

## Transgenic mouse model: a new approach for the investigation of endocrine pancreatic B-cell growth

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**SUMMARY** The transformation and adaptation of pancreatic insulin-producing (B) cells has been studied in a transgenic mouse model using a panel of antisera recognising peptides and general neuroendocrine markers at both light and electron microscopical levels. Stages of tumour genesis in the transgenic mouse model from hyperplasia to neoplasia, have been compared with human B-cell tumours. A normal complement of peptide containing cells was seen in the transgenic mouse pancreas, but cells containing pro-insulin-derived peptides became more numerous as hyperplasia commenced. The transgenic mouse tumours were composed of B cells, although 30–35% of the tumours were also found to contain PP cells – a finding which is directly comparable with that in human insulin-producing tumours. NSE, 7B2 and chromogranin immunoreactivities were found in most cells from all the tumours examined. Antisera to PGP 9.5, a novel marker for elements of the neuroendocrine system, were found to stain hyperplastic and neoplastic B-cells intensely. In contrast, normal mouse B-cells did not show PGP 9.5 immunoreactivity thus it appears that PGP 9.5 is differentially expressed in transformed and/or growing mouse B-cells and hence may be used as an indicator in studies of early tumour growth.

Although the pathology of pancreatic islet B-cell tumours is well described, the adaptive changes from cell transformation to the fully differentiated tumour mass are largely unknown. Indeed, this statement holds true for all endocrine neoplasms as they tend to be slow growing tumours for which no successful experimental model has yet been established.<sup>1</sup> Studies of cultured cells have not met with much success, largely because of the slow growth rate and the atypical differentiation of the cells in culture. Likewise, tumour fragments transplanted into immune suppressed recipients frequently show abnormal adaptational patterns.

The molecular genetics of cell transformation at the onset of tumour genesis has been studied extensively in recent years. This research led to the identification and isolation of genes (called oncogenes) as causative agents in the development of some tumours.<sup>2</sup> It has become possible recently to study tumour genesis in a

new way, using a technique for gene transfer which serves to establish oncogenes in the mouse germ line (transgenic mice) and then examine the morphological changes associated with their presence. The production of transgenic mice involves the microinjection of a solution of DNA into one of the pronuclei of a fertilised mouse oocyte, after which the injected embryos are reimplanted into the oviducts of pseudo-pregnant female mice and allowed to develop.<sup>3,4</sup> About 20% of the mice born carry the injected DNA as a heritable genetic element, which they then transmit to offspring, thus generating a lineage of transgenic mice which generally manifest the phenotype endowed by the new gene—for example, ref.<sup>5</sup> Transgenic mice have proved useful for studying oncogenesis, in particular through the use of oncogenes which are manipulated to express specifically in a certain cell type, thereby eliciting a particular type of tumour (some applications of targetted oncogenesis in transgenic mice are reviewed in refs.<sup>6–8</sup>

A hybrid gene containing the simian virus 40 (SV40) early (transforming) region under control of

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the rat insulin II gene regulatory region was recently established in the mouse germ line.<sup>8</sup> It was found that tissue specific, pancreatic insulin producing (B) cell tumours were heritably produced in the transgenic offspring. The transformed B-cells were identified by their proliferation and by expression of large T-antigen (of the oncogene encoded in simian virus 40).<sup>9</sup> Temporal adaptive changes from normal islets, through B-cell hyperplasia to the formation of solid B-cell tumours from a few of the hyperplastic islets were noted.<sup>8</sup> We have investigated B-cell tumour formation in these transgenic mice from neonates to animals with advanced neoplasia, using both light and electron microscopical immunocytochemistry in an attempt to identify morphological or antigenic markers which may be associated consistently with tumour induction and/or differentiation. It was also our aim to compare the transgenic mouse model with parallel studies of insulin-producing tumours in man. In this way we have attempted to evaluate the potential contribution of transgenic systems to our understanding of tumour development.

## Methods

### ANIMALS

A total of 25 transgenic mice and two normal mice from the B6D2F1 (C57BL/6J × DBA/2J) strain have been investigated.

### FIXATION

Whole pancreata from 18 mice were fixed in Bouin's solution for two hours and thereafter washed in 30% ethanol and embedded in paraffin wax (56 °C mp). Human B-cell tumours (n = 23) were collected from the departmental files. The tumour tissues had been fixed by a variety of methods, including 10% buffered formalin and Bouin's solution, processed in the usual manner and embedded in paraffin wax. All tissues were sectioned at 5 µm.

For electron microscopy, nine mice were fixed by intracardiac perfusion with 4% paraformaldehyde plus 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 after exsanguination with 0.1 M phosphate-buffered 0.15 M saline (PBS) pH 7.4. The pancreata were then dissected free and immersion fixation was continued for up to two hours. Each pancreas was subdivided for electron microscopy and half of the blocks were osmicated (1% osmium tetroxide in 0.1 M phosphate buffer pH 7.2) for one hour before ethanol dehydration and embedding in Araldite epoxy resin. Specimens from eight of the 23 human tumours were processed according to the same schedule after immersion fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for two hours.

### LIGHT MICROSCOPY

The tissues were examined by haematoxylin and eosin, Grimelius' silver impregnation technique<sup>10,11</sup> and immunocytochemistry.

### Immunocytochemistry

Antisera to 17 different antigens, including peptides and general neuroendocrine markers, were used and their characteristics are given in Table 1. Antibodies to neuroendocrine markers included neurone-specific enolase (NSE),<sup>12-14</sup> the secretory granule matrix component protein chromogranin,<sup>15-17</sup> protein gene product (PGP 9.5)<sup>18</sup>—a brain derived protein (M<sub>r</sub> = 27000) which has been identified in many neuroendocrine tissues<sup>19,20</sup> and 7B2 (originally named APPG by Hsi and coworkers)<sup>21,22</sup> a protein with structural similarities to insulin growth factor<sup>23</sup> and found to be costored with insulin in normal, hyperplastic and tumorous B-cells.<sup>24</sup>

The peroxidase-antiperoxidase method<sup>25</sup> was used throughout this study and the sections were lightly counterstained with haematoxylin.

### ELECTRON MICROSCOPY

Ultrathin sections showing silver to silver-gold interference colours were collected on cleaned, uncoated 200-mesh hexagonal nickel grids.

### Immunocytochemistry

The antisera used for electron microscopy are indicated in Table 1.

Antigenic sites were localised with the modified indirect gold labelled immunoglobulin procedure<sup>26</sup> using 5, 10, or 15 nm colloidal gold. Osmicated tissue sections were pretreated with sodium metaperiodate for 60 minutes at room temperature before immunostaining.<sup>27</sup> All preparations were counterstained with 5% methanolic uranyl acetate and aqueous lead citrate before viewing in the transmission electron microscope.

## Results

The goals at the outset of this study were: (i) to characterise the morphology of the islets and islet cells in the pancreas of transgenic mice using histological and immunocytochemical techniques, and to compare these findings with human insulin producing tumours; (ii) to evaluate the expression of general neuroendocrine antigens in the transformed B-cells.

From these observations it would be possible to assess the suitability of the transgenic mouse model in studies of endocrine tumour genesis.

Table 1 Characteristics of the antisera used.

Description	Antibody to:	Type	Donor species	Immunocyto-chemistry*		Source	
				LM	EM		
Family							
P	Pro-insulin	Insulin	Polyclonal	Guinea pig	+	+	Miles Labs; Wellcome
R		C-peptide	Polyclonal	Rabbit	+	+	Novo Research; Prof. N. Yanaihara
E	Pro-glucagon	Pro-insulin	Monoclonal	Mouse	+	+	Hagedorn Labs; Novo Labs
C		Glucagon	Polyclonal	Rabbit	+	+	HH
U		Glicentin	Polyclonal	Rabbit	+	+	A Moody
R		Glucagon-like peptides 1 & 2	Polyclonals	Rabbit	+	+	HH
S	Pro-somato-	Somatostatin	Polyclonal	Rabbit	+	+	HH
O	statin	Rat somatostatin cryptic pep. [RSCP]	Polyclonal	Rabbit	+	+	R H Goodman
R	Other peptides	Pancreatic polypeptide	Polyclonal	Rabbit	+	+	Eli Lilly Inc.
		Gastrin	Polyclonal	Rabbit	+	NT	HH
		VIP	Polyclonal	Rabbit	+	NT	HH
		Epidermal growth factor (EGF)	Polyclonal	Rabbit	+	NT	H Gregory
General neuroendocrine markers		Neuron-specific enolase (NSE)	Polyclonal	Rabbit	+	NT	P J Marangos
		Chromagranin	Polyclonal	Rabbit	+	+	Immuno Nuclear Corp.
		7B2	Polyclonal	Rabbit	+	NT	HH
		PGP 9.5	Polyclonal	Rabbit	+	NT	R Thompson

Key: \*Satisfactory results obtained from PAP (LM) and immunogold (EM) staining procedures completed using optimal antibody titres, compared against appropriate positive and negative controls. NT – not tested.

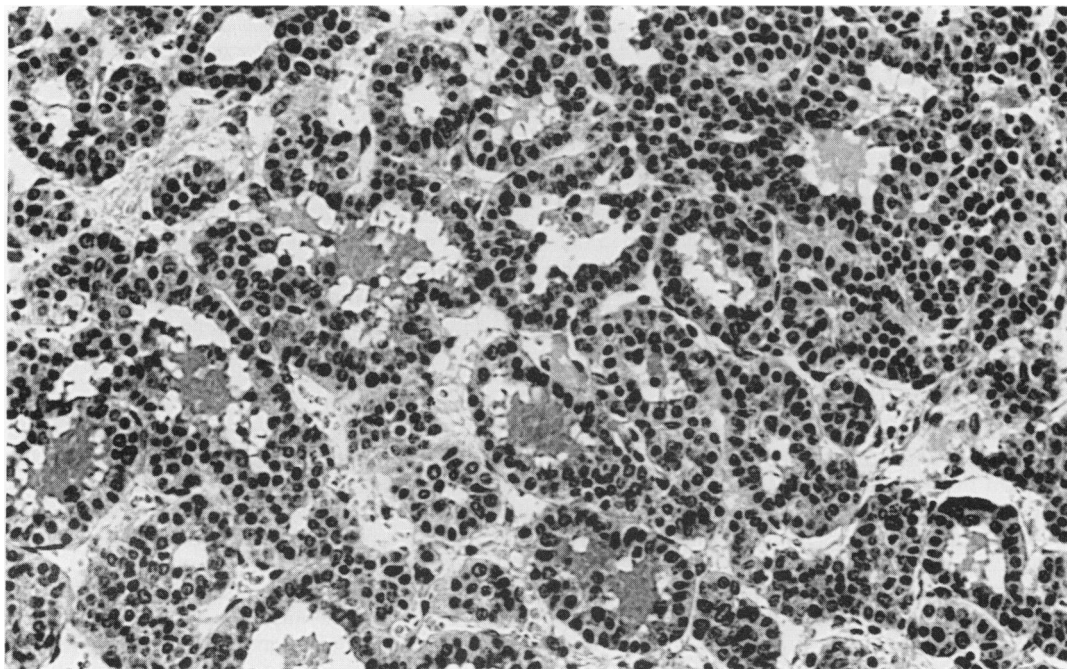


Fig. 1 Haematoxylin and eosin stain of a tumour in a transgenic mouse. Cells of a regular size are arranged in a trabecular formation. Bouin's-fixed tissue, 5  $\mu$ m wax section,  $\times 280$ .

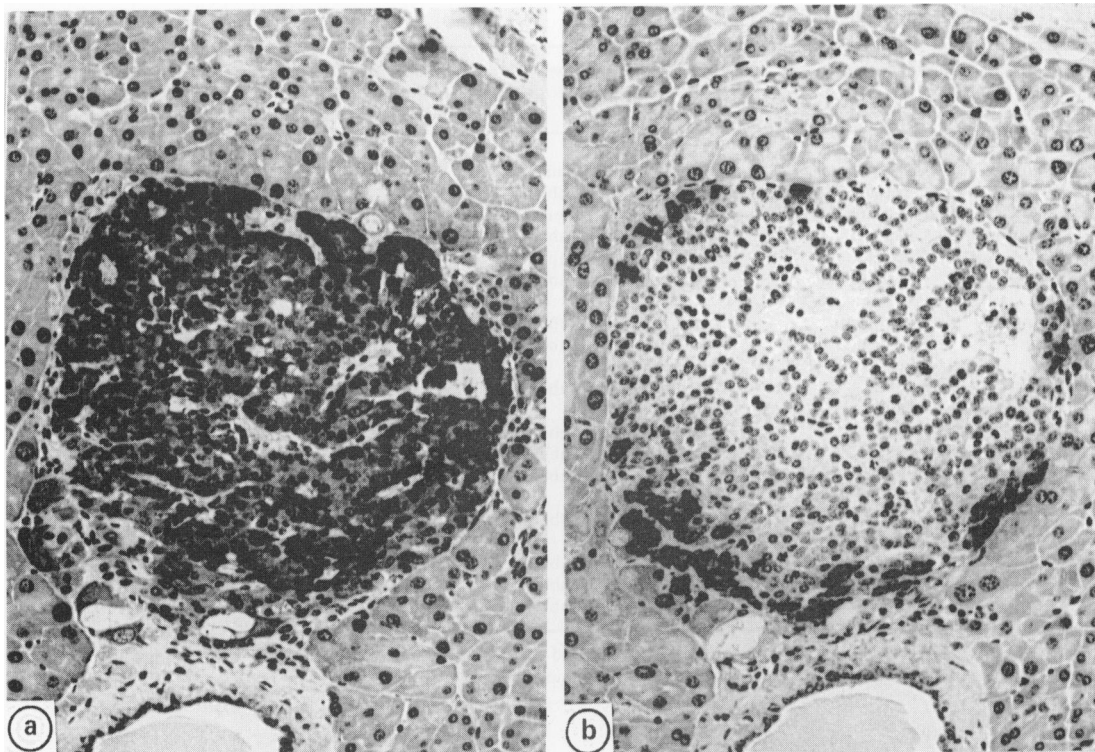


Fig. 2 (a) Hyperplastic islet in a transgenic mouse immunostained for insulin. The majority of the cells show strong immunoreactivity and are, therefore, B cells. Bouin's-fixed tissue, 5  $\mu$ m wax section, PAP immunostain with weak haematoxylin counterstain.  $\times 200$ . (b) Similar islet to that shown in Fig. 2a immunostained for somatostatin. Only a few D cells can be discerned, towards the periphery of the islet. Same procedure as in (a) above.  $\times 200$ .

#### LIGHT MICROSCOPY

The pancreata of transgenic mice showed a spectrum of normal islets, through hyperplasia to neoplasia. Hyperplasia of islets was defined as an increase in the size of the islets and number of B-cells with accompanying cytological atypia, nuclear pleomorphism and hyperchromatism with an increase in the number of mitoses.

The tumours were well circumscribed, however, occasional infiltration into the exocrine pancreas was seen. The predominant pattern was trabecular (Fig. 1), with much ductule formation, but solid and acinar areas could also be seen. Occasional tumours were associated with a peripheral aggregate of lymphoid cells, suggesting an immune response to the tumours. Tumour cells were round to oval in shape with varying amounts of cytoplasm. Considerable nuclear pleomorphism and hyperchromatism were evident and mitoses were frequent. The tumours were vascular and large numbers of extravasated red blood cells could be seen in ductules. Stromal fibrosis was not seen.

In all human B-cell tumours mixtures of the classical solid, trabecular and acinar patterns could be seen.<sup>28</sup> The tumour cells were similar in size and shape to those in normal islets; the nuclei were round to oval in shape with varying amounts of eosinophilic cytoplasm. Nuclear pleomorphism was not significant and mitoses were sparse. Various degrees of fibrosis and hyalinisation were seen in the stroma.

#### Immunocytochemistry

In the pancreata from transgenic mice normal islets showed a typical endocrine cell profile. As the islets became hyperplastic the number of cells immunoreactive for insulin (Fig. 2a) and C-peptide increased with a corresponding decrease in glucagon and somatostatin (Fig. 2b) immunoreactants.

The majority of the tumour cells were immunoreactive to insulin and C-peptide antisera. The staining was non-polar, similar to that seen in normal mouse B-cells. Neither glucagon and somatostatin immunoreactants, gastrin, VIP nor EGF were identified in any of the tumour cells. Pancreatic polypeptide-

Table 2 Immunostaining results using antisera recognising general neuroendocrine characteristics.

Antisera to:	Mouse pancreatic cells Non-transgenic Normal	Transgenic Normal	Hyperplastic	Tumour
NSE	+	+	+	+
Chromogranin	+	+	+	+
PGP 9.5	+*	+*	++	+++
7B2	+++	+++	+++	+++

+ - +++ cells weakly (+) to strongly (+++) immunoreactive. \* - staining also includes non-B cells (see text).

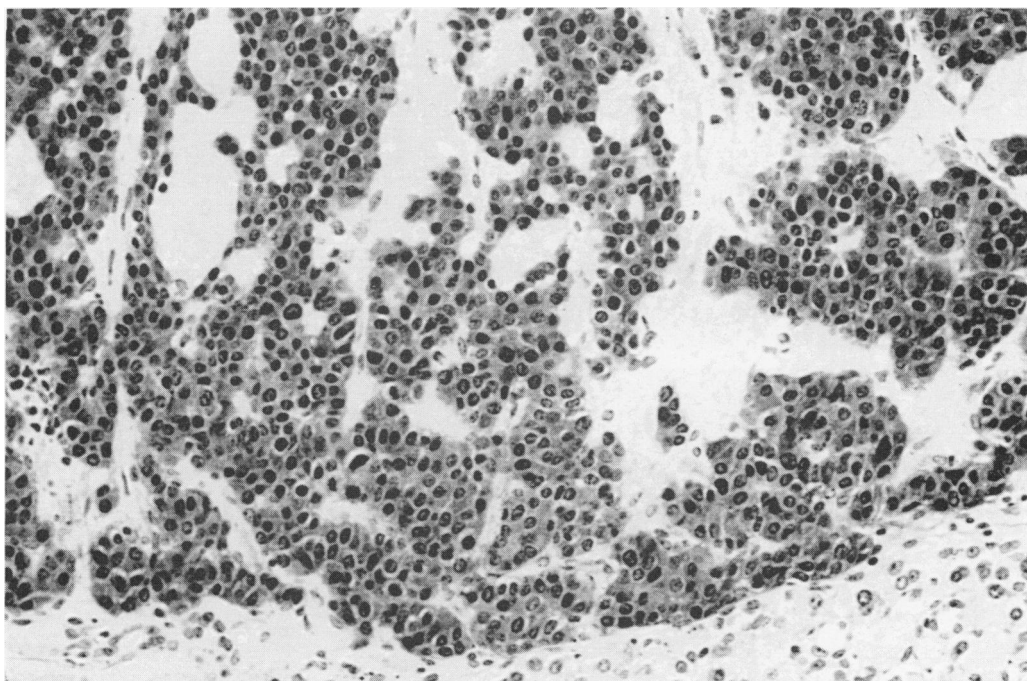


Fig. 3 Protein gene product (PGP 9.5) immunoreactivity in all cells of a tumour from a transgenic mouse. Same procedure as Fig. 2a.  $\times 280$ .

containing (PP) cells were identified in approximately 30% of the tumours, although it was not possible to determine whether these represented tumour, or included, cells.

Immunostaining with antisera to the neuroendocrine markers is given in Table 2. The reaction with both NSE and chromogranin antisera was very variable and often weak. With antisera to the brain-derived protein PGP 9.5 (17) non-B-cells in normal islets, usually at the periphery, were immunoreactive. Hyperplastic islets and tumours B-cells exhibited strong PGP 9.5 immunoreactivity (Fig. 3). 7B2 immunostaining was observed in normal, hyperplastic and tumour cells.

Human tumours were immunoreactive for the neuroendocrine markers NSE and PGP 9.5. The majority of the tumour cells were immunoreactive with antisera to pro-insulin, insulin, C-peptide and 7B2. Pro-insulin immunostaining was restricted to the perinuclear region, which would be consistent with the normal distribution of Golgi cisternae. Glucagon and somatostatin immunoreactants were identified in a small proportion of the tumours. Pancreatic polypeptide containing cells were seen in 8 of the tumours (approx 35%). Thus, a number of secretory (insulin/C-peptide/proinsulin) and non-secretory (NSE, 7B2) antigenic determinants are expressed by both mouse and human tumour B-cells. Likewise,

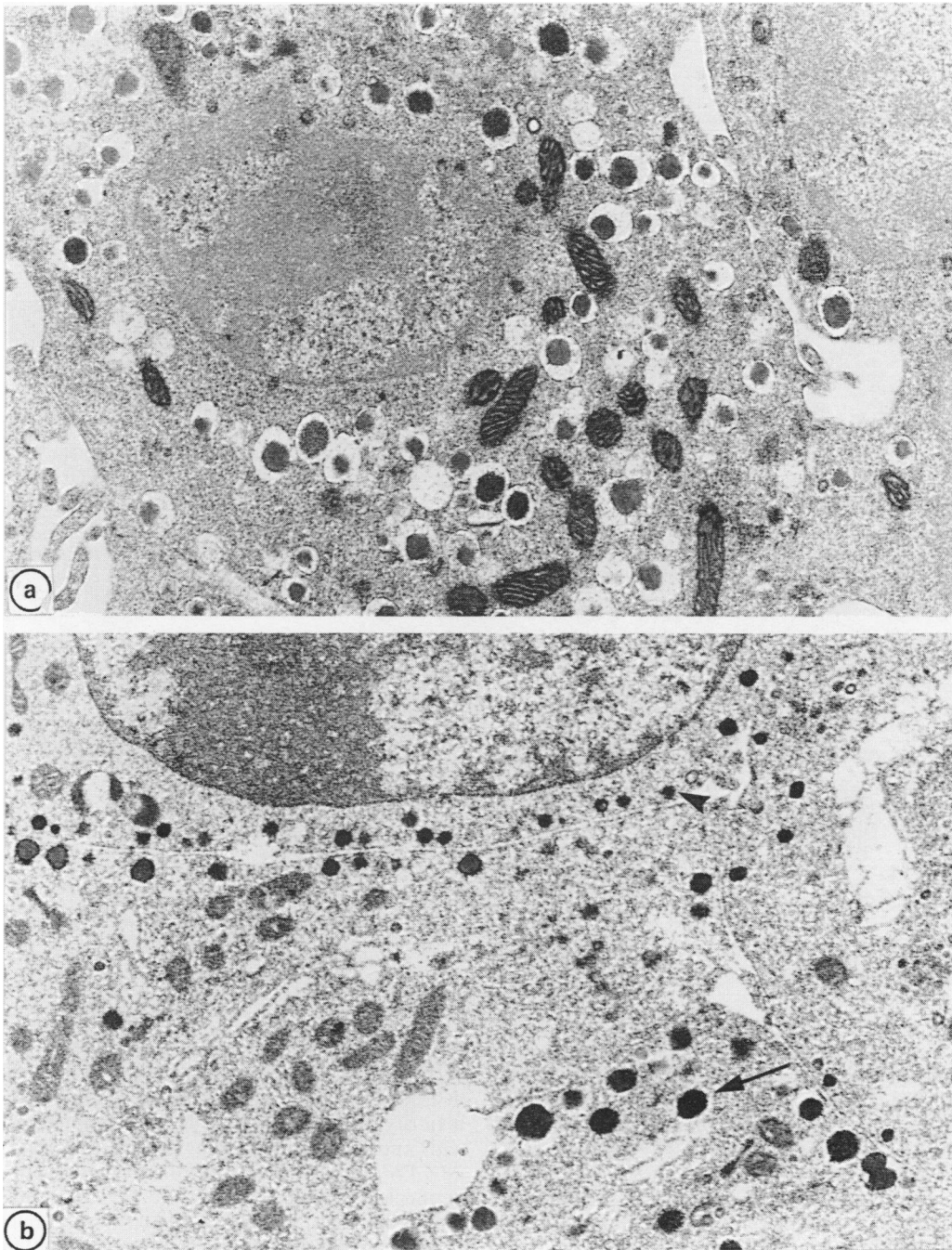
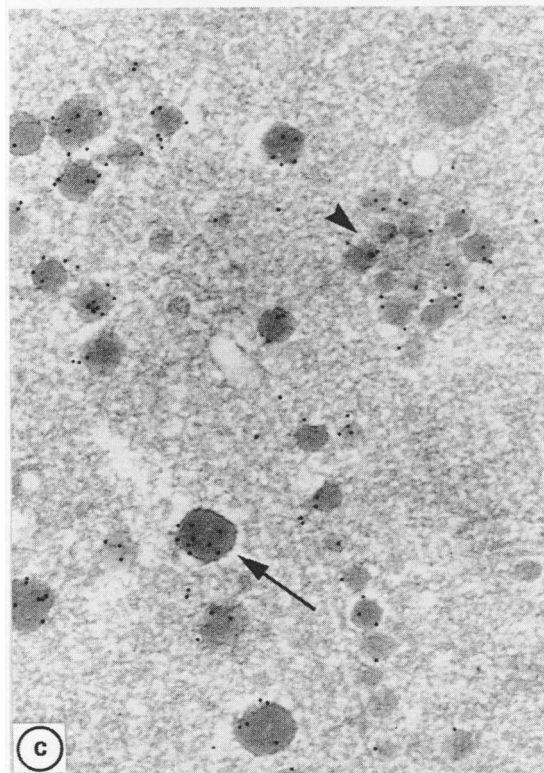
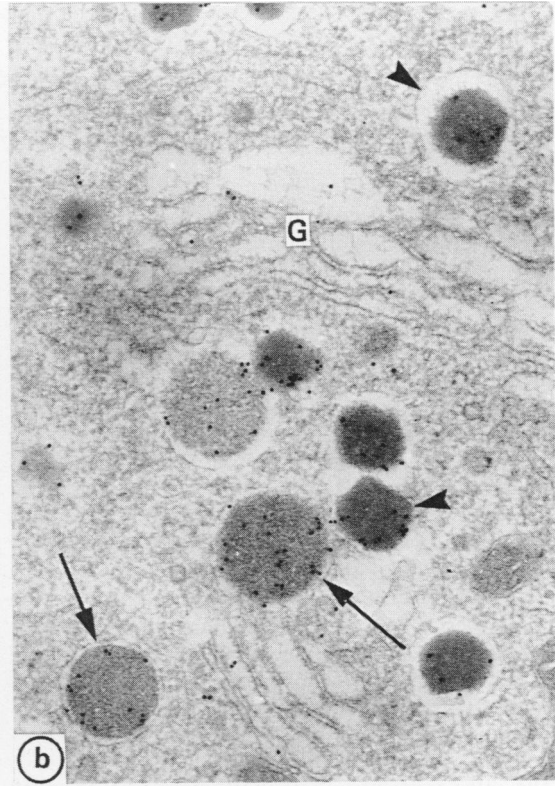
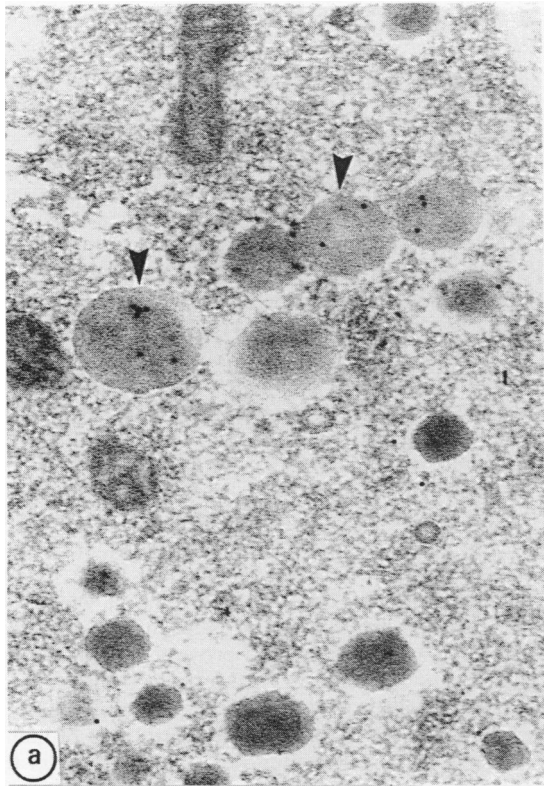


Fig. 4 Electron micrograph of tumour cells from transgenic mouse. (a) Heavily granulated cell with typical  $\beta$ -granules. (b) Typical  $\beta$ -granules (arrow) and atypical granules (arrowhead). Uranyl acetate and lead citrate,  $\times 20\ 000$ .



**Fig. 5** Tumour cell from transgenic mouse immunostained with antisera to pro-insulin (a), C-peptide (b) and insulin (c). (a) Immature granules immunostained (arrowheads);  $\times 63000$ . (b) Immature granules (arrows) and mature granules (arrowheads), G - Golgi complex;  $\times 63000$ . (c) Typical  $\beta$ -granules (arrow) and atypical granules (arrowheads);  $\times 40000$

mouse and human B-cell tumours exhibit intense PGP 9.5 immunostaining, although PGP 9.5 is apparently absent from normal mouse B-cells.

#### ELECTRON MICROSCOPY

Tumour cells and normal cells from transgenic mice pancreata contained the same types of typical secretory granules as found in normal mouse B-cells—that is, 220–300 nm  $d_{max}$ ; spherical electron dense core, occasionally acentric within a large halo. All tumour cells were heavily granulated (Fig. 4a). In about 5% of the tumour cells, the secretory granules were present at the periphery of the cell. Atypical secretory granules (small [200 nm diameter], electron-dense without a distinct halo) were observed in about 1% of the tumour cells (Fig. 4b).

In seven of the human tumours the cells contained typical secretory granules with characteristics similar to those described above. Secretory granules having a distinct crystalline core, however, were also observed in these cases. In three cases of human B-cell tumour, a mixed (typical and atypical secretory granules) cell nature was observed, whilst only in one case were the secretory granules morphologically 'atypical'.

#### Immunocytochemistry

The same pattern of immunostaining was observed for the secretory granule types in normal B-cells, transgenic mouse and in human tumour cells. Pro-insulin immunoreactivity was found to be restricted to immature secretory granules localised generally within the Golgi region of the tumour cells (Fig. 5a). Insulin and C-peptide immunoreactivities were found in immature, mature and in the atypical granule types (Figs 5b and 5c). One consistent finding in the tumours was of a significantly more intense immunoreaction when C-peptide antisera were used, compared to the result obtained using anti-insulin sera.

#### Discussion

The transgenic mouse model for heritable pancreatic B-cell tumour formation appears to be an excellent system for studying the induction, cellular adaptation and growth of this endocrine tumour type. Light microscopical immunostaining of the mouse and human normal and neoplastic tissue revealed an expected pattern for antigens such as insulin, C-peptide, pro-insulin, glucagon, somatostatin, chromogranin and NSE. At the electron microscopical level pro-insulin immunoreactivity was found to be restricted to immature secretory granules, whereas insulin and C-peptide immunoreactivities were found in all secretory granule types. The perinuclear staining with pro-insulin sera is consistent with the immature granule localisation close to the Golgi complex. Only

in the transgenic mouse tumours was C-peptide immunoreactivity consistently more intense than the immunostaining for insulin. This may reflect the probability that our anti-C-peptide cross-reacts extensively with pro-insulin as well as with free C-peptide. This in turn could indicate, however, an inefficient conversion of proinsulin into insulin which would be consistent with the finding of unexpectedly large tumours in some mice before death associated with hypoglycaemia. Pancreatic polypeptide (PP) cells were found in about 30–35% of both the mouse and human tumours and it will be intriguing to discover whether they represent included, or true tumour, cells. Pancreatic polypeptide cells are commonly encountered in human pancreatic islet B-cell tumours.<sup>29,30</sup>

Our results show that PGP 9.5 immunoreactivity may be readily detected in hyperplastic and neoplastic tumour cells from transgenic mice and human pancreatic tissue. Antisera to PGP 9.5, however, did not react with adult mouse pancreatic B-cells. We suggest that PGP 9.5 could prove to become recognised as an antigen expressed in transformed and/or growing B-cells. As PGP 9.5 is known to be a cytoplasmic, or at least a non-granular, protein<sup>31</sup> the presence of this immunoreactivity may reflect the degree of cellular transformation from the normal state without necessarily indicating the secretory or storage capacity of the cells.

In conclusion, our studies show the transgenic mouse model to be a useful system for studying endocrine cell transformation and adaptation.

The glucagon, GLP-1, GLP-2, somatostatin, gastrin, VIP and 7B2 antisera used in this study were raised in collaboration with Professor S R Bloom, Department of Medicine, RPMS, London, UK. Submitted by invitation to: Third International Conference on Intestinal and Pancreatic Adaptation, Titisee, FDR, June 1986. The authors are grateful to the following for the provision of antisera used during this study: Professors R H Goodman (Boston, USA; anti-RSCP), N Yanaihara (Shizuoka, Japan; anti-C-peptide), P J Marangos (Bethesda, USA; anti-NSE), Drs R Thompson (Cambridge, UK; anti-PGP 9.5), R Chance (Indianapolis, USA; anti-PP), H Gregory (Macclesfield, UK; anti-EGF), O Madsen (Copenhagen, Denmark; anti-pro-insulin) and A Moody (Bagsvaerd, Denmark; anti-glicentin).

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