 and 18 weeks of gestational age single-pancreatic-cell suspensions were prepared. These have the advantage of unequivocal detection of double-positive cells and are easily quantified. Pancreata were minced into small fragments, which were incubated with collagenase (SIGMA, St. Louis, MO, 1 mg/ml) in Earle's balanced salt solution (EBSS, GIBCO, Paisley, UK) for two 10-min periods. In between, fresh collagenase was added and single cells were removed by centrifugation. Cells were centrifuged over a Percoll gradient with layers of 1.004 (EBSS), 1.030, and 1.063 g/ml density. The 1.030-1.063 g/ml interphase, containing 83% viable cells, was cultured overnight in RPMI 1640 (GIBCO) supplemented with 10% pooled, heat-inactivated human serum (Bloodbank, Academic Hospital Leiden, The Netherlands), 1.5 U/ml dispase I (Boehringer Mannheim, Mannheim, FRG), and 10 ug/ml DNase (SIGMA).

Bromodeoxyuridine incorporation. Small tail fragments of the human fetal pancreas and single-cell preparations were cultured for 1 hr in the presence of 10uM 5-bromo-2-deoxyuridine (SIGMA) in RPMI 1640, supplemented with 10% human serum. Some fractions were cultured for 2,4,8, or 16 hours to observe the frequency of cells that incorporated BrdU. Since there

was no large variation in this frequency, we chose to incorporate for 1 hr (data not shown). The fragments were fixed in 4% buffered formalin or in 0.4% glutaraldehyde and 1% acrolein for light or electron microscopy.

Immunohistochemistry (sections). Paraffin sections of the tail parts of human fetal and newborn pancreata were deparaffinized using xylene and a graded alcohol series and washed in phosphate-buffered saline (PBS). They were then incubated for 30 min at room temperature with a combination of two primary antibodies (three sections per pancreas for three possible combinations), washed, and incubated for 30 min with a combination of two secondary antibodies conjugated to either FITC or TRITC. After a final wash, sections were mounted and stored at -20°C.
For BrdU immunohistochemistry the sections of a series of pancreata (n=20) were pretreated with 0.1% pronase (SIGMA) for 5 min at 37°C and 2N HCl for 30 min at 37°C followed by neutralization with a borate buffer (pH 8.5). These steps were done after deparaffination and before progressing to the application of the primary antibodies.
We used monoclonal antibodies to insulin (HUI-018, cross-reactivity with proinsulin 100%, 1:50, Novo, Bagsvaerd, Denmark), glucagon (GLU-001, cross-reactivity with glicentin 100%, 1:30, Novo), somatostatin (SOM-018, 1:1000, Novo) and 5-bromo-2-deoxyuridine (1:50, Becton Dickinson, Mountain View, CA). Each of these antibodies was combined with polyclonal antibodies (Dako, Glostrup, Denmark) to insulin (1:1000, guinea-pig origin), glucagon (1:300, rabbit origin), or somatostatin (1:800, rabbit origin). In addition, a polyclonal antibody to pancreatic polypeptide was used (1:20, rabbit origin, Dako). Second antibodies were goat-anti-mouse FITC and TRITC (1:150 and 1:60), goat-anti-rabbit FITC and TRITC (1:200 and 1:200) (Nordic Immunological Laboratories, Tilburg, The Netherlands), and goat-anti-guinea-pig FITC and TRITC (1:60 and 1:300) (Southern Biotechnology, Birmingham, UK). Control experiments consisted of replacement of each primary antiserum with a preimmune serum or with PBS/bovine serum albumin (BSA). Background staining was minimal and spectral overlap was not observed. Furthermore, the fact that both single- and double-positive cells could be observed, indicated that there was no cross-reactivity between the different hormone antibodies.

Immunocytochemistry (single cells). Following cell culture for at least 18 hr, cytospins from pancreatic cell suspensions were prepared (n=15 pancreatic suspensions; 2x12, 1x13, 4x14, 5x15, 2x16, and 1x18 weeks of gestation). Staining and control experiments were similar to

that described for paraffin sections. Instead of deparaffination, cytospins were fixed in acetone and washed in PBS before applying the primary antibodies. For quantification of results, 100 endocrine cells, recognizable by their fluorescence, were counted. Disrupted cells were excluded.

Immunoelectron microscopy. After fixation for 24-72 hr, pancreatic fragments were embedded in Lowicryl K4M (Roth et al., 1981). Ultrathin sections were prepared on a Reichert Om U3 (Reichert, Vienna, Austria), ultramicrotome and placed on Formvar-coated copper grids. Incubation times were 1-2 hr for the combinations of monoclonal and polyclonal primary antibodies [to insulin, glucagon, and somatostatin, see Immunohistochemistry] and 1 hr for the secondary antibodies. The latter were conjugated to colloidal gold of 10 or 15 nm (Aurion, Wageningen, The Netherlands). For BrdU immunoelectron microscopy we used the method described by Thiry and Dombrowicz (1988). Sections were floated on a 5 M HCl solution for 30 min and neutralized with a 1 M borax solution before mounting. The anti-BrdU antibody (1:50) was applied in combination with polyclonal antibodies to insulin, glucagon, or somatostatin [see Immunohistochemistry] for 3-4 hr. Finally, the sections were contrasted with uranyl acetate for 20 min and with lead citrate for 30 sec. Sections were analysed on a Philips EM400 electron microscope (Eindhoven, The Netherlands).
In each section 100 cells, detectable by their endocrine granule content, were counted. Controls consisted of replacement of primary antibodies by preimmune serum or PBS. Background reactivity was less than 0.5 gold particle per secretory granule, whereas in the case of a positive signal an average of 5 gold particles per secretory granule was observed. Cross-reactivity of primary antibodies with each of the other two hormones was analyzed by single staining and observation of gold particles over morphologically different types of secretory granules. The use of Lowicryl embedding medium, necessary for immunoelectron microscopy, results in negative membrane contrasting which makes detection of intra- and intercellular membranes difficult.

Statistics. The association between the gestational age of the pancreatic specimen and the percentage of multiple-hormone-containing cells and that between gestational age and percentage of BrdU-positive cells in light microscopical analysis of single-cell suspensions and sections was evaluated according to Spearman's rank correlation test.

RESULTS

Multiple-hormone expression in human midgestational and newborn pancreata and midgestational endocrine pancreatic cells.

Immunohistochemistry. Eight human fetal pancreata (12-18 weeks) and six newborn pancreata (22-40 weeks) were analyzed by fluorescence microscopy. In the fetal pancreata, endocrine cells reactive with antibodies to insulin, glucagon, somatostatin, or pancreatic polypeptide appeared as single cells, scattered in the pancreatic parenchyma, and as endocrine cell clusters of varying sizes (Figs. 1A,B). This pattern was found at all gestational ages studied, although the number and size of the cell clusters increased with age. Single cells, containing one of the hormones, were found in the proximity of ducts (Figs. 1C,D). Pancreatic polypeptide-containing cells were observed very infrequently, as tail parts of the pancreas were used, and this hormone was omitted from the double-incubation studies. In all fetal pancreata studied we found one or more cells coexpressing two of the three hormones tested (insulin, glucagon, somatostatin) among a majority of cells containing only a single hormone. Figures 1E and 1F show the coexpression of insulin and glucagon. Newborn pancreata (22-40 weeks), which had no morphological signs of proteolysis, presented with numerous endocrine cell clusters and islets. In contrast to the midgestational pancreata, we never observed cells containing more than one hormone.

Immunocytochemistry. Single-cell preparations of fetal midgestational pancreata were used to assess the frequency of multiple-hormone-containing cells. Table 1 summarizes the data on 15 human fetal pancreatic single-cell suspensions (2x12, 1x13, 4x14, 5x15, 2x16, and 1x18 weeks of gestation). Predominantly, the combination of insulin and glucagon expression in one cell was found. However, the combination of insulin and somatostatin or glucagon and somatostatin could also be identified. One of the 15 pancreatic suspensions (14 weeks of gestational age) did not contain any cells with two hormones. In two pancreatic cell suspensions all three hormone combinations could be found. No statistically significant association was observed between gestational age and the percentage of any hormone combination ($P > 0.05$). The frequency of pancreatic polypeptide-containing cells was too low ($< 0.1\%$ of all cells) to study its coexpression in other endocrine cells.

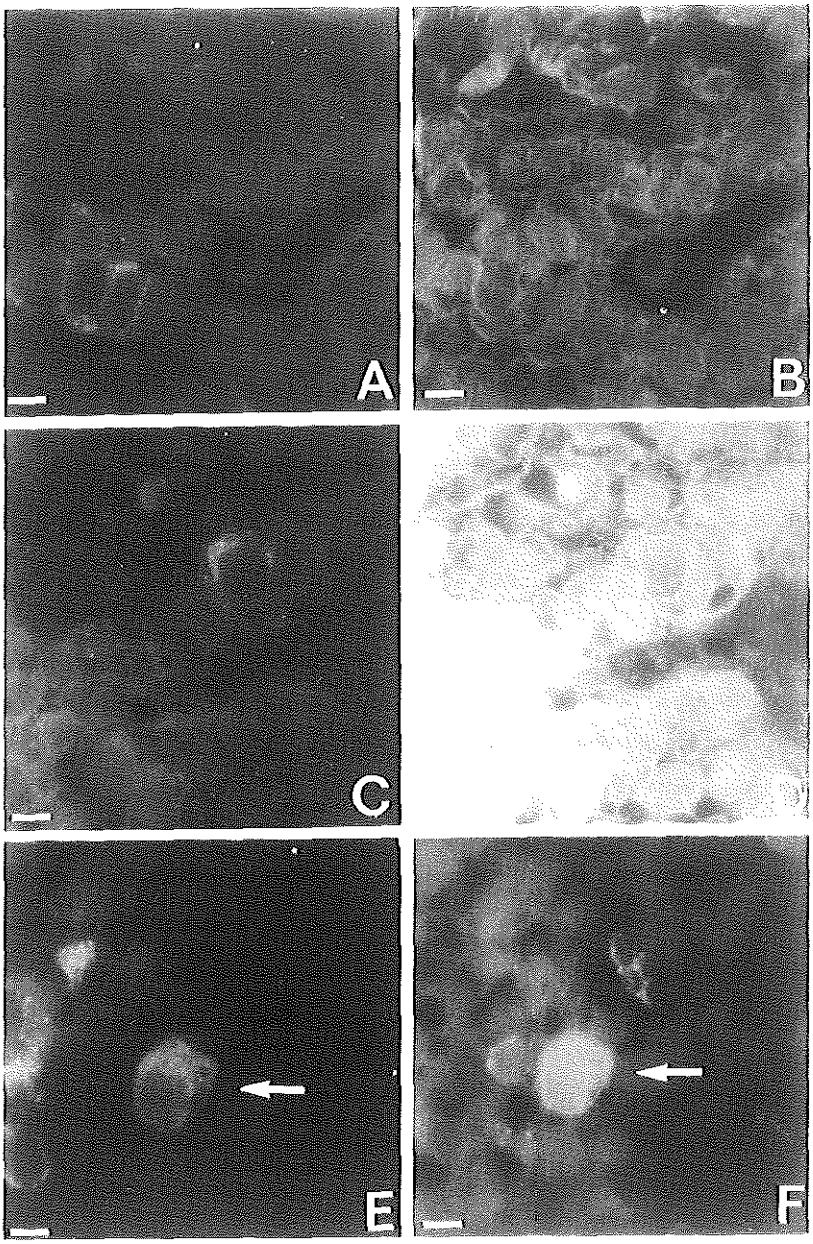


Fig. 1. Sections of a midgestational pancreas (A-F) stained for insulin (A,C,E) and glucagon (F). Insulin-positive cells can be present as endocrine cell clusters (A) or as single cells (C). They are often located in the proximity of ducts, which can be identified in the corresponding UV-filter exposures (B,D). Double labeling with insulin (E) and glucagon (F) shows a cell that displays both insulin and glucagon reactivity (arrow). Bar represents 5 μm in A to D, 5μ in E, and 6 μm in F.

Table 1. Double hormone containing cells in the population of endocrine cells in the human fetal pancreas between 12 and 18 weeks of gestational age.

hormone combination	number of pancreases with double+ cells	percentage of cells co-expressing hormones		
		ins+	glu+	sms+
ins/glu	11/15	2.5	8.6	-
ins/sms	9/14	3.9	-	8.3
glu/sms	4/15	-	5.1	1.6

ins = insulin; glu = glucagon; sms = somatostatin

Electron microscopy. Four fetal pancreatic fragments and six newborn pancreatic fragments were processed for immunoelectron microscopy. In the midgestational fetal pancreata three different endocrine cell types (alpha, beta and delta cells) could be distinguished by the presence of ultrastructurally typical secretory granules. Fragments of the pancreatic tail were taken to obtain a relatively high proportion of endocrine cells, in which pancreatic polypeptide-containing cells were not studied because of their low frequency in this area. The ultrastructure of the different granule types was identical to that in adult endocrine pancreatic cells and was confirmed by the presence of the corresponding immunoreactivity (Like and Orci, 1972; Dudek and Boyne 1986). Alpha cell granules were black, electron dense, sometimes with a dark grey outer rim; beta cell granules had crystallized black cores surrounded by a white halo or were uniformly black to grey; delta cell granules were the largest in size, relatively electron lucent, and light grey.

In the fetal pancreata we were able to identify cells containing two or three different granule types within the same cell (Figs. 2A-C). These morphological findings could be confirmed by immunoreactivity over the secretory granules, each granule labelled by a single hormone antibody. We found cells coexpressing glucagon and insulin in the corresponding granule types, and cells coexpressing glucagon and somatostatin. In addition cells containing all three hormones

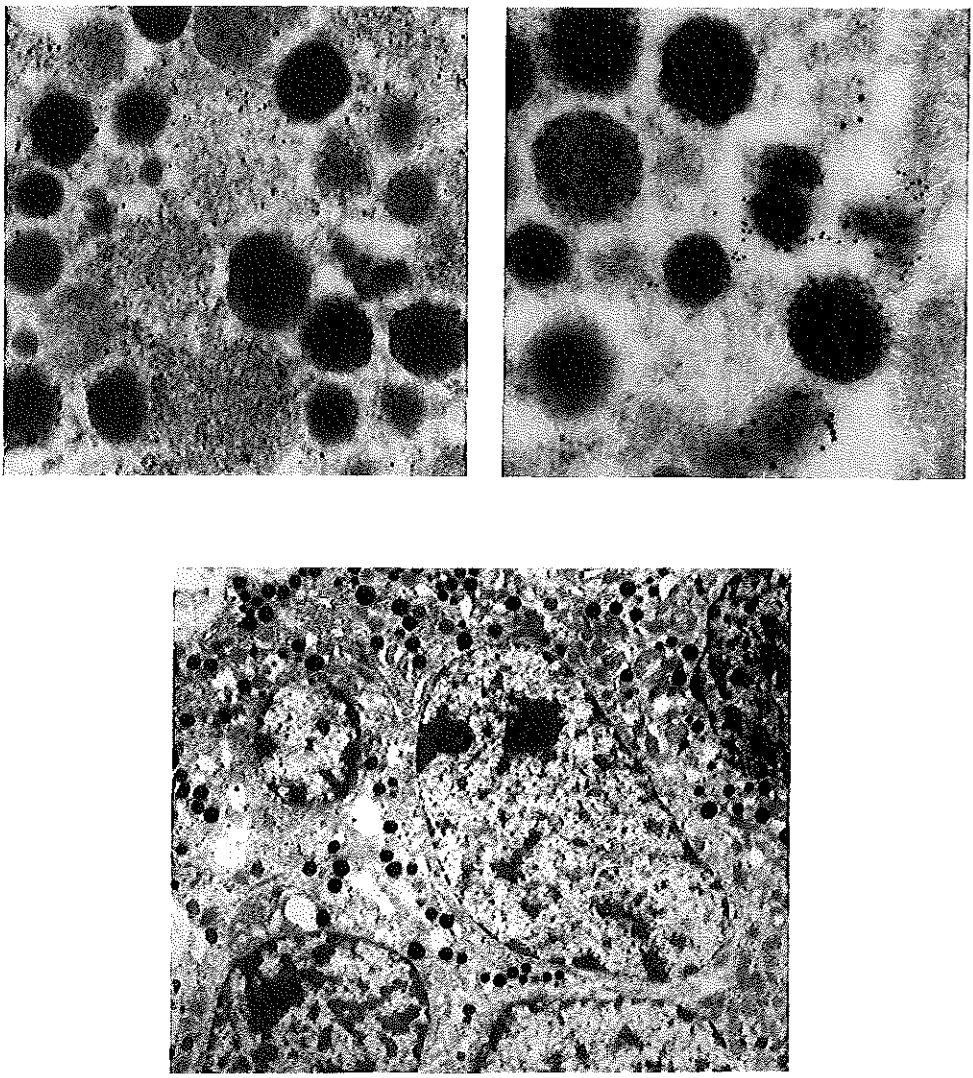


Fig. 2. Electron micrograph showing endocrine cells of 15 weeks gestation. (A) Section stained for glucagon and somatostatin. (B) Section stained for insulin and somatostatin. (C) An overview of a cluster of endocrine cells, some of which contain more than one hormone. The use of Lowicryl embedding medium for immunoelectron microscopy results in a negative contrasting of the membranes, which are therefore not visible in these pictures. (A) Granules with alpha and delta morphology present in the same cell, containing glucagon (large, 15-nm gold particles) or somatostatin (small, 10-nm gold particles), respectively. Arrows show an area with cytoplasmic somatostatin immunoreactivity. Bar represents 150 nm. (B) Immunoreactivity to insulin (small, 10-nm gold particles) and to somatostatin (large, 15-nm gold particles) can be found in one cell in granules with beta and delta morphology. Furthermore, unlabeled granules of the alpha type can be seen (arrow). Bar represents 110 nm. (C) Arrows and arrowheads indicate glucagon- and somatostatin-containing granules respectively. They can be distinguished morphologically. At this magnification immunoreactivity cannot be seen. Bar represents 680 nm.

in three distinct granule types were observed. A small number of cells contained granules with alpha cell morphology and both glucagon and insulin immunoreactivity.

Reactivity over background levels was also found outside secretory granules in the endoplasmic reticulum and in the cytoplasm for insulin, glucagon, and somatostatin (figure 2a). Nuclei and mitochondria displayed minimal background staining, as did nonendocrine cells. In control experiments, omitting primary antibodies, only occasional gold particles were found over all cell types and subcellular organelles. Cross-reactivity of the primary antibodies was found to be absent.

Newborn pancreatic fragments presented with poor morphological preservation. This was probably the result of the interval between death of the infant and start of autopsy/fixation. Consequently, these fragments could not be used for ultrastructural studies.

Assessment of proliferating cells in the midgestational human fetal pancreas and endocrine pancreatic cells.

In pancreatic fragments from 20 human fetal pancreata (12-18 weeks) processed for BrdU detection at the light microscopic level, a similar distribution pattern for insulin-, glucagon-, and somatostatin-positive cells was observed as in sections processed by routine immunohistochemistry. The frequency of BrdU positive nuclei, occurring in 0.9% to 6.4% of all cells, was negatively correlated with the age of the tissue fragments (r_x = -0.79, $p < 0.001$). BrdU-positive cells might be found close to ductal structures as well as close to endocrine hormone-containing cells, but we never observed a BrdU-positive cell that contained insulin, glucagon, or somatostatin (Figs. 3A-C).

Human fetal single-cell preparations ($n = 18$) confirmed these findings. There was a negative correlation between age of the pancreatic specimen and the percentage of BrdU-positive cells (r_x = -0.643, $p = 0.05$). No BrdU-positive cells could be found to contain insulin, glucagon, or somatostatin. The percentages of the hormone-containing cell types in the cytospins prepared for BrdU immunocytochemistry were not significantly different from non-BrdU cytospins ($p > 0.05$). At the electron microscopical level we found that 2.8% to 5.8% of the cells had incorporated BrdU (12 grids from six pancreata), evidenced by heavy labeling over the nucleus. Many hormone-containing cells were identified, either by the specific ultrastructural granulation, or by specific

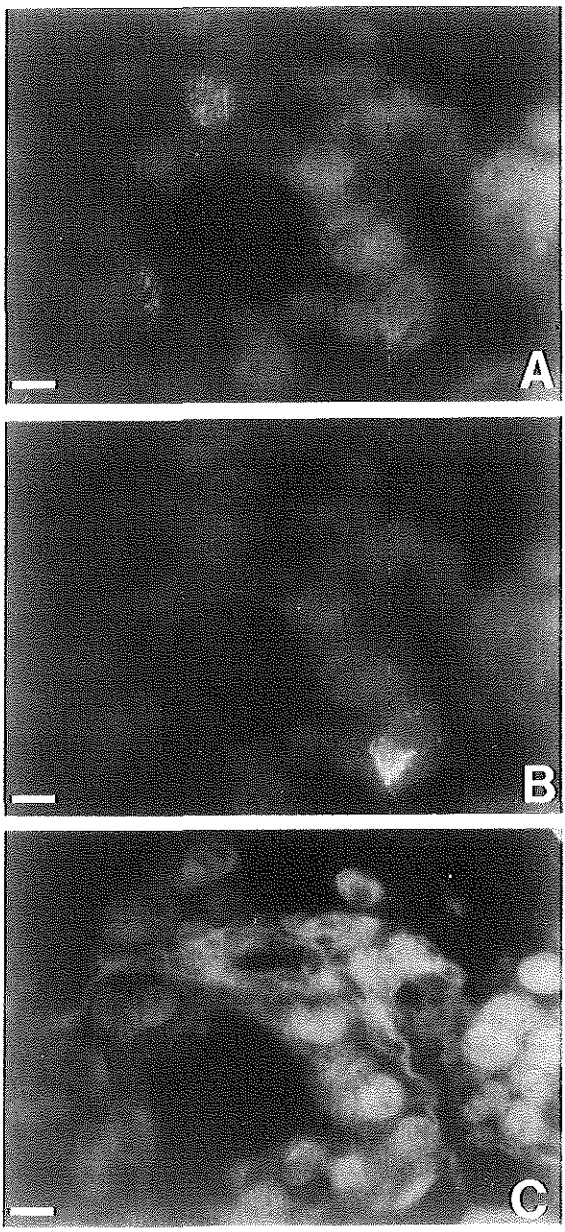


Fig. 3. Section of a 15-week pancreas stained for (A) bromodeoxyuridine and (B) insulin. Cells coexpressing BrdU and insulin could not be found. (C) A corresponding UV filter exposure, allowing the identification of pancreatic structures. Bars represents 10 μm.

immunolabelling against insulin, glucagon, or somatostatin. However, none of the BrdU-positive cells contained secretory granules nor cytoplasmic immunoreactivity to insulin, glucagon, or somatostatin.

DISCUSSION

The midgestational human fetal pancreas contains endocrine cells expressing multiple hormones simultaneously.

This study shows the presence of multiple-hormone-containing cells in the developing human pancreas between 12 and 18 weeks of gestation, but not in fetal/newborn pancreata between 22 and 40 weeks. The onset of hormone expression at the protein level in the human fetalpancreas has been demonstrated at week 7 for glucagon (Assan et al., 1973), and at week 8-10 for insulin and somatostatin (Stefan et al., 1983). Pancreatic polypeptide expression has also been found at week 8-10, but was not evaluated electron microscopically in this study. Alpert et al. (1988) described features of the ontogeny of pancreatic hormone expression in transgenic mice and showed the existence of multiple-hormone-containing cells. Based on these findings, they proposed a developmental scheme, where glucagon is the first hormone to be expressed. These cells subsequently differentiated, through multiple-hormone-expressing stages, into mature endocrine hormone cells that contained only one type of hormone. In our study, assessing three hormones, we found all three possible hormone combinations at the light microscopic level. At the electron microscopic level, however, we found glucagon expression at the protein level in all cells that expressed more than one hormone. This is in agreement with the developmental scheme of Alpert et al. (1988) where glucagon expression occurs as the first step in the differentiation of endocrine pancreatic cells. The multiple-hormone-expressing cells might then be the next stage before cells are committed to the production of a single hormone.

Since in pancreata between 22 and 40 weeks of gestation and in adult human pancreata only endocrine cells expressing a single hormone were found, the differentiation process of endocrine pancreatic cells may be limited to the period at the end of the first and the beginning of the second trimester of pregnancy. However, we were not able to confirm the absence of multiple-hormone-containing cells electron microscopically in premature newborn pancreata.

The electron microscopic data supported the light microscopic data on multiple hormone expression. Immunoreactivity for each hormone (insulin, glucagon, or somatostatin) was only found in granules with a corresponding morphology. The finding that in multiple-hormone-containing cells, two or three types of granules are morphologically and immunologically evident, shows that different hormones are produced and stored in parallel. Regulatory functions to produce and process only one hormone are apparently not yet functional at this stage of development. Some immunoreactivity over background levels for insulin, glucagon, and somatostatin was present over the cytoplasm and endoplasmic reticulum. The cytoplasmic labeling may result from synthesized hormone that has escaped packaging.

Midgestational human fetal pancreatic cells expressing multiple hormones do not proliferate under basal conditions.

Our data showed that no bromodeoxyuridine was incorporated in hormone-containing cells after a 1-hr culture period. Neither was such incorporation found after 48 hr of labeling or after 48 hr of follow-up of BrdU-labeled cell cultures (data not shown). In addition, it would not be possible to distinguish a cycling (BrdU-incorporating), hormone-containing cell from a cycling cell that incorporated BrdU, left the cell cycle, and started to differentiate and produce hormone. The method of BrdU incorporation has a slightly lower sensitivity than ^{3}H-thymidine incorporation. Furthermore, the ischemia time, although only 15-25 minutes, may interfere with the detection of proliferating hormonal cells. However, numerous BrdU-positive cells could be found in tissue sections. Also, the percentage of BrdU-positive cells is comparable in tissue sections and single-cell preparations. In one study (Nielsen et al., 1989) 0.9% of a population of newborn rat beta cells incorporated BrdU under nonstimulating conditions. We should have been able to pick up such a low frequency in our study since each paraffin section contained 100-500 beta cells. Swenne (1982) estimated the S-phase and cell cycle time of fetal rat beta cells to be 6.4 and 14.9 hr, respectively, and suggested the proliferative compartment of beta cells to be 10%. If these data are comparable to the human situation, our labeling periods would allow detection of almost 50% of actively cycling cells, being 5-25 BrdU-positive beta cells in our sections. If the cell cycle of fetal endocrine pancreatic cells was longer, e.g. 50-60 hours, 10% of the cycling cells could still be detected, i.e. 1-5 BrdU-positive beta cells per section. However, in this study we could not identify (multiple) hormone-containing cells that

showed proliferation by incorporating BrdU.

Earlier studies (Nielsen et al., 1989; Popiela and Moore, 1989; Popiela et al., 1988; Davidson et al., 1989; Swenne, 1982; Swenne, 1983; Swenne et al., 1980, 1987, 1988) suggested that rodent endocrine hormone-containing cells (both fetal and adult) do proliferate, but only under stimulating conditions.

The absence of proliferation in endocrine hormone-containing cells and thus in multiple-hormone-containing cells suggests that once hormone production has started, cells are committed and stop proliferating. This does not exclude that endocrine cells might be induced to proliferate in vitro by the addition of growth factors. However, pancreatectomy studies in adult mice indicated that newly formed endocrine cells may originate from precursors in the ductal epithelium (Smith et al., 1991). The present finding of BrdU-positive cells in or adjacent to ductal structures would be in keeping with this.

We conclude that multiple-hormone-containing cells are present in the midgestional human fetal pancreas, but most likely not in premature newborn pancreata between 22 and 40 weeks of gestational age. Unfortunately, procurement of these older pancreata is hampered due to longer warm ischemia times. The absence of BrdU incorporation supports the idea that multiple-hormone-containing cells may be an intermediate stage toward full commitment to single hormone production and secretion. Thus, endocrine pancreatic cells in the human fetal pancreas would arise from precursor cells that do not express hormones.

ACKNOWLEDGEMENTS

The authors thank the Division of Neonatology of the Sophia Childrens Hospital for their help and assistance in obtaining the pancreatic tissues and Dr. J. den Hollander of the Department of Pathology for the preparation of the newborn specimens. They also thank Mr. M. Kuit for excellent photograpy. R.R. de K. and G.K. are supported by a grant from the Stichting Diabetes Fonds Nederland, The Netherlands. H.J.A. is supported by a grant from the Ter Meulen Foundation of the Dutch Royal Academy of Sciences, the A. Renold Fellowship of the European Association for the Study of Diabetes. M.R. is supported by the Short Term Research Training Program, University of Maryland/Erasmus University Rotterdam Exchange Program.

REFERENCES

ALPERT, S., HANAHAN, D., and TEITELMAN, G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell **53**, 295-308

ASSAN, R., and BOILLOT, J. (1973). Pancreatic glucagon and glucagon-like material in tissues and plasma from human fetuses 6-26 weeks old. Pathol Biol **21**, 149-155

DAVIDSON, P.M., CAMPBELL, I.L., OXBROW, L., HUTSON, J.M., and HARRISON, L.C. (1989). Pancreatic beta cell proliferation in rabbits demonstrated by bromodeoxyuridine labeling. Pancreas **4**, 594-600

DUDEK, R.W., and BOYNE, A.F. (1986). An excursion through the ultrastructural world of quick-frozen pancreatic islets. Am J Anat **175**, 217-243

LARSSON, L.I. (1978). Endocrine pancreatic tumors. Hum Pathol **9**, 401-416

LIKE, A.A., and ORCI, L. (1972). Embryogenesis of the human pancreatic islets: a light and electron microscopic study. Diabetes **21 (suppl.2)**, 511

NIELSEN, J.H., LINDE, S., WEILINDER, B.S., BILLESTRUP, N., and MADSEN, O.D. (1989). Growth hormone is a growth factor for the differentiated pancreatic beta cell. Mol Endocrinol **13**, 165-173

POPIELA, H., TOMITA, T., and MOORE, W. (1988). Clonal growth of immunocytochemically identified islet beta and alpha cells in culture. Pancreas **3**, 413-420

POPIELA, H., and MOORE, W. (1989). Proliferation of immunocytochemically identified islet beta cells in culture: effect of growth factors, hormones and nutrients. Pancreas **4**, 244-248

ROTH, J., BENDAYAN, M., CARLEMALM, E., VILLIGER, W., and GARAVITO, M. (1981). Enhancement of structural preservation and immunocytochemical structures in low temperature embedded pancreatic tissue. J Histochem Cytochem **29**, 663-671

SMITH, F.E., ROSEN, K.M., VILLA-KOMAROFF L., WEIR, G., and BONNER-WEIR, S. (1991). Enhanced insulin-like growth factor I gene expression in regenerating rat pancreas. Proc Natl Acad Sci **88**, 6152-6156

STEFAN, Y., GRASSO, S., PERRELET, A., and ORCI, L. (1983). A quantitative immunofluorescent study of the endocrine cell populations in the developing human pancreas. Diabetes **32**, 293-301

SWENNE I., BONE, A.J., HOWELL, S.L., and HELLERSTROM, C. (1980). Effects of glucose and amino acids on the biosynthesis of DNA and insulin in fetal rat islets maintained in tissue culture. Diabetes **29**, 686-692

SWENNE, I. (1982). The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. Diabetes **31**, 754-760

SWENNE, I. (1983). Effects of aging on the regenerative capacity of the pancreatic B-cell of the rat. Diabetes **32**, 14-19

SWENNE, I., HILL, D.J., STRAIN, A.J., and MILNER, R.D.G. (1987). Growth hormone regulation of somatomedin C/insulin-like growth factor I production and DNA replication in fetal rat islets in tissue culture. Diabetes **36**, 288-294

SWENNE, I., HELDIN, C.H., HILL, D.J., and HELLERSTROM, C. (1988). Effects of platelet-derived growth factor and somatomedin-C/insulin-like growth factor I on the deoxyribonucleic acid replication of fetal rat islets of Langerhans in tissue culture. Endocrinology **122**, 214-218

THIRY, M., and DOMBROWICZ, D. (1988). Anti-bromodeoxyuridine monoclonal antibody: an alternative tool for the identification of replicated DNA at the electron microscope level. Biol Cell **62**, 99-102

Neuroendocrine Markers N1 and HNK-1 Indicate Potential Endocrine Precursor Cells in the Midgestational Human Fetal Pancreas.

Ronald R. De Krijger, Henk-Jan Aanstoot, Greetje Kranenburg, Michael Reinhard, and G. Jan Bruining

Department of Paediatrics, Erasmus University and University Hospital/Sophia Children's Hospital, Rotterdam, The Netherlands

Submitted

SUMMARY

Two monoclonal antibodies, N1 and HNK-1, were used to identify potential endocrine precursor cells and to study their expression in hormone containing islet cells in the human fetal pancreas. Double label immunohistochemical experiments were performed using tissue sections and single cell preparations of human fetal pancreata of 12 to 18 weeks gestation and tissue sections of human adult control pancreata. In these experiments, neuroendocrine markers (N1 and HNK-1) were combined with insulin, glucagon, and somatostatin. N1 has been detected before on fetal and adult α, ß, and δ cells. To identify potential precursor cells, cell suspensions were incubated with 5-bromo-2-deoxyuridine (BrdU), a marker of cell proliferation. Both N1 and HNK-1 were expressed on (BrdU-positive) proliferating cells. HNK-1-positive but not N1-positive proliferating cells were present until 15 weeks gestation. HNK-1 was exclusively present on islet δ cells throughout development. These data suggest that two populations of potential endocrine precursor cells exist: N1/BrdU cells, giving rise to at least islet α and ß cells, and N1/HNK-1/BrdU cells, giving rise to islet δ cells.

In conclusion, two neuroendocrine markers, N1 and HNK-1, identify two lineages of potential endocrine precursor cells and suggest phenotypic differentiation of δ cells before hormone expression starts.

INTRODUCTION

The pancreas contains at least four endocrine cell types: α, ß, δ, and PP-cells, producing and secreting glucagon (GLU), insulin (INS), somatostatin (SOM), and pancreatic polypeptide (PP). Endocrine islet cells share many features with neuronal cells, but are considered to be of endodermal origin [12,19]. Hormone expressing cells can be found from 6-8 weeks gestation onwards in human embryos [13, 23]. Studies in the mouse indicate that islet hormone mRNA can be detected at day E9 (20 somite stage) of embryonic development [8]. It is unclear whether these cells represent precursor cells or early differentiated cells without any proliferative capacity. In fully differentiated islets, a single hormone is produced per islet cell type. During islet development, multiple hormone expressing cells are present in mice [1,24] and humans [5,14]. Multiple hormone containing cells may represent an intermediate phase between proliferating cells and

full islet cell differentiation. Previous experiments have shown that the proliferative capacity under basal conditions, measured by BrdU incorporation, in multiple and single hormone containing islet cells is either below the detection limit of this assay or absent [5,7]. Thus, hormone expression at the protein level cannot help in the identification of endocrine precursor cells.

We therefore searched for other potential markers of early human islet cell development, aiming to detect endocrine precursor cells and analyzing the expression patterns of such markers in human fetal islet cells. Monoclonal antibodies N1 (directed against an epitope present on human fetal islet cells), and HNK-1 (directed against a glycolipid/glycoprotein moiety present on several cell types, including neural crest cells [10]), have been detected before in islet cells [6,22]. Expression of these markers in BrdU-positive proliferating cells and coexpression with hormones was studied in tissue sections and single cell preparations of human fetal pancreata and adult control pancreata.

Recently, early expression of PP was suggested in mouse endocrine cells [9]. Subsequent studies indicated that this reactivity in fact may be caused by neuropeptide Y or by peptide YY [25,26]. In our study, PP was not analyzed because of the very low frequency of PP cells in the developmental period studied.

MATERIALS AND METHODS

Tissues and cells. Human fetal pancreata from 12-18 weeks gestational age (n=25) were obtained from abortions, after signed informed consent. Human adult pancreata were obtained from multiorgan donors and used as controls (n=2). The study was approved by the local ethical committee. Gestational age was assessed by echographic measurement of the biparietal diameter of the head. Abortions were induced mechanically resulting in warm ischemia times of 15-25 minutes, and an improved viability compared to prostaglandin induced abortion [16]. For immunohistochemistry pancreata were snap frozen in liquid nitrogen (n=25). Pancreatic single cell suspensions were prepared as described [6].

BrdU incorporation. Single cell preparations (after 18 hours of culture) were cultured for 1 hour in the presence of 10 μM 5-bromo-2-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO, USA) in RPMI 1640 (Gibco, Paisley, UK), supplemented with 10% pooled human serum (Bloodbank, Academic Hospital Leiden, The Netherlands), as described before [5].

Immunocytochemistry. Cell surface labelling and cytospin preparation, after cell culture of total pancreatic cell suspensions for 18 hours, was performed as described previously [6]. For BrdU immunocytochemistry, cytospins were treated, after fixation, with 2 N HCl for 30 minutes at 37°C and neutralized with a borate buffer (pH 8.5). Subsequently, they were incubated with primary and secondary antibodies. Cytospins were incubated for 30 minutes at room temperature with one or two primary antibodies (single or double staining), washed in phosphate buffered saline (PBS), pH 7.4, and incubated for another 30 minutes with one or two secondary antibodies conjugated to fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC). After a final wash in PBS, sections were mounted and stored at -20°C until analysis.
Primary antibodies are specified in table 1. Secondary antibodies were goat-anti-mouse-FITC and -TRITC (1:150 and 1:60), goat-anti-mouse IgG1-TRITC (1:50), goat-anti-mouse IgG2-FITC and -TRITC (both 1:25), and goat-anti-mouse IgM-FITC and -TRITC (both 1:25), goat-anti-rabbit-FITC and -TRITC (1:200 and 1:200) (Nordic Immunological Laboratories, Tilburg, The Netherlands), goat-anti-guinea-pig-FITC and -TRITC (1:60 and 1:300) (Southern Biotechnology, Birmingham, UK). For double label experiments primary antibodies from different species were used. If this was not possible, Ig subclass specific second antibodies were applied. In control cytospins the primary antisera were replaced by PBS/bovine serum albumin. Background staining was minimal, spectral overlap was not observed. To exclude staining differences due to fixation techniques all antibodies were tested in 4% paraformaldehyde fixed and acetone fixed tissues. No differences in staining were found. For quantification of results, 250-500 cells were counted.

Immunohistochemistry. Cryostat sections were fixed in acetone or 4% paraformaldehyde for 15 minutes. and washed in PBS. Primary and secondary antibodies were applied as described above. Control preparations were similar as for cytospins. In some cases the indirect peroxidase technique was used instead of the indirect fluorescence technique. No differences in staining were found for either of the fixation techniques.

Staining of viable cells. Fluorescein diacetate (FDA, SIGMA) was used for the determination of cell viability [21]. A stock solution of 1 mg/ml FDA in acetone was made and stored at -20°C. Cell suspensions were incubated with a 1:10,000 dilution of this stock for 5 minutes. at 37°C, followed by centrifugation and analysis on a FACScan (Becton Dickinson).

Table 1. Primary antibodies used in this study.

ANTIGEN	SOURCE	DILUTION	SUPPLIER	REMARKS
A. Polyclonal antibodies				
insulin	guinea-pig	1:1500	Dako	
glucagon	rabbit	1:750	Dako	
somatostatin	rabbit	1:800	Dako	
pancreatic polypeptide	rabbit	1:1000	Dako	
neurofilament	rabbit	1:10	Sanbio	clone 2F11 reacting to 70 and 200kD fragments
B. Monoclonal antibodies				
insulin	mouse	1:50	Novo/Nordisk	HUI-018 proinsulin crossreaction 100%
proinsulin	mouse	1:10	Dr. Madsen	GS4G9
glucagon	mouse	1:30	Novo/Nordisk	GLU-001 glicentin crossreaction 100%
somatostatin	mouse	1:1000	Novo/Nordisk	SOM-018
N1	mouse	1:500		ascites
HNK-1	mouse	1:10	ATCC	ascites
BrdU	mouse	1:100	BD	

ATCC = American Tissue Culture Collection; BD = Becton Dickinson

Statistics. The association between gestational age of the pancreata and the percentage of marker-positive cells was evaluated according to Spearman's rank correlation test. All data are presented as mean ± SD.

RESULTS

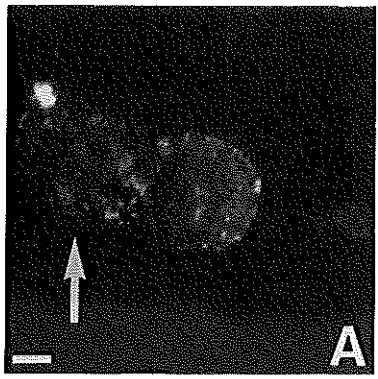
Detection of potential endocrine precursor cells using neuroendocrine markers.

BrdU incorporation was assessed in total human fetal single cell preparations. Processing cytospins for BrdU detection did not affect the percentage of hormone and marker positive cells, compared to cytospins processed for detection of hormones and markers only [5]. An inverse relationship between gestational age (range: 12 to 18 weeks gestation) and the percentage of BrdU-positive cells was found (range: 0.4-6.9%, n=21, r_s= -0.53, $p<0.01$). BrdU was never present in hormone containing cells. In 4 ± 1% of N1-positive cells BrdU incorporation was observed (n=7). The age of the specimens did not have an effect on the percentage of N1/BrdU double labeled cells. In 5 pancreata of 12-14 weeks gestation 8 ± 3% of HNK-1 positive cells showed BrdU incorporation (Fig 1A,B). None of 5 pancreata of more than 14 weeks gestation contained HNK-1/BrdU double labeled cells.

Thus, N1 positive proliferating cells were observed at all gestational ages. Until 15 weeks gestation, but not thereafter, proliferating cells expressing HNK-1 on their surface were also present. Neither of these cells did coexpress pancreatic hormones.

Expression of neuroendocrine markers in hormone-containing islet cells.

Cells containing INS, GLU, SOM, and PP were present in all fetal and adult pancreata studied. The low frequency of PP-positive cells in human fetal pancreata (confirmed to be 0.1 ± 0.1% (n=10) in single cell preparations) did not allow further analysis. The other three hormones were seen in single cells, small endocrine cell clusters, and larger islet-like cell clusters. Such islet-like cell clusters showed the typical cell distribution of islets, with a ß cell core and a peripheral mantle of α and δ cells.



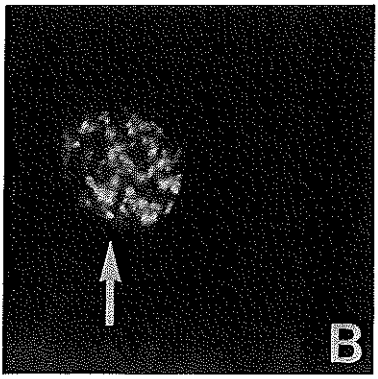


Figure 1. Immunofluorescent double staining with HNK-1 (A) and BrdU (B) of a cytospin preparation of a 15 week human fetal pancreas. Cells were first incubated with bromodeoxyuridine for 1 hour in culture. HNK-1 was identified as membrane fluorescence, BrdU by nuclear fluorescence in the same cell (arrow). The indirect fluorescence technique was used. Bar represents 5 μm.

N1 reactivity was similar as described [6]. The majority of INS, GLU, and SOM containing cells in tissue sections and single cell preparations reacted with N1. N1-positive cells constituted 15.6 $\pm$ 5.7% of all fetal pancreatic cells, half of which contain either INS, GLU, or SOM. The majority of non-hormone containing N1-positive cells contained another neuroendocrine marker. There was a significant increase in the percentage of N1 positive cells with gestational age ($n=29$, $r_s=0.57$, $p<0.01$) [6].

HNK-1-positive cells were located around the insulin-positive cell core of small endocrine cell clusters and larger islet-like cell clusters (Fig. 2A,B) in fetal pancreata of all gestational ages. The majority of these HNK-1 cells were SOM positive in double label experiments. No HNK-1 reactivity was seen in INS or GLU cells. In adult islets HNK-1 positive cells were generally located around the central ß cell core of the islets and were always SOM positive. However, they were less abundant than HNK-1/SOM cells in fetal islets (Fig. 2C,D). Other HNK-1-positive, SOM negative cells in the human fetal pancreas were found in bundle-like structures. These cells were costained by antibodies to neurofilament (70 kD and 200 kD), suggesting that they were intrapancreatic nerve fibers. No neurofilament reactivity was seen within fetal or adult islet cells.

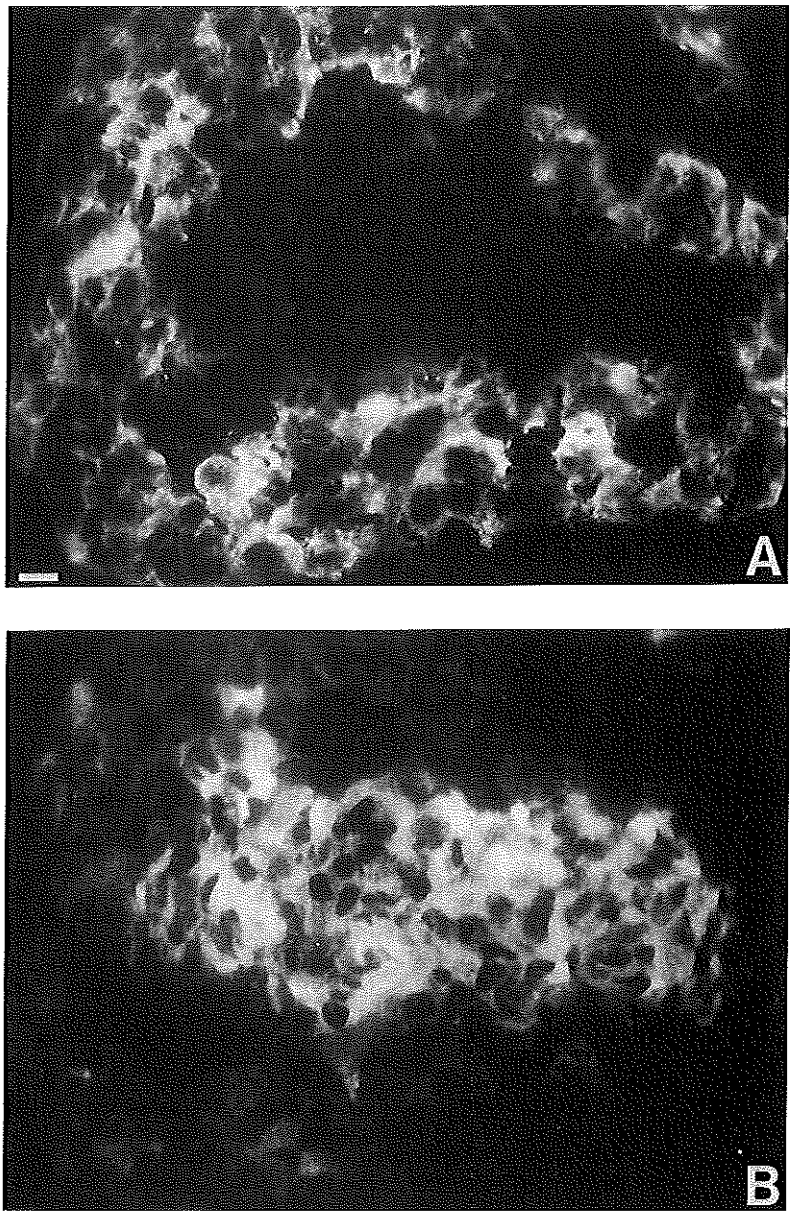


Figure 2. Double labeling of a pancreatic tissue section of 15 weeks gestation with HNK-1 (A) and proinsulin (B). No cells coexpressing HNK-1 and proinsulin are seen. HNK-1-positive cells are located peripheral to the ß cell core. Staining was performed with the indirect fluorescence technique (A and B). Bar represents 10 μm.

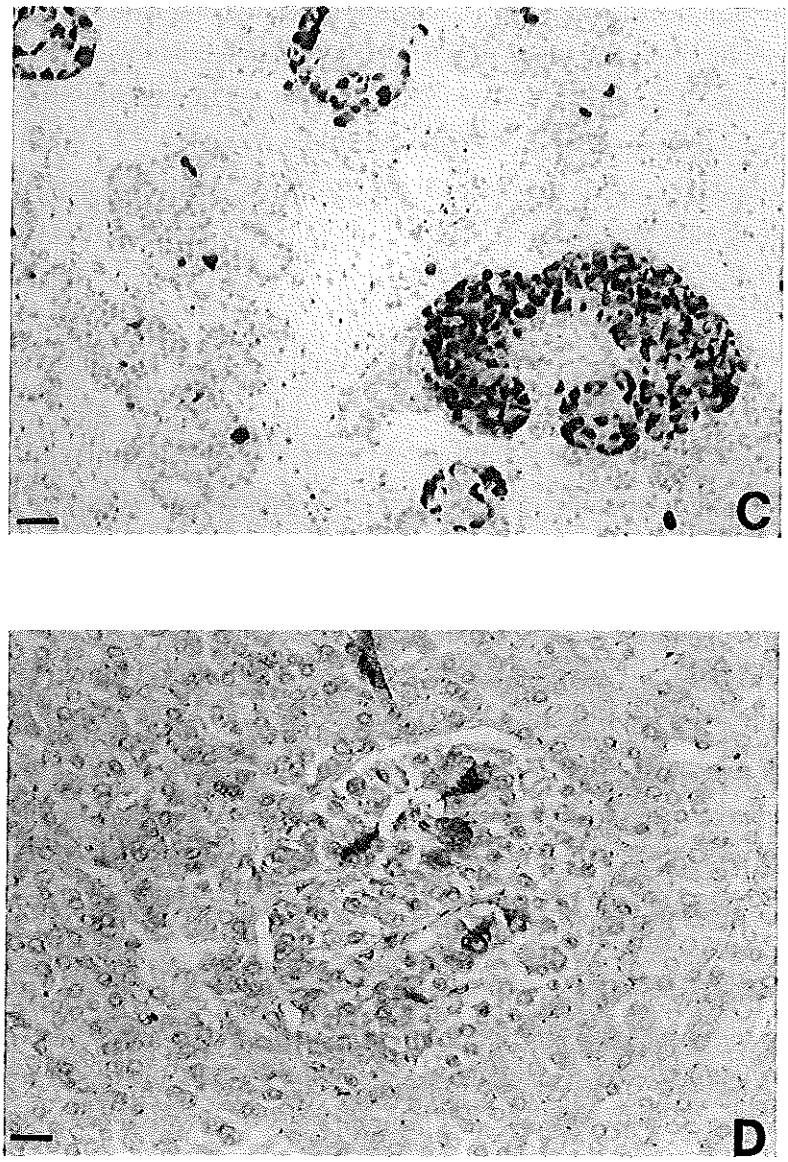


Figure 2. Tissue sections of a 14 week old fetal pancreas (C) and of an adult pancreas (D), respectively, stained with an antibody to somatostatin (SOM). Single cells and endocrine cell clusters with an abundant peripheral ring of SOM cells can be observed in C. In the adult islet in D few SOM cells are found. Staining was performed with the indirect peroxidase technique (C and D). Bar represents 30 μm in C, and 20 μm in D.

In human fetal single cell suspensions 11.2 ± 5.7% of the cells were HNK-1 positive. There was a significant decrease in the percentage of HNK-1 reactive cells with increasing age of the pancreata ($n=15$, $r_s=-0.511$, $p<0.05$). Of the δ cells 81 ± 12% presented HNK-1 surface reactivity, whereas none of the α or ß cells were stained.

Coexpression of N1 and HNK-1 was shown: 9.4 ± 7.8% was also HNK-1 positive, and 9.0 ± 6.6% of the HNK-1 cells was N1 positive.

DISCUSSION

Studies on growth and development of islet cells are important for the understanding and treatment of diseases where ß cells are underrepresented, such as type-I diabetes mellitus, or overrepresented, such as nesidioblastosis. Adult endocrine pancreatic cells have little proliferative capacity under basal conditions, but may be stimulated to proliferation by partial pancreatectomy [2]. Few data exist about proliferation in human fetal islet cells. In human fetal hormone containing cells no BrdU uptake was detected in our previous studies, indicating a low proliferative capacity of such cells [5]. This is confirmed in the present study.

Thus, other markers are needed to detect proliferating endocrine precursor cells. Such markers should be present in proliferating cells and should continue to be expressed in hormone containing cells. In this study we analyzed two neuroendocrine markers, N1 and HNK-1, for their colocalization with BrdU. The use of BrdU as an indicator for in vitro islet cell proliferation has been validated before [3,15].

N1/BrdU cells can be found at all gestational ages. This indicates that human fetal islet cell development is a repetitive event. N1 is also expressed in adult islet cells as well as in cells that line the lumen of the human fetal stomach, duodenum, and bronchi. This N1 expression pattern supports an endodermal origin of islet cells. In the developing pancreas, as opposed to the adult pancreas, proliferating (BrdU-positive) N1-positive cells exist, which indicates that N1 expression precedes hormone expression. Coexpression of N1 and the endocrine hormones in the human fetal pancreas has been shown [6]. N1-positive cells without BrdU and without hormone expression costain with the neuroendocrine markers synaptophysin and chromogranin-A. These markers are not present in proliferating cells (data not shown). We conclude that, during human development, a pool of hormone-negative N1 expressing cells are able to proliferate

and represent an early endocrine precursor cell type, which gives rise to at least α, ß, and δ cells (Fig. 3). The finding of proliferating N1 reactive cells is compatible with a significant increase of N1 cells between 12 and 18 weeks gestation [6].

The absence of BrdU in hormone expressing cells contrasts with earlier in vivo and in vitro findings in the rat [2,15]. In human islet cells the high degree of specialization needed for hormone synthesis and secretion seems to be non-concomitant with proliferation.

Proliferating HNK-1 cells (HNK-1/BrdU) were exclusively found until 15 weeks gestation. Although triple labelling was not performed, it is conceivable that a pool of HNK-1/N1/BrdU cells exist. This is based on the presence of N1/HNK-1 coexpressing cells as well as the finding of N1 expression on δ cells, both in the fetal and adult pancreas. The lack of HNK-1/BrdU cells after week 14 indicates the existence of two subsets of precursor cells: N1/BrdU cells and N1/HNK-1/BrdU cells (Fig. 3). Possibly, HNK-1 expression is lost early and thus N1/BrdU cells may represent a later stage of development. The selective presence of HNK-1 in islet δ cells suggests that N1/HNK-1/BrdU cells are a separate lineage and HNK-1 expression would indicate an early segregation of the δ cell lineage. The amount of HNK-1 positive δ cells in islets of the fetal pancreas is far larger than in adult islets. This confirms previous studies [4,6,20] in which a higher relative amount of δ cells was found during development, compared with adult pancreas. The physiological role of this high proportion of δ cells in the fetal pancreas is poorly understood. We speculate that somatostatin expression is a prerequisite for islet growth and development. An inhibitory effect of somatostatin on insulin release and an inability of glucose stimulated insulin release have been described in human fetal islets [17,18]. HNK-1 expression may be important for the three dimensional architecture of islets, since this molecule is also involved in the organization of neural crest cells [11]. HNK-1 is an important neural crest cell marker. Some of the HNK-1 cells in the pancreas are located in nerve-like bundles and coexpress neurofilament. Since these cells do not express SOM (data not shown), it is unlikely that they are endocrine cells.

In conclusion, two monoclonal antibodies, N1 and HNK-1, identify proliferating cells in the human fetal pancreas. N1 is found in hormone containing islet cells, including INS, GLU and SOM. HNK-1 is exclusively expressed on SOM cells (Fig. 3). Further evidence of a relationship between proliferating N1 or HNK-1 cells and hormone expressing islet cells may come from culture experiments with human fetal pancreatic cell suspensions enriched for N1 positive cells by fluorescence activated cell sorting [6].

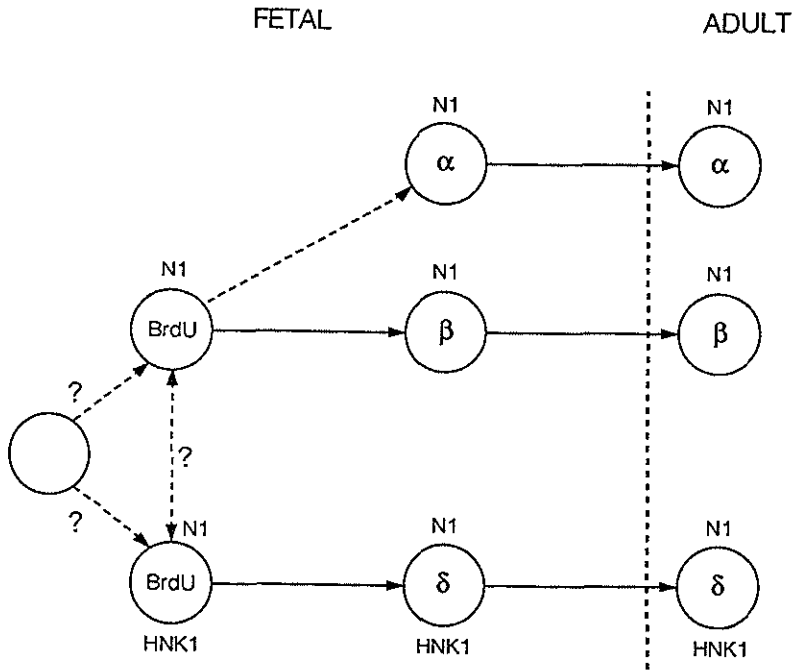


Figure 3. Proposed developmental scheme for human fetal islet cells. A pluripotent precursor cell gives rise to two lineages of proliferating precursor cells: N1/BrdU cells and N1/HNK-1/BrdU cells. N1/BrdU cells give rise to α and ß cells, N1/HNK-1/BrdU cells give rise to δ cells. Multiple hormone expression may occur in an intermediate stage which is not indicated in this scheme.

ACKNOWLEDGEMENTS

The authors thank Dr. O.D. Madsen (Hagedorn Research Laboratory, Gentofte, Denmark) for his generous gift of the proinsulin antibody. R.R. de K. and G.K. were supported by a grant from the "Diabetes Fonds Nederland", The Netherlands. H.J.A. was supported by a grant from the "Ter Meulen Foundation" of the Royal Dutch Academy of Sciences, the A. Renold Fellowship of the European Association for the Study of Diabetes, and Novo/Nordisk A/S, Denmark. M.R. was supported by the Short Term Research Training Program, University of Maryland/Erasmus University Rotterdam Exchange Program.

REFERENCES

1. Alpert S, Hanahan D, Teitelman G (1988) Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 53:295-308

2. Bonner-Weir S, Baxter LA, Schuppin GT, Smith FE (1993) A second pathway for regeneration of adult exocrine and endocrine pancreas. Diabetes 42:1715-1720

3. Brelje TC, Sorenson RL (1991) Role of prolactin versus growth hormone on islet ß-cell proliferation in vitro: implications for pregnancy. Endocrinology 128:45-57

4. Clark A, Grant AM (1983) Quantitative morphology of endocrine cells in human fetal pancreas. Diabetologia 25:31-35

5. De Krijger RR, Aanstoot HJ, Kranenburg G, Reinhard M, Visser WJ, Bruining GJ (1992a) The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev Biol 153:368-375

6. De Krijger RR, Aanstoot HJ, Kranenburg G, Verkerk A, Jongkind JF, Van Strik R, Lafferty K, Bruining GJ (1992b) Enrichment of beta cells from the human fetal pancreas by fluorescent activated cell sorting with a new monoclonal antibody. Diabetologia 35:436-443

7. De Vroede MA, In 't Veld PA, and Pipeleers DG (1990) Deoxyribonucleic acid synthesis in cultured adult rat pancreatic B cells. Endocrinology 127:1510-1516

8. Gittes GK, Rutter WJ (1992) Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci USA 89:1128-1132

9. Herrera PL, Huarte J, Sanvito F, Meda P, Orci L, Vassalli JD (1991) Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development 113:1257-1265

10. Jungalwala FB, Chou DKH, Suzuki Y, Maxwell GD (1992) Temporal expression of HNK-1-reactive sulfoglucuronyl glycolipid in cultured quail trunk neural crest cells: comparison with other developmentally regulated glycolipids. J Neurochem 58:1045-1051

11. Künemund V, Jungalwala FB, Fischer G, Chou DKH, Keilhauer G, Schachner M (1988) The L2/HNK-1 carbohydrate of neural cell adhesion molecules is involved in cell interactions. J Cell Biol 106:213-223

12. Le Douarin N (1978) The embryological origin of the endocrine cells associated with the digestive tract: experimental analysis based on the use of a stable cell marking technique. In: Bloom SR (ed) Gut Hormones, Churchill Livingstone, London, pp 49-56

13. Like AA, Orci L (1972) Embryogenesis of the human pancreatic islets: a light and electron microscopic study. Diabetes 21 (suppl.2):511-534

14. Lukinius A, Ericsson JLE, Grimelius L, Korsgren O (1992) Electron microscopic immunocytochemical study of the ontogeny of fetal human and porcine endocrine pancreas, with special reference to co-localization of the four major islet hormones. Dev Biol 153:376-390

15. Nielsen JH, Linde S, Welinder BS, Billestrup N, Madsen OD (1989) Growth hormone is a growth factor for the differentiated pancreatic ß cell. Mol Endocrinol 13:165-173

16. Otonkoski T, Knip M, Panula P, Andersson S, Wong I, Goldman H, Simell O (1988a) Morphology, yield and functional integrity of islet-like cell clusters in tissue culture of human fetal pancreas obtained after different means of abortion. Acta Endocrinologica 118:68-76

17. Otonkoski T (1988b) Insulin and glucagon secretory responses to arginine, glucagon, and theophylline during perifusion of human fetal islet-like cell clusters. J Clin Endocrinol Metab 67:734-740

18. Otonkoski T, Andersson S, Simell O (1993) Somatostatin regulation of ß-cell function in the normal human fetuses and in neonates with persistent hyperinsulinemic hypoglycemia. J Clin Endocrinol Metab 76:184-188

19. Pictet R, Rutter WJ (1972) Development of the embryonic endocrine pancreas. In: Steiner D, Freinkel N (eds) Handbook of Physiology, vol. 1. Williams and Wilkins, Baltimore, pp 25-66

20. Rahier J, Wallon J, Henquin JC (1981) Cell populations in the endocrine pancreas of human neonates and infants. Diabetologia 20:540-546

21. Rotman B, Papermaster BW (1966) Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA 55:134-141

22. Shioda Y, Nagura H, Tsutsumi Y, Shimamura K, Tamaoki N (1984) Distribution of Leu 7 (HNK-1) antigen in human digestive organs: an immunohistochemical study with monoclonal antibody. Histochem J 16:843-854

23. Stefan Y, Grasso S, Perrelet A, Orci L (1983) A quantitative immunofluorescent study of the endocrine cell populations in the developing human pancreas. Diabetes 32:293-301

24. Teitelman G, Alpert S, Hanahan D (1988) Proliferation, senescence, and neoplastic progression of ß cells in hyperplastic pancreatic islets. Cell 52:97-105

25. Teitelman G, Alpert S, Polak JM, Martinez A, Hanahan D (1993) Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 118:1031-1039

26. Upchurch BH, Aponte GW, Leiter AB (1994) Expression of peptide YY in all four islet cell types in the developing mouse pancreas suggests a common peptide YY-producing progenitor. Development 120:245-252

Enrichment of Beta cells from the human fetal pancreas by fluorescent activated cell sorting with a new monoclonal antibody.

R.R. de Krijger[1], H.J. Aanstoot[1], G. Kranenburg[1], A. Verkerk[2], J.F. Jongkind[2], R. van Strik[3], K.J. Lafferty[4], and G.J. Bruining[1]

Department of [1]Pediatrics, Erasmus University Rotterdam and University Hospital/Sophia Childrens Hospital, Department of [2]Cell Biology and [3]Epidemiology/Biostatics, Erasmus University Rotterdam, Rotterdam, The Netherlands, and [4]Barbara Davis Center, University of Colorado, Denver, Colorado, USA

Diabetologia 35:436-443 (1992)

SUMMARY

The aim of this study was to produce an antibody reactive to the surface of endocrine pancreatic cells and use this antibody for the purification of endocrine cells from the human fetal pancreas by fluorescence activated cell sorting. We describe such an antibody, called N1, reacting with the surface and cytoplasm of endocrine cells in the adult and fetal human pancreas (12 to 18 weeks gestational age). While unreactive to exocrine and mesenchymal cells, it was not specific for endocrine cells, as evidenced by its staining pattern in tissues other than pancreas. Almost 40% of the N1-positive pancreatic cells contained either insulin, glucagon or somatostatin. Conversely, more than 90% of each of the hormone-containing cells was N1 positive. An additional 40% of N1-positive cells, not containing other pancreatic hormones, was shown to contain islet amyloid polypeptide, synaptophysin, chromogranin, tyrosin hydroxylase or CA812. A two-step collagenase digestion protocol yielded $1.29 \pm 0.17 \times 10^5$ cells per mg pancreatic tissue. After Percoll gradient centrifugation, the suspension contained $15.6 \pm 5.7\%$ ($n=25$, mean $\pm$ SD) cells reactive with N1. By fluorescence activated cell sorting using the antibody N1, the single-cell suspension was enriched from $3.0 \pm 1.4\%$ to $16.2 \pm 4.8\%$ ($n=10$, $p<0.01$) Beta cells. Alpha and Delta cells were also enriched significantly by this procedure. The percentage of N1-positive cells increased from $17 \pm 4\%$ to $83 \pm 6\%$. This preparation enriched for endocrine cells allows future studies on possible endocrine precursor cells.

INTRODUCTION

Several antibodies have been reported to react with Beta cells or endocrine pancreatic cells [1-6]. Rat Beta cells or islets were usually the substrate when determining the specificity of these antibodies. Islets of Langerhans constitute 1-2% of the adult human pancreas. Their recovery is based on enzymatic digestion of the pancreatic glands [7]. Recovery of single endocrine pancreatic cells has been described by physicochemical separation procedures [8]. In the human fetal pancreas, a large proportion of the endocrine cells is located outside islets, complicating islet isolation procedures [9].

The aim of the present study was to produce antibodies, which after reacting to the surface of human fetal endocrine pancreatic cells, could be used for selective enrichment of these cells

by fluorescence activated cell sorting. Such enriched cell preparations could be an important tool in the study of differentiation and proliferation of endocrine pancreatic cells. We describe the production and staining pattern of an antibody, N1, reactive to the surface of Beta cells. Subsequently, a method for the production of single-cell suspensions from the human fetal pancreas is described. These were labelled with N1 and subjected to fluorescence activated cell sorting. We show a substantial enrichment of Beta cells, Alpha cells and Delta cells.

MATERIALS AND METHODS

Tissue pre-treatment for immunization. Human fetal and adult pancreata were obtained with permission of the local ethical committee. Abortions were performed mechanically, resulting in a warm ischaemia time of 10-20 min. The specimens were between 12 and 18 weeks gestational age. For immunization, fetal pancreata were cut into 1 mm^3 pieces and cultured for 14 days in serum-free medium HB104 (Hana Biologics, Berkeley, Calif., USA) supplemented with 20 mmol/l HEPES, 100 IU/ml penicillin, 100 U/ml neomycin, 100 mg/ml streptomycin, 1 mmol/l sodium pyruvate and 20 mmol/l L-glutamine. The tissue was cultured under 95% O_2 and 5% CO_2 at 37°C. Following culture, the tissue fragments were homogenized, centrifuged at 12,000x g for 5 min to remove nuclei and other particles, and stored at -20°C until use.

Immunization. C57Bl/6J and SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, Me., USA). NZB/N mice were obtained from National Institutes of Health (NIH, Bethesda, Md., USA). The mice were injected intraperitoneally three times with 500 μg (wet weight) of homogenized tissue in complete Freund's adjuvant, incomplete Freund's adjuvant and phosphate buffered saline (137 mmol/l NaCl, 8 mmol/l $Na_2HPO_4.2H_2O$, 2 mmol/l KH_2PO_4, 3 mmol/l KCl; PBS), pH 7.4, respectively. The mice received an intravenous boost of 50 μg homogenized tissue 3 days before spleen cell harvesting.

Production and screening of antibodies. Spleen cells of immunized animals were fused with P3X63-AG8.653 myeloma cells according to Galfre and Milstein [10] and cultured in hypoxanthine-aminopterin-thymidine (HAT) medium with syngeneic peritoneal macrophages as feeders. After culture at 37°C in 5% CO_2 for 14 days, hybridomas were screened by staining cryostat sections

of adult human pancreata with the indirect fluorescence technique mentioned below, using culture supernatants as the first antibody step. Positive clones were expanded, subcloned by limiting dilutions and re-tested on human fetal pancreatic sections.

Dispersion. Human fetal pancreata were minced with scissors to fragments of 1 mm^3. The pieces were centrifuged at 100x g for 1 min. A maximum of 0.4 ml tissue was resuspended per 4 ml Earle's balanced salt solution without Ca/Mg (EBSS; Gibco, Paisley, UK) containing collagenase type I (Sigma Chemical Co., St. Louis, Mo., USA, 1 mg/ml), and DNase (10 μg/ml, Sigma) and placed in a shaker bath (GFL, Burgwedel, FRG) at 37°C, for 10 min. The suspension was pipetted up and down for 1-2 min with a glass pipette and centrifuged at 100x g for 1 min. The pellet was re-incubated with a fresh collagenase solution for another 10 min and centrifuged again. All glassware was siliconized. The 100x g supernatants, containing single cells, were washed, passed through a 100 μm nylon mesh (van Wijk, Santpoort, The Netherlands), resuspended and counted in a Burker-Turk counting chamber. Cell numbers were expressed as 10^6/mg tissue.

Overnight culture. After dispersion cells were suspended in RPMI 1640 at a concentration of 1-2 x 10^6 cells/ml. The medium was supplemented with 2 mmol/l L-glutamine (Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml Fungizone (Gibco), 25 mmol/l HEPES (Merck, Darmstadt, FRG), 10% pooled, heat-inactivated human serum (Bloodbank, University Hospital Leiden, The Netherlands), 1.5 U/ml dispase I (Boehringer Mannheim, Mannheim, FRG) and 10 μg/ml DNase (Sigma). The enzymes were added to prevent cell aggregation or cells attaching to the bottom of the dish. The cells were cultured in a CO_2 incubator (Heraeus, Osterode, FRG) at 37°C and 5% CO_2 for 12-15 h.

Gradient centrifugation. An iso-osmotic Percoll solution (SIP) was prepared by mixing 90 parts of Percoll (Pharmacia, Uppsala, Sweden) with ten parts 10 times concentrated Hanks' balanced salt solution (HBSS; Gibco) and 10 mmol/l HEPES. Working solutions for Percoll gradients were prepared by mixing fixed ratios of SIP and EBSS. A gradient with layers of 1.004 (EBSS), 1.030 and 1.063 g/ml was constructed. Following the 12-15 h culture period, the cells were suspended in the 1.063 g/ml Percoll solution and subjected to floatation centrifugation for 25 min at 400x g at 20°C in a Heraeus Varifuge RF (Heraeus) without braking. Subsequently,

cells were collected from the interphases and washed three times in EBSS, supplemented with 2.5% pooled human serum. Finally, the cells were counted, viability and the percentage of insulin-positive cells was assessed.

Immunohistochemistry. Pancreata from mouse, rat, monkey and men (adult and fetal) and control organs were snap-frozen through isopentane in liquid nitrogen and stored until use. Cryostat sections were prepared at 5μm thickness and stained after fixation with 4% buffered formalin or without fixation by an indirect labelling technique, using either fluorescence- or peroxidase-conjugated second antibodies [11,12]. N1 was used at a dilution of 1:2000 (ascites) or 1:10 (culture supernatant). Furthermore, an anti-insulin mouse monoclonal antibody [13] was used at a titre of 1:300. Both antibodies were combined with a goat-anti-mouse fluorescein- or tetramethyl rhodamine-isothiocyanate (FITC or TRITC) labelled antibody (Nordic Immunological Laboratories, Tilburg, The Netherlands) at a dilution of 1:60 and 1:15 respectively or a rabbit-anti-mouse peroxidase conjugated second antibody (Dako, Glostrup, Denmark) at a dilution of 1:100.

Double-staining on sections was performed using the fluorescence procedure. H37, an exocrine-specific rat monoclonal antibody [5] (a gift from Dr.O.D. Madsen, Gentofte, Denmark) was used at a dilution of 1:10, and combined with N1. As second antibody rabbit-anti-rat FITC (Dako) at a dilution of 1:80 was used. Both primary and secondary antibodies were applied together. Controls included replacement of the first antibody with either single- or double-staining using the corresponding animal serum or PBS.

Immunocytochemistry. These experiments were carried out on cells after Percoll gradient centrifugation. For surface labelling cells were labelled in suspension with a primary antibody in PBS/bovine serum albumin for 30 min at 4°C at a concentration of 1 x 10^7 per ml. Then they were washed, centrifuged and incubated with a fluorescence-conjugated second antibody for 30 min at 4°C. Finally, they were washed once more, cytospins were prepared and fixed in acetone for 15 min at room temperature. Subsequently, for cytoplasmic labelling, primary and secondary antibodies were applied on the cytospins for 30 min at room temperature. In between, the cytospins were washed with PBS.

The following surface monoclonal antibodies were used: N1, dilution 1:2000, BE2 and 1B2, against pancreatic acinar cells, dilutions 1:10 and 1:1 respectively (a gift from Dr.H. Clausen,

Seattle, Wash., USA), ER-Pr7, against pancreatic ductal and acinar cells, 1:5 (a gift from Dr. Th.H. van der Kwast, Rotterdam, The Netherlands). All monoclonal antibodies were of mouse origin. Cytoplasmic antisera used were anti-somatostatin 1:800, anti-vimentin 1:50 and anti-glucagon 1:300 (Dako), and anti-islet amyloid polypeptide 1:90 (Peninsula, Belmont, Calif., USA), all were of rabbit origin. A guinea-pig anti-insulin antiserum (Dako) was used at a dilution of 1:1000. The following cytoplasmic monoclonal antibodies were used: mouse anti-synaptophysin 1:10 and anti-tyrosin hydroxylase 1:100 (Boehringer Mannheim), mouse anti-chromogranin A 1:400 (a gift from Dr.J.R.D. Rahier, Brussels, Belgium) and CA812, a BB-rat monoclonal autoantibody, 1:100 (a gift from Dr. O.D. Madsen) [5]. Second antibodies were goat-anti-mouse-FITC and TRITC (1:60 and 1:15), goat-anti-rabbit-FITC and TRITC (1:80 and 1:80) (Nordic), goat-anti-guinea-pig-FITC and TRITC (1:60 and 1:15) (Southern Biotechnology, Birmingham, UK) and goat-anti-rat-FITC 1:250. Control experiments were performed in single- and double-staining experiments, replacing the primary antiserum with a pre-immune serum or with PBS. No background staining or spectral overlap was observed. Typically 500 to 1000 cells were counted by fluorescence microscopy, excluding disrupted cells from the counting.

Typing and subtyping. For the determination of the antibody class of N1, goat-anti-mouse antibodies specific for IgM (1:20), IgG1 (1:20), IgG2 (1:10), IgG3 (1:10) and IgG4 (1:20) were used (Nordic). Stainings were performed as described above.

Staining of viable cells. Fluorescein diacetate (FDA) is a fluorogenic substrate for the determination of cell viability [14]. A stock solution of 1 mg/ml FDA in acetone was made and stored at -20°C. Cell suspensions were incubated with a 1:10,000 dilution of this stock for 5 min. Then, they were centrifuged and analysed on a FACScan (Becton Dickinson).

Cell sorting. Cell sorting experiments were performed on a FACS II cell sorter (Becton Dickinson), equipped with a 5 W argon laser (Spectra Physics, Mountain View, Calif., USA). Laser power was 250 mW during all experiments. Cells were labelled for sorting with N1 (titre 1:500) as described. A goat-anti-mouse antibody conjugated to phycoerythrin (PE) at a dilution of 1:20 (Caltag Laboratories, San Francisco, Calif., USA) was used as a second step, since the autofluorescence of the cells interfered with an FITC stain. With excitation at 488 nm, emission was recorded above 580 nm for PE. For sterile sorting the system was flushed with 70% ethanol

for 15 min, followed by a 15-min wash with sterile distilled water.
The percentage of N1-positive cells, confirmed by immunocytochemistry, was used to set a window when sorting N1-positive cells. Dead cells, while potentially reactive with N1, could largely be eliminated on the basis of their different light scattering properties. This difference was confirmed by the FDA viability assay.

Statistical analysis. The relationship between age and the percentage of cells of a certain type was evaluated according to Spearman's rank correlation. The significance of differences between viability assays before and after Percoll gradient centrifugation was determined by Wilcoxon's signed rank test. All data are given as mean ± SD.

RESULTS

Production and screening of monoclonal antibody N1. One of the hybridoma supernatants stained islets in human adult pancreatic sections. The supernatant was retested on human fetal pancreas and showed positive staining as well. The hybridoma was cloned by limiting dilutions and cloning was repeated to ensure the purity of the cell line. The resulting monoclonal antibody was named N1 and identified as an IgG1 antibody.

Immunohistochemistry. N1 immunoreactivity withstands fixation by 4% buffered formalin, 1% paraformaldehyde or acetone. Paraffin embedding deletes N1 reactivity. Human and monkey pancreas showed a positive staining reaction, mouse and rat tissue did not. In adult human and monkey pancreas all islet cells were cytoplasmically stained by N1 (Fig. 1a). This was confirmed by staining serial sections with N1 and with insulin. Double-staining with N1 and H37, a marker for exocrine pancreatic cells, showed no overlap between these markers in the human adult pancreas. In the human fetal pancreas groups of cells and single cells, dispersed throughout the parenchyma were stained by N1 (Fig. 1b). Double-staining showed that some of these cells contained insulin. The reactivity of N1 in other human fetal organs is summarized in Table 1.

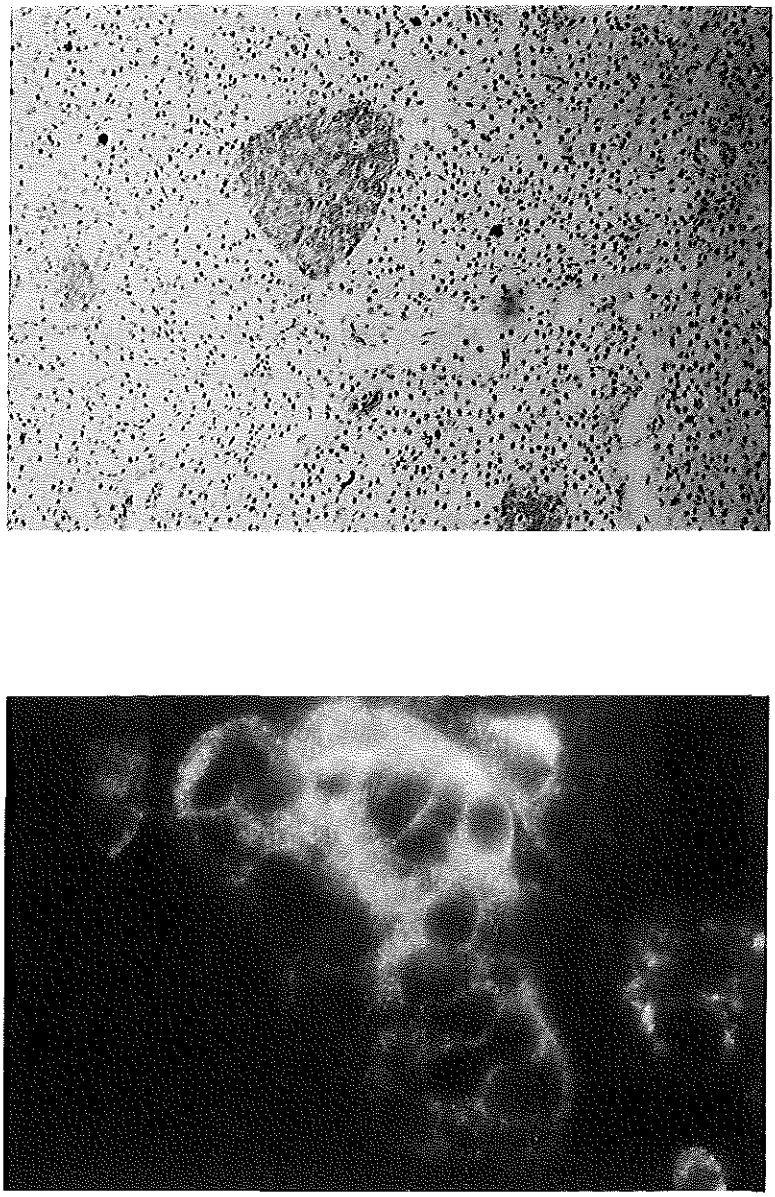


Fig. 1. Sections of human adult (a) or human fetal (b) pancreas stained with N1. In (a) the second antibody is peroxidase-linked and developed with diamino-benzidine, in (b) a fluorescein isothiocyanate-conjugated second antibody was used. In both sections N1 stains a cluster of cells representing the islet of Langerhans.

Table 1. Staining pattern of the monoclonal antibody N1 on several human fetal tissues.

Tissue	Cell type	Reaction
pancreas	islet cells	++
kidney	epithelial cells lining proximal tubules	++
parathyroid	chief cells	++
duodenum	all cells lining the lumen	++
stomach	all cells lining the lumen	+
lung	all cells lining the lumen of bronchi	+
liver	hepatocytes	+
brain	neuronal cells	+

A strongly positive reaction is indicated by ++, a positive reaction by +.

Immunocytochemistry. Human fetal pancreatic cell suspensions showed N1 reactivity on the surface of cells (Fig. 2a). No overlap was seen between N1 and vimentin, a marker of mesenchymal cells. There were no insulin-positive cells staining with exocrine cell monoclonal antibodies, specific for acinar as well as ductal cells. Double-staining for N1 and hormonal markers (insulin, glucagon, somatostatin) or for N1 and either synaptophysin, tyrosin hydroxylase, CA812, chromogranin A or islet amyloid polypeptide further characterized the population of N1-positive cells (Table 2, Fig. 2a,b). In some cases two mouse monoclonal antibodies were used subsequently. Incubation with N1 and its conjugate was done in suspension, as described, followed by the preparation of cytospins and fixation. Because of this procedure, and the spatial difference of the staining pattern (surface labelling for N1 vs cytoplasmic labelling for the other antibody) cells could be recognized and scored without interference of cross-reactivity. Double-stainings performed with each of the five markers above combined with each of the three hormone antibodies showed that 40% of synaptophysin-positive cells, 50% of chromogranin A-positive cells, 50% of CA812-positive cells, 60% of islet amyloid polypeptide-positive cells and 100% of tyrosin hydroxylase-positive cells did not contain one of the three hormones (unpublished results).

Table 2. Simultaneous positivity of N1 and exocrine, hormonal and other markers in human fetal pancreatic cell preparations.

Antibody marker	n	% positive cells	% of N1-positive cells, also positive for the marker indicated
N1	25	15.6 ± 5.7	100.0
BE2	5	21.2 ± 6.6	ND
1B2	5	11.0 ± 7.1	ND
ER-Pr7	5	8.2 ± 1.3	ND
vimentin	5	45.1 ± 9.9	0.0
insulin[a]	25	3.0 ± 1.4	16.9 ± 4.9
glucagon[a]	14	1.2 ± 0.7	6.1 ± 3.1
somatostatin[a]	14	1.9 ± 0.7	14.7 ± 6.0
synaptophysine[a]	12	4.1 ± 1.5	24.9 ± 11.8
tyrosine hydrox.	6	0.3 ± 0.2	1.7 ± 1.7
CA812[a]	5	2.0 ± 0.5	12.6 ± 2.7
chromogranin[a]	7	1.4 ± 0.6	9.4 ± 3.7
islet amyloid polypeptide[a]	9	4.0 ± 1.9	22.8 ± 12.2

[a]these antibody markers react with a population of cells of which 80-95% is also reacting with N1
n = number of fetal pancreata (12-18 weeks gestational age)
Results are given as mean ± SD; ND = not done

Hence, the five subpopulations added to characterize N1-positive cells. Together they contributed 35-40% of the population of N1-positive cells. Overlap between the populations stained by each of the five markers remains possible, reducing this percentage. Between 12 and 18 weeks gestational age the percentage of N1-positive cells increased from 10 to 19% ($n=29$, $r_s=0.57$, $p<0.01$). At the same time there was a similar increase of insulin-positive cells ($n=21$, $r_s=0.45$, $p<0.05$), but not of Alpha and Delta cells. The proportion of hormone-containing cells in the N1 population did not significantly change.

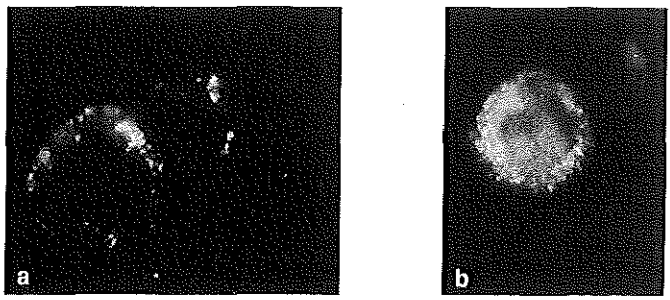


Fig. 2a,b. Pancreatic cell suspension double-stained with N1 (a) and insulin (b). N1 reactivity can be seen on the surface of cells, whereas insulin reactivity is cytoplasmic.

Dispersion. Because pancreatic weight increased from 50 mg at 12 weeks gestational age to 250 mg at 18 weeks, there was a considerable variation in the cell yield between pancreata (Table 3). The yield per amount of tissue was relatively constant throughout this age range: $1.29 \pm 0.17 \times 10^5$ cells per mg pancreatic tissue (n=10, mean ± SD). There was virtually no tissue remaining after the second collagenase digestion step and the passage through the 100 μm nylon mesh. There was no correlation between cell yield per weight and the age of the processed specimen (r_s=0.18).

Table 3. Comparison between human fetal pancreatic cell suspensions at various stages of the procedure for enrichment of single endocrine pancreatic cells: after dispersion and after a 16-h culture period in RPMI 1640 with 10% human serum.

parameter	n	post-dispersion	post-culture	p-value
yield per pancreas	15	13.3 x 10^5 cells	7.9 x 10^5 cells	-
[range]		[5.6 - 24.7]	[4.5 - 14.4]	
viable cells (%)	15	47 ± 9	56 ± 13	p<0.02
single cells (%)	18	64 ± 13	89 ± 10	p<0.01
insulin cells (%)	18	2.2 ± 1.2	3.3 ± 1.3	p<0.02

n = number of pancreata

Data presented (mean ± SD) in Table 3,4 and 5 come from different series of experiments

Overnight culture. Recovery of the cell number following culture in RPMI 1640 plus 10% human serum and 1.5 U/ml dispase was 66 ± 28% (n=15). At the same time the percentage of single cells and the percentage of insulin-positive cells increased (Table 3). Cells were not attached to the dishes, nor was there any clumping. Omission of dispase resulted in extensive clumping and cell attachment at the present cell concentration.

Gradient centrifugation. Percoll gradient centrifugation resulted in two interphases and a pellet. The viability of the 1.004-1.030 g/ml interphase was 48 ± 22%, that of the 1.030-1.063 g/ml interphase was 83 ± 5% and that of the pellet was 20 ± 8%, as compared with 53 ± 8% in the initial cell suspension (n=7). There was a large number of erythrocytes present in the pellet. The difference in viability between the 1.030-1.063 g/ml interphase and the initial cell suspension was significant ($p<0.01$) (Table 4). The recovery of cells in the viable interphase was 55 ± 12% (n=18).

Table 4. Comparison between human fetal pancreatic cell suspensions at various stages of the procedure for enrichment of single endocrine pancreatic cells: after a 16-h culture period in RPMI 1640 with 10% human serum and after Percoll gradient centrifugation.

parameter	n	post-culture	post-Percoll	p-value
yield per pancreas	18	13.4 x 10^5 cells	7.2 x 10^5 cells	-
[range]		[6.0 - 23.6]	[2.7 - 12.4]	
viable cells (%)	19	54 ± 8	81 ± 4	$p<0.01$
insulin cells (%)	13	2.1 ± 0.6	2.8 ± 1.1	NS

n = number of pancreata

Data presented (mean ± SD) in Table 3,4 and 5 come from different series of experiments

Cell sorting. Fluorescence activated cell sorting of N1-positive cells (Fig. 3) resulted in an increase of the percentage of insulin-containing cells (Fig. 4). Also glucagon- and somatostatin-positive cells were enriched significantly (Table 5). After sorting, 6.2 ± 2.7% (n=10) of the cells which were obtained after Percoll gradient centrifugation, were recovered in the enriched suspension.

During the sorting procedure the viability decreased (Table 5). The final recovery of Beta cells after all the procedures (from dispersion to sorting) was 11.3 ± 3.6% (n = 10) as compared to a recovery of 2.2 ± 1.1% for all cells.

Table 5. Comparison between human fetal pancreatic cell suspensions at various stages of the procedure for enrichment of single endocrine pancreatic cells: after Percoll gradient centrifugation and after fluorescence activated cell sorting of N1-labelled cells.

parameter	n	post-Percoll	post-sorting	p-value
yield per pancreas	10	7.4 x 10^5 cells	0.5 x 10^5 cells	-
[range]		[3.2 - 13.0]	[0.2 - 0.7]	
viable cells (%)	5	80 ± 6	63 ± 10	$p<0.05$
insulin cells (%)	10	3.0 ± 1.4	16.2 ± 4.8	$p<0.01$
glucagon cells (%)	8	1.2 ± 0.7	5.7 ± 2.0	$p<0.01$
somatostatin cells (%)	8	1.9 ± 0.7	6.8 ± 2.1	$p<0.01$
N1 cells (%)	5	17 ± 4	83 ± 6	$p<0.05$
vimentin cells (%)	6	47 ± 10	26 ± 7	$p<0.02$

n = number of pancreata

Data presented (mean ± SD) in Table 3,4 and 5 come from different series of experiments

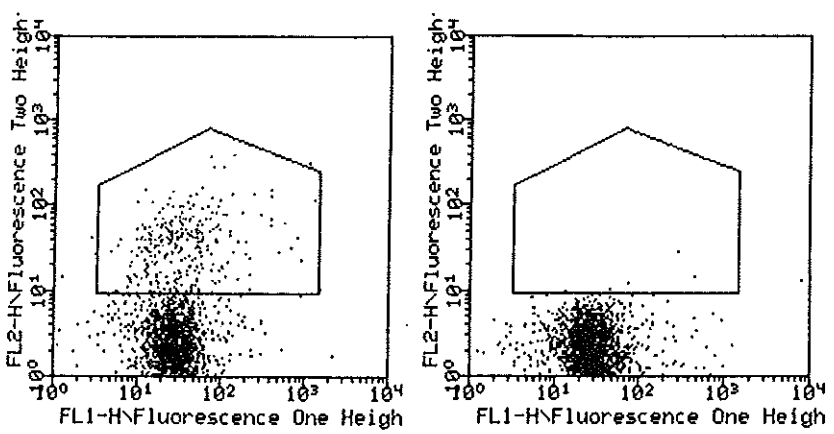


Fig. 3. Dot plots showing the fluorescence distribution of a pancreatic cell suspension labelled with N1 (left panel) and a pancreatic cell suspension labelled with an irrelevant antibody (right panel). This picture was produced on a FACStar-plus cell sorter and attached software, courtesy of Beckton Dickinson, Erembodegem, Belgium.

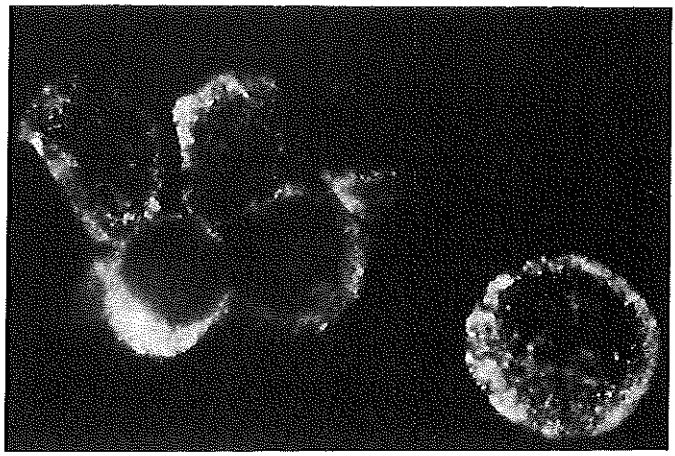


Fig. 4. Fluorescent picture of a pancreatic cell preparation following cell sorting. Numerous insulin-positive cells can be seen.

DISCUSSION

We describe the production of a monoclonal antibody, N1, reactive to the surface and cytoplasm of islet cells in the human fetal and adult pancreas. Apparently, its epitope is present early in development and persists throughout adulthood. N1 does not react to exocrine or mesenchymal cells, confirming its endocrine specificity within the pancreas. Outside the pancreas, N1 reactivity was found in several other tissues and was not restricted to endocrine cells.

Almost 40% of N1-positive cells in the fetal pancreas contained either insulin, glucagon or somatostatin. Since cells were cultured for at least 16 h before staining, it is unlikely that this binding pattern is influenced by the dispersion process. Five other markers (islet amyloid polypeptide, synaptophysin, chromogranin A, tyrosine hydroxylase and CA812) also constitute 35-40% of N1-positive cells. By combining these markers with antibodies to insulin, glucagon and somatostatin, overlapping populations were observed. Only hormone-negative subpopulations of cells positive with one of the five markers were used for further characterization of N1-positive cells. Synaptophysin is present on the membrane of secretory granules of islet cells [16] or in synaptic-like microvesicles [17]. Chromogranin A is converted to betagranin and co-secreted with insulin [18]. Islet amyloid polypeptide is co-localized with insulin in Beta-cell secretory granules [19].

Recently, it has been suggested that islet amyloid polypeptide is present in more immature cells [20,21]. The expression of synaptophysin, chromogranin A and islet amyloid polypeptide in cells not containing insulin, glucagon or somatostatin is intriguing. Apparently, cells with (neuro)endocrine characteristics are present which do not contain hormones. Alternatively, these cells may have lost their hormone or hormone production in vitro or through the various experimental procedures.

The increase in the percentage of N1-positive cells from 10 to 19%, between 12 and 18 weeks gestational age might represent a period with rapid endocrine cell proliferation and/or differentiation relative to the rest of the pancreas. A concomitant increase in the percentage of insulin-positive cells resulted in an unaltered ratio of insulin- vs N1-positive cells.

Many procedures exist for the recovery of islets from the adult pancreas [22-27]. The scattered distribution of endocrine cells in the human fetal pancreas excludes standard islet isolation procedures [9]. Instead we dispersed the whole pancreas into single cells allowing cell sorting. Our method resulted in considerable cell death, probably due to the use of collagenase. Therefore, Percoll gradient centrifugation was performed to increase the percentage of viable cells before antibody labelling and cell sorting. The clumping tendency of the preparations results in a suboptimal percentage of viable cells.

For the enrichment of Beta cells, van de Winkel et al. [28] were able to sort rat Beta cells from other endocrine pancreatic cells on the basis of flavin adenine dinucleotide (FAD) content [29] resulting in autofluorescence. We could not increase the percentage of fetal Beta cells using this method (unpublished results), or by using forward light scatter as a sorting parameter as previously described for rat islet cells [30,31]. The observed differences may be the result of the human or fetal character of these pancreatic cells.

Therefore we labelled the surface of the cells with N1, while in suspension. Enrichment is limited by the proportion of Beta cells in the N1-positive cell population ($16.9 \pm 4.9\%$). Some contamination with mesenchymal cells is present. This may be caused by non-specific binding of label to these cells or the formation of doublets with N1-positive cells. The decrease in viability after sorting may result from dead cells with false positive N1 reactivity, although care was taken to eliminate these on the basis of light scattering properties. Moreover, the stress of the sorting procedure might result in a decreased viability.

Preliminary experiments have shown that the sorted cell suspension can be cultured for up to 1 week, without a decrease in viability or the percentage of endocrine cells (unpublished

results). The enriched suspensions may be used to further address questions about the exact development of endocrine pancreatic cells and the order of expression of relevant markers. In conclusion, an antibody was produced which reacts to the surface and cytoplasm of islet cells in the human fetal pancreas. With this antibody we have been able to enrich Beta cells and other endocrine cells from a total pancreatic cell preparation by fluorescence activated cell sorting.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Mr. M. Kuit for photographical assistance. They thank Dr. O. Madsen and Mrs. E.E. Pedersen for their excellent immunohistochemical stainings. H.J.A. is supported by a stipend from the "Ter Meulen Foundation". R.R. de K. and G.K. are supported by a grant from the Dutch Diabetes Research Fund.

REFERENCES

1. Ziegler B, Witt S, Waterstradt B, Schlosser M, Ziegler M (1989) Heterogeneity of monoclonal antibodies against pancreatic Beta cells. Exp Clin Endocrinol 93: 173-180

2. Witt S, Ziegler B, Waterstradt B, Besch W, Hehmke B, Ziegler M (1987) Generation and partial characterization of monoclonal antibodies reactive with islet cell antigens. Exp Clin Endocrinol 89: 276-282

3. Vissing H, Papadopoulos G, Lernmark Å (1986) Monoclonal antibodies against pancreatic islet-cell-surface antigens selected by flow cytofluorometry. Scand J Immunol 23: 425-433

4. Brogren C-H, Hirsch F, Wood P, Druet P, Poussier P (1986) Production and characterization of a monoclonal islet cell surface autoantibody from the BB rat. Diabetologia 29: 330-333

5. Contreas G, Jorgensen J, Madsen OD (1990) Novel islet, duct, and acinar cell markers defined by monoclonal autoantibodies from prediabetic BB rats. Pancreas 5:540-547

6. Gerbitz KD, Boenke B, Paprotta A, Chemnitz U (1988) Human nesidioblastosis tissue as an immunogen for generation of islet cell specific monoclonal antibodies. J Clin Chem Clin Biochem 26:659-666

7. Warnock GL, Kneteman NM, Evans MG, Dabbs KD, Rajotte RV (1990) Comparison of automated and manual methods for islet isolation. Can J Surg 33:368-371

8. Van de Winkel M, Maes E, Pipeleers DG (1982) Islet cell analysis and purification by light scatter and autofluorescence. Biochem Biophys Res Comm 107:525-532

9. Like AA, Orci L (1972) Embryogenesis of the human pancreatic islets: a light and electron microscopic study. Diabetes 21 [Suppl 2]: 511-534

10. Milstein C, Galfre G, Secher DS, Springer T (1979) Monoclonal antibodies and cell surface antigens. Cell Biol Int Rep 3:1-16

11. Nakane PK, Pierce GB (1967) Enzyme-labelled antibodies for the light and electron microscopic localization of tissue antigens. J Cell Biol 33:307-318

12. Wicker R, Avrameas S (1969) Localization of virus antigens by enzyme-labelled antibodies. J Gen Virol 4:465-471

13. Keilacker H, Dietz H, Witt S, Woltanski KP, Berling R, Ziegler M (1986) Kinetic properties of monoclonal insulin antibodies. Biomed Biochim Acta 45: 1093-1102

14. Rotman B, Papermaster BW (1966) Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters. Proc Natl Acad Sci USA 55:134-141

15. Roth J, Bendayan M, Carlemalm E, Villiger W, Garavitto M (1981) Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J Histochem Cytochem 29:663-671

16. Kalina M, Lukinius A, Grimelius L (1991) Ultrastructural localization of synaptophysin to the secretory granules of normal glucagon and insulin cells in human islets of Langerhans. Ultrastruct Pathol 15:215-219

17. Navone F, Jahn R, Di Giola G, Stukenbrok H, Greengard P, De Camilli P (1986) Protein p38: an integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J Cell Biol 103:2511-2527

18. Hutton JC (1989) The insulin secretory granule. Diabetologia 32:271-281

19. Lukinius A, Wilander E, Westermark GT, Engström U, Westermark P (1989) Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets. Diabetologia 32:240-244

20. Madsen OD, Blume N, Petersen JE, Kofod H, Dyrberg T (1990) Specific antisera to IAPP, proinsulin C-peptide 1, and 2: probes to study murine islet Beta-cell differentiation. Diabetologia 33 [suppl]: A108 (Abstract)

21. Madsen OD, Nielsen JH, Michelsen B et al. (1991) Islet amyloid polypeptide and insulin expression are controlled differently in primary and transformed islet cells. Mol Endocrinol 5:143-148

22. Hegre OD, Marshall S, Schulte BA, Hickey GE, Williams F, Sorenson RL, Serie JR (1983) Nonenzymic in vitro isolation of perinatal islets of Langerhans. In Vitro 19:611-620

23. Ono J, Takaki R, Fukuma M (1977) Preparation of single cells from pancreatic islets of adult rat by the use of dispase. Endocrinol Japon 24:265-270

24. Pipeleers DG, In 't Veld PA, Van de Winkel M, Maes E, Schuit FC, Gepts W (1985) A new in vitro model for the study of pancreatic A and B cells. Endocrinology 117:806-816

25. Kover K, Moore WV (1989) Development of a method for isolation of islets from human fetal pancreas. Diabetes 38:917-924

26. Hellerström C, Lewis NJ, Borg H, Johnson R, Freinkel N (1979) Method for large-scale isolation of pancreatic islets by tissue culture of fetal rat pancreas. Diabetes 28:769-776

27. Korsgren O, Sandler S, Schnell Landstrom A, Jansson L, Andersson A (1988) Large-scale production of fetal porcine pancreatic isletlike cell clusters. An experimental tool for studies of islet cell differentiation and xenotransplantation. Transplantation 45:509-514

28. Van de Winkel M, Maes E, Pipeleers DG (1982) Islet cell analysis and purification by light scatter and autofluorescence. Biochem Biophys Res Comm 107:525-532

29. MacDonald MJ (1981) Flavin content of intracellular compartments of pancreatic islets compared with acinar tissue and liver. Endocrinology 108:1899-1902

30. Rabinovitch A, Russell T, Shienvold F, Noel J, Files N, Patel Y, Ingram M (1982) Preparation of rat islet B-cell-enriched fractions by light-scatter flow cytometry. Diabetes 31:939-943

31. Nielsen DA, Lernmark Å, Berelowitz M, Bloom GD, Steiner DF (1982) Sorting of pancreatic islet cell subpopulations by light scattering using a fluorescence activated cell sorter. Diabetes 31:299-306

Islet Cell Cytoplasmic Antibody Reactivity in Midgestational Human Fetal Pancreas

R.R. de Krijger, M.V. van Krugten, G. Kranenburg, H.J. Aanstoot, J.L. Molenaar[1], and G.J. Bruining

Department of Pediatrics and Clinical Genetics, Erasmus University and University Hospital/Sophia Children's Hospital Rotterdam, Department of Clinical Immunology[1], SSDZ Delft, The Netherlands

Acta Diabetologica (in press)

SUMMARY

The reactivity of ICA-positive and ICA-negative sera of recent onset type-I diabetic patients was studied in human fetal pancreata of 12-18 weeks gestation and compared with reactivity of these sera in adult human control pancreata. The aims of the study were: 1) to observe the presence of ICA staining in human fetal islet cells; 2) to compare end-point titres (in JDF units) of ICA-positive patient sera in fetal pancreata and adult human control pancreata. Ten ICA-positive sera and 8 ICA-negative sera from newly diagnosed diabetic patients, and 4 sera from healthy controls were tested on 3 human adult and 8 human fetal pancreata. As in the adult control pancreata, ICA-positive sera reacted to insulin-, glucagon-, and somatostatin-positive cells of fetal pancreata of all gestational ages. This was observed both in single cells and in cells in islet-like cell clusters. Dilution of a reference serum gave similar results in both adult and fetal pancreata. In contrast, the ICA-positive patient sera yielded a striking heterogeneity in fetal as well as in adult pancreata. However, end-point titres between adult and fetal pancreata did not differ significantly ($p > 0.05$). In conclusion, ICA-positive sera from recent onset diabetic patients show that the expression of molecules to which ICA react is present in all islet cells and starts before week 12 of gestation.

INTRODUCTION

Humoral autoimmunity to pancreatic islet cells can be detected in sera of recent-onset type-I (insulin-dependent) diabetic patients and in sera of prediabetic individuals before clinical onset of type-I diabetes (1). Islet cell cytoplasmic antibodies (ICA) have been studied extensively. They are detected by indirect immunofluorescence of cryostat sections of bloodgroup O human adult pancreata. ICA-positive sera usually stain α, ß, and δ cells in the islets of Langerhans ("whole islet staining pattern") (2). The assay has been standardized through the use of reference sera (3). Multiple islet cell antigens have been suggested as ICA targets including glutamic acid decarboxylase (GAD) (4-6). Apart from the whole islet staining pattern of ICA-positive sera, a restricted pattern, in which only ß cells are stained, has been observed (7).
In experimental studies it has been shown that the onset of autoimmunity will depend on the timing of antigen expression during fetal development (8). It is therefore of interest to study

human fetal antigen expression, using ICA-positive patient sera. Limited information is available on the reactivity of ICA on human fetal pancreatic tissue (9). This tissue contains endocrine cells at various stages of development, being present as single cells or organized in smaller or larger endocrine cell clusters (10). In one study a single ICA-positive serum and a single ICA-negative serum were tested (9). Only insulin/ICA double staining was evaluated. The other endocrine cell types were not analyzed. No comparison was made with adult pancreata. In the present study, the ICA staining pattern in all major islet cell types was investigated. End-point titres (in Juvenile Diabetes Foundation (JDF) units) of a series of ICA positive sera were determined in fetal and adult control pancreata.

MATERIALS AND METHODS

Sera. Serum samples were obtained within 24 hours of the first insulin injection from 18 type-I diabetic patients (aged 8-20 years, mean: 14 years; four boys and six girls). The ICA-positive samples (n=10) were selected to represent a range from weakly to strongly positive for ICA, as tested on adult pancreas. The cut-off point for the distinction between an ICA-positive from an ICA-negative patient serum was 0.63 JDF unit. All sera were ICA-IgG positive and 6 showed complement fixation ability (CF-ICA) (11). Insulin autoantibodies (IAA) in these samples were absent by RIA (12) as well as by ELISA (13). Control sera were obtained from four healthy individuals. No control had a first degree relative with insulin dependency. Serum samples were stored at -80°C.

Human fetal pancreata. Human fetal pancreata (bloodgroup O) between 12 and 18 weeks gestation (n=8) were obtained from mechanical abortion, approved by the local ethical committee, and with signed informed consent. The warm ischemia time was less than 20 minutes. After collection, the pancreata were snapfrozen in liquid nitrogen and stored at -80°C. Bloodgroup typing was performed on spleen cells.

Human adult pancreata. Bloodgroup O pancreata were obtained from 3 organ donors. The pancreas was removed first. The adult pancreata were divided into pieces of 0.5 cm^3, snapfrozen in liquid nitrogen and stored at -80°C.

Immunohistochemistry. The methods used have been described previously (14,15). Briefly, cryostat sections were incubated for 18 hours with serum (diluted 1:2 to 1:1024) in phosphate buffered saline (PBS). Aprotinin (Trasylol, Sigma Chemical Co., St. Louis, MO, USA) 0.47 mg/ml was added to prevent proteolytic degradation of pancreatic tissue. Incubations were performed in a dark-moist chamber at room temperature. Slides were washed in PBS and double staining was carried out with a mouse anti-human proinsulin monoclonal antibody (GS4G9, dilution 1:10, a gift of Dr. O.D. Madsen, Gentofte, Denmark) for 30 min. Slides were again washed in PBS and the sections were incubated with a TRITC-labelled rabbit anti-mouse IgG 1:100 (PBS) (Dako, Copenhagen, Denmark) for 30 min. Finally, fluorescein isothiocyanate (FITC) conjugated rabbit anti-human IgG (Dako) was added in a dilution of 1:100 for 30 min, to detect ICA. Antibodies to glucagon (GLU-001, 1:30, Novo, Bagsvaerd, Denmark) or somatostatin (SOM-018, 1:1000, Novo) were used for double staining instead of the proinsulin antibody to detect the other endocrine cell types. Both were mouse monoclonal antibodies.

Sera were diluted stepwise, until reciprocal titers of 1024, generating 10 point titration curves. The reciprocal end-point titre was defined as the maximal dilution at which fluorescence could be detected by two independent observers. A reference serum (80 JDF units/ml) was used on all pancreata studied (3 adult, 8 fetal), by diluting to 4 different concentrations 1:2, 1:8, 1:32, and 1:128 in normal ICA-negative serum (3). These four concentrations were further diluted to obtain final reciprocal titers between 1:4 and 1:16384. Linear titration curves were obtained.

The limited amount of fetal tissue available and the unknown sensitivity of these pancreata for the ICA sera were reasons to deviate from ICA workshop recommendations. Transformation from end-point titre to JDF units was based on reciprocal end-point titre of the reference serum on each pancreas.

Statistical analysis. Differences in end-point titre between adult and fetal pancreata were analyzed by the Mann-Whitney U-test.

RESULTS

We have previously described histologic features of the human midgestational pancreas using antibodies to insulin, glucagon, and somatostatin (10). In accordance with that study, positive single cells and cell clusters of varying sizes were observed for all three hormones tested and in all 8 fetal pancreata used for this study.

Reciprocal end-point titres and corresponding JDF units of ICA on the adult and fetal pancreata are indicated in table 1. In three fetal pancreata (F1,F2,F6) the limited amount of tissue prohibited the testing of some of the sera. In pancreata F2, F3, F7, and F8 one ICA-positive serum did not react, whereas in pancreas F1 two sera did not react (see table 1). In the adult pancreata, two sera did not react to pancreas A3, whereas all sera showed ICA staining on the other two adult pancreata.

Double staining of sections, combining ICA sera with antibodies to insulin, glucagon, and somatostatin showed that all three cell types express ICA targets (fig. 1). In addition to ICA reactive endocrine cell clusters, single hormone containing cells were also stained (fig. 2). In all pancreata and with all ICA-positive sera the "whole islet staining pattern" was observed (2), whereas ß cell restricted ICA staining was not seen in this study. No hormone negative cells reacted with ICA-positive sera in either fetal or adult pancreata.

The results of testing and titrating the reference serum and its dilutions are shown in table 2. All three adult pancreata had identical reciprocal end-point titres. The end-point titre of the reference serum in fetal pancreas F1, F4, and F5 was one dilution step lower, whereas the difference was two steps in pancreata F7 and F8.

The results of the 10 patient sera were highly heterogeneous, on the three adult as well as on the 8 fetal pancreata. With sera 6, 7, and 10, pancreas A1 showed titres which were 5, 4, and 4 dilution steps lower than for the same sera with pancreas A2, respectively. With sera 3, 4, 8, and 9, however, titres were similar or even 1 or 2 dilution steps higher. Serum 9 gave a 5 dilution steps lower titre in pancreas F2 than in pancreas F3, whereas with serum 7 pancreas F2 had a two step higher titre. No significant differences in end-point titre between adult and fetal pancreata could be detected ($p > 0.05$).

Of the 8 sera of recent onset diabetic patients, which tested ICA-negative on adult pancreata, 2 were found reactive to pancreas F3 and F5, 1 other serum reacted to pancreas F6, all at a dilution of 1:2 (0.63 JDF units). None of the four healthy control sera reacted to either adult or fetal pancreata.

Table 1. Reciprocal end-point titres and transformation into JDF units (indicated in brackets) of 10 sera of recent onset diabetic patients on 3 adult and 8 fetal human pancreata

pancreas	serum number									
	1	2	3	4	5	6	7	8	9	10
A1	4 (1.25)	4 (1.25)	8 (2.5)	16 (5)	4 (1.25)	32 (10)	8 (2.5)	32 (10)	256 (80)	8 (2.5)
A2	16 (5)	16 (5)	4 (1.25)	64 (20)	16 (5)	1024 (320)	128 (40)	32 (10)	256 (80)	128 (40)
A3	0 (0)	0 (0)	2 (0.63)	64 (20)	8 (2.5)	512 (160)	128 (40)	8 (2.5)	32 (10)	32 (10)
F1 (12)	0 (0)	0 (0)	NT	8 (2.5)	8 (2.5)	NT	8 (10)	NT	NT	NT
F2 (13)	0 (0)	NT	4 (1.25)	16 (5)	NT	256 (80)	16 (5)	2 (0.63)	4 (1.25)	16 (5)
F3 (13)	2 (0.63)	2 (0.63)	8 (2.5)	16 (5)	16 (5)	256 (80)	4 (1.25)	8 (2.5)	128 (40)	0 (0)
F4 (13)	8 (2.5)	8 (2.5)	8 (2.5)	32 (10)	8 (2.5)	128 (40)	16 (5)	4 (1.25)	64 (20)	32 (10)
F5 (14)	4 (1.25)	8 (2.5)	8 (2.5)	64 (20)	4 (1.25)	128 (40)	64 (20)	32 (10)	128 (40)	16 (5)
F6 (14)	8 (2.5)	8 (2.5)	16 (5)	32 (10)	16 (5)	NT	NT	NT	NT	NT
F7 (15)	0 (0)	8 (2.5)	4 (1.25)	16 (5)	8 (2.5)	128 (40)	32 (10)	4 (1.25)	64 (20)	2 (0.63)
F8 (17)	8 (2.5)	8 (2.5)	4 (1.25)	32 (10)	8 (2.5)	128 (40)	32 (10)	16 (5)	64 (20)	0 (0)

0 = negative; NT = not tested; A = adult pancreas; F = fetal pancreas (gestational age in weeks indicated in brackets)

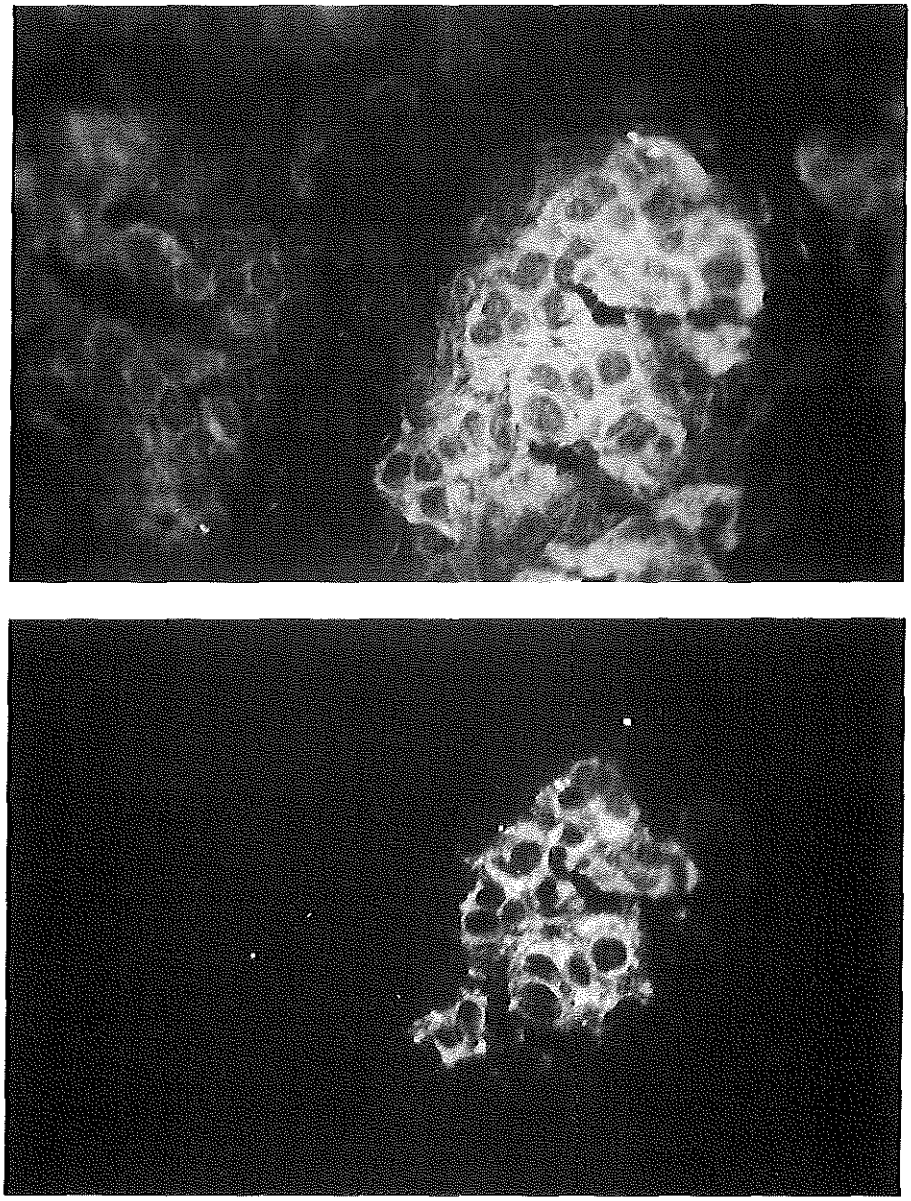


Figure 1. Immunofluorescence photographs of an identical area in a human fetal pancreatic section double stained with an ICA-positive serum (upper panel), and with a monoclonal proinsulin antibody (lower panel). Note that ICA reactivity is present in cells surrounding the proinsulin positive cell core.

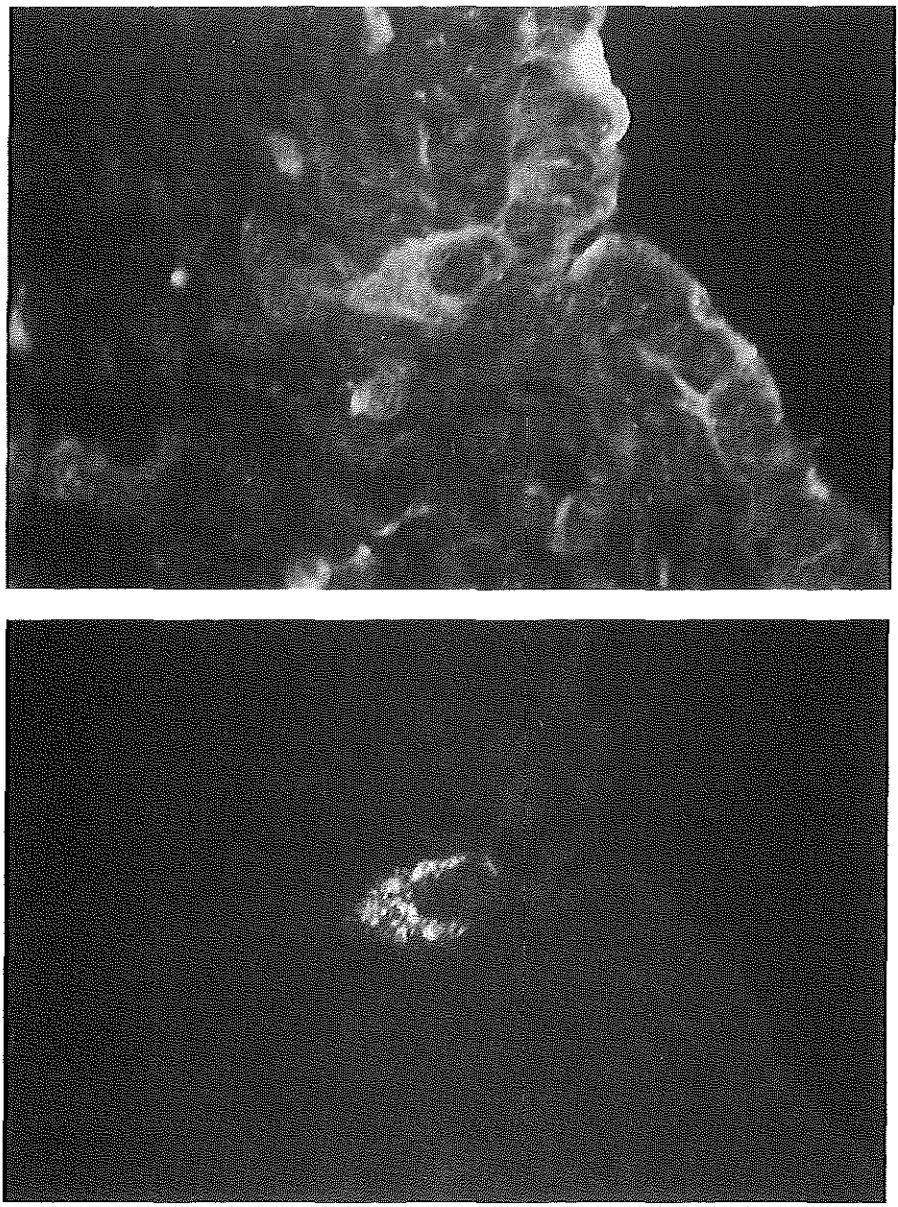


Figure 2. Immunofluorescence photographs of an identical area in a human fetal pancreatic section double stained with an ICA-positive serum (upper panel), and with a monoclonal proinsulin antibody (lower panel). Coexpression of proinsulin and ICA reactivity can be observed in a single endocrine cell.

Table 2. Reciprocal end-point titres of the reference serum on adult (A) and fetal (F) pancreata.

		reference serum dilution			
Pancreas (weeks gestation)	reference serum	1:2	1:8	1:32	1:128
A1	256	128	32	8	2
A2	256	128	32	8	2
A3	256	128	32	8	2
F1 (12)	64	32	8	2	0
F2 (13)	256	128	32	32	8
F3 (13)	256	128	32	8	2
F4 (13)	64	32	8	2	2
F5 (14)	64	32	8	2	2
F6 (14)	256	128	32	2	2
F7 (15)	16	8	2	2	0
F8 (17)	16	8	2	2	0

DISCUSSION

We confirm the observation of Sundkvist et al. (9) that ICA reactivity is present in the human fetal pancreas. ICA-reactivity is found in all fetal pancreata between 12 and 18 weeks gestation, indicating that expression of molecules to which ICA react starts before week 12 of gestation. The ICA reactivity includes all insulin, glucagon, as well as somatostatin positive cells, consistent with findings in the adult pancreas (16). In this study no ß-cell restricted ICA reactivity pattern is found. In human fetal pancreata, ICA reactivity is seen in endocrine cell clusters and in single hormone containing cells. Thus, ICA reactivity does not mark the different stages of endocrine cell development during the 12-18 week period. This is supported by the finding that the staining pattern is quite similar in the different fetal pancreata. We have not examined hormone co-expressing cells, in which two or three hormones are present simultaneously, as we have shown to be present during endocrine cell ontogeny (10). Triple labelling studies should be used to analyze if the multiple hormone expressing cells also contain ICA reactivity. Analysis of reference serum reactivity on adult pancreata gives an identical result at each dilution

in each of the three pancreata. When our 10 diabetic patient sera are applied, however, heterogeneous end-point titres and corresponding JDF units are obtained. This variability in adult pancreata has been described before (17).

In the fetal pancreata dilution of the reference serum yields end-point titres comparable to those of the adult pancreata. Heterogeneous results are obtained in the fetal pancreata with the patient sera. This does not lead to significant differences in end-point titres between adult and fetal pancreata. The differences in reactivity of ICA positive sera between fetal pancreata may be explained by the fact that ICA react to multiple target molecules, which are not present in equal amounts during development (7,18). During fetal development islet cells are present at differing developmental stages, which may affect the antigenic profile qualitatively and quantitatively. In addition, the metabolic state of endocrine cells, which is unknown in our fetal islet cells, has been shown to influence antigen expression (19). No conclusions can be drawn with regard to the effect of antigenic heterogeneity in fetal pancreata on ICA titre, because this study does not address ICA reactivity to defined antigens. Further molecular identification of ICA epitopes in the human fetal pancreas is essential.

ACKNOWLEDGEMENTS

H.J.A. is supported by a grant from the "Ter Meulen Foundation" of the Royal Dutch Academy of Sciences and the A. Renold Fellowship of the European Association for the Study of Diabetes. M.V. van K. took part in the Erasmus University Medical Student Research Training Program.

REFERENCES

1. Bruining GJ, Molenaar JM, Grobbee DE, Hofman A, Scheffer GJ, Bruining HA, De Bruyn AM, Valkenburg HA, Ten-year follow-up study of islet cell antibodies and childhood diabetes mellitus. Lancet i:1100-1103, 1989

2. Atkinson MA, Kaufman DL, Newman D, Tobin AJ, Maclaren NK, Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. J Clin Invest 91:350-356, 1993

3. Greenbaum C, Palmer J, Nagataki S, Yamaguchi Y, Molenaar JL, Van Beers WAM, MacLaren NK, Lernmark Å, and participating laboratories, Improved specificity of ICA assays in the fourth international immunology of diabetes serum exchange workshop. Diabetes 41:1570-1574, 1992

4. Nayak RC, Omar AK, Rabizadeh A, Srikanta S, Eisenbarth GS, Cytoplasmic islet cell antibodies: evidence that the target antigen is a sialoglyco-conjugate. Diabetes 34:617-619, 1985

5. Colman PG, Nayak RC, Campbell IL, Eisenbarth GS, Binding of cytoplasmic islet cell antibodies is blocked by human pancreatic glycolipid extracts. Diabetes 37:645-652, 1988

6. Pietropaolo M, Castaño L, Babu S, Buelow R, Kuo YL, Martin S, Martin A, Powers AC, Prochazka M, Naggert J, Leiter EH, Eisenbarth GS, Islet cell autoantigen 69 kD (ICA69). Molecular cloning and characterization of a novel diabetes-associated autoantigen. J Clin Invest 92:359-371, 1993

7. Genovese S, Bonifacio E, McNally JM, Dean BM, Wagner R, Bosi E, Gale EAM, Botazzo GF, Distinct cytoplasmic islet cell antibodies with different risks for type I (insulin-dependent) diabetes mellitus. Diabetologia 35:385-388, 1992

8. Adams TE, Alpert S, and Hanahan D, Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic beta cells. Nature 325:223-228, 1987

9. Sundkvist G, Bergqvist A, Weibull H, Bergqvist D, Fält K, Landin Olsson M, Lernmark Å, Islet cell antibody reactivity with human fetal pancreatic islets. Diab Res Clin Pract 14:1-8, 1991

10. De Krijger RR, Aanstoot HJ, Kranenburg G, Reinhard M, Visser WJ, Bruining GJ, The midgestational human fetal pancreas contains cells coexpressing islet hormones. Dev Biol 153:368-375, 1992

11. Bruining GJ, Molenaar J, Tuk CW, Lindeman J, Bruining HA, Marner B, Clinical time-course and characteristics of islet-cell cytoplasmic antibodies in childhood diabetes. Diabetologia 26:24-29, 1984

12. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paguette TL, Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 222:1337-1339, 1983

13. Wilkin T, Armitage M, Casey C, Pyke DA, Hoskins PJ, Rodier M, Diaz JC, Leslie RDG, Value of insulin auto-antibodies as serum markers for insulin-dependent diabetes mellitus. Lancet 1:480-482, 1985

14. Madsen OD, Landin-Olsson M, Bille G, Sundkvist G, Lernmark Å, Dahlqvist G, Ludvigsson J, A two-colour immunofluorescence test with a monoclonal human proinsulin antibody improves the assay for islet cell antibodies. Diabetologia 29:115-118, 1986

15. Landin-Olsson M, Sundkvist G, Lernmark Å, Prolonged incubation in the two-colour immunofluorescence test increases the prevalence and titre of islet cell antibodies in Type 1 (insulin-dependent) diabetes mellitus. Diabetologia 30:327-332, 1987

16. Bottazzo GF, Doniach D, Islet-cell antibodies (ICA) in diabetes mellitus. Evidence of an autoantigen common to all cells in the islet of Langerhans. Ric Lab 8:29-39, 1978

17. Landin-Olsson M, Sundkvist G, Lernmark Å, Precision of islet-cell antibody assay depends on the pancreas. Diabetologia 31:511(A), 1988

18. Nayak RC, Omar MAK, Rabizadeh A, Srikanta S, Eisenbarth GS, "Cytoplasmic" islet cell antibodies: evidence that the target antigen is a sialoglycoconjugate. Diabetes 28:617-619, 1985

19. McCulloch DK, Barmeier H, Neifing JL, Palmer JPP, Metabolic state of the pancreas affects end-point titre in the islet cell antibody assay. Diabetologia 34:622-625, 1991

Usage of *TCRAV* and *TCRBV* gene families in human fetal and adult *TCR* rearrangements.

Frank M. Raaphorst[1,2], Jeroen van Bergen[1], Renée Langlois van den Bergh[2], Maarten van der Keur[3], Ronald de Krijger[4], Jan Bruining[4], Maarten J.D. van Tol[2], Jaak M. Vossen[2] and Peter J. van den Elsen[1].

[1]Department of Immunohematology and Bloodbank, and [2]Department of Pediatrics, Leiden University Hospital, Leiden, P.O. Box 9600, 2300 RC, Leiden, The Netherlands. [3]Department of Cytochemistry and Cytometry, Sylvius Laboratories, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. [4]Department of Pediatrics, Subdivision of Clinical Genetics, Erasmus University/Sophia Children's Hospital, Dr. Molenwaterplein 60, 3015 GJ Rotterdam, The Netherlands.

Immunogenetics 39:343-350 (1994)

ABSTRACT

We have investigated fetal and adult T-cell receptor (*TCR*) *A* and *B V*-gene repertoires both by FACS analysis with the available *TCR V* region-specific mAbs and by the polymerase chain reaction (PCR) with *TCR V* gene family-specific oligonucleotides. Among the low number of $CD3^+$ T cells, most of the *TCR V* regions tested for could be detected by FACS analysis in liver, bone marrow and spleen derived from a 14-week-old fetus and two 15-week-old fetuses. Similarly, the PCR analysis showed that the majority of the *TCRAV* and *TCRBV* families were expressed in the peripheral organs of the 13-week-old fetus, although an apparent absence of particular *TCR V* families was found in liver and bone marrow. This was most probably the consequence of the low number of $CD3^+$ T cells in these organs. In 17-week-old fetal thymi the level of expression of some *TCRAV* and *TCRBV* gene families, in particular those that contain a single member, was lower compared to post-partum thymi and adult peripheral blood mononuclear cells. The combined data of FACS and PCR analysis demonstrate that *TCR V* genes belonging to the majority of *TCR V* gene families can be used in *TCR* α and ß chain rearrangements during early human fetal life. Our data also suggest that the expression levels of some of the single member *TCR V* gene families may be influenced by the developmental stage.

INTRODUCTION

T-lymphocytes specifically recognize processed peptide antigens, presented by major histocompatibility complex molecules, via the T-cell receptor (*TCR*; reviewed in Strominger 1989). *TCRs* can be divided into two classes: the $\alpha\beta$ *TCR*, expressed on the majority of peripheral T-lymphocytes, and the $\gamma\delta$ *TCR* (Brenner et al. 1986, 1987; Strominger 1989). Both chains of these heterodimeric *TCRs* are composed of variable and constant regions. Variable regions are assembled from germ-line encoded variable (*V*), diversity (*D*; only present in ß chains) and joining (*J*) elements through a process called *V(D)J* recombination (reviewed in Schatz et al. 1992). The currently known human *TCR V* elements have been grouped into 29 *TCRAV* families and 24 *TCRBV* families; the majority of these contain only a single member (Concannon et al. 1986; Kimura et al. 1987; Wilson et al. 1988; Ferradini et al. 1991; Plaza et al. 1991; Robinson 1991; Roman-

Roman et al. 1991).

Cells committed to the T-cell lineage are detectable in human fetal liver as early as the eighth week of gestation, although at this stage of fetal life rearrangement of the *TCR* genes is undetectable (Asma et al. 1983; Sánchez et al. 1993). From 12 weeks of gestation onwards the bone marrow is the primary site of lymphopoiesis (Asma et al. 1983, 1984; Gale 1987; Abe 1989). The thymus is colonized by (pre) T cells, generated in the hematopoietic organs, at 8 or 9 weeks of gestation and remains the major site for T-cell differentiation and selection of the *TCR* repertoire (Asma et al. 1983, 1984; Gale 1987; Haynes et al. 1988; Abe 1989; Sánchez et al. 1993). *TCRA* and *TCRB* gene rearrangements have been detected in the human fetal thymus as early as 15 weeks of gestation (Campana et al. 1989) but are likely to be initiated at an earlier stage. Rearrangement of *TCR* gene elements is an ordered process. For instance, studies of mouse and human T-cell development (Born et al. 1985; Samelson et al. 1985) and analysis of human $CD3^-$ T-cell acute lymphoblastic leukemias (Furley et al. 1986; van Dongen et al. 1987) have shown that the *TCRB* and *TCRG* loci rearrange before the *TCRA* and *TCRD* loci. Previously, we and others have demonstrated that the employment of the various *TCR V* gene elements in mouse and human *TCR* δ chain rearrangements is under developmental control. Furthermore, the overall usage of *TCRDV* elements is influenced by post-thymic peripheral modification (Elliot et al. 1988; Ito et al. 1989; Lafaille et al. 1989; Krangel et al. 1990; van der Stoep et al. 1990). The usage of *TCRGV* and *TCRGJ* gene elements was found to be tissue-specific during mouse fetal life (Lafaille et al. 1989). Some of these observations have also been made regarding the usage pattern of *TCRAJ* elements during mouse development (Roth et al. 1991). To gain an insight into the generation of the repertoire of $\alpha\beta$ chain *TCRs* and possible developmental influences on the usage of *TCR V* gene families during the establishment of the human *TCR A* and *B* repertoire, we have investigated the usage of *TCRAV* and *TCRBV* families in *TCR* rearrangements derived from fetal liver, bone marrow and spleen obtained at 14 (n=1) and 15 weeks (n=2) of gestation using fluorescence-activated cell sorter (FACS) analysis. As the number of monoclonal antibodies specific for human *TCR V*-regions is limited and fetal liver and bone marrow contain extremely low numbers of T cells, we have also determined *TCRAV* and *TCRBV* gene family expression in adult peripheral blood mononuclear cells (PBMC), two human post-partum thymi, two human fetal thymi at 17 weeks of gestation and in human fetal liver, bone marrow, spleen, gut and cord blood at 13 weeks of gestation using the polymerase chain reaction (PCR).

MATERIALS AND METHODS

Human organs. Human fetal tissue was acquired by interruption of pregnancy on non-medical grounds and used for the experiments after informed consent. The gestational age was determined by measurement of the foot length (Moore 1988). As a consequence of the suction procedure we were unable to locate the thymi of the younger fetuses. The use of this material for research purposes was approved by the Ethical Research Committees of the University Hospitals of Leiden and Rotterdam under strict conditions.

To obtain fetal bone marrow, long bones were flushed with phosphate buffered saline containing 5% bovine serum albumin (BSA) and 0.25% Na-ethylenediaminetetraacetate (EDTA) and the cells were pelleted. Bone marrow of the 11-week-old fetus was not investigated, as lymphopoiesis is marginal at this point of gestation. For PCR analysis, pelleted bone marrow and the other fetal organs were immediately frozen in liquid nitrogen and stored at -80° C. For FACS analysis, single-cell suspensions were prepared from liver, bone marrow and spleen as previously described (Asma et al. 1983, 1984). Mononuclear cells were isolated by Ficoll/Isopaque density gradient centrifugation. Post-partum thymi were obtained from pediatric patients undergoing cardiac surgery. Adult peripheral blood was obtained by venepuncture from a healthy donor; PBMC were isolated by Ficoll/Isopaque density gradient centrifugation.

Characterization of fetal T cells by immunological staining. *TCR V* region usage of fetal T cells was analyzed by double staining for CD3 with Leu4-PE (Becton Dickinson, Mountain View, CA) or OKT3-PE (Ortho Diagnostic Systems, Raritan, NJ) combined with Vß2-, Vß3-, Vß8-, Vß17- and Vß19-specific monoclonal antibodies (mAbs; Immunotech, Marseille, France) and Vß5a-, Vß5b-, Vß5c-, Vß6-, Vß8b-, Vß12-, Vα2- and Vα12-specific mAbs (T cell Sciences, Cambridge, MA), respectively. After washing the cells, we detected membrane-bound unconjugated *TCR V* region-specific mAbs with the FITC-labelled IgG fraction of goat antisera (Nordic Immunological Laboratories, Tilburg, the Netherlands) against the IgG subclass of the particular mAb.

FACS analysis. Stained cells were analyzed with a FACS (FACStar, Becton Dickinson) equipped with an argon ion laser tuned at 300 mW of 488 nm excitation light. The forward light scatter was detected with a photodiode, whereas the orthogonal light scatter and red and green fluorescence

emission signals were detected by photomultiplier tubes. The green and red emission were measured with band-pass type interference filters (530/30; type no. 19-62774-00, and 585/42; type no. 19-62774-03, Becton Dickinson, respectively). A 560 nm dichroic mirror (type no. 19-62772-00, Becton Dickinson) was used to separate spectrally the FITC and the PE emission. All signals were recorded as 256 channel histograms and stored into list-mode using a consort-30 computer program (Becton Dickinson) on a HP-300 computer system (Hewlett Packard). In samples from liver and bone marrow at least 150,000 nucleated cells were analyzed because of the low percentages of $CD3^+$ cells; for the spleen this figure was at least 50,000 nucleated cells. The data obtained using the unconjugated *TCR V* region-specific mAbs followed by mouse IgG subclass-specific conjugate were corrected for background staining by application of the latter antibody only.

Optimal setting of the lymphocyte (and blast) gate for cell suspensions of the different organs was based on the fluorescence pattern obtained after staining of the cells with a cocktail of mAbs against CD45 (Becton Dickinson), CD33 (Becton Dickinson) and Glycophorin A (Immunotech).

Method validation. As the amount of T cells in fetal liver and bone marrow was extremely low, we assessed whether the percentage of T cells expressing the *TCRBV3* family as determined by our assay was influenced by the number of $CD3^+$ T cells in a given sample. We therefore mixed adult PBMC with a $CD3^-$ $CD10^+$ pre-B leukemic cell line in several ratios (100:0, 75:25, 50:50, 25:75). The results demonstrated that, irrespective of the ratio, the percentage of $TCRBV3^+$ T cells within the $CD3^+$ population remained constant. Therefore, detection of this *TCRBV* family was not influenced by the amount of $CD3^+$ T cells present in the sample (data not shown).

RNA extraction, cDNA synthesis, PCR analysis and Southern blotting. RNA was extracted with guanidinium salts using standard laboratory methods (Sambrook et al. 1989) and dissolved into 40 μl of DEPC-treated water. Five μg of RNA was used for oligo dT-primed cDNA synthesis using reverse transcriptase (Riboclone cDNA synthesis system, Promega Corp., Madison, WI). Following phenol extraction, the double-stranded cDNA was dissolved in 100 μl TE. The expression of *TCRAV* and *TCRBV* gene families was analyzed using PCR amplification as previously described by our laboratory (Lambert et al. 1992; van Eggermond et al. 1993; Hawes et al. 1993; Struyk et al. 1993) with minor modifications. Briefly, 0.5 μl cDNA was amplified using 20 pmol *TCRAV* and *TCRBV* specific 5' sense primers, 20 pmol 3' antisense

TCRAC primer or a *TCRBC* primer specific for both *TCRBC1* and *TCRBC2* loci, and 2.5 units *Taq* DNA polymerase (Boehringer, Mannheim, Germany) in the presence of 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 4 mM $MgCl_2$, 0.5 mM of each dNTP, and 0.06 mg/ml BSA in a final volume of 100 μl. PCR cycles consisted of 1 min denaturation at 97° C, 1 min annealing at 54° C and 1 min extension at 74° C in a Bio-Med Thermocycler 60. As the amount of *TCR* message within an organ (liver, bone marrow, gut and cord blood as opposed to thymus and spleen) varied greatly, an internal control reaction for the total amount of *TCR* product detected in each cDNA preparation was included in the experiments. To this end, a PCR reaction was performed using the 3' antisense *TCR C* primer mentioned above and a sense primer specific for the 5' region of the constant gene element. PCR reactions were also performed without template cDNA as a negative control. In order to assess the efficiency of the *TCRAV* and *TCRBV* primers, adult PBMC were included in all experiments. The analysis of the *TCRAV* and *TCRBV* repertoires was repeated at least three times for each organ. The possibility of maternal peripheral blood contamination was excluded by the lack of detection of IgG-encoding transcripts in RNA obtained from the fetal organs (Raaphorst et al. 1992).

In order to maximize the chance of detecting *TCR* rearrangements that might be present at trace levels, the PCR reactions were performed for up to 40 cycles. The sequences of the primers have been described before (Choi et al. 1989; Oksenberg et al. 1990; Lambert et al. 1992; Hawes et al. 1993; Struyk et al. 1993).

Ten μl of the PCR reactions and serial dilutions (undiluted, 5 times and 25 times diluted) of the internal control amplifications were fractionated on a 1% agarose gel in 0.5x tris-borate, blotted onto nylon membranes (Biotrace HP, Gelman Sciences, Ann Arbor, MI) and screened with ^{32}P-labelled probes specific for the *TCRAC* or *TCRBC* region, according to protocols supplied by the manufacturer of the membranes. Autoradiography was performed using Kodak XAR films (Rochester, NY).

RESULTS

FACS analysis of TCR V gene expression in fetal liver, bone marrow and spleen. In Table 1 the results of the FACS analysis of *TCR V* gene usage by human fetal T cells, obtained at 14 (n = 1) and 15 weeks (n=2; fetus 1 and 2) of gestation, are shown. A representative example

of the FACS profiles obtained for detection of *TCR V* gene expression within various fetal tissues is presented in Figure 1. $CD3^+$ T cells expressing the various *TCRAV* and *TCRBV* gene elements were detectable in all fetal tissues analyzed but were, in contrast to spleen, present at extremely low numbers in liver and bone marrow. For instance, in the liver and bone marrow of 14- and 15-week-old fetuses approximately 0.35% to 2.5% of the gated nucleated cells were $CD3^+$ (data not shown). Most *TCRAV* and *TCRBV* gene families tested for were expressed in these fetal organs, albeit at different levels. As shown in Table 1, the degree of expression of these *TCRAV* and *TCRBV* gene families appeared to be subject to individual differences. This is exemplified by the expression level of the TCRBV2 element, which differed when the liver and spleen obtained from two age-matched fetuses (fetus 1 and 2) were compared. Variable patterns of *TCR V*-gene usage could be discerned for other *V* elements analyzed as well.

PCR analysis of TCR V gene expression in thymi. To expand our studies with mAbs, we subsequently determined the expression of *TCRBV* and *TCRAV* elements by PCR. In Figures 2 and 3 the Southern analyses of the expression of *TCRBV* and *TCRAV* families in two human fetal thymi at 17 weeks of gestation (FT1 and FT2) and two post-partum thymi (AT1 and AT2) in comparison to adult PBMC are shown. The majority of the *TCRBV* and the *TCRAV* families were detectable in the post-partum thymi and adult PBMC. The expression level of *TCRBV* and *TCRAV* families in the fetal thymi was subject to more variation: although the frequency of usage of the majority of the multi-member families (*TCRBV1-TCRBV8* and *TCRBV10-TCRBV13*) was comparable in FT1, FT2, AT1 and AT2, the majority of the single-member families (for instance the *TCRBV15-TCRBV18* and the recently identified *TCRAV24-TCRAV29* gene families) appeared to be expressed at a lower level in both fetal thymi.

Table 1. FACS analysis of membrane-expressed *TCR V* gene elements

	V-chain	14 wk fetus	15 wk fetus (1)	15 wk fetus (2)
L	Vß2	5.3	7.5	1.9
	Vß3	4.5	4.3	1.8
	Vß5a	4.3	2.8	2.0
	Vß5b	4.6	2.7	0.8
	Vß5c	6.7	5.3	2.7
	Vß6	5.0	1.8	2.1
	Vß8	7.0	-	2.7
	Vß8b	5.5	-	2.0
	Vß12	7.6	2.5	2.7
	Vß17	0.7	-	0.4
	Vß19	6.5	4.8	1.5
	Vα2	2.5	6.1	1.8
	Vα12	4.1	-	1.4
BM	Vß2	10.9	8.0	7.7
	Vß3	0.0	4.0	7.4
	Vß5a	2.0	-	2.0
	Vß5b	0.7	4.0	5.7
	Vß5c	1.8	8.6	5.0
	Vß6	4.2	4.3	4.0
	Vß8	7.9	-	4.8
	Vß8b	4.5	-	5.5
	Vß12	5.4	-	6.2
	Vß17	0.0	-	5.2
	Vß19	7.2	-	7.4
	Vα2	0.8	-	2.9
	Vα12	3.3	-	5.0
S	Vß2	4.2	5.7	10.2
	Vß3	9.2	3.6	1.1
	Vß5a	3.1	-	2.6
	Vß5b	2.6	0.8	0.9
	Vß5c	6.7	-	3.5
	Vß6	2.5	-	1.3
	Vß8	7.5	-	3.4
	Vß8b	3.2	-	2.3
	Vß12	5.2	-	4.6
	Vß17	0.0	-	0.3
	Vß19	3.4	-	2.8
	Vα2	2.8	-	1.8
	Vα12	4.0	-	1.8

FACS analysis of membrane-expressed *TCR V* gene elements by $CD3^+$ T cells isolated from liver (L), bone marrow (BM) and spleen (S) of a 14-week-old human fetus and two 15-week-old fetuses (Fetus 1 and Fetus 2). Values are expressed as the percentage of $CD3^+$ T cells expressing the indicated *V* chain. - = not done.

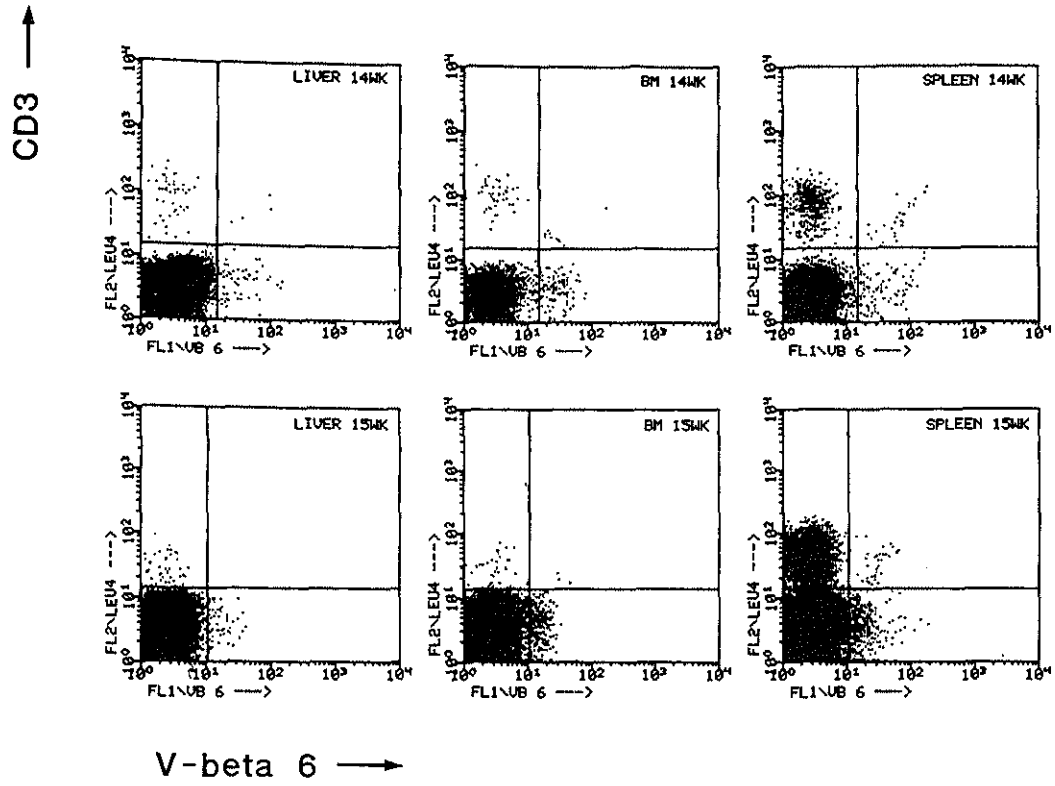


Fig. 1. FACS profiles of the detection of CD3(Leu4)$^+$Vß6$^+$ T cells stained by an FITC-labelled *TCRBV6*-specific mAb in fetal liver, bone marrow (BM) and spleen obtained at 14 and 15 weeks (Fetus 2) of gestation.

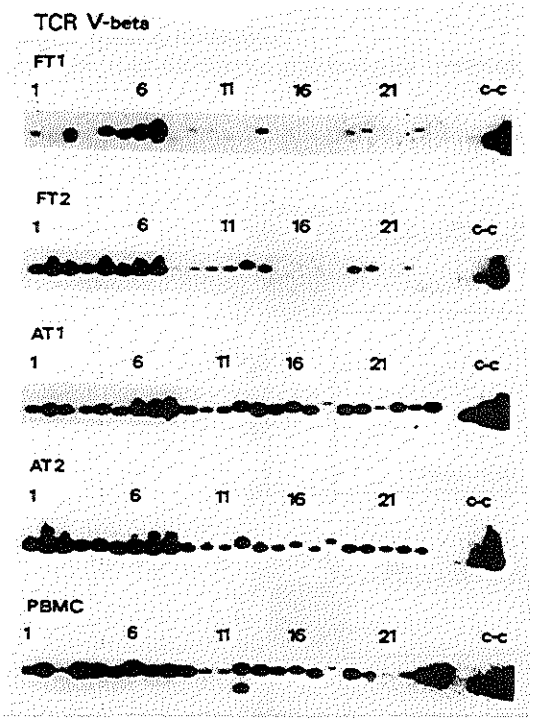


Fig. 2. *TCRBV* gene family repertoire analyzed by PCR in two human fetal thymi (17 weeks of gestation; FT1 and FT2), two post-partum thymi (AT1 and AT2) and adult PBMC (all 26 cycles). C-C is the serially diluted control amplification of the constant locus of the *TCR*. Note: *TCRBV5* rearrangements were detected in two separate PCR reactions, using different primers (*TCRBV5a* and *TCRBV5b*)

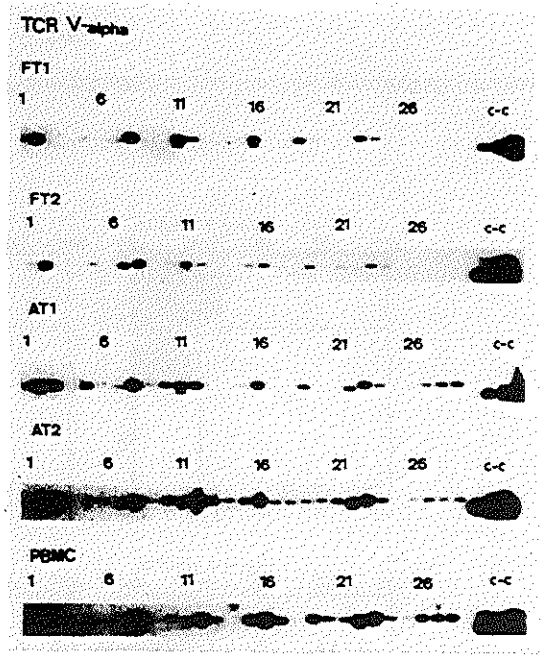


Fig. 3. *TCRAV* repertoire analyzed by PCR following 30 cycles in two human fetal thymi (17 weeks of gestation; FT1 and FT2), two post-partum thymi (AT1 and AT2) and adult PBMC.

PCR analysis of TCR V gene expression in fetal liver, bone marrow, spleen, gut and cord blood. Determination of the *TCRBV* repertoire in the 13-week-old fetus showed that almost all of the *TCRBV* families were detectable in cord blood, gut and spleen, whereas *TCRBV* gene usage appeared to be restricted in liver and bone marrow (Fig. 4). Similarly, most of the *TCRAV* families tested for were readily detectable in the peripheral organs of the 13-week-old fetus with the exception of the *TCRAV24-TCRAV29* single member gene families (Fig. 5). As found in the analysis of *TCRBV* gene families, the level of expression of the various *TCRAV* gene families differed markedly in 13-week-old fetal liver and bone marrow compared to the expression of these *TCRAV* gene families in the periphery. In the 11-week-old fetus, the *TCRBV* gene family usage profiles exhibited a restricted pattern in both hematopoietic and peripheral organs which was most likely related to the low percentage of $CD3^+$ T cells in fetal organs at this gestational age. In addition, *TCRAV* rearrangements were barely detectable in the investigated organs of this fetus (data not shown).

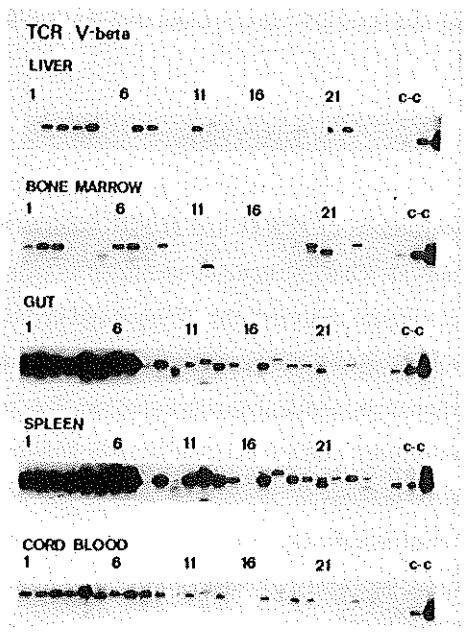


Fig. 4. *TCRBV* repertoire analyzed by PCR in human fetal liver (35 cycles), bone marrow (35 cycles), gut (30 cycles), spleen (30 cycles) and cord blood (35 cycles) obtained from a human fetus at 13 weeks of gestation. Note: *TCRBV5* rearrangements were detected in two separate PCR reactions, using two different primers (*TCRBV5a* and *TCRBV5b*).

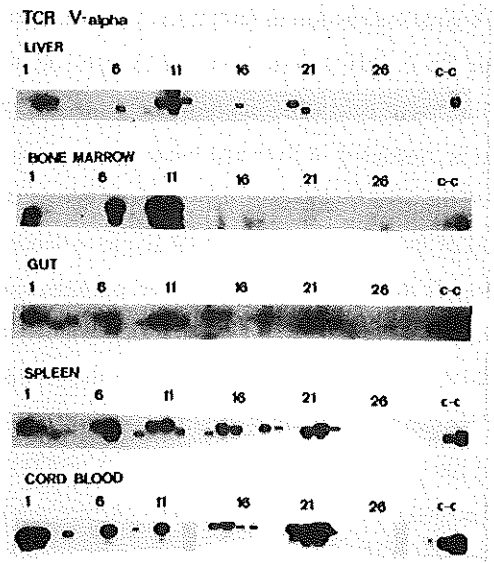


Fig. 5. *TCRAV* repertoire analyzed by PCR in human fetal liver (37 cycles), bone marrow (38 cycles), gut (35 cycles), spleen (35 cycles) and cord blood (35 cycles) obtained from the same fetus at 13 weeks of gestation.

DISCUSSION

PCR analysis of the usage patterns of *TCRAV* and *TCRBV* families by T cells in adult PBMC and post-partum thymi indicated that all families identified to date were detectable in these tissues. In the oldest fetal samples analyzed, the 17-week-old thymi, the majority of the *TCR V* gene families were detectable as well, although some families appeared to be underrepresented in comparison to post-partum thymi and adult peripheral blood. This was most clearly illustrated by considering the single-member *TCRAV* and *TCRBV* gene families. The employment of the various *TCRAV* and *TCRBV* families as detected by PCR in the 13-week-old peripheral fetal tissues (gut, spleen and cord blood) was extensive, and more diverse than the repertoire of the T cells in the corresponding primary hematopoietic organs (liver and bone marrow).

In analogy with mouse fetal T-cell development (Roth et al. 1991) and human fetal B-cell development (Schroeder and Wang 1990; Raaphorst et al. 1992), the apparent restrictions in human *TCRAV* and *TCRBV* family expression could reflect actual preferential usage of *TCR V* gene elements in T-cell precursors derived from liver and bone marrow at early fetal ages. However, considering the more diverse *TCR* repertoire in the periphery of these fetuses, the low number of immature

and mature $CD3^+$ T cells, especially in the hematopoietic tissues, probably lies at the heart of the apparent restricted phenotype of *TCR V* family usage. This was reflected by the number of cycles of PCR required to obtain a comparable level of detection in the various tissues (Figs. 2-5). Also, FACS analysis of the expression of *TCRAV* and *TCRBV* gene products of $CD3^+$ T-cells in 14- and 15-week-old fetal liver, bone marrow and spleen (Fig. 1; Table 1) indicated that the majority of the *TCR V* regions tested for were detectable, although sometimes at an extremely low level. The percentage of staining of $CD3^+$ T cells in fetal tissue for each individual mAb was in a range similar to the staining patterns in PBMC, as previously reported by our group (Lambert et al. 1992; Hawes et al. 1993). The mAb staining profiles suggested that the patterns of expression of *TCR V* families were subject to individual-specific variation, which has recently also been described in several other studies of *TCR V* gene expression in unrelated individuals (Akolkar et al. 1992; Robinson 1992; Rosenberg et al. 1992; Hawes et al. 1993) and T cell subsets (Davey et al. 1991; Grunewald et al. 1992). It is of note that *TCR V* gene family-specific PCR reactions were designed to detect all known members of a given family. Consequently, this may explain why the FACS and PCR analyses were not completely overlapping, because the mAbs used are specific for a single member only.

As the peripheral fetal *TCR V* gene repertoire was extensive both in PCR and FACS analysis, our data show that in early human fetal *TCR* rearrangements *V* elements belonging to the majority of *TCR V* gene families were used by fetal T cells. Recently reported data obtained by other groups support this conclusion: studies of *TCRBV* expression in human fetal thymus and spleen at 15 to 22 weeks of gestation also demonstrated that all of the 20 tested *TCRBV* gene families can be used in *TCR* rearrangements at this stage of fetal development (Doherty et al. 1991; Bonati et al. 1992). In addition, transplantation of 18- to 20-week-old human fetal thymus and liver in SCID-hu mice demonstrated that all investigated *TCRBV* elements were expressed by the repopulating T cells (VandeKerckhove et al. 1992). Furthermore, the relative expression levels of *TCRBV* elements expressed by T cells in the SCID-hu fetal thymus differed in comparison to the *TCRBV* usage patterns of T cells in human post-partum thymi (Doherty et al. 1991; VandeKerckhove et al. 1992), which is compatible with our results.

Taken together, our data indicate that there are no apparent major limitations in the usage of *TCRAV* and *TCRBV* gene families in the fetal and adult tissues analyzed.

ACKNOWLEDGMENTS

We thank Drs R.R.P. de Vries, C.J.M. Melief, J. Henwood and J. Teale for critically reading the manuscript, J.L.M. Waaijer for her assistance in the FACS analyses, H. Tanke for fruitful discussions and H.J. van der Bijl for creating a stimulating environment.

REFERENCES

Abe, J. Immunocytochemical characterization of lymphocyte development in human embryonic and fetal livers. *Clin Immunol Immunopathol 51*: 13-21, 1989

Akolkar, P.N., Gulwani-Akolkar, B., Pergolizzi, R., Bigler, R.D., and Silver, J. Influence of HLA genes on T cell receptor V segment frequencies and expression levels in peripheral blood lymphocytes. *J Immunol 150*: 2761-2773, 1992

Asma, G.E.M., Langlois van den Bergh, R., and Vossen, J.M. Use of monoclonal antibodies in a study of the development of T lymphocytes in the human fetus. *Clin Exp Immunol 53*: 429-436, 1983

Asma, G.E.M., Langlois van den Bergh, R., and Vossen, J.M. Development of pre-B and B-lymphocytes in the human fetus. *Clin Exp Immunol 56*: 407-414, 1984

Bonati, A., Zanelli, P., Ferrari, S., Plebani, A., Starcich, B., Savi, M., and Neri, T.M. T-cell receptor ß chain gene rearrangement and expression during human thymic ontogenesis. *Blood 79*: 1472-1483, 1992

Born, W., Yagüe, J., Palmer, E., Kappler, J., and Marrack, P. Rearrangement of T cell receptor ß chain genes during T-cell development. *Proc Natl Acad Sci USA 82*: 2925-2929, 1985

Brenner, M.B., McLean, J., Dialynas, D.P., Strominger, J.L., Smith, J.A., Owen, F.L., Seidman, J.G., Ip, S., Rosen, S., and Krangel, M.S. Identification of a putative second T cell receptor. *Nature 322*: 145-149, 1986

Brenner, M.B., McLean, J., Scheft, H., Warnke, R.A., Jores, N., and Strominger, J.L. Characterization and expression of human α/ß T cell receptor by using a framework monoclonal antibody. *J Immunol 138*: 1502-1509, 1987

Campana, D., Janossy, G., Coustan-Smith, E., Amlot, P.L., Tian, W.-T., Ip, S., and Wong, L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. *J Immunol 142*: 57-66, 1989

Choi, Y., Kotzin, B., Herron, L., Callahan, J., Marrack, P., and Kappler, J. Interaction of *Staphylococcus aureus* toxin "superantigens" with human T-cells. *Proc Natl Acad Sci USA 86*: 8941-8945, 1989

Concannon, P., Pickering, L.A., Kung, P., and Hood, L. Diversity and structure of human T cell receptor ß chain variable genes. *Proc Natl Acad Sci USA 83*: 6598-6602, 1986

Davey, M.P., Meyer, M.M., Munkirs, D.D., Babcock, D., Braun, M.P., Hayden, J.B., and Bakke, A.C. T-cell receptor variable region ß chain genes show differential expression in CD4 and CD8 cells. *Human Immunol 32*: 194-202, 1991

Doherty, P.J., Roifman, C.M., Pan, S., Cymerman, U., Ho, S.K.W., Thompson, E., Kamel-Reid, S., and Cohen, A. Expression of the human T cell receptor Vß repertoire. *Mol Immunol 28*: 607-612, 1991

Elliot, J.F., Rock, E.P., Patten, P.A., Davis, M.M., and Chien, Y.H. The adult T cell receptor delta-chain is diverse and distinct from that of fetal thymocytes. *Nature 331*: 627-631, 1988

Ferradini, L., Roman-Roman, S., Azocar, J., Michalaki, H., Triebel, F., and Hercend, T. Studies on the human T cell receptor α/ß variable region genes. II. Identification of four additional Vß subfamilies. *Eur J Immunol 21*: 935-942, 1991

Furley, A.J., Mitzutani, S., Weilbaecher, K., Dhaliwal, H.S., Ford, A.M., Chan, L.C., Molgaard, H.V., Toyonaga, B., Mak, T.W., Elsen, P.J. van den, Gold, D., Terhorst, C., and Greaves, M.F. Developmentally regulated rearrangement and expression of genes encoding the T-cell receptor-T3 complex. *Cell 46*: 75-87, 1986

Gale, R.P. Development of the immune system in human fetal liver. *Thymus 10*: 45-56, 1987

Grunewald, J., Jeddi-Tehrani, M., Pisa, E., Janson, C.H., Andersson, R., and Wigzell, H. Analysis of Jß gene segment usage by $CD4^+$ and $CD8^+$ human peripheral blood T lymphocytes. *Int Immunol 4*: 643-650, 1992

Hawes, G.E., Struyk, L., and Elsen, P.J. van den. Differential usage of T-cell receptor V-gene segments in $CD4^+$ and $CD8^+$ subsets of T lymphocytes in monozygotic twins. *J Immunol 150*: 2033-2045, 1993

Haynes, B.F., Singer, K.H., Denning, S.M., and Martin, M.E. Analysis of expression of CD2, CD3 and T cell antigen receptor molecules during early human fetal thymic development. *J Immunol 141*: 3776-3784, 1988

Ito, K., Bonneville, M., Takagaki, Y., and Tonegawa, S. Different $\gamma\delta$ receptors are expressed on thymocytes at different stages of development. *Proc Natl Acad Sci USA 86*: 631-635, 1989

Kimura, N., Toyonaga, B., Yoshikai, Y., Triebel, F., Debre, P., Minden, P.D., and Mak, T.W. Sequences and repertoire of human T cell receptor α and ß chain variable region genes in thymocytes. *Eur J Immunol 17*: 375-383, 1987

Krangel, M.S., Yssel, H., Brocklehurst, C., and Spits, H. A distinct wave of human T cell receptor $\gamma\delta$ lymphocytes in the early fetal thymus: evidence for controlled gene rearrangement and cytokine production. *J Exp Med 172*: 847-859, 1990

Lafaille, J.J., DeCloux, A., Bonneville, M., Takagaki, Y., and Tonegawa, S. Junctional sequences of T cell receptor $\gamma\delta$ genes: implications for $\gamma\delta$ cell lineages and for a novel intermediate of V-(D)-J joining. *Cell 59*: 859-870, 1989

Lambert, M., Eggermond, M. van, Mascart, F., DuPont, E., and Elsen, P.J. van den. TCR Vα- and Vß-gene segment use in T-cell subcultures derived from a type III bare lymphocyte syndrome patient deficient in MHC class II expression. *Dev Immunol 2*: 227-236, 1992

Moore, K.L. The developing human. Clinically oriented embryology, 4th edition, W.B. Saunders Company, Philadelphia, 1988

Oksenberg, J.R., Stuart, S., Begovich, A.B., Bell, R., Erlich, H.A., Steinman, L., and Bernard, C.C.A. Limited heterogeneity of rearranged T-cell receptor Vα transcripts in brains of multiple sclerosis patients. *Nature 345*: 344-346, 1990

Plaza, A., Kono, D.H., and Theofilopoulos, A.N. New human Vß genes and ploymorphic variants. *J Immunol 147*: 4360-4365, 1991

Raaphorst, F.M., Timmers, E., Kenter, M., Tol, M.J.D. van, Vossen, J. M., and Schuurman, R.K.B. Preferential utilization of germ-line V_H3 elements and short diverse third complementarity determining (CDR3) regions in human fetal B lymphocyte immunoglobulin heavy chain rearrangements. *Eur J Immunol 22*: 247-251, 1992

Robinson, M.A. The human T cell receptor ß-chain complex contains at least 57 variable gene segments. *J Immunol 146*: 4392-4397, 1991

Robinson, M.A. Usage of human T cell receptor Vß, Jß, Cß and Vα gene segments is not proportional to gene number. *Human Immunol 35*: 60-67, 1992

Roman-Roman, S., Ferradini, L., Azocar, J., Genevee, C., Hercend, T., and Triebel, F. Studies on the human T cell receptor α/ß variable region genes I. Identification of seven additional Vα subfamilies and 14 Jα gene segments. *Eur J Immunol 21*: 927-933, 1991

Rosenberg, W.M.C., Moss, P.A.H., and Bell, J.I. Variation in human T-cell receptor Vß and Jß repertoire: analysis using anchor polymerase chain reaction. *Eur J Immunol 22*: 541-549, 1992

Roth, M.E., Holman, P.O., and Kranz, D.M. Non-random use of Jα gene segments. Influence of Vα and Jα localization. *J Immunol 147*: 1075-1081, 1991

Sambrook, J., Fritsch, E.F., and Maniatis, T. *Molecular cloning, a laboratory manual*, 3rd edition. Cold Spring Harbor Laboratory Press, New York, 1989

Samelson, L.E., Lindsten, T., Fowlkes, B.J., Elsen, P.J. van den, Terhorst, C., Davis, M.M., Germain, R.N., and Schwartz, R.H. Expression of genes of the T cell antigen receptor complex in precursor thymocytes. *Nature 315*: 765-768, 1985

Sánchez, M.J., Gutiérrez-Ramos, J.C., Fernández, E., Leonardo, E., Lozano, J., Martínez-A, C., and Toribio, M.L. Putative pre-thymic T cell precursors within the early human embryonic liver: a molecular and functional analysis. *J Exp Med 177*: 19-33, 1993

Schatz, D.G., Oettinger, M.A., and Schlissel, M.S. V(D)J recombination: molecular biology and regulation. *Annu Rev Immunol 10*: 359-383, 1992

Schroeder, H.W., Jr., and Wang, J.Y. Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. *Proc Natl Acad Sci USA 87*: 6146-6150, 1990

Strominger, J.L. Developmental biology of T cell receptors. *Science 244*: 943-950, 1989

Struyk, L., Kurnick, J.T., Hawes, G.E., Laar, J.M. van, Schipper, R., Oksenberg, J.R., Steinman, L., Vries, R.R.P. de, Breedveld, F., and Elsen, P.J. van den. TCR V-gene usage in synovial fluid lymphocytes of patients with chronic arthritis. *Human Immunol*, 1993, in press

VandeKerckhove, B.A.E., Baccala, R., Jones, D., Kono, D.H., Theofilopoulos, A.N., and Roncarolo, M.-G. Thymic selection of human T cell receptor Vß repertoire in SCID-hu mice. *J Exp Med 176*: 1619-1624, 1992

Van der Stoep, N., Krijger, R. de, Bruining, J., Koning, F., and Elsen, P.J. van den. Analysis of early fetal T-cell receptor δ chain in humans. *Immunogenetics 32*: 331-336, 1990

Van Dongen, J.J.M., Quertermous, T., Bartram, C.R., Gold, D.P., Wolvers-Tetteroo, I.L.M., Comans-Bitter, W.M., Hooijkaas, H., Adriaansen, H.J., Klein, A. de, Raghavachar, A., Ganser, A., Duby, A.D., Seidman, J.G., Elsen, P.J. van den, and Terhorst, C. T-cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA and cell membrane level. *J Immunol 138*: 1260-1269, 1987

Van Eggermond, M.C.J.A., Rijkers, G.T., Kuis, W., Zegers, B.J.M., and Elsen, P.J. van den. T cell development in a major histocompatibility complex class II-deficient patient. *Eur J Immunol 23*: 2585-2591, 1993

Wilson, R.K., Lai, E., Concannon, P., Barth, R.K., and Hood, L.E. Structure, organization and polymorphism of murine and human T-cell receptor alpha and beta chain families. *Immunol Rev 101*: 149-172, 1988

Chapter 8. General discussion and future perspectives.

This thesis aims at increasing the understanding of endocrine cell development in the human fetal pancreas. Firstly, identification of islet stem cells allows attempts for in vitro replication and multiplication of islet cells. Eventually, this could lead to transplantation of cells in type-I diabetic patients. Secondly, ß cell (auto)antigen expression during fetal development seems important in the pathogenesis of type-I diabetes mellitus, in which these cells are selectively destroyed by the immune system, possibly resulting from defective (fetal) tolerance development. Coexpression of insulin, glucagon, and somatostatin within one cell occurs between 12 and 18 weeks gestation but not between 22 and 40 weeks gestation. Different combinations of these hormones are present in ultrastructurally distinct secretory granules (Chapter 3). Hormone containing cells do not proliferate. Subsequently, neuroendocrine markers have been analyzed for their presence in replicating cells. Two markers, N1 and HNK-1, are expressed on DNA-synthesizing cells, indicating that these cells may be endocrine precursor cells. In addition, early phenotypic differentiation of islet δ cells is suggested by the presence of HNK-1 on proliferating cells and on δ cells (Chapter 4). N1 is described in Chapter 5 as a new monoclonal antibody to an unknown surface determinant of islet cells. Because of its surface expression, N1 was used in the separation of endocrine from non-endocrine cells in pancreatic single cell suspensions, prepared by a collagenase digestion technique.

Islet cell antibody (ICA) reactivity was investigated in the human fetal pancreas. Targets of type-I diabetes mellitus related humoral autoimmunity were found to be present in midgestational human fetal islet cells. The heterogeneous findings obtained with these polyclonal autoantibodies suggest that interpancreatic differences exist in fetal antigen expression (Chapter 6). In the following sections, the results presented in this thesis will be discussed. Future perspectives will be concentrated on two topics: 1) further analysis of islet cell interrelationships and identification of islet stem cells; 2) the immunological relevance of fetal islet antigen expression for type-I diabetes.

*** islet cell interrelationships.**

The present finding of all combinations of coexpression of insulin, glucagon, and somatostatin, even triple expression, suggests a common ancestry for islet α, ß, and δ cells. This is in accordance with earlier findings in the embryonal mouse pancreas, in which sequential activation of glucagon, insulin, somatostatin, and pancreatic polypeptide (PP) protein expression respectively was suggested (Alpert et al., 1988). Another study found that PP was the earliest expressed hormone during mouse development (Herrera et al., 1991). This has been shown, however, to result from antibody crossreactivity between PP and neuropeptide Y (NPY) (Teitelman et al., 1993). However, peptide YY, an intestinal hormone which has been localized to α cells, was recently detected in all four islet cell types as they emerge during mouse embryogenesis (Upchurch et al., 1994). Peptide YY shares 50% and 70% amino acid identity with PP and NPY respectively. By absorption studies of antisera with relevant or irrelevant antigens it was shown that the PP and NPY reactivity observed in the earlier studies in fact was peptide YY reactivity. These data contain two important implications: 1) the occurrence of peptide YY in all islet cell types supports the idea of a common ancestor cell, which may express peptide YY; 2) the presence of an intestinal hormone, peptide YY, in islet cells suggests studies to further investigate similarities between islet cells and other cells of the gastrointestinal tract.

The abovementioned studies have all been performed by double immunohistochemistry (IHC). Such studies have their limitations: 1) a sufficient amount of protein must be present in order to be detected; 2) antibody crossreactivity must be carefully checked by absorption studies and may not always be eliminated. Detection of mRNA by reverse transcriptase-PCR (RT-PCR) is superior with regard to sensitivity and specificity, but does not allow conclusions about the localization of expression. Using this technique, hormone gene expression of insulin and glucagon appeared simultaneously (Gittes and Rutter, 1992). In situ hybridization (ISH) for the detection of mRNA, though less sensitive than RT-PCR, does allow the analysis of positive or negative cells in their histological context, and may be combined with IHC. We performed double ISH and a combination of ISH and IHC for insulin, glucagon, and somatostatin. The preliminary results show that hormone coexpression also occurs at the mRNA level, confirming a presumed common ancestry for α, ß, and δ cells (**FIGURE 6**). Hormone mRNA expression may be found without (probably prior to) protein expression. To observe if translation to the corresponding protein occurs ISH for the detection of mRNA should always be combined with IHC for the detection of proteins.

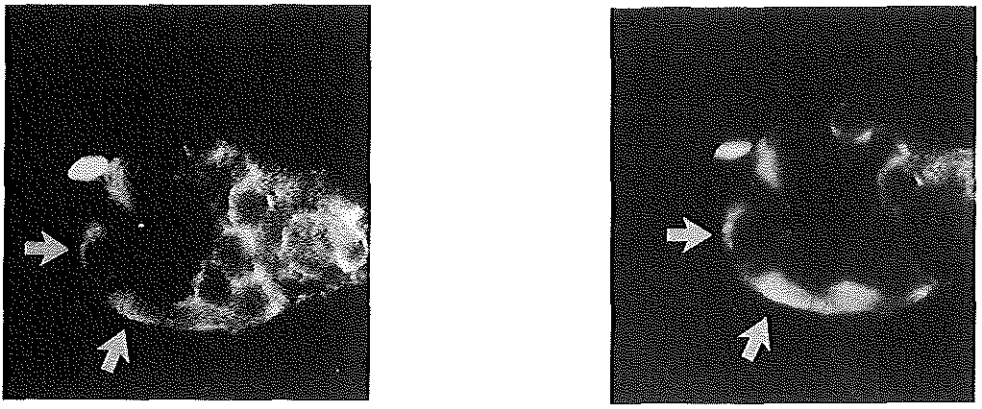


Figure 6. Double in situ hybridization, using biotin and digoxygenin-labeled probes against insulin (left panel) and glucagon (right panel). The arrows point at cells that contain both insulin and glucagon mRNA. Cells containing insulin only can also be observed.

In Chapter 4 islet cell interrelationships were analyzed using neuroendocrine proteins as markers. The concept of using neuroendocrine markers has been applied before (Teitelman et al. 1987, 1993, Upchurch et al., 1994). Tyrosine hydroxylase (TH), NPY, and peptide YY were detected in all islet cell types. This suggested that islet cells may arise from a common progenitor, which expresses one or more of these neuroendocrine marker proteins. Because all islet cells were stained, no conclusions could be drawn about the phenotypic differentiation of the islet cell types. Similarly, N1, present in α, ß, and δ cells, may be a candidate islet progenitor cell protein. The selective presence of HNK-1 in proliferating cells and δ cells in the human fetal pancreas suggests a partial δ cell differentiation early in ontogeny. The HNK-1 epitope has been described on several nervous system specific molecules, including isoforms of neural cell adhesion molecules (NCAMs), a matrix protein of neurosecretory granules, and an acidic glycolipid in human peripheral nerves (Kruse et al., 1984, Tischler et al., 1986, Chou et al., 1985). It also stains a subpopulation of GABAergic neurons in the rat cerebral cortex (Kosaka et al., 1990). Both adhesion molecules and GABA production have been shown in islet cells. It will be of interest to identify which protein(s) is (are) the HNK-1-target on δ cells. In **FIGURE 7** a developmental scheme is proposed, based on the findings described in Chapters 3 and 4. In conclusion, neuroendocrine and gastrointestinal marker proteins allow detection of islet cell interrelationships. Protein or mRNA expression of such markers in **all** islet cell types supports

the idea of a common ancestry for all islet cells. Thus, these markers may also be present in proliferating islet progenitor cells. To prove a relationship between proliferating progenitor cells and islet cells, both expressing neuroendocrine or gastrointestinal marker proteins, culture experiments and in vivo analyses are needed (see next section: "islet precursor cells"). **Differential** expression of markers in islet cells indicates differentiation to a specialized islet cell phenotype (α, ß, δ, or PP cell). The occurrence of differentially expressed markers may be taken as a sign of phenotypic differentiation of a cell to one of the islet cell types. The onset of expression of such markers in relation to proliferation or to the onset of hormone expression may reveal how early in ontogeny these events occur. Again, culture experiments and in vivo analyses (in transgenic mice) are needed.

In the following paragraphs, two groups of marker molecules, transcription regulation factors and adhesion molecules, are described that may aid in the elucidation of islet cell interrelationships, both in terms of a common ancestry and in terms of phenotypic differentiation. For both groups of molecules, studies have indicated their common and/or differential and early expression in islet cells.

For the control of cell type specific hormone expression, specific transcription regulatory proteins and corresponding regulatory sequences are needed. A region of 350 base pairs of 5' flanking DNA of the rat insulin I gene has been shown to contain several regulatory sequences which influence insulin gene transcription in cell lines and transgenic mice (Edlund et al., 1985, Walker et al., 1983, Alpert et al., 1988). More recently, islet cell type specific transcription regulation factors have been identified. An insulin enhancer binding factor (IEF2) and an insulin promotor binding factor (IPF1) are present in nuclear extracts of ßTC1 cells, a transgenically derived insulin-producing ß-cell line (Efrat et al., 1988a), and of other insulin-producing cell lines (Ohlsson et al., 1991), but not in nuclear extracts of αTC1 cells, a transgenically derived glucagon-producing cell line (Efrat et al., 1988b). IPF1 is also expressed in adult mouse ß cells (Ohlsson et al., 1993). Thus, IEF2 and IPF1 may be regarded ß cell specific, in contrast to another insulin enhancer binding factor (IEF1), which is also expressed in αTC1 cells (Ohlsson et al., 1991). The homeodomain-containing LIM protein Isl-1 (Karlsson et al., 1990) is found in all four islet cell types, in endocrine pancreatic tumors, and some other rat tissues (Dong et al., 1991). All results derive from the study of rodent ß cells or cell lines.

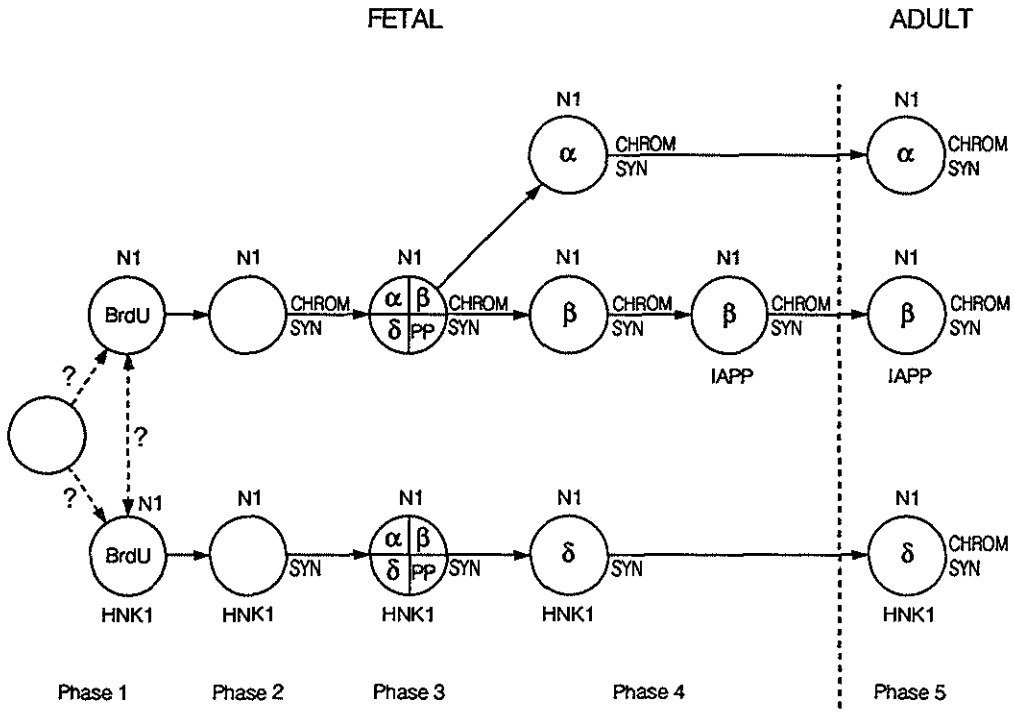


Figure 7. Proposed developmental pathway of human fetal islet cells, based on the findings presented in Chapters 3 and 4. At the very left of the scheme a common stem cell is suggested to give rise to at least two groups of proliferating precursor cells: N1 cells, and HNK-1/N1 cells. Islet δ cells develop from HNK-1/N1 precursor cells, but may express multiple hormones before hormone expression becomes limited to somatostatin. The expression of chromogranin-A starts after 18 weeks gestation in these cells. N1 cells give rise α and ß cells. Again, multiple hormone expression may occur first. Islet ß cells are also characterized by the onset of IAPP expression from 12 weeks gestation onwards. The methods used in our experimental work do not allow to analyze the transition between the different proposed cell types.

Apparently, both general and islet cell type specific factors exist. It is likely that the cellular profile of transcription regulation factors will determine the phenotype of a cell. This hypothesis may be tested by using the available probes and antisera to general and specific islet transcription regulation factors in human fetal islet cells. It is conceivable that these experiments will allow further insight into the events of phenotypic islet cell development and differentiation of islet cell types. For example, IPF1 is found in the ventral and dorsal wall of the primitive foregut of 13 somite mouse embryos (around day E8.5). This suggests ß cell commitment of precursor cells before pancreas morphogenesis occurs. The detection of peptide sequences of unknown transcription regulation factors, which are present in low copy numbers, may be facilitated by ultrasensitive microsequencing techniques, which are currently developed (Hood, 1994). By this technique 5 femtomoles of peptide are sufficient to analyze its amino acid sequence. This enables the identification of factors involved in early developmental stages.
The simultaneous regulation of gene sets may be studied by the analysis of similar or identical DNA sequences. In the rat insulin-I, glucagon, and somatostatin genes, a common enhancer region binding islet cell-type specific transcription factors has been described (Knepel et al., 1991). By combining regulatory sequence information of a series of genes and target sequences of transcription regulation factors, the hypothesis that certain genes are co-regulated may be tested. Clearly, this will be a very complex area of investigation.

Cell adhesion molecules are thought to be involved in (fetal) islet development, because of the non-random distribution of the different islet cell types within an islet. When adult rat islets are dispersed into single cells, they spontaneously form aggregates with an identical three-dimensional architecture as the original islets (Halban et al., 1987). From this study it was concluded that even adult islet cells possess the information necessary to obtain an appropriate spatial orientation. The 135 kD form of the neural cell adhesion molecule (N-CAM) is expressed in neonatal and adult rat islet cells, with higher expression on non-ß-cells than on ß cells (Möller et al., 1992). The differential expression of N-CAM suggests a role in the calcium-independent aggregation of islet cells and in the formation of a mantle of non-ß-cells (Rouiller et al., 1990, 1991). Cadherins are a family of calcium dependent cell adhesion molecules that play an important role in cellular differentiation and organ morphogenesis. Three cadherins, E-CAD, N-CAD, and R-CAD, have been detected on pancreatic islet cells. E-CAD is found on all islet cells as well as on exocrine cells and it is thus less likely to play a role in the formation or morphological

arrangement of islet cells (Möller et al., 1992). The islet specificity of N-CAD and R-CAD suggests a role in the interaction and communication of the different islet cell types (Hutton et al., 1993). It has been hypothesized that N-CAD and R-CAD, expressed both in the retina and in pancreatic islets, are required for the organization of a heterogeneous mass of cells into a highly ordered structure (Inuzuka et al., 1991). In mice, the onset of mRNA expression of N-CAD and R-CAD is around day E13, while the onset of hormone mRNA expression in mouse embryos is at day E9 (Gittes and Rutter, 1992). Thus, these molecules may be involved in the formation of islets or in the terminal differentiation of islet precursor cells. In humans, fetal cell adhesion molecule expression has not been studied. The expression of these molecules may provide evidence for islet cell precursors or for differentiation into phenotypically different islet cells. By the introduction of genes for adhesion factors under control of hormone gene promotors in transgenic mice, the importance of each adhesion molecule on three-dimensional islet development may be studied.

*** endocrine stem cells.**

A second issue of the studies in Chapters 3 and 4 is the identification of potential endocrine precursor cells. The rapid increase of islet cells during human fetal development indicates that endocrine stem cells and precursor cells are present in this tissue. Recognition of such cells allows attempts to regulate their reproduction in vitro. This, in turn, offers therapeutic possibilities in cases where ß cells are over- or underrepresented.

Proliferation was not detected in human fetal endocrine hormone containing cells, whether they express a single or multiple hormones (Chapter 3). This is in accordance with studies in adult rat ß cells (De Vroede et al., 1990), but not with studies in newborn rat ß cells, in which a low percentage of replicating cells is found under basal conditions (Nielsen et al., 1989). In midgestational fetal pig islets 0.4-6 per thousand cells proliferate (Andersson, personal communication). At least, ß cell replicatory potential is severely limited after the onset of hormone expression, and can only be stimulated by the appropriate growth factors (see Chapter 1). For example, normal mouse islets, devoid of stem cells, show a 4-fold increase in cell number when transplanted in obese hyperglycaemic hyperinsulinaemic mice. This occurs only when both hyperinsulinaemia and hyperglycaemia are present and is also dependent on the genetic

background of the obese mouse (Andersson, personal communication).
In the human fetal pancreas, multiple hormone expression does not result in the identification of potential endocrine precursor cells. However, two neuroendocrine markers, N1 and HNK1, colocalize with BrdU, a marker of cell replication. We suggest that these cells are candidate endocrine precursor cells, since N1 and HNK1 continue to be expressed on hormone containing cells. Another neuroendocrine marker, TH, is also found in proliferating cells in mouse embryos, but adult TH cells did not proliferate (Teitelman and Lee, 1987, Teitelman et al., 1988). The similarities in protein expression pattern between neuronal cells and islet cells indicate that other neuroendocrine marker proteins may be succesfully employed in the analysis of islet stem cells. Other potential islet stem cell markers may derive from the ductal origin of islet cells and from the similarities with gastrointestinal hormone producing cells. An adult rat ductal epithelial marker, cytokeratin 20 (CK20), identifies proliferating cells in the periphery of fetal rat islets. In addition, CK20 is present in fetal rat α, ß, and δ cells, suggesting that proliferating CK20 cells give rise to hormone expressing islet cells. Thus, CK20 may identify islet precursor cells in the fetal rat pancreas (Bouwens, personal communication).

Transcription regulating proteins may also be of help in the identification of precursor cells, because they are among the earliest proteins that determine cell type specific differentiation (see above). However, such cells may have limited proliferative potential, because they are committed to become an endocrine cell.
Identification of stem cells is a first step. For the in vitro maintenance of endocrine stem cells, knowledge of growth factors and growth factor receptors that govern cell type specific proliferation and differentiation is essential. Only limited immunohistochemical information is available about growth factors and their receptors in the human fetal pancreas (Chapter 1). They may be important in the formation of individual islets and in the determination of the ß cell reserve. Influence on growth factor promoted endocrine cell growth may be exerted by genetic and environmental factors. A link between malnutrition during pregnancy and ß cell mass (Van Assche and Aerts, 1979) and between birth weight and glucose intolerance (Hales et al., 1991) has been established in clinical studies. Knowledge of fetal endocrine growth factors and their receptors allows testing of these factors in culture experiments. In preliminary experiments, cytoplasmic TGFα reactivity is seen in human fetal pancreatic cell suspensions. TGFα is coexpressed in glucagon containing cells. EGF and TGFß-3 are not found in human fetal islet cells. EGF

and NGF receptors (NGFr) are found on a proportion of human fetal pancreatic cells. NGFr positive cells are located peripheral to the insulin positive core of endocrine cell clusters. This localization shows similarities with the pattern of HNK1 positive cells and suggests coexpression of NGFr, HNK1 and somatostatin. The low affinity NGFr is shown to be involved in apoptosis (Rabizadeh et al., 1993). Therefore, part of the abundant fetal δ cells may be subject to apoptosis during fetal development. From these preliminary findings it seems likely that several growth factors (TGFα, NGF) and their respective receptors (EGF, NGF) play a role in midgestational islet cell development (De Krijger et al., unpublished observations). Recently, it has been reported that fetal rat islets and ß cell lines express the high affinity NGF receptor (TRK-A) on their surface (Scharfmann et al., 1993). In 16 and 21 day old fetal rat islets, none of the α or ß cells express this receptor, but around the islets and in the wall of ductules numerous TRK-A positive cells were observed. Also, such peripheral cells expressed NGF. During a 6 day culture period of fetal rat islets an increasing proportion of the cells became insulin and TRK-A positive. This apparent maturation could be blocked by the addition of a tyrosine kinase inhibitor that blocked the TRK-A receptor but not by an anti-NGF antibody (Kanaka and Scharfmann, personal communication). These findings confirm that NGF and its receptor may play an important role in the development of islet stem cells to differentiated islet cells.

In conclusion, the protein and mRNA expression of factors involved in fetal cell growth and differentiation must be studied by immunohistochemistry and in situ hybridization. Subsequently, growth factors should be added in cell culture to test their effect in a dynamic system. In vitro culture experiments can prove the capability of endocrine stem cells in fetal pancreatic cell suspensions to self-renewal and to differentiation into islet cells. Neuroendocrine and gastrointestinal marker proteins are candidates to monitor these events.

In Chapter 5, a dispersion protocol is described to obtain human fetal pancreatic single cell suspensions. Any marker protein expressed on the surface of islet cells or of potential progenitor cells (e.g. N1) can be used to select this cell population by fluorescence activated cell sorting (see Chapter 5). Preliminary culture experiments of N1 positive cell suspensions have been performed, but fibroblast overgrowth constituted a major problem in culturing fetal endocrine cells. Cells were cultured in DMEM or medium 199, supplemented with 10% FCS at a density of 1-1.5 x 10^5 cells/well in wells coated with bovine cornea endothelial cell matrix. The viability decreased in both cases from 66 $\pm$ 10% immediately after cell sorting (= day 0), to 45 $\pm$

12% on day 1, and 32 ± 6% on day 2 (n=5). Cell coating did not have a beneficial effect. When DMEM was diluted 1:1 with medium conditioned by ßTC3 cells, viability at day 2 increased. Recently, HIT cell (a transformed hamster ß cell line) culture medium was succesfully used for the culture of ßTC1 cells. In conclusion, further efforts are needed to obtain a culture system for sorted human fetal cell suspensions. As outlined above and in the previous section, islet cell interrelationships, including islet progenitor cells, must be investigated in a dynamic cell culture system. Two obstacles should be overcome. First, fibroblast overgrowth should be slowed down by fibroblast-selective drugs, such as 2-deoxyglucose. Second, cell viability should be increased by the addition of conditioned media of ß cell lines, or by the addition of relevant growth factors. Cell suspensions labelled with surface monoclonal antibodies (such as N1 or HNK-1) may then be cultured after fluorescence activated cell sorting. The effect of differing concentrations of growth factors on the proliferative fraction, on islet cell phenotype and on expression of genes may then be evaluated.

Data from the literature and the work presented in this thesis allow several conclusions to be drawn for the future study of endocrine pancreatic growth and differentiation. First, the tissue substrate used is important. Results obtained with ß (or α) cell lines must be interpreted with caution, because of the effect of transformation on the ß cell specific machinery and resulting protein expression. For the study of growth and differentiation **fetal** pancreatic tissue is indispensable. Fetal mouse, rat and pig islets are available in sufficient quantities. In relation to human disease, such as type-I diabetes mellitus, human fetal tissue is preferable, but not always available. It is important to note that rat and mouse are born after 19 and 21 days respectively in a much more immature condition than humans. In addition, in rodents islet cell development seems to occur in a limited period of time, whereas in the human fetal pancreas repetitive differentiation from precursor cells can be observed throughout the entire 12-18 week period. This must be taken into account when comparing data from rodents with those from human studies. Transgenic and knock-out mouse studies (e.g. the introduction of transcription regulation factor or growth factor (receptor) transgenes under control of endocrine hormone promotors or the deletion of such genes on islet cell development) may yield important results. In the past, however, such studies sometimes had unexpected outcomes, possibly through unknown compensation mechanisms, and left the investigators with more questions than answers.

Second, the picture emerging for islet cell development is one of a complex interaction of growth factors and their receptors. The effects of growth factors may depend on their concentration, but also on the concentration of their receptors (Nielsen, personal communication). Thus, carefully controlled experiments, introducing not more than one variable, are needed to dissect the exact role of each component in islet growth.
Finally, similarities of islet cells with neuronal cells and with other gastrointestinal cells should be kept in mind in the design of experiments. For example, expression of the hepatocyte growth factor (HGF) has been shown in adult rat α cells and in mouse embryos (Svensson, personal communication). Also, islets transplanted in the liver or under the kidney capsule proliferate after partial hepatectomy or nephrectomy, along with hepatocytes and renal cells respectively. Native pancreatic islets do not proliferate under these conditions. This indicates that islet cells respond to local factors which induce adaptive proliferation.

*** human fetal development in relation to type-I diabetes mellitus.**

In Chapter 2 a number of studies has been presented which indicate that the development of the fetal immune system and the fetal pancreas may be important for the pathogenesis of type-I diabetes mellitus. The first question to be answered for the human fetal pancreas is: are targets of autoimmune destruction, few of which have been characterized at the molecular level, present at this stage of development? This question may be answered by analyzing the reactivity of autoantibodies of type-I diabetic patients (or the reactivity of monoclonal antibodies in the case of GAD) and of autoreactive T cells to fetal islet cells.
In Chapter 6, the reactivity of ICA to islet cells in the human fetal pancreas is shown. It is suggested that multiple target molecules are found during fetal development and that there may be autoantigenic differences between fetal pancreata. However, ICA positive sera are polyclonal, and only one ICA target (glutamic acid decarboxylase (GAD), see below) has been characterized at the molecular level. Two things can be learned from this study: 1) targets of humoral autoimmunity in type-I diabetes are found at this stage of fetal islet cell development; 2) it is important to work with molecularly defined antigens and with monoclonal antibodies instead of with polyclonal patient sera responding to a diverse array of proteins.
The best example of a molecularly defined antigen is GAD, a target of autoimmune destruction

in type-I diabetes, of which protein and DNA sequences are known (Baekkeskov et al., 1990). GAD is encoded for by two non-allelic genes, resulting in the production of GAD65 and GAD67 (Erlander et al., 1991). Both isoforms are involved in the production of gamma-amino butyric acid (GABA), a major inhibitory neurotransmitter. Autoantibodies to GAD65 are found in the majority of recent onset diabetic patients (Atkinson et al., 1990). T-cell autoimmunity against GAD has been detected in NOD mice (Kaufman et al., 1993, Tisch et al., 1993). This indicates that GAD may be a pathogenetically relevant target, because ß cell destruction is thought to be caused by cellular autoimmunity. In human adult islets only GAD65 is found (Petersen et al., 1993, Kim et al., 1993). Preliminary experiments in human fetal islets confirm the presence of GAD65 between 12 and 18 weeks gestation. The majority of δ cells and approximately half of the α and ß cells show GAD reactivity, in contrast to adult islets, in which the majority of ß cells and few other islet cells are GAD-reactive (De Krijger et al., unpublished observations).

In future experiments, which may also be applied to other molecularly defined targets of autoimmunity in type-I diabetes, it will be important to determine: 1) the distribution of protein and mRNA expression in fetal pancreatic (islet) cells; 2) the subcellular site of expression and the molecular configuration of proteins, which will determine the possibility and nature of immunological recognition. In this context it must be noted that truncated forms of the GAD protein occur in the embryonic rat brain (Bond et al., 1990); 3) the onset of expression in relation to the major endocrine hormones and to other islet (precursor) cell markers.
The analysis of targets of autoreactive T-cells has been described before (Van Vliet et al., 1989, Roep et al., 1990). In these studies, insulinoma cells or their subfractions, which are abundantly available, were the substrate. Human fetal pancreatic cell suspensions contain many irrelevant cells, which may even inhibit T-cell proliferation. Cells selected by fluorescence activated cell sorting with N1 are probably the best subpopulation of human fetal pancreatic cells to be analysed, because within the pancreas N1 is selectively expressed on endocrine cells (Chapter 5). Available diabetes-related T-cell clones may be tested in a stimulation assay with the corresponding antigen presenting cells to detect their reactivity against fetal pancreatic subpopulations. Subfractionation of these cells may be hampered, however, by the limited amount of such cells (maximally 1-2 million per fetal pancreas).

The fact that relevant antigens are expressed during fetal islet cell development merely indicates that autoimmunity might start at this stage, but might start at any later timepoint, if triggered appropriately. As mentioned before (Chapter 2), fetal tolerance induction might be decisive for later autoimmune disease (Adams et al., 1987). When does tolerance development occur during human embryology? To answer this question human fetal thymic tissue must be studied (the majority of research on tolerance development so far has been done with fetal mouse thymus). Thus, the formation of the T-cell repertoire of the fetal immune system has been studied in the human fetal thymus between 12 and 18 weeks gestation (as this tissue was available) in collaborative experiments (Chapter 7). In this study, which analyzed both thymic and peripheral T-cells (in liver, bone marrow and spleen), T-cell receptor (TCR) rearrangements were observed using the majority of TCR V gene families. The expression level of TCR V gene families may depend on the developmental stage. However, these findings do not yet allow conclusions with regard to the onset and duration of tolerance induction in human fetal development.
A second question is whether tolerance induction to ß cell antigens occurs in the thymus or in the periphery.
The presence of ß cell antigens, GAD being an interesting candidate antigen, in the human fetal thymus should be investigated. It must be noted that the molecules in the thymus may not present in their native configuration, which may abolish regular antibody reactivity.
Present knowledge, however, suggests that peripheral antigens may induce tolerance peripherally. At least for $CD8^+$ T cell tolerance this is strongly suggested (Heath et al., 1993). Histologically, large organized lymphoid cell infiltrates, containing T-cell zones, interdigitating dendritic cells and macrophages, have been found in the periphery of the midgestational human fetal pancreas (Jansen et al., 1993, De Krijger, unpublished). Within the pancreas small clusters of lymphatic cells are found. These authors speculate that the lymphoid infiltrates may serve peripheral tolerance induction. Phenotypic characterization of pancreatic T cells and antigen presenting cells may be done by using the available series of CD marker antibodies.
Subsequently, functional in vitro experiments should be directed at the interaction between pancreatic (or thymic) immunecompetent cells and islet (ß) cell antigens. By fluorescence activated cell sorting (using the abovementioned CD marker antibodies) subpopulations of T-lymphocytes and antigen presenting cells can be obtained. Purified target antigens of diabetic autoimmune destruction or human fetal pancreatic subpopulations sorted with N1 (see above) could serve as substrates. These experiments may be done with tissues of different gestational ages, and

thus at differing developmental stages. The conditions for such experiments have been described before (Van Vliet et al., 1989, Roep et al., 1990). In this context it is important to mention that fetal thymus and pancreas can be obtained from the same fetus and that HLA typing can be performed on fetal splenocytes (De Krijger et al., unpublished). Thus, the outcome of stimulation experiments with susceptible vs. resistant HLA types may be compared. Recent experiments have suggested that, in addition to MHC, other unknown genetic polymorphisms may determine the differentiation of T-lymphocyte phenotypes. This resulted in pathogenic or non-pathogenic effector cells (Scott et al., 1994).

Finally, to extend the in vitro findings to an in vivo situation, SCID mice may be repopulated with human fetal thymic or peripheral T-cells or subpopulations. It will be of interest to see if autoreactive T-cells, determined in in vitro assays indeed cause ß cell destruction and type-I diabetes in these animals, and if non-autoreactive T-cells do not.

Adams TE, Alpert S, and Hanahan D. (1987). Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic beta cells. Nature 325:223-228.

Alpert S, Hanahan D, and Teitelman G. (1988). Hybrid insulin genes reveal a developmental lineage for pancreatic endocrine cells and imply a relationship with neurons. Cell 53:295-308.

Atkinson MA, MacLaren NK, Scharp DW, Lacy PE, and Riley WL. (1990). 64000 M, autoantibodies as predictors of insulin-dependent diabetes. Lancet 335:1357-1360.

Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, and DeCamilli P. (1990). Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151-156.

Bond RW, Wyborski RJ, and Gottlieb DI. (1990). Developmentally regulated expression of an exon containing a stop codon in the gene for glutamic acid decarboxylase. Proc Natl Acad Sci USA 87:8771-8775.

Chou KH, Ilyas AA, Evans JE, Quarles RH, and Jungalwala FB. (1985). Structure of a glycoprotein reacting with monoclonal IgM in neuropathy and with HNK-1. Biochem Biophys Res Commun 128:383-388.

De Krijger RR, Aanstoot HJ, Kranenburg G, Verkerk A, Jongkind JF, Van Strik R, Lafferty K, and Bruining GJ. (1992). Enrichment of beta cells from the human fetal pancreas by fluorescent activated cell sorting with a new monoclonal antibody. Diabetologia 35:436-443.

De Vroede MA, In 't Veld PA, and Pipeleers DG. (1990). Dexoyribonucleic acid synthesis in cultured adult rat pancreatic B cells. Endocrinology 127:1510-1516.

Dong J, Asa SL, and Drucker DJ. (1991). Islet cell and extrapancreatic expression of the LIM domain homeobox gene isl-1. Mol Endocrinol 5:1633-1641.

Edlund T, Walker MD, Barr PJ, and Rutter WJ. (1985). Cell-specific expression of the rat insulin gene: evidence for role of two distinct 5' flanking elements. Science 230:912-916.

Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, and Baekkeskov S. (1988a). Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. Proc Natl Acad Sci USA 85:9037-9041.

Efrat S, Teitelman G, Anwar M, Ruggiero D, and Hanahan D. (1988b). Glucagon gene regulatory region directs oncoprotein expression to neurons and pancreatic α cells. Neuron 1:605-613.

Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, and Tobin AJ. (1991). Two genes encode distinct glutamate decarboxylases. Neuron 7:91-100.

Gittes GK, and Rutter WJ. (1992). Onset of cell-specific gene expression in the developing mouse pancreas. Proc Natl Acad Sci USA 89:1128-1132.

Halban P, Powers S, George K, and Bonner-Weir S. (1987). Spontaneous reassociation of dispersed adult rat pancreatic islet cells into aggregates with three-dimensional architecture typical of native islets. Diabetes 36:783-790.

Hales CN, Barker DJP, Clark PMS, Cox LJ, Fall C, Osmond C, and Winter PD. (1991). Fetal and infant growth and impaired glucose tolerance at age 64 years. Br Med J 303:1019-1022.

Heath WR, Allison J, and Miller JFAP. $CD8^+$ T cell tolerance and autoimmunity to extra-thymic antigens. Autoimmunity 15:293-298.

Herrera PL, Huarte J, Sanvito F, Meda P, Orci L, and Vassalli JD. (1991). Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development 113:1257-1265.

Hood L. (1994). Thinking big in Seattle (special news report). Science 264:206-208.

Hutton JC, Christofori G, Chi WY, Edman U, Guest PC, Hanahan D, and Kelly RB. (1993). Molecular cloning of mouse pancreatic islet R-cadherin: differential expression in endocrine and exocrine tissue. Mol Endocrinol 7:1151-1160.

Inuzuka H, Redies C, and Takeichi M. (1991). Differential expression of R- and N-cadherin in neural and mesodermal tissues during early chicken development. Development 113:959-967.

Jansen A, Voorbij HAM, Jeucken PHM, Bruining GJ, Hooijkaas H, and Drexhage HA. (1993). An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases. Autoimmunity 15:31-38.

Karlsson O, Thor S, Norberg T, Ohlsson H, and Edlund T. (1990). Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. Nature 344:879-882.

Kaufman DL, Clare-Salzer M, Tian J, Forsthuber T, Ting GSP, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, and Lehmann PV. (1993). Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 366:69-72.

Kim J, Richter W, Aanstoot HJ, Shi Y, Fu G, Rajotte R, Warnock G, and Baekkeskov S. (1993). Differential expression of GAD_{65} and GAD_{67} in human, rat, and mouse pancreatic islets. Diabetes 42:1799-1808.

Knepel W, Vallejo M, Chafitz JA, and Habener JF. (1991). The pancreatic islet-specific glucagon G3 transcription factors recognize control elements in the rat somatostatin and insulin-I genes. Mol Endocrinol 5:1457-1466.

Kosaka T, Isogai K, Barnstable CJ, and Heizmann CW. (1990). Monoclonal antibody HNK-1 selectively stains a subpopulation of GABAergic neurons containing the calcium-binding protein parvalbumin in the rat cerebral cortex. Exp Brain Res 82:566-574.

Kruse J, Mailhammer R, Wernecke H, Faissner A, Sommer I, Goridis C, and Schachner M. (1984). Neural cell adhesion molecules and myelin-associated glycoprotein share a common carbohydrate moiety recognized by monoclonal antibodies L2 and HNK1. Nature 311:153-155.

Möller CJ, Christgau S, Williamson MR, Madsen OD, Zhan-Po N, Bock E, and Baekkeskov S. (1992). Differential expression of NCAM and cadherins in pancreatic islets, glucagonomas and insulinomas. Mol Endocrinol 6:1332-1342.

Nielsen JH, Linde S, Welinder BS, Billestrup N, and Madsen OD. (1989). Growth hormone is a growth factor for the differentiated pancreatic beta cell. Mol Endocrinol 3:165-173.

Ohlsson H, Thor S, and Edlund T. (1991). Novel insulin promoter-and enhancer-binding proteins that discriminate between pancreatic alpha and beta cells. Mol Endocrinol 5:897-904.

Ohlsson H, Karlsson K, and Edlund T. (1993). IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J 12:4251-4259.

Petersen JS, Russel S, Marshall MO, Kofod H, Buschard K, Cambon N, Karlsen AE, Boel E, Hagopian WA, Hejnæs K, Moody A, Dyrberg T, Lernmark Å, Madsen OD, and Michelsen BK. (1993). Differential expression of glutamic acid decarboxylase in rat and human islets. Diabetes 42:484-495.

Raaphorst FM, Van Bergen J, Langlois van de Bergh R, Van der Keur M, De Krijger RR, Bruining GJ, Van Tol MJD, Vossen JM, and Van den Elsen PJ. (1994). Usage of TCRAV and TCRBV gene families in human fetal and adult TCR rearrangements. Immunogenetics, in press.

Rabizadeh S, Oh J, Zhong L, Yang J, Bitler CM, Butcher LL, and Bredesen DE. (1993). Induction of apoptosis by the low-affinity NGF receptor. Science 261:345-348.

Roep, BO, Arden SD, de Vries RRP, and Hutton JC. (1990). T cell clones from a type 1 diabetes patient respond to insulin secretory granule proteins. Nature 345:632-634.

Rouiller D, Cirulli V, and Halban P. (1990). Differences in aggregation properties and levels of the neuronal adhesion molecule (NCAM) between islet cell types. Exp Cell Res 191:305-312.

Rouiller D, Cirulli V, and Halban P. (1991). Uvomorulin mediates calcium dependent aggregation of islet cells, whereas calcium independent cell adhesion molecules distinguish between islet cell types. Dev Biol 148:233-242.

Scharfmann R, Tazi A, Polak M, Kanaka C, and Czernichow P. (1993). Expression of functiional nerve growth factor receptors in pancreatic ß-cell lines and fetal rat islets in primary culture. Diabetes 42:1829-1836.

Scott B, Liblau R, Degermann S, Marconi LA, Ogata L, Caton AJ, McDevitt HO, and Lo D. (1994). A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity 1:73-82.

Teitelman G, and Lee JK. (1987). Cell lineage analysis of pancreatic islet cell development: glucagon and insulin cells arise from catecholaminergic precursors present in the pancreatic duct. Dev Biol 121:454-466.

Teitelman G, Alpert S, and Hanahan D. (1988). Proliferation, senescence, and neoplastic progression of beta cells in hyperplastic pancreatic islets. Cell 52:97-105.

Teitelman G. (1993). On the origin of pancreatic endocrine cells, proliferation and neoplastic transformation. Tumor Biol 14:167-173.

Teitelman G, Alpert S, Polak JM, Martinez A, and Hanahan D. (1993). Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development 118:1031-1039.

Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, and McDevitt HO. (1993). Immune responses to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 366:72-75.

Tischler AS, Mobtaker H, Mann K, Nunnemacher G, Jason WJ, Dayal Y, Delellis RA, Adelman L, and Wolfe HJ. (1986). Anti-lymphocyte antibody Leu-7 (HNK-1) recognizes a constituent of neuroendocrine granule matrix. J Histochem Cytochem 34:1213-1216.

Upchurch BH, Aponte GW, and Leiter AB. (1994). Expression of peptide YY in all four islet cell types in the developing mouse pancreas suggests a common peptide YY-producing progenitor. Development 120:245-252.

Van Assche FA, and Aerts L. (1979). The fetal endocrine pancreas. Contrib Gynaecol Obstet 5:44-57.

Van Vliet E, Roep BO, Meulenbroek L, Bruining GJ, de Vries RRP. (1989). Human T cell clones with specificity for insulinoma cell antigens. Eur J Immunol 19:213-216.

Walker MC, Edlund T, Boulet AM, and Rutter WJ. (1983). Cell-specific expression controlled by the 5' flanking region of insulin and chymotypsin. Nature 306:537-541.

SUMMARY

The aim of this thesis was to study the development of pancreatic islet cells in the midgestational human fetal pancreas and to assess the importance of islet cell development for type-I diabetes mellitus. The difference between human and rodent development was a major reason to study the human fetal pancreas. At the start of these studies little was known about human fetal endocrine cell development, except a number of morphological studies.

In Chapter 1 an overview is given of adult and fetal islet anatomy and physiology and fetal islet development. Islets of Langerhans are clusters of endocrine cells, dispersed in the exocrine pancreatic parenchyma. The four major endocrine cell types are α, ß, δ, and PP cells, producing and secreting glucagon, insulin, somatostatin and pancreatic polypeptide, respectively. During fetal development endocrine cells appear from 7 weeks gestation onwards. The first mature islets are found at 15 weeks gestation. All islet cells are thought to originate from a common endodermal precursor cell. This precursor cell, however, remains elusive. Hormone containing islet cells have a limited proliferative potential under baseline conditions, but may be stimulated to proliferation by growth factors. Recently, the presence of these factors and their receptors has been studied extensively in fetal and adult islet cells.

Another important reason to study the development of the human fetal pancreas is the notion that tolerance development probably occurs during fetal development. The mechanisms of tolerance induction are reviewed in Chapter 2. Autoimmune diseases, such as type-I (insulin-dependent) diabetes mellitus, are thought to result from defective tolerance development. In such diseases the immune system attacks self-antigens. In type-I diabetes the pancreatic ß cells are selectively destructed, but the target antigen(s) is (are) unknown. Histologically, a cellular infiltrate, consisting of macrophages and T-lymphocytes, can be identified in the islets of Langerhans. Both $CD4^+$ and $CD8^+$ autoreactive T-lymphocytes are required for the development of type-I diabetes. Autoantibodies have been found in sera of type-I diabetic patients. Islet cell antibodies (ICA) and antibodies to glutamic acid decarboxylase (GAD) are present in 80% of the patients. The cause of the ß cell destruction in type-I diabetes is unknown. Susceptibility to the disease is genetically determined, but environmental factors have also been implicated.

In Chapter 3 the coexpression of islet hormones in human fetal endocrine cells is described. Two or three hormones are found in morphologically distinct granule types within one cell. Cells containing hormones do not proliferate.

Therefore, proliferating precursor cells cannot be identified using hormones as markers. No conclusions can be drawn with regard to islet cell interrelationships, because all combinations of coexpression occur. Hormone coexpression at the mRNA level was confirmed by in situ hybridization. Combination of in situ hybridization with immunohistochemistry showed that cells may contain mRNA without the corresponding hormone protein.

In Chapter 4, potential islet precursor cells are identified using two neuroendocrine markers, N1 and HNK-1. Early phenotypical differentiation of δ cells is suggested by expression of HNK-1 on islet δ cells and on proliferating cells. Such proliferating cells, as well as multiple hormone containing cells, are observed in all fetal pancreata between 12 and 18 weeks gestation, indicating that islet cell development is a continuously repeating event.

In Chapter 5, the characterization of the monoclonal antibody N1 is described. Fetal endocrine cells can be separated from exocrine cells, using N1, with the aim of culturing the endocrine cells. However, preliminary culture experiments have shown that this is hampered by fibroblast overgrowth and suboptimal culture medium conditions.

In Chapter 6, the reactivity of islet cell antibody (ICA) positive sera of recent onset diabetic patients to fetal islet cells is studied. All sera reacted to all fetal pancreata, but quantitatively the results are heterogeneous. This is probably due to the polyclonal nature of ICA and the fetal interpancreatic differences in expression of ICA target antigens. Preliminary experiments show expression of GAD65, a target of autoimmune destruction in type-I diabetes, in α, ß, and δ cells in the human fetal pancreas. In Chapter 7, the ontogeny of the T-cell repertoire is studied, by analyzing T-cell receptor rearrangements in the thymus and in peripheral lymphatic organs (liver, spleen, and bone marrow). It is suggested that the expression of T-cell receptor V gene families depends on the developmental stage. These findings may have important implications for the occurrence of tolerance development.

Finally, in Chapter 8 the results are discussed and suggestions for further experiments are given. It can be concluded that the human fetal pancreas can be used to study islet cell development, in particular for the detection of endocrine precursor cells. The presence of targets of autoimmune destruction in type-I diabetes makes the human fetal pancreas suitable for studies on the ontogeny of these molecules. Fetal thymic tissue may be used to study the development of the fetal immune system, including tolerance induction.

SAMENVATTING

Het doel van dit proefschrift was het bestuderen van de ontwikkeling van eilandjescellen in het humane foetale pancreas (alvleesklier) tussen 12 en 18 weken gestatieduur (de eerste helft van het tweede trimester van de zwangerschap) en het bepalen van het belang van de ontwikkeling van deze cellen voor type-I (insuline afhankelijke) diabetes mellitus (suikerziekte). Het verschil tussen de ontwikkeling in de mens en in knaagdieren was een belangrijke reden om dit werk met behulp van humaan foetaal pancreasweefsel uit te voeren. Aan het begin van het onderzoek was er weinig bekend over de ontwikkeling van endocriene eilandjescellen in het humane foetale pancreas, behalve een aantal beschrijvende, morfologische studies.

In Hoofdstuk 1 wordt een overzicht gegeven van de anatomie en fysiologie van volwassen en foetale eilandjes en van de foetale ontwikkeling van eilandjes. Deze eilandjes van Langerhans zijn groepjes endocriene cellen, die verspreid in het exocriene, spijsverteringsenzymen producerende, pancreasweefsel voorkomen. De vier belangrijkste endocriene celtypen zijn α, ß, δ, en PP cellen, die respectievelijk glucagon, insuline, somatostatine, en pancreatisch polypeptide produceren en afgeven aan de bloedbaan. Tijdens de humane foetale ontwikkeling komen vanaf 7 weken gestatieduur endocriene cellen voor. De eerste morfologisch volwassen eilandjes zijn vanaf 15 weken aanwezig. Alle eilandjescellen zijn waarschijnlijk afkomstig uit een gemeenschappelijke endodermale voorlopercel. De kenmerken van deze voorlopercel(len) zijn vooralsnog onbekend. Hormoon producerende eilandjescellen zijn sterk beperkt in de mogelijkheid zich te delen onder normale omstandigheden, maar kunnen hiertoe wel aangezet worden door bijvoorbeeld groeifactoren. In het recente verleden is de aanwezigheid van groeifactoren en hun receptoren uitgebreid bestudeerd in foetale en volwassen eilandjescellen.

Een andere belangrijke reden om de ontwikkeling van het humane foetale pancreas te bestuderen is de wetenschap dat tolerantie ontwikkeling waarschijnlijk ook tijdens de foetale onwikkeling gebeurt. De mechanismen die een rol spelen bij tolerantie ontwikkeling worden in Hoofdstuk 2 besproken. Autoimmuunziekten, zoals type-I (insuline-afhankelijke) diabetes mellitus, ontstaan mogelijk door een stoornis in de tolerantie ontwikkeling. In dat geval worden lichaamseigen cellen aangevallen. Bij type-I diabetes gaat het om de ß cellen in de eilandjes van Langerhans. Het precieze doelwit (of doelwitten) van de aanval is (zijn) nog onbekend. Histologisch is er een cellulair infiltraat, bestaande uit macrofagen en T-lymfocyten (bepaalde witte bloedcellen), aanwezig in de eilandjes van Langerhans.

Zowel CD4- als CD8-positieve T-lymfocyten zijn nodig voor het ontstaan van type-I diabetes. Autoantilichamen zijn aangetoond in het serum van patienten met type-I diabetes. Antistoffen tegen eilandjescellen (ICA) en antistoffen tegen glutaminezuur decarboxylase (GAD) worden aangetroffen bij 80% van de patienten. Ondanks deze kennis is de oorzaak van het vernietigen van de ß cellen bij type-I diabetes nog steeds onbekend. De gevoeligheid voor de ziekte is genetisch bepaald, maar ook omgevingsfactoren spelen een rol.

In hoofdstuk 3 wordt aangetoond dat in humane foetale eilandjescellen meer dan 1 hormoon tegelijk aanwezig kan zijn, iets wat in volwassen eilandjescellen niet voorkomt. Twee, en soms drie, hormonen worden aangetroffen in morfologisch verschillende blaasjes in de eilandjescellen. Geen van de hormoonbevattende cellen vertoonde nog delingsactiviteit. Hieruit kan worden afgeleid dat delende voorlopercellen niet met behulp van deze hormonen kunnen worden opgespoord. Bovendien kunnen geen conclusies worden getrokken over de onderlinge relaties van de verschillende endocriene celtypen, omdat alle combinaties van 2 hormonen in 1 cel voorkomen. Het gezamenlijk voorkomen van meerdere hormonen in 1 cel werd ook aangetoond met in situ hybridizatie, een techniek die laat zien dat in een cel mRNA (een afschrift van het DNA), wordt gemaakt, dat als "mal" dienst doet om eiwitten te maken. Bij het gelijktijdig aantonen van mRNA en eiwit (betrekking hebbend op hetzelfde hormoon) werd duidelijk dat mRNA aanwezig kan zijn, zonder dat er al eiwit gevormd was.

In Hoofdstuk 4 worden potentiele voorlopercellen van eilandjescellen geidentificeerd met behulp van 2 merker antistoffen, N1 en HNK-1. Vroege uitrijping van δ cellen wordt verondersteld door de aanwezigheid van HNK-1 in zowel voorlopercellen als in de δ cellen in eilandjes. De rijping van eilandjescellen uit voorlopercellen komt gedurende deze periode van ontwikkeling continu, getuige het feit dat in alle pancreata in de periode tussen 12 en 18 weken gestatieduur zowel prolifererende cellen, gemerkt met N1 of HNK-1, als cellen met meer dan 1 hormoon voorkomen.

In Hoofdstuk 5 wordt het monoclonale antilichaam N1 gekarakteriseerd. Foetale eilandjescellen kunnen worden gescheiden van andere, exocriene, pancreascellen door ze te merken met N1 en dan daaraan een fluorescerende stof te verbinden. Met behulp van een apparaat dat reageert op fluorescentie kunnen de met N1 gemerkte cellen apart worden opgevangen. Het kweken van deze cellen is nog erg moeilijk omdat bindweefselcellen de endocriene cellen in weefselkweek overgroeien en doordat de kweekomstandigheden voor de endocriene cellen niet optimaal zijn.

In Hoofdstuk 6 wordt de reactiviteit van patientensera met daarin eilandcel-antistoffen (ICA) tegen foetale eilandjescellen bestudeerd. Deze experimenten laten zien dat de doelwitten waartegen deze antistoffen gericht zijn, reeds in deze fase van ontwikkeling aanwezig zijn. Voorts blijken de resultaten quantitatief heterogeen, waarschijnlijk omdat eilandcel-antistoffen niet tegen één, maar tegen meerdere doelwitten reageren. Een tweede reden voor de heterogeniteit kan veroorzaakt worden door een verschil in de aanwezigheid van de doelwit antigenen in de foetale eilandjescellen. In voorlopige experimenten werd de aanwezigheid van GAD, een doelwit van autoimmuniteit in type-I diabetes, in humane foetale α, ß, en δ cellen aangetoond.
In Hoofdstuk 7 wordt het ontstaan van T-lymfocyten repertoire bestudeerd, door te kijken naar herschikking van T-cel receptoren in de thymus en in perifere lymfatische organen (lever, milt, en beenmerg). Door dit proces is het lichaam later in staat te reageren tegen allerlei soorten indringers. Het is waarschijnlijk dat het voorkomen van genfamilies, die coderen voor bepaalde delen van de T-cel receptor, afhangt van het foetale ontwikkelingsstadium. Deze bevinding kan van groot belang zijn voor de tolerantie ontwikkeling.
Tenslotte worden in Hoofdstuk 8 de resultaten bediscussieerd en worden voorstellen voor toekomstige experimenten gedaan. Er kan worden geconcludeerd dat het humane foetale pancreas kan worden gebruikt voor het bestuderen van eilandcel ontwikkeling, vooral voor het opsporen van endocriene voorlopercellen. De aanwezigheid van doelwitten van autoimmuundestructie in type-I diabetes maakt humaan foetaal pancreasweefsel geschikt voor het bestuderen van de ontogenie van deze eiwitten. Foetaal thymusweefsel kan gebruikt worden voor de foetale ontwikkeling van het immuunsysteem, inclusief tolerantie inductie.

DANKWOORD

Aan dit proefschrift hebben velen op uiteenlopende manieren bijgedragen. In den beginne was er Mu Bruining. Mu, ik herinner me nog de middag dat ik je vroeg om "wat onderzoek" te mogen doen, en jouw bevestigende antwoord daarop. Je bent al die jaren een zeer stimulerende factor geweest, tevens zorgde je voor alle randvoorwaarden van het onderzoek. Daarvoor wil ik je buitengewoon hartelijk danken. Overigens hoop ik dat we in de toekomst nog vaak plannetjes zullen bespreken.

Henk-Jan, ook jij dient op deze plaats genoemd. Toen ik nog niet zo lang bezig was, wist Mu een kinderarts-in-opleiding met een typische naam, die in diabetes geinteresseerd was. Vanaf dat moment liepen onze wegen min of meer parallel. Ik vond en vind je gezelschap altijd buitengewoon inspirerend. Nooit was het je teveel moeite om een manuscript nog eens als advocaat van de duivel door te nemen. Bedankt!

Greetje, mijn lievelingsanaliste. Ruim 2½ jaar hebben we onafscheidelijk gewerkt aan al onze foetale pancreasproefjes. Je stond altijd klaar, was nooit te beroerd om wat langer te blijven, had altijd het laatste woord, kortom: je was (bijna) perfect.

Mijn twee promotoren, Professor Visser en Professor Galjaard wil ik danken voor de mogelijkheid die ze mij geboden hebben om dit onderzoek uit te voeren, voor hun belangstelling, en voor hun snelle beoordeling van het manuscript. Tevens wil ik Professor Bosman, mijn huidige opleider, en Professor Lamberts bedanken voor het beoordelen van dit manuscript.

Bij het doen van onderzoek en met name bij de afronding in de vorm van een proefschrift speelt het thuisfront een zeer belangrijke rol. Hiervoor wil ik Annet en mijn ouders dan ook danken. Zij zorgden ervoor dat ik vele avonden en weekenden "naar boven" kon, en dat bovendien zonder morren.

Dit onderzoek had niet kunnen plaatsvinden zonder geschikt materiaal. Gedurende vele jaren heeft Dr. D. Schipper van de Bloemenhovekliniek in Heemstede, evenals alle andere artsen en medewerkers voor dit superieure materiaal en voor een uitstekende sfeer gezorgd.

Het is onmogelijk om op deze plaats iedereen te noemen, die mij tijdens het in dit proefschrift beschreven onderzoek met raad en daad terzijde heeft gestaan. Toch wil ik al die mensen, met name op de Klinische Genetica en de Celbiologie, maar ook op een behoorlijk aantal andere afdelingen, die altijd wel 5 minuten of dat ontbrekende stofje hadden, bij deze hartelijk danken.

Tenslotte wil ik nog twee personen expliciet bedanken. Ten eerste Ton Verkerk, de onvermoeibare "trouble shooter" van de FACS-II, het oude beestje, dat we gebruikten voor de cel-sorteer-proeven. Geen moeite was je te veel, als wij er weer eens niet uitkwamen; en zelfs als ik het apparaat had laten "droogkoken" werd je niet (althans niet zichtbaar) kwaad.
Ten tweede Frans, die er menigmaal voor heeft gezorgd dat wp-dokumenten, die door eigen of computerstommiteiten door mij reeds verloren waren gewaand, toch weer tevoorschijn werden getoverd uit de krochten van de harde schijf. Chapeau!

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 4 april 1966 geboren te Rotterdam. Hier volgde hij de lagere en middelbare school (Citycollege Emmaus-Franciscus, ongedeeld VWO). In 1983 startte hij met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het doctoraalexamen werd behaald op 12 november 1987. Het als student-assistent begonnen onderzoek naar de ontwikkeling van humane foetale endocriene pancreascellen werd onder leiding van Dr. G.J. Bruining voortgezet als promotie-onderzoek aan de afdeling Kindergeneeskunde (Hoofd: Prof. Dr. H.K.A. Visser) en uitgevoerd op de afdeling Pathologie (toenmalig hoofd: Prof. Dr. R.O. van der Heul) en op de afdeling Klinische Genetica (Hoofd: Prof. Dr. H. Galjaard). Nadat medio 1992 het experimentele werk voltooid was, startte hij met de senior co-schappen en het arts-examen werd behaald op 17 december 1993. Van 1 januari t/m 30 juni 1994 werkte hij voor de afdeling Plastische Chirurgie bij mw. Dr. Chr. Vermeij-Keers aan een project over de detectie van parvovirus B19 infecties in foetaal en placenta materiaal afkomstig van spontane abortus en intrauteriene vruchtdood. Op 1 juli 1994 is hij gestart met de opleiding tot patholoog aan de afdeling Pathologie (Hoofd: Prof. Dr. F.T. Bosman) van de Erasmus Universiteit.