Evidence for enhanced rates of complement activation in serum from patients with newly diagnosed insulin-dependent diabetes mellitus exposed to rat islet cells and complement-dependent induction of islet cell apoptosis

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Abstract

In this paper we report the concentration of terminal complement complexes (TCCs, SC5b-9, an index of complement activation) in newly diagnosed insulin-dependent diabetes mellitus (IDDM) patient serum and normal human serum. In the nine patients studied, levels of serum soluble TCCs were approximately 1.6-fold higher than in sera obtained from normal control individuals. On incubation of rat islet cells with diluted serum (10%, v/v, concentration), complement activation was increased at a significantly faster rate and the total TCC concentration was significantly higher in culture medium containing IDDM patient serum than in medium containing control serum. The concentration of anti-(glutamic acid decarboxylase) autoantibodies in newly diagnosed IDDM patient serum was on average 60-fold higher than in normal human control serum.

IDDM patient serum (10%, v/v) induced apoptosis in islet cells, as determined by islet cell density changes and

DNA fragmentation patterns. However, serum from IDDM patients was not able to induce apoptosis of the cells when complement components (C1q and C3) or antibodies were depleted. In addition, glutamine and the potent antioxidant 1-pyrrolidinedicarbothioic acid partially reversed cell death induced by IDDM patient serum in a concentration-dependent manner. The ATP concentration in islet cells incubated for 24 h in the presence of diluted IDDM patient serum was reduced to 4.4% of that observed in islet cells incubated in fetal calf serum or 7.3% of that observed in islet cells incubated in normal human serum. On the basis of these observations, we suggest that the pathway of IDDM patient serum-induced islet cell apoptosis may involve antibody-dependent complement activation, free radical generation and a precipitous fall in ATP levels.

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Introduction

The disease human insulin-dependent diabetes mellitus (IDDM) is characterised by β -cell death in the islets of Langerhans, resulting in a catastrophic loss of insulin-secreting capacity. Both cell-mediated and humoral immune mechanisms have been implicated in the pathogenesis of human IDDM (Eisenbarth 1986, Atkinson & Maclaren 1994). Animal models of IDDM have demonstrated a role for T-lymphocytes in promoting β -cell death (Bach 1988, Castano & Eisenbarth 1990). Human IDDM may also involve T-lymphocyte-induced β -cell death, as many pancreatic islets of newly diagnosed patients demonstrate insulinitis with T-cell infiltration. However, Somoza *et al.* (1994) failed to detect any message for interleukin (IL)-1, tumour necrosis factor (TNF) or interferon (IFN)- γ in pancreas from two patients with IDDM. O'Brien *et al.* (1997) have, in addition, reported that

detectable levels of β -cell apoptosis occurred before lymphocytic infiltration of islets in the spontaneously diabetic NOD/Lt mouse. It is also known that autoantibody titres are increased in the serum of individuals with IDDM months to years before the clinical onset of diabetes (Bingley *et al.* 1994, Bonifacio *et al.* 1995). Bottazzo *et al.* (1980) have reported that complement-fixing islet cell antibodies exist in the serum of IDDM patients and that they are a more reliable marker of pancreatic β -cell damage than conventional islet-cell antibodies. In addition, they reported deposition of the complement component C9 on some cells of pancreatic islets from a newly diagnosed IDDM patient (Bottazzo *et al.* 1985). However, it was not until recently that firm evidence emerged in support of a direct role for complement in islet cell death, indicating that complement-fixing islet cell antibodies in serum isolated from IDDM patients could trigger the assembly of terminal complement complexes

(TCCs) on human islet cells (Radillo *et al.* 1996). As the pancreatic donor in the latter study was non-diabetic, strong evidence for complement activation initiating β -cell damage rather than being a reaction to it was provided. The mechanism of islet cell death was not assessed by Radillo *et al.*

The mechanism of β -cell death responsible for the development of IDDM in mouse models (NOD (O'Brien *et al.* 1997) or low-dose streptozotocin (O'Brien *et al.* 1996)) or *in vitro* studies with isolated rat cells (L-1 β -, TNF- α - or IFN- γ -treated pancreatic islet or HIT β -cell line (Rabinovitch *et al.* 1994) or amylin-treated islet cells (Lorenzo *et al.* 1994)) have been described. All studies describe an apoptotic mechanism of β -cell death. The parameters measured include internucleosomal DNA cleavage, DNA strand breakage, cell volume decrease, condensation of nuclear chromatin and apoptotic body formation. In the study described here, we provide evidence for complement activation in serum from nine newly diagnosed IDDM patients, and assess biochemical and morphological parameters of apoptosis in rat islet cells exposed to newly diagnosed IDDM patient serum.

Materials and Methods

Unless otherwise stated all reagents were of analytical quality and were obtained from Sigma Chemical Company, Poole, Dorset, UK.

Human serum samples

Samples were obtained by venepuncture from nine consenting newly diagnosed IDDM patients and nine age-matched control subjects with no history of diabetes and confirmed normal glucose tolerance. Blood samples were collected in silicone-coated evacuated blood tubes and allowed to clot for 20 min at room temperature. The samples were then centrifuged at 400 *g* for 10 min and the supernatant removed for storage at -70°C until used.

The characteristics of the IDDM patient blood samples were as follows: seven male and two female patients, age 17–39 years. Serum samples were obtained between 3 days and 4 months after the diagnosis and at least 11 h after the last insulin injection. All patients were in a fasted state at the time the blood sample was taken.

Anti-(glutamic acid decarboxylase (GAD)) assay

Anti-GAD autoantibodies were measured in serum samples by using a 'Diaplets' anti-GAD kit supplied by Boehringer-Mannheim, according to the manufacturer's instructions.

Removal of complement components C1q and C3 from serum

Anti-C1q or anti-C3 antibodies (Sigma) were diluted in 10 mM phosphate buffer containing 145 mM NaCl, pH

7.2 (44 mg/ml anti-human C1q or 38 mg/ml anti-human C3). A 100 μl sample of diluted anti-C1q antibodies was added to columns 1–6 of a 96-well microtitre plate, and 100 μl of diluted anti-C3 antibodies to columns 7–12. After incubation at 4°C overnight, the buffer was removed and the wells washed three times with 10 mM phosphate buffer containing 500 mM NaCl and 0.1% (w/v) Tween 20 (pH 7.2). Then 100 μl of serum was added to column 1 and the plate was incubated at room temperature for 40 min. After this time, the serum samples were removed and transferred to column 2, where they were incubated for 40 min and then transferred to column 3, and so on. By the time the serum samples had reached column 12, they were depleted of complement (C1q and C3) as assessed by cell viability studies.

Control plates were set up with 0.1% BSA replacing anti-C1q and anti-C3 antibodies. The sera obtained from the control plates had similar complement-activation profiles to those not added to control plates.

Isolation and culture of rat islets

Male Wistar rats, 8–10 weeks old, were obtained from the Biomedical Facility, University College Dublin. Islet cells were obtained by collagenase digestion of the pancreas as previously described (Appels *et al.* 1989). Some 1×10^6 cells were seeded into each well of a 24-well plate in RPMI 1640 medium containing antibiotics and 10% serum (human or fetal calf serum (FCS)). In some experiments (where indicated) RPMI medium initially depleted of both glucose and glutamine was used for cell culture, but with the subsequent addition of 0, 1.0, 2.0 or 5.0 mM glutamine.

Culture of cell lines

The rat clonal β -cell line BRIN BD11 was kindly donated by Professor P. Flatt, University of Ulster, Coleraine, N Ireland and was cultured as previously described (McClenaghan *et al.* 1996). The pig kidney epithelial cell line, LLC-DK1, was generously provided by Professor M. Ryan, Department of Pharmacology, University College Dublin and was cultured as previously described (Hull *et al.* 1976).

Determination of SC5b-9 (TCC) formation in serum and culture medium from rat islet cells exposed to various serum types

Serum samples were obtained as described above and diluted for the SC5b-9 assay. Islet cells were prepared as previously described and plated at a density of 1×10^5 per well in a 24-well tissue culture plate; they were subsequently cultured with 10% (v/v) human serum (IDDM patient or normal) or 10% FCS over a time period of up to 24 h. SC5b-9 production was determined using an

enzyme immunoassay kit (Quidel, San Diego, CA, USA) as follows. The microassay wells coated with a mouse monoclonal antibody specific for human SC5b-9 were rehydrated by incubation with 250 µl wash solution (PBS, pH 7.4, containing 0.05% Tween 20 and 0.01% thiomersol) for 2 min at room temperature (20–26 °C). After incubation, the wash buffer was removed and the wells blotted dry on absorbent paper. Then 100 µl standards (human serum containing known amounts of SC5b-9) or appropriately diluted samples were added, followed by incubation at room temperature for 1 h. After incubation, the wells were washed five times with wash buffer and blotted dry as before. Then 50 µl SC5b-9 conjugate (horseradish peroxidase-conjugated goat antibodies to antigens of SC5b-9 diluted in PBS containing protein stabilizers and 0.01% thiomersol) were added, followed by incubation for 1 h at room temperature. After this period the mixture was decanted and the wells were washed four or five times as before. Immediately after the wash procedure, 100 µl substrate solution (50 µl of 0.7% 2,2'-azino-di-(3-ethylbenzthiazolinesulphonic acid) diammonium salt per ml of 0.1 M citrate buffer containing 0.05% H₂O₂) was added to each well. After 30 min incubation at room temperature, the reaction was terminated by the addition of 50 µl 250 nM oxalic acid to each well. The absorbance was determined at 405 nm within 1 h.

Percoll density-gradient centrifugation

Percoll solutions (1.08, 1.078, 1.076, 1.074, 1.072, 1.07, 1.06 g/ml) were prepared as previously described (Cotter & Martin 1996) and layered into an appropriate centrifuge tube. Islet cells (suspended in 2 ml Percoll solution (1.06 g/ml)) were carefully layered on top of the gradient before centrifugation at 400 *g* for 30 min.

Detection of islet cell cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleotides) by ELISA and DNA strand breaks by in situ nick translation

Islet cells were cultured in RPMI 1640 medium supplemented with 10% serum (IDDM patient, normal human or FCS). After 24 or 48 h the culture plate was centrifuged for 10 min at 200 *g*. The supernatant was removed, and cell pellets were resuspended in lysis buffer, centrifuged to remove cell debris and the supernatant transferred to a prepared microtitre plate for determination of DNA fragments using a cell death detection ELISA kit (Boehringer-Mannheim) according to the manufacturer's instructions.

To detect DNA strand breaks *in situ*, rat islet cells were incubated in the presence of IDDM serum or normal human serum for 24 h and then acetone-fixed. Cells were subsequently washed and exposed to nick-translation mixture (Feshel *et al.* 1994). They were finally exposed to

peroxidase-labelled avidin to allow immunocytochemical detection of biotin-labelled dUTP. Cells with strand breaks stained brown.

Determination of cell viability

Islet cell viability was assayed via exclusion of the vital dye, trypan blue, which is taken up by cells when loss of membrane integrity occurs. Cell viability of both islet cells and kidney epithelial cells was also assessed via cell lactate dehydrogenase content and agreed well with the trypan blue-exclusion studies.

Determination of ATP concentration

The bioluminescent somatic cell assay kit (Sigma) was used to determine ATP released from a suspension of islet cells after culture in the presence of 10% IDDM patient serum, normal human serum or FCS. ATP concentration was determined in accordance with the manufacturer's instructions using a Turner luminometer.

Statistical analysis

The results are presented as mean $\pm$ s.e.m. Groups of data were compared using unpaired Student's *t*-test where appropriate. Differences were considered significant if $P < 0.01$ or $P < 0.001$.

Results

SC5b-9 concentration in serum obtained from newly diagnosed IDDM patients and normal human control subjects and in serum diluted in cell culture medium and exposed to rat islet cells for up to 24 h

Levels of soluble TCCs (SC5b-9) were determined in serum from both IDDM patients and non-diabetic control individuals. Nine newly diagnosed IDDM patients gave a blood sample between 3 days and 5 months after the initial diagnosis. The IDDM patients had a mean age of 26.8 ± 2.4 years (mean $\pm$ s.e.m.). The TCC level in these individuals was 3.69 ± 0.59 µg/ml. Nine non-diabetic individuals (sex-matched) mean age 25.8 ± 1.2 years had a mean TCC concentration of 2.28 ± 0.75 µg/ml (Fig. 1*a*). The difference in TCC concentration between IDDM and non-diabetic individuals was not significantly different ($0.1 < P < 0.2$). However, exposure of rat pancreatic islet cells to 10% IDDM patient serum (from here on called IDDM serum) caused substantial activation of complement compared with cells incubated in the presence of normal human serum, resulting in a significantly higher TCC concentration in the medium of cells exposed to IDDM serum at all time points measured (Fig. 1*b*). In addition, significantly higher levels of TCC were found in

Figure 1 Determination of level of complement activation in human sera and islet cell incubation medium. Patient and normal human sera were obtained as described in the Materials and Methods section. (a) SC5b-9 was determined in diluted serum samples. (b) 1×10^6 islet cells were incubated in the presence of 10% (v/v) IDDM serum (□) or normal human serum (NHS) (◇) for up to 24 h. SC5b-9 concentrations were determined. They were significantly higher in medium containing IDDM serum than in medium containing normal human serum where indicated (** $P < 0.01$).

medium supplemented with IDDM serum after exposure to the rat clonal β -cell line BRIN BD11 than in medium supplemented with normal human serum (Table 1). The concentration of TCC was not significantly elevated in culture medium containing IDDM serum above that observed in medium containing normal human serum when exposed to either of two unrelated cell types, the pig kidney epithelial cell line, LLC-DK1, or the murine resident peritoneal macrophage (Table 1).

Anti-GAD concentration in newly diagnosed IDDM patient serum

The presence of antibodies to GAD is an indication of an autoimmune process which, in the majority of cases, leads to IDDM. A positive test result with an anti-GAD concentration of >32 ng/ml indicates manifest IDDM or an autoimmune process that is likely to lead to IDDM. Using a Boehringer-Mannheim ‘Diaplets’ assay kit,

Table 1 The concentration of SC5b-9 (TCC, Terminal Complement Complex) after 24 h in cell culture medium supplemented with either normal human or IDDM patient serum (10%, v/v) was determined. *P*-values indicate the level of significant difference between TCC concentration in medium containing IDDM serum compared with medium containing normal human serum after 24 h of cell culture. Results are expressed as mean $\pm$ S.E.M.

Cell type	Serum type	TCC formation after 24 h culture	
		(ng per 1×10^6 cells)	<i>P</i> value
Primary rat islets	Normal human	2838 $\pm$ 450	<0.01
	IDDM patient	6424 $\pm$ 435	
BRIN BD11 rat pancreatic β -cells	Normal human	3534 $\pm$ 334	<0.01
	IDDM patient	6213 $\pm$ 453	
LLC-DK1 pig kidney epithelial cells	Normal human	1572 $\pm$ 197	=0.031
	IDDM patient	1007 $\pm$ 95	
Primary rat peritoneal macrophages	Normal human	1930 $\pm$ 83	>0.05
	IDDM patient	2233 $\pm$ 232

Figure 2 Determination of changes in islet cell buoyant density. Islet cells (1×10^6) were incubated in the presence of 10% (v/v) IDDM serum, heat-treated IDDM serum, C1q- and C3-depleted IDDM serum or FCS for 4 or 8 h. Islet cell buoyant density was then determined. The change in islet cell density was significantly different for cells incubated in IDDM serum from that in cells incubated in either heat-treated or C1q- and C3-depleted IDDM serum (** $P < 0.001$, after 4 h incubation and $\Delta\Delta\Delta P < 0.001$ after 8 h incubation).

we determined a mean anti-GAD concentration of 925.6 ± 190.5 ng/ml in newly diagnosed IDDM patient serum ($n=15$) which is approximately 60-fold higher than that in normal human serum (15.4 ± 1.5 ng/ml; $n=12$).

Assessment of buoyant cell density after 4 or 8 h exposure to IDDM serum, complement-depleted IDDM serum, heat-treated IDDM serum, non-diabetic serum or FCS

As early apoptotic cells shrink, there is an increase in their buoyant cell density. They can be resolved from their non-apoptotic neighbours by centrifugation after layering on to a 1.06–1.08 g/ml Percoll gradient (Appels *et al.* 1989). After centrifugation, non-apoptotic cells are found at the 1.06/1.07 g/ml interface, whereas the cells that form the pellet are apoptotic. Exposure of rat islets for 4 or 8 h to IDDM serum resulted in a large change in buoyant cell density, with approximately 60% and 80% of cells detected as apoptotic after 4 h and 8 h respectively (Fig. 2). The cells detected in the non-apoptotic layer were not

insulin containing as determined by immunofluorescence and immunostaining (insulin) methodologies. Depletion of IDDM serum complement components C1q and C3 or heat treatment (denaturing heat-labile complement components) resulted in a large decrease in the amount of apoptotic cells detected (approximately 30% after 4 and 8 h; Fig. 2). The latter value was close to the level of apoptosis detected in cells exposed to normal human serum or FCS.

Measurement of DNA fragmentation in rat islet cells exposed to IDDM serum, heat-treated IDDM serum, normal human serum or FCS

One of the hallmarks of apoptotic death is early DNA fragmentation. It is possible to detect histone-associated DNA fragments (mono- and oligo-nucleosomes) released from the fragmenting nuclei of cells undergoing apoptosis, by an ELISA method. We determined that there was an approximately 600% increase in DNA fragmentation in rat islet cells exposed to IDDM serum for 24 h compared with cells exposed to heat-treated IDDM serum or normal human serum (Fig. 3a). Similar results were obtained for rat islet cells exposed to 10% serum for 48 h.

To detect DNA strand breaks *in situ*, we incubated rat islet cells in the presence of IDDM serum for 24 h and then acetone-fixed the cells. Cells were subsequently washed and exposed to nick-translation mixture (Feshel *et al.* 1994). They were finally exposed to peroxidase-labelled avidin to allow immunocytochemical detection of biotin-labelled dUTP. Those with strand breaks stained brown. There was an approximately twofold increase in cells containing DNA strand breaks after 24 h incubation in IDDM serum compared with cells incubated in normal human serum (Fig. 3b).

Cell viability in cells exposed to newly diagnosed IDDM patient serum, heat-treated IDDM serum, normal human serum or FCS, assessed by trypan blue exclusion

One of the major distinguishing morphological features of cell death is a loss of membrane integrity which may be detected by failure to exclude trypan blue. Both necrotic and apoptotic cells undergoing secondary necrosis will fail to exclude trypan blue. When rat islet cells were exposed to IDDM serum for 24 h, approximately $65.0 \pm 2.9\%$ remained viable, whereas only $50.0 \pm 1.7\%$ were viable after 48 h (Fig. 4). The number of viable cells remaining after exposure to 10% heat-treated IDDM serum or normal human serum for 24 h was $89.0 \pm 2.3\%$ and $80.8 \pm 5.8\%$ respectively (Fig. 4), whereas viability after 48 h incubation was determined to be $82.6 \pm 4.1\%$ or $72.1 \pm 2.9\%$ respectively (Fig. 4).

The difference in loss of viability between cells cultured in untreated IDDM serum compared with those cultured in heat-treated IDDM serum, normal human serum or

Figure 3 Measurement of islet cell DNA fragmentation and single-cell DNA strand breaks. (a) Islet cells were incubated in the presence of 10% (v/v) IDDM, heat-treated IDDM or normal human serum, or FCS for 24 or 48 h. DNA fragmentation was determined using a Boehringer-Mannheim Cell death detection ELISA. Islet cells cultured in untreated IDDM serum had a significantly higher level of fragmented DNA compared with those cells cultured in heat-treated IDDM serum or normal human serum ($***P < 0.001$ after 24 h incubation and $^{***}P < 0.001$ after 48 h incubation). (b) To detect DNA strand breaks *in situ*, rat islet cells were incubated in the presence of IDDM serum or normal human serum for 24 h and then acetone-fixed. Cells were subsequently washed and exposed to nick-translation mixture (Feshel *et al.* 1994). They were finally exposed to peroxidase-labelled avidin to allow immunocytochemical detection of biotin-labelled dUTP. Cells with strand breaks stained brown. ($**P < 0.01$ after 24 h incubation compared with untreated IDDM serum).

FCS was significant ($P < 0.01$) after 24 and 48 h (Fig. 4). However, loss in viability can be both IDDM serum-dependent and IDDM serum-independent, as illustrated by the results presented in Fig. 4 (compare loss of viability of cells cultured in untreated IDDM serum, normal human serum and FCS). The degree of non-specific serum-induced cell death increased substantially after 72 h, so that there was no difference in loss of viability of cells cultured in IDDM serum or normal human serum after 96 h of culture (results not shown).

The viability of an unrelated cell type, the pig kidney epithelial cell line, LLC-DK1, was reduced to $87.4 \pm 2.4\%$ after 24 h incubation in the presence of IDDM serum. In addition, the viability of murine resident peritoneal macrophages was not significantly reduced by 24 h of culture in IDDM serum compared with culture in normal human serum (results not shown). The loss of viability of islet cells cultured in IDDM serum is thus cell specific.

Effect of depletion of complement components C1q and C3 or Protein A immunoprecipitation of immunoglobulins, from IDDM patient serum on cell viability

To determine the importance of complement pathway activation or the presence of immunoglobulins of the IgG

class to the loss of islet cell viability induced by IDDM serum, we depleted serum of either complement components C1q and C3 or IgG as described in the Materials and Methods section. The substantial loss of islet cell viability observed on exposure of islet cells to IDDM serum (compared with normal human serum or FCS) was abolished when C1q and C3 were removed from the serum (Fig. 5a) or IgG was removed from the serum (Fig. 5b) after both 24 and 48 h of incubation.

Effect of the antioxidant 1-pyrrolidinedithiocarbamic acid, ammonium salt (PDTC) on islet cell viability after exposure to IL-1 β or IDDM serum

IL-1 β can potentially induce death in islet cells: 10 ng/ml reduced islet cell viability from $83.1 \pm 3.5\%$ to $40.1 \pm 3.5\%$. The cytotoxic effect of 10 ng/ml IL-1 β can be partially reversed by incubation of the islet cells with the potent antioxidant PDTC; the recovery occurs in a concentration-dependent manner, reaching a value of $63.4 \pm 2.9\%$ in the presence of 500 μ M PDTC (Fig. 6, top). When rat islet cells were incubated with IDDM serum for 48 h, the substantial loss of viability ($55.8 \pm 3.5\%$) could also be partially reversed, in a

Figure 4 Effect of incubation in various serum types on islet cell viability. Islet cells were incubated in the presence of 10% (v/v) IDDM, heat-treated IDDM or normal human serum for 24 or 48 h. Cell viability was determined by trypan blue exclusion. Islet cells cultured in untreated IDDM serum had a significantly lower viability than those cells cultured in heat-treated IDDM serum or normal human serum (** $P < 0.01$ after 24 h incubation and $\Delta\Delta P < 0.01$ after 48 h incubation).

concentration-dependent manner, by incubation in the presence of PDTC (to $72.7 \pm 2.3\%$; Fig. 6, bottom).

Effect of glutamine concentration on islet cell viability after exposure to IDDM serum, normal human serum or FCS

When rat islet cells were incubated in the presence of IDDM serum for 24 or 48 h, the substantial loss of viability (compared with control serum) could be reversed by incubation in the presence of 5 mM glutamine (50% viability after 48 h incubation was increased to $76.2 \pm 1.7\%$ in the presence of 5 mM glutamine; Fig. 7). The addition of 5 mM glutamine had no effect on viability of cells incubated in heat-treated IDDM serum, normal human serum or FCS (results not shown).

Additional experiments demonstrated that the substantial loss of cell viability (compared with control serum) of rat islets incubated in the presence of IDDM serum for 48 h could be partially reversed by incubation in the presence of $1 \mu\text{M}$ nifedipine, an L-type Ca^{2+} channel

antagonist (from 54.1% in the absence to 62.8% in the presence of $1 \mu\text{M}$ nifedipine after 48 h incubation; results not shown).

Measurement of cell ATP concentration after exposure to IDDM serum, normal human serum or FCS

Islet cells were incubated in the presence of IDDM serum, normal human serum or FCS for 24 and 48 h. ATP concentration in cells exposed to IDDM serum was reduced to 4.4% of that found in cells incubated in FCS or 7.3% of that found in cells incubated in normal human serum after 24 h (Table 2). A similar precipitous fall in ATP concentration was observed in cells incubated in IDDM serum for 48 h (Table 2).

Discussion

Evidence for complement activation, e.g. increased circulating levels of C4a and C3a but no detectable change in TCC concentration in serum from IDDM patients compared with serum samples from patients with non-insulin-dependent diabetes mellitus and age-matched controls, has been previously reported (Bergamaschini *et al.* 1991). However, the IDDM patients used in the latter study had been diagnosed some years earlier (mean 3 years, range 2–23 years), thus it is probable that reduced circulating autoantibody and activated complement components in these patients may explain the low levels reported (Bergamaschini *et al.* 1991) compared with those reported here for newly diagnosed patients. Other reports have shown that complement-fixing islet cell antibodies are increased in the serum of IDDM individuals (Bonifacio *et al.* 1995, Dobersen *et al.* 1980). The previous studies may have been unable to detect significant levels of activated complement in serum from IDDM patients, because of lack of availability or poor specificity of antibodies used for the TCC ELISA. Here we report that levels of serum soluble TCCs are approximately 1.6-fold higher in serum from newly diagnosed IDDM patients than age-matched non-diabetic control individuals. Complement activation in IDDM individuals may initiate β -cell damage rather than occur in response to damage caused by other immune mechanisms, thus a number of studies have attempted to demonstrate complement-mediated β -cell cytotoxicity after exposure to IDDM patient serum. IDDM patient serum was found to be cytotoxic to rat islet cells (Feshel *et al.* 1994), hamster islet cells (Rittenhouse *et al.* 1980) and cloned rat islet cells (Eisenbarth *et al.* 1981) at levels significantly above those found when cells were incubated in serum from patients with non-insulin-dependent diabetes mellitus or normal human serum. However, one report has shown that normal human serum (heat-inactivated) can cause similar levels of cytotoxicity to newly diagnosed IDDM serum in

Figure 5 Effect of incubation in standard serum, complement-depleted IDDM serum, Protein A-treated serum, normal human serum or FCS on islet cell viability. (a) Islet cells were incubated in the presence of 10% (v/v) IDDM, C1q- and C3-depleted IDDM serum or normal human serum for 24 or 48 h. Cell viability was determined by trypan blue exclusion. Islet cells cultured in C1q- and C3-depleted IDDM serum had significantly greater viability than those cells cultured in untreated IDDM serum (** $P < 0.01$ after 24 h incubation and $\Delta\Delta P < 0.01$ after 48 h incubation). (b) Islet cells were incubated in the presence of 10% (v/v) IDDM, Protein A-treated IDDM serum or normal human serum for 24 or 48 h. Cell viability was determined by trypan blue exclusion. Islet cells cultured in Protein A-treated IDDM serum had significantly greater viability than those cells cultured in untreated IDDM serum (** $P < 0.01$ after 24 h incubation and $\Delta\Delta P < 0.01$ after 48 h incubation).

the presence of rabbit serum (Cavender *et al.* 1986). The problems associated with heterologous activation of complement may have contributed to the latter results. However, more recently, Radillo *et al.* (1996) have reported that the cytotoxic activity of IDDM serum towards human pancreatic islets is dependent on TCC formation. In this study, we have shown that complement activation occurs in culture medium supplemented with IDDM or control serum exposed to rat islet cells, but the level of TCC in medium supplemented with serum from IDDM patients was significantly higher at all time points measured. In addition, we have reported that the level of TCC in cell culture medium after exposure to the clonal β -cell line, BRIN BD11, was significantly higher in medium supplemented with IDDM serum than in normal human serum (Table 1; Conroy *et al.* 1998). The level of TCC in medium supplemented with IDDM serum was not significantly different from medium supplemented with normal human serum when exposed to either primary murine macrophages or the pig kidney epithelial cell line, LLC-DK1 (Table 1). We have demonstrated that there is a relatively high concentration of autoantibodies in IDDM patient serum (45-fold above normal human serum) and we speculate that elevated complement

activation occurs after the autoantibodies have bound to their specific autoantigen target on the β -cell surface.

The mechanism of complement-mediated cytotoxicity in islets exposed to IDDM serum has only been alluded to in one recent study, where the process of apoptosis was implied on the basis of DNA fragmentation patterns (Juntti-Berggren *et al.* 1993). In this study we have provided evidence that, on exposure to islet cells, complement is activated at a faster rate in IDDM serum than in non-diabetic control serum, and that complement is responsible for initiating apoptosis in islet cells. Our cell viability assays also demonstrate that both complement and antibody components in serum are involved in the process of cell death, probably via classical complement pathway activation. The mechanism by which apoptosis is initiated by complement activation remains unknown. However, evidence is provided in this paper that oxidant generation may be involved, as loss of viability may be partially reversed by addition of the antioxidant, PDTC. As IL-1 β -induced cell death may also be partially reversed by PDTC, the pathways of complement- and IL-1 β -induced death may share common steps. IL-1 β is known to upregulate the expression of inducible nitric oxide synthase in HIT-T15 cells (Eizirik *et al.* 1992) as well as

Figure 6 Effect of the antioxidant PDTC on IL-1 β - or IDDM serum-induced islet cell cytotoxicity. Top, 1×10^6 islet cells were incubated with 10% (v/v) FCS, 10 ng/ml IL-1 β and various concentrations of PDTC for 24 h. PDTC significantly inhibited IL-1 β -induced cytotoxicity at 500 μ M (* $P < 0.01$ after 24 h incubation). Cell viability was determined by trypan blue exclusion. Bottom, 1×10^6 islet cells were incubated with 10% (v/v) IDDM serum and various concentrations of PDTC for 24 and 48 h. Cell viability was determined by trypan blue exclusion.

Figure 7 Effect of extracellular L-glutamine concentration on IDDM serum-induced islet cell cytotoxicity. Islet cells (1×10^6) were cultured in glucose- and glutamine-deficient RPMI medium (Gibco) and were additionally incubated with 10% (v/v) IDDM serum and various concentrations of L-glutamine for 24 and 48 h. Cell viability was determined by trypan blue exclusion. L-Glutamine significantly inhibited IDDM serum-induced cytotoxicity at 5 mM (** $P < 0.01$ after 24 h incubation and $\Delta\Delta P < 0.01$ after 48 h incubation).

stimulating NO production in mouse pancreatic islets (Welsh & Sandler 1992) and rat pancreatic islets (Corbett *et al.* 1991). IL-1 β also causes significant DNA damage in islet cells and HIT-T15 cells via NO generation (Delaney *et al.* 1993, Kaneto *et al.* 1995). Thus NO generation may

Table 2 ATP concentration in rat islet cells exposed to 10% IDDM patient, normal human serum or FCS for 24 or 48 h. Islet cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) IDDM or normal human serum or FCS. After 24 or 48 h ATP concentration was determined using a bioluminescent somatic cell assay kit (Sigma), according to the manufacturer's instructions. ATP concentration as fmol/100 000 cells

Serum type	ATP concentration	
	24 h	48 h
IDDM (untreated)	0.11	0.07
IDDM (heat treated)	0.90	0.86
Normal human (untreated)	1.50	1.32
Normal human (heat treated)	1.43	1.21
FCS	2.50	1.90

play a key role in IL-1 β -stimulated cell death. The antioxidant PDTC may act upstream of NF κ B, i.e. before transcription of inducible nitric oxide synthase, as oxidants can be potent stimulators of the transcription factor (Schreck *et al.* 1992), or may act by scavenging NO radicals after translation of inducible nitric oxide synthase. The mechanism of complement-stimulated islet-cell death and the mechanism described above for IL-1 β -stimulated death may or may not share common components.

We have determined that provision of L-glutamine can be cytoprotective to islet cells exposed to IDDM serum. L-Glutamine can be utilized at high rates by islet cells (Malaisse *et al.* 1982) and its metabolism may be connected to the pathways controlling insulin secretion. It is possible that oxidation of L-glutamine can elevate the ATP/ADP ratio sufficiently to block a catastrophic depletion of ATP which may occur after DNA damage and activation of poly(ADP-ribose) polymerase (Okamoto 1992). We have demonstrated that ATP levels in islet cells incubated in the presence of IDDM serum are reduced to 4.4% of those in islet cells incubated in FCS or 7.3% of those in islets incubated in normal human serum after 24 h (Table 2). The L-type Ca²⁺ channel antagonist, nifedipine, partially reversed the cytotoxic effects of IDDM serum on islet cells (results not shown), suggesting that Ca²⁺ influx may contribute to cell death-promoting pathways. Our data suggesting that activation of L-type Ca²⁺ channels may be important in the mechanism of death support those of Juntti-Berggren *et al.* (1993) who provided evidence for the involvement of L-type Ca²⁺ channels in the pathway of IDDM serum-induced death of RINm5F cells.

The involvement of autoantibodies and complement in β -cell apoptosis *in vivo* may occur downstream of a switch in activated T-lymphocyte stimulation of B-lymphocytes such that the B-lymphocytes subsequently produce complement-fixing anti- β -cell antibodies. The relatively low level of complement activation that we report here in the serum of newly diagnosed IDDM patients (the level of 5 μ g/ml TCC in IDDM serum represents about 2% of total circulating C5 to C9 components) suggests a 'low-grade' assault *in vivo* that results in a relatively slow rate of apoptosis, which must exceed the rate of β -cell regeneration. This hypothesis would fit with the known slow pathogenesis of most types of IDDM, from initial β -cell destruction to clinical diagnosis. Previous experiments in which autoantibodies were injected into healthy animals without subsequent development of diabetes may indicate that the initial autoantibodies produced in diabetes-prone animals are non-complement-fixing. These autoantibodies would not be able to precipitate diabetes in recipient animals. A switch in autoantibody isotype in response to a developmental/environmental stimulus may result in circulating complement-fixing autoantibodies being produced from B-lymphocytes, leading to enhanced rates of β -cell destruction. We speculate that complement-fixing

autoantibodies will be present at high concentrations at the time of diagnosis.

Although cytotoxic T-lymphocytes and macrophages may play a significant role in late β -cell destruction, a paper by Radillo *et al.* (1996) has described detectable levels of β -cell apoptosis before lymphocytic infiltration of islets in the NOD/Lt mouse, suggesting that humoral factors may be important early on in disease pathogenesis.

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