Original Article

Patients With Chronic Pancreatitis Have Islet Progenitor Cells in Their Ducts, but Reversal of Overt Diabetes in NOD Mice by Anti-CD3 Shows No Evidence for Islet Regeneration

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Monoclonal antibodies to T-cell coreceptors have been shown to tolerise autoreactive T-cells and prevent or even reverse autoimmune pathology. In type 1 diabetes, there is a loss of insulin-secreting β-cells, and a cure for type 1 diabetes would require not only tolerance induction but also recovery of the functional β-cell mass. Although we have previously shown that diabetic mice have increased numbers of ductal progenitors in the pancreas, there is no evidence of any increase of insulin-secreting cells in the ducts. In contrast, in the adult human pancreas of patients with chronic pancreatitis, we can demonstrate, in the ducts, increased numbers of insulin-containing cells, as well as cells containing other endocrine and exocrine markers. There are also significantly increased numbers of cells expressing the homeodomain protein, pancreatic duodenal homeobox-1. Anti-CD3 has been shown to reverse overt diabetes in NOD mice; thus, we have used this model to ask whether monoclonal antibody–mediated inhibition of ongoing β-cell destruction enables islet regeneration to occur. We find no evidence that such monoclonal antibody therapy results in either regeneration of insulin-secreting β-cells or of increased proliferation of islet β-cells. *Diabetes* 56:634–640, 2007

Type 1 diabetes is characterized by selective immune-mediated destruction of the pancreatic β-cells, and disease is manifest when there is no longer enough insulin being secreted by the remaining β-cell mass to maintain glucose homeostasis. At the time of disease onset, although there will be extensive β-cell destruction, residual β-cells will be evident; albeit, many will have impaired function. This impaired ability to produce and secrete insulin may be due to cytokine-mediated effects (1,2). A cure for type 1 diabetes, therefore, requires both tolerance induction in the β-cell–specific T-cells and recovery of the functional islet mass (3).

Recovery of the functional islet mass could be achieved by recovery of residual β-cells from cytokine-mediated effects, proliferation of residual β-cells, and also islet neogenesis from precursors in the pancreatic ducts. While ductal ligation (4) and partial pancreatectomy (5,6) studies have indicated the presence of ductal progenitors capable of regenerating β-cell mass, recent studies using lineage tracing have highlighted a role for proliferation of residual β-cells as the main source of new β-cells following pancreatectomy (7). The advent of good tolerogenic protocols, which are capable of halting ongoing β-cell destruction, makes recovery of the β-cell mass in a diabetic individual a possibility. Monoclonal antibodies to coreceptors on T-cells have been shown to induce tolerance and prevent autoimmunity in a range of experimental models, as well as in graft rejection (8–17). Monoclonal antibodies to CD4 and CD3 have been shown to halt effector cell-mediated destruction of β-cells. (18,19). Recent clinical trials using humanized anti-CD3 antibodies in newly diagnosed type 1 diabetic patients have shown that C-peptide levels are maintained (or even improved) in some treated individuals, indicating no further loss of β-cells (20,21). To evaluate whether monoclonal antibody–mediated inhibition of ongoing β-cell destruction enables islet regeneration to occur, we have used the NOD mouse model of type 1 diabetes. We felt that this would be an appropriate model, as the early data preceding the translation to the clinic of anti-CD3 treatment had been carried out in NOD mice (18). Furthermore, there is evidence for the presence of ductal progenitors in the pancreas of diabetic NOD mice (22). These cells were identified by expression of the homeodomain protein pancreatic duodenal homeobox (PDX)-1, a factor shown to be essential for the development of both the exocrine and endocrine pancreas and expressed in pancreatic progenitors (23). PDX-1 is also expressed in adult β-cells where it plays a role as a transactivator of insulin transcription (24). Interestingly, although increased numbers of glucagon-containing cells were identified in the pancreatic ducts of diabetic NOD mice, there was no evidence of an increase in insulin-containing cells. One possible explanation for this was that insulin-containing cells were not detected because ductal cells in an adult animal may be unable to undergo full differentiation to produce insulin. An alternative explanation is that differentiated ductal cells become the target of further autoimmune attack in the diabetic

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Received for publication 19 June 2006 and accepted in revised form 30 November 2006.

BrdU, bromodeoxyuridine; PDX, pancreatic duodenal homeobox.

DOI: 10.2337/db06-0832

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TABLE 1
Details of chronic pancreatitis and control patients

Diagnosis	Age (years)	Sex
Chronic pancreatitis	55	M
Chronic pancreatitis	34	F
Chronic pancreatitis	53	F
Chronic pancreatitis	29	M
Chronic pancreatitis	33	F
Chronic pancreatitis	43	M
Chronic pancreatitis	52	F
Chronic pancreatitis	44	M
Chronic pancreatitis	45	M
Chronic pancreatitis	35	M
Chronic pancreatitis	34	M
Age-matched control subjects		
Islet cell tumour (insulinoma)	62	F
Islet cell tumour (insulinoma)	50	M
Pancreatic adenocarcinoma	38	M
Islet cell tumour (insulinoma)	29	F
Islet cell tumour (insulinoma)	53	F
Pancreatic adenocarcinoma	49	M
Pancreatic adenocarcinoma	56	F
Normal pancreas	40	F

mouse. There are some data suggesting that ductal progenitors are present in the pancreas in some pathological situations such as nesidioblastosis (25) and chronic pancreatitis (3). To determine whether the failure to detect insulin-containing ductal cells in adult diabetic NOD mice was an age-related differentiation problem or was due to autoimmune attack, we have examined adult human pancreas material where we had preliminary data suggesting that neogenesis could occur. We have carried out a detailed analysis of sections of pancreas from patients with chronic pancreatitis and present here evidence for regeneration from ductal progenitors in the adult human.

To determine whether the failure to detect insulin-containing cells in the ducts of diabetic NOD mice was due to autoimmune destruction, we have also carried out a series of experiments using monoclonal antibodies in NOD mice to determine whether, following tolerance induction, the reversal or improvement in diabetic symptoms is correlated with a recovery of the β-cell mass through neogenesis, recovery, or replication of residual cells. Our data suggests that these agents do not facilitate neogenesis but act by enabling recovery of residual β-cell function.

RESEARCH DESIGN AND METHODS

Human pancreas samples. Pancreas sections were obtained from 11 patients with chronic pancreatitis. There were seven males and four females. Control human pancreatic tissue was from eight patients (three male and five female) with either insulinomas or adenocarcinomas but who did not suffer from pancreatitis (Table 1). For study, normal control pancreata from children and young adults (age range 2.5–21 years) were obtained at autopsy from accident victims. The data from the latter control group are not described in detail but were similar to the former group of normal control subjects. Tissue samples were fixed in 10% neutral buffered formalin and paraffin embedded for histological analysis. Ethical permission was granted (to A.K.F.) for the study of endocrine cell proliferation in cases of chronic pancreatitis.

Mice. NOD mice were maintained in the Biological Services facility of the Department of Pathology at the University of Cambridge. They received standard laboratory food and water ad libitum. All animal experiments were approved by the ethical review committee of the University of Cambridge.

Antibodies for in vivo treatment. Hamster anti-CD3 antibody, clone 145 2C11, was obtained from Pharmingen (Becton Dickinson, Oxford, U.K.). Normal hamster immunoglobulin was from Rockland Immunochemicals (Gilbertsville, PA).

In vivo antibody treatment. Female NOD mice were tested twice per week for the presence of urinary glucose using Diastix (Bayer, Newbury, U.K.) test strips. Mice showing positive urinary glucose on two sequential occasions were considered diabetic and were then randomized to be given 5 μg/day i.v. for 5 consecutive days of either anti-CD3 or of normal hamster immunoglobulins.

Bromodeoxyuridine incorporation. To identify proliferating cells, mice were given a single dose of bromodeoxyuridine (BrdU) (Accurate Chemical and Scientific, Hicksville, NY) (100 μg/g body wt i.p.), dissolved in 0.007 N NaOH in normal saline. BrdU is a thymidine analog that becomes incorporated into the DNA of dividing cells. After 24 h of incorporation, mice were killed. Pancreases were fixed in Bouin's fluid and processed for paraffin embedding.

Immunohistochemistry. Immunostaining was performed according to the protocol of Gu and Sarvetnick (26). Briefly, paraffin sections were deparaffinised in xylene and rehydrated in graded ethanols to distilled water; excessive aldehydes in the fixed sections were quenched in 0.2 mol/l glycine for 30 min. Sections were then treated for 30 min with 0.3% hydrogen peroxide to block endogenous peroxidase, followed by a 30-min incubation in 10% normal goat serum (Vector Laboratories, Peterborough, U.K.) to block nonspecific binding sites. All antibody incubations were for 30 min, followed by washings with PBS/1% BSA.

To stain the human sections, the primary antibodies were mouse anti-Ki:67 (present in the G1, S, G2, and M phases of the cell cycle and absent from resting G0 cells), rabbit anti-glucagon (Chemicon International, Southampton, U.K.), guinea pig anti-insulin (Dako), and rabbit anti-amylase (Sigma, Gillingham, U.K.). Rabbit anti–PDX-1 was a gift from Helena Edlund (Umea, Sweden). The secondary antibodies were biotinylated anti-mouse Ig, anti-rabbit Ig, or anti– guinea pig Ig (Vector Laboratories). For the Ki67 and PDX-1 staining, microwave antigen retrieval was utilized in which paraffin sections were microwaved in 10 mmol/l citric acid monohydrate (pH 6.0) and processed for 3 × 5 min at high power before blocking in glycine.

To stain the mouse tissues with BrdU, sections were pretreated with 1.4 N HCl for 2 h, followed by antibody staining with rat anti-BrdU (Sera-Lab, Sussex, U.K.) and biotinylated anti-rat Ig (Vector Laboratories). For insulin and glucagon staining of mouse tissues, the same antibody combinations described above were used.

The sections were then incubated for 30 min in avidin-biotin peroxidase complex (ABC kit; Vector Laboratories), followed by detection with diaminobenzidene in 0.07% hydrogen peroxide as the substrate. Sections were counterstained with hematoxylin, dehydrated in alcohols and xylene, and mounted in DPX (VWR International, Poole, U.K.).

To stain for both glucagon and insulin, sections were first stained with glucagon as described above. After diaminobenzidene substrate, sections were incubated with guinea pig anti-insulin followed by anti– guinea pig fluorescein isothiocyanate (Dako, Glostrup, Denmark), counterstained and mounted in anti-quench medium.

Statistics. Statistical analyses were performed with GraphPad Prism using a Mann-Whitney U t test. Results were considered significant if P values were ≤ 0.05.

RESULTS

Ductal cell differentiation in the adult human pancreas. We had carried out preliminary studies suggesting that some ductal cells in the pancreas of patients with chronic pancreatitis had the capacity to differentiate into cells capable of producing proteins normally associated with either pancreatic endocrine or exocrine tissue (3). We therefore carried out a more detailed analysis of sections of pancreas from either human control or chronic pancreatitis patients to determine whether there were indeed statistically significant differences in the numbers of ductal cells apparently differentiating in these two groups. We used Ki67 to identify proliferating cells and antibodies to glucagon, insulin, and amylase to identify the cells that were undergoing differentiation. The homeodomain protein PDX-1 has been shown to be expressed in the pancreatic ducts following partial pancreatectomy in rats (27), as well as in the ducts of diabetic NOD mice (22). Its presence in pancreatic ducts has been shown to follow proliferation, leading to the suggestion that its expression in the ducts may reflect a pluripotent state enabling further differentiation (27). Antibodies to PDX-1 were therefore also used to stain sections in the two groups. Some of the

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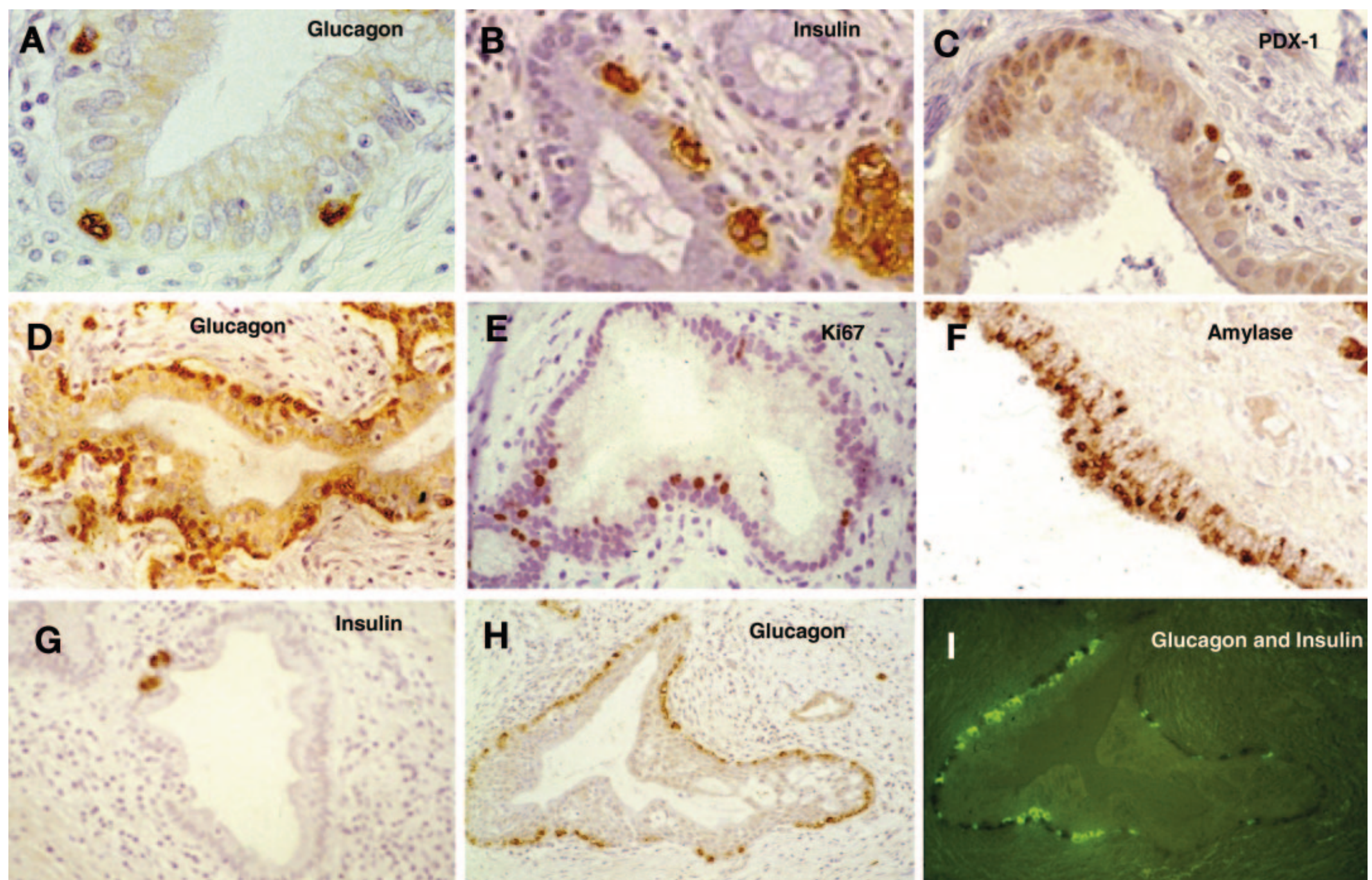

FIG. 1. Representative sections of pancreatic ducts from patients with chronic pancreatitis stained with primary antibodies to glucagon (*A*, *D*, and *H*), insulin (*B* and *G*), PDX-1 (*C*), amylase (*F*), and Ki67 (*E*). *I*: Stained for glucagon (brown) and insulin (green). Images *A*–*C* were taken at a magnification ×100. Images *D*–*I* were taken at ×40.

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observed staining patterns are shown in Fig. 1. We found that some ducts in patients with chronic pancreatitis had many cells staining, but we could find no evidence that individual cells expressed more than one hormone. An example of a duct containing many glucagon- and insulin-staining cells is shown (Fig. 1*I*).

The number of pancreatic ducts containing cells that were proliferating or expressing endocrine or exocrine proteins were enumerated (Fig. 2). Significant differences were found in the pancreatic ducts of chronic pancreatitis patients compared with control subjects. There were increased numbers of proliferating cells ($P < 0.0005$), and increased numbers of cells containing glucagon ($P < 0.005$), amylase ($P < 0.0003$), PDX-1 ($P < 0.0006$), or insulin ($P < 0.05$) were observed. In summary, these studies show that ductal cells in the adult pancreas have the capacity to undergo altered differentiation (metaplasia).

Does T-cell–targeted monoclonal antibody treatment influence ductal cell differentiation? Having established that ductal cells in the adult human pancreas have the capacity to express insulin protein, we examined whether this could be observed in the adult mouse pancreas once autoimmune-mediated β-cell destruction was halted. Monoclonal antibodies to CD4 or CD3 have been shown to halt primed effector cells from mediating tissue destruction, with antibodies to CD3 being particularly potent at reversing new-onset diabetes in NOD mice (18). This provides an ideal model to assess the capacity for regeneration in the adult diabetic pancreas following immune tolerance induction. We therefore treated groups of newly diagnosed diabetic NOD mice with anti-CD3 antibody for 5 days. As with the previously published data, this reversed diabetes in approximately two-thirds of the treated mice. Control mice received normal hamster immunoglobulin, and their diabetes progressed inexorably (Fig. 3*A*). Sections of pancreas from anti-CD3–treated mice showed the presence of recovered islets with peri-islet infiltrates (Fig. 3*B* and *C*). At three different time points (8–11, 17–20, and 28–40 days) after antibody therapy and 24 h before being killed, groups of control and anti-CD3–treated mice were injected with BrdU for subsequent analysis of the extent of cell proliferation. Sections of pancreas were then analyzed by immunohistochemistry for cell division and expression of glucagon and insulin. While there was evidence of glucagon-containing cells and cell division in the ducts, we could not find insulin-containing cells in the ducts of either diabetic or nondiabetic anti-CD3–treated mice (Fig. 4). Further groups of 12 control and 13 anti-CD3–treated mice were killed during the treatment period—after 2 days of treatment. Sections of pancreas were examined, but, again, we found no insulin-staining cells in the ducts of control or anti-CD3–treated mice (data not shown). This confirms our observations made at the later time points that there is no evidence for regeneration of β-cells from ductal cells in this treatment model. Thus, immune tolerance induction using this monoclonal antibody did not facilitate differentiation of ductal progenitor cells into insulin or, indeed, glucagon-producing cells. Furthermore, there was some indication that anti-CD3 treatment had significantly de-

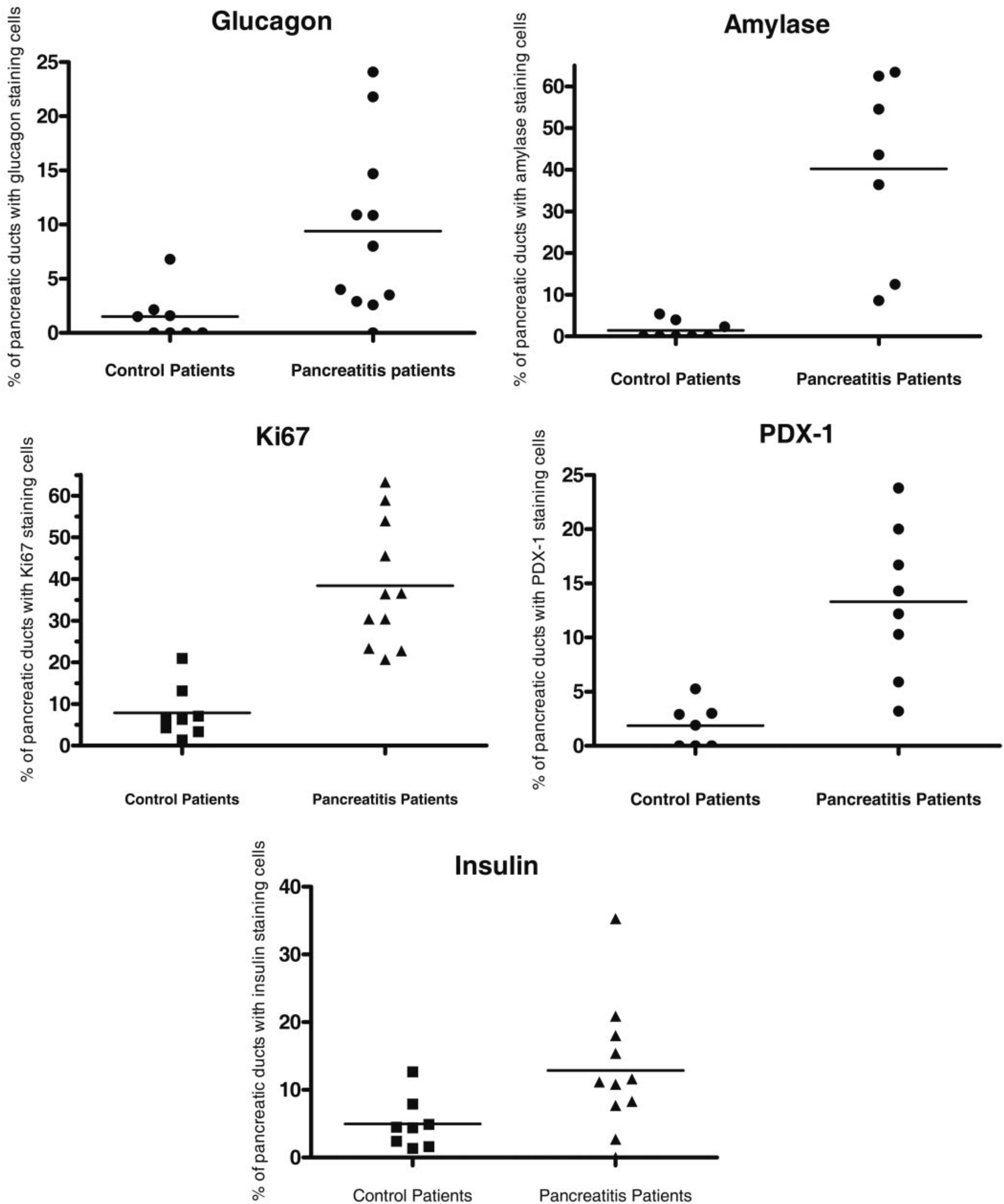

FIG. 2. Comparison of the percentages of ducts in the pancreas containing cells showing positive staining for the different markers between control patients and those with chronic pancreatitis. Each point represents an individual patient and is usually the mean of several sections analyzed. All ducts on each section were counted (on average ~100 ducts per patient). There are significant differences between these two groups for glucagon ($P < 0.005$), amylase ($P < 0.0003$), Ki67 ($P < 0.0005$), PDX-1 ($P < 0.0005$), and insulin ($P < 0.05$).

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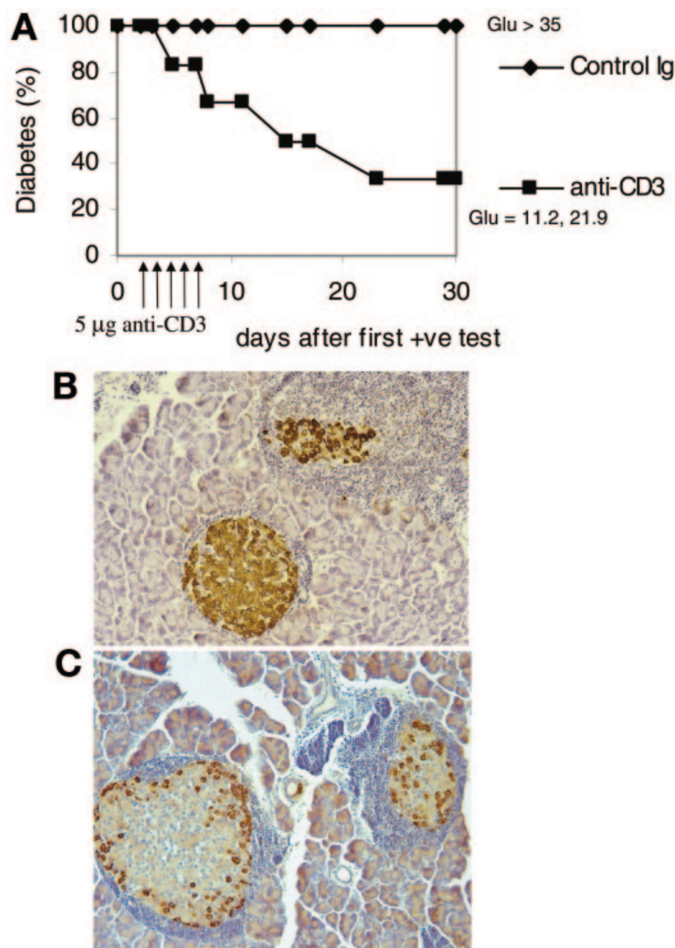

FIG. 3. Reversal of overt diabetes by anti-CD3 antibody. *A*: Overtly diabetic NOD mice were randomized to receive either anti-CD3 or control hamster antibody. Thirty days after the first positive test for glucosuria (+ve test), there was reversal to negative glycosuria in four of six anti-CD3–treated mice, and in the remaining two, blood glucose (Glu) levels were 11.2 and 21.9 mmol/l. Levels in the recipients of control hamster Ig were all >35 mmol/l. *B* and *C*: A representative example of insulin staining (*B*) and glucagon staining (*C*) in the pancreas of an anti-CD3–treated mouse, 40 days after the first positive test for glucosuria. This mouse had reverted to a negative urinary glucose and a blood glucose reading of 8.9 mmol/l. The remaining islets have a predominantly peri-islet infiltrate, although in some cases, this is quite extensive. Between the two islets in *C* is a small duct with glucagon-stained cells.

pressed ductal cell proliferation ($P < 0.02$) at the early (8–11 days) point of assessment.

The effect of monoclonal antibody therapy on proliferation of residual pancreatic β-cells. Our data suggest that there is no contribution of islet neogenesis from ductal progenitors in the reversal of diabetes by monoclonal antibody therapy seen in spontaneously diabetic NOD females. However, it is possible that new β-cells could arise by proliferation of residual β-cells in the diabetic pancreas. There is experimental data, albeit in a nonautoimmune situation, that adult β-cells can arise by proliferation of existing β-cells rather than arising from endogenous stem cells (7). To determine whether this reversal of diabetes, by immune tolerance through anti-CD3 therapy, was associated with replication of residual pancreatic β-cells, we compared the levels of BrdU incorporation in the pancreatic islets of diabetic and tolerized NOD mice. The times from first diagnosis were 8–11 and 17–20 days. Mice treated with control hamster Ig were killed 8–11 days after first diagnosis. No remaining islets, containing cells staining positive for insulin, could be found in the control group killed 17–20 days after first diagnosis. The number of cells in the insulin-containing islets staining for BrdU in the control hamster Ig group was 2.2 ± 0.8; in the anti-CD3–treated group (8–11 days from first diagnosis), this was 2.3 ± 0.6; and in BrdU-stained cells (17–20 days from first diagnosis), this was 4.5 ± 2.8. Again, we could find no evidence of an increased proliferation in the islets of tolerized NOD mice (Fig. 5).

DISCUSSION

Our studies in the adult human pancreas have shown that there is the potential for ductal cells to differentiate into cells capable of producing insulin. This was seen in patients with chronic pancreatitis where there is destruction of pancreatic tissue, and there was clear evidence of a significantly increased presence of ductal cells containing either glucagon or insulin. In contrast, our studies in spontaneously diabetic NOD mice, while showing increased development of glucagon-containing cells in the pancreatic ducts, did not show an increase in cells containing insulin. Indeed, there was a significant reduction in the presence of such cells in the adult diabetic mouse pancreas (22). This was consistent with an autoimmune destruction targeting presumptive new β-cells. Monoclonal antibodies to CD3 have been shown not only to reverse diabetes in NOD mice but also to significantly improve β-cell function in newly diagnosed diabetic patients (20,21,28). This antibody treatment is thought to establish immune tolerance to islet antigens in treated individuals and provided us with an ideal system to determine whether the observed lack of insulin-containing ductal cells is indeed due to autoimmune destruction. However, detailed analysis of the pancreas of treated mice at different time points after anti-CD3 therapy provided no evidence for β-cell neogenesis from ductal progenitors.

Why should there be such a disparate finding in the diabetic pancreas, even after tolerance induction, to that seen with chronic pancreatitis? The etiology of diabetes and chronic pancreatitis is clearly very different; in chronic pancreatitis, the exocrine pancreas is mainly affected and the process is usually nonautoimmune, whereas in type 1 diabetes, only the β-cells are targeted by an autoimmune process. The factors driving tissue repair and regeneration in the two different situations may have some overlap but also may be distinct as the patterns of destruction are so different.

There is good evidence suggesting that autoimmune destruction of the β-cell may be achieved through the combined activities of several cells. Studies in NOD mice have implicated $CD4^+$ T-cells, $CD8^+$ T-cells, and macrophages in the final demise of the β-cell. It is clear that pro-inflammatory cytokines adversely affect β-cell function (29–31), and neutralization or scavenging of such cytokines markedly improve β-cell survival (32). It is known that tolerogenic monoclonal antibody–based therapies inhibit the pro-inflammatory response in the pancreas of NOD mice (19), and it may be that by administering such tolerogenic antibodies, the immediate relief from a cytokine-mediated insult allows recovery of existing β-cells. This would explain not only diabetes reversal in NOD mice but also the observation of no further loss of C-peptide in type 1 diabetic patients treated with anti-CD3 (20,21,28). In the mouse, successful reversal

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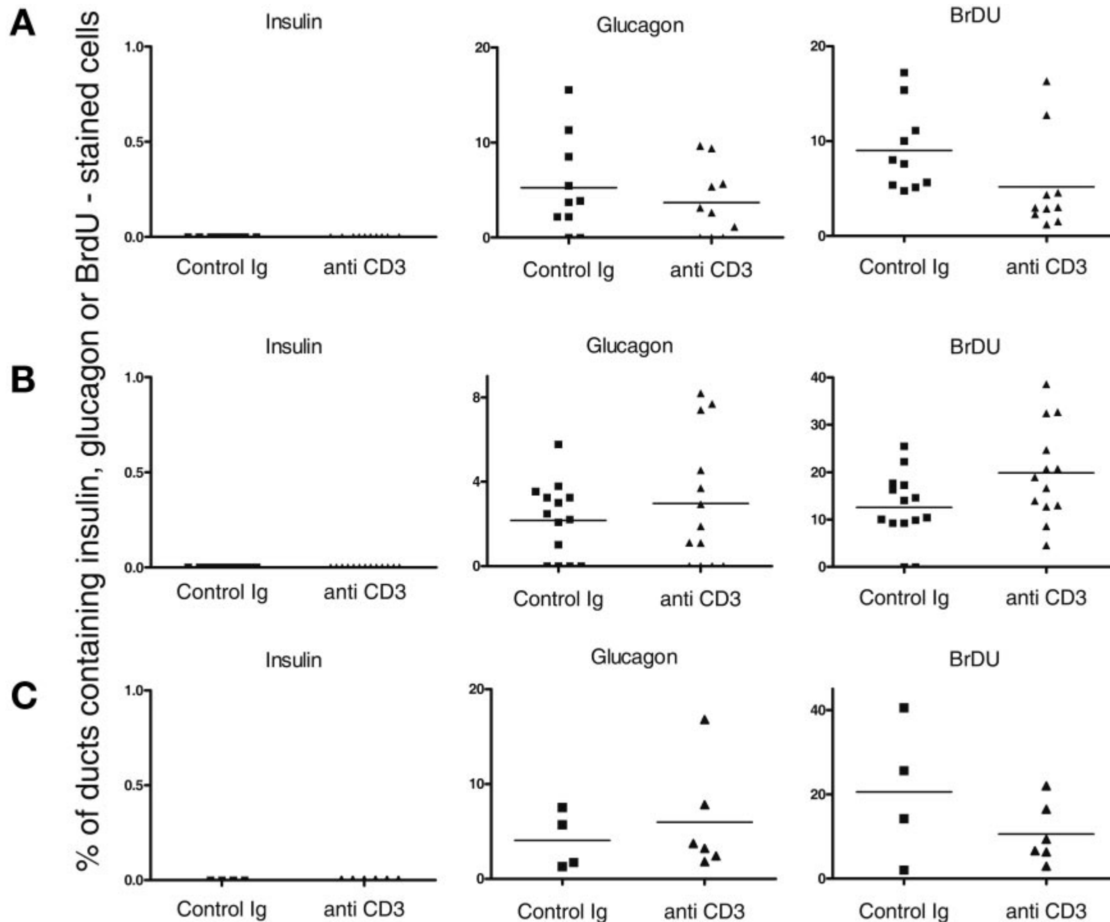

FIG. 4. Comparison of the percentages of ducts in the pancreas containing cells showing positive staining for the different markers between control hamster Ig– and anti-CD3–treated mice. Mice were killed at varying times after first diagnosis and subsequent treatment. The times from first diagnosis were 8–11 (*A*), 17–20 (*B*), and 28–40 (*C*) days. The points on the graphs represent individual mice, and the analysis was done counting all the ducts on at least three sections. In some cases, up to 12 sections per mouse were counted. On average, for each staining, ~100 ducts per individual mouse were counted. There was no evidence for regeneration of β-cells. There were no cells detected in the ducts of either group that stained positive for insulin. There was no difference between numbers of the glucagon-stained cells at any of the time points. The dividing cells that had taken up BrdU were in fact significantly fewer in the anti-CD3–treated group at the earliest time point, and there was no significant difference at the later time points.

of diabetes with anti-CD3 occurs only if animals are treated at the time of onset; it is ineffective if diabetes has been manifest for some time. This might suggest that although the induction of tolerance with monoclonal antibodies ensures that there is no further autoreactive response, there is no replacement of the destroyed β-cell mass. Indeed, we could find no evidence for restoration of the β-cell mass following tolerance induction either through regeneration from ductal progenitors or through replication of residual β-cells. Our inability to detect any evidence for β-cell replacement, following tolerance induction by anti-CD3, may reflect a lack of the growth and differentiation factors that play a role in neogenesis (rev. in 33). Regenerative and repair processes are elicited by stimuli such as inflammation or surgical interventions that target exocrine and endocrine tissue, whereas in type 1 diabetes, although inflammation is present, only the β-cell is targeted. Thus, signals arising from different modes of pancreas destruction may have very different outcomes.

Detailed analyses of the conditions (chronic pancreatitis or partial pancreatectomy) where regeneration occurs, either from ductal cells or through β-cell replication, will enable the characterization of factors that may eventually be used therapeutically in a coupled protocol with anti-CD3 antibody.

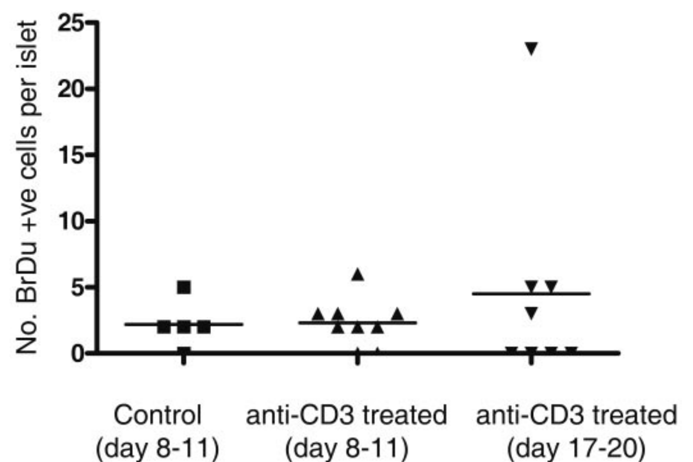

FIG. 5. Dividing cells in the insulin-staining islets of anti-CD3–treated mice. Number of BrdU-positive cells was counted in the islets, which on the sequential section were insulin positive of anti-CD3–treated mice. Mice were killed at varying times after first diagnosis and subsequent treatment. The times from first diagnosis were 8–11 and 17–20 days. Mice treated with control hamster Ig were killed 8–11 days after first diagnosis. No remaining islets, containing insulin-staining cells, could be found in the control group killed 17–20 days after first diagnosis. There was no significant difference between any of the groups.

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ACKNOWLEDGMENTS

This work was funded by a grant from the Wellcome Trust.

We thank Helena Edlund for the gift of the PDX-1 antibody.

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