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Effects of islet cell surface antibodies and complement on the release of insulin and chromium from perifused β cells

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SUMMARY

The dynamics of insulin and chromium release from prelabelled rat pancreatic islet cells were studied by perifusion of cells supported in a column of Bio-Gel P-2 polyacrylamide beads. The column-perifused β cells released insulin in a biphasic pattern in response to 30 mmol/l D-glucose and in a monophasic pattern to 20 mmol/l L-arginine. Rat islet cells, first exposed to a rabbit anti-rat islet cell surface serum and complement and then added to the column, were unable to release insulin in response to 30 mmol/l D-glucose and 0·1 mmol/l 3-isobutyl-1-methylxanthine (IBMX). In order to study cytotoxicity by an additional approach, islet cells were prelabelled with radioactive chromium ($Na_2{}^{51}CrO_4$). These cells did not release either insulin or ^{51}Cr in response to glucose. Furthermore, exposure of the cells to surface antiserum and complement before perifusion did not induce either chromium or insulin release. Similar results were obtained when glucose alone or combined with surface antiserum was added to the perifusate bathing rat islet cells incubated with 0·1 mmol/l non-radioactive Na_2CrO_4 before perifusion. However, a transient, dramatic release of insulin from these cells was induced by adding complement to the perifusion medium (containing surface antibodies). These results indicate that complement-dependent cytotoxicity of islet cell surface antibodies involves different phenomena. Firstly, the cytotoxic reaction results in a transient release of insulin whether the physiological release mechanisms were blocked by chromium or not. Secondly, in cells not treated with chromium the cytotoxic reactions renders the β cells unable to release insulin in response to glucose.

INTRODUCTION

Islet cell antibodies are organ-specific, species-non-specific antibodies which react with either cytoplasmic or cell surface components of pancreatic islet cells (Bottazzo, Florin-Christensen & Doniach, 1974; MacCuish *et al.*, 1974; Lendrum, Walker & Gamble, 1975; Lendrum *et al.*, 1976; Irvine *et al.*, 1977; Del Prete *et al.*, 1977; Lernmark *et al.*, 1978, 1981). Islet cell cytoplasmic (ICA) antibodies were first detected in patients with insulin-dependent diabetes and polyendocrine autoimmune disorders (Bottazzo *et al.*, 1974; MacCuish *et al.*, 1974). Subsequently, ICA were identified at the time of diagnosis in 65–85% of insulin-dependent diabetics (Lendrum *et al.*, 1975, 1976; Irvine *et al.*, 1977; Del Prete *et al.*, 1977). ICA were also found in non-insulin-dependent diabetics treated with oral hypoglycaemic agents and healthy first-degree relatives of diabetic patients, who later developed insulin-dependent diabetes (Irvine *et al.*, 1977; Del Prete *et al.*, 1977).

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These observations suggest the possible aetiological involvement of autoantibodies in the pathogenesis of insulin-dependent diabetes.

In order for antibodies to mediate either antibody-dependent cellular cytotoxicity or complement-mediated cytotoxicity, it is probable that they would have to interact with the cell membrane. In this regard, we have recently identified circulating antibodies in insulin-dependent diabetics which react with surface antigens of living pancreatic islet cells (Lernmark *et al.*, 1978, 1981) and have also produced similar antibodies in experimental animals (Lernmark *et al.*, 1980). Utilizing the technique of cell column perifusion (Lowry & McMartin, 1974) to examine the dynamics of insulin and chromium release from dispersed rat pancreatic islet cells, we observed that islet cell surface antibodies together with complement increase chromium release from prelabelled cells and block insulin secretion in response to glucose (Kanatsuna *et al.*, 1981).

In the present study we report that cytotoxicity measured by chromium release can be differentiated from that measured by release of insulin.

MATERIALS AND METHODS

Preparation of rat islet cells. Pancreatic islets from male Sprague–Dawley rats weighing approximately 200 g, prepared by collagenase digestion and Ficoll gradient centrifugation, were further incubated with collagenase for 5 min and dispersed into free cells by mechanical shaking (Lernmark *et al.*, 1980). Islets and dispersed cells were maintained at pH 7·4 in Swim's S-77 medium as described elsewhere (Lernmark *et al.*, 1978; Kanatsuna *et al.*, 1981). Cell numbers were determined in a haemocytometer, $3{\cdot}1 \pm 0{\cdot}3 \times 10^5$ islet cells being obtained from a single rat pancreas (mean ± s.e.m. for eight different experiments). In the same experiments, the number of single cells averaged $55 \pm 3\%$, the remainder occurring in clumps of two to 10 cells. Exclusion of trypan blue indicated a viability of $98 \pm 0{\cdot}3\%$.

Preparation of antiserum and complement. Four female New Zealand rabbits (1·5 kg body weight) were immunized with living dispersed rat islet cells as described previously (Lernmark *et al.*, 1980). Intravenous booster injections of $0{\cdot}5\text{–}1{\cdot}0 \times 10^6$ islet cells in 1 ml 0·9% NaCl were administered two to five times at 2-week intervals. The rabbits were bled 1 week after each booster injection. Serum was heat-inactivated (56°C, 30 min), filtered (45-μm Millipore filters) and the islet cell surface antibody titre determined using a ^{125}I-protein A radioligand assay (Lernmark *et al.*, 1980). Normal guinea-pigs were bled by heart puncture. The plasma was filtered (0·45 μm) and used as a source of complement.

Column perifusion of rat islet cells. Living rat islet cells ($1\text{–}5 \times 10^5$ cells) were perifused on columns of Bio-Gel P-2 as described elsewhere (Kanatsuna *et al.*, 1981). Two columns kept at 37°C were perifused in parallel with Swim's medium at a flow rate of 0·6 ml/min. Fractions were collected at 1–10-min intervals and frozen immediately for insulin assay. Insulin was measured by a double-antibody method using rat insulin as standard (Morgan & Lazarow, 1963). Further experimental details are given in the legends to the figures.

Labelling of islet cells with ^{51}Cr. Rat pancreatic islet cell suspensions (10^6 cells/ml) were incubated at 37°C for 75 min in Swim's medium with 40 g/l BSA and 100 c.p.m. per cell of $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Massachusetts). In one series of experiments the cells were incubated with 0·1 mmol/l non-radioactive Na_2CrO_4. The cells were then washed twice by centrifugation (50 ***g*** for 5 min at room temperature) in Swim's medium with 40 g/l BSA.

RESULTS

Insulin release from column-perifused islet cells

Cells were mixed with the beads in the column and a wash-out period lasting 15 min was carried out until a stable baseline of secretion was obtained (Fig. 1). In 11 different perifusion experiments using 30 mmol/l D-glucose and 0·1 mmol/l IBMX as the stimulus, there was a marked increase in the rate of insulin release within 30 sec (taking the 1·5-min lag period of the system into account). The rate of

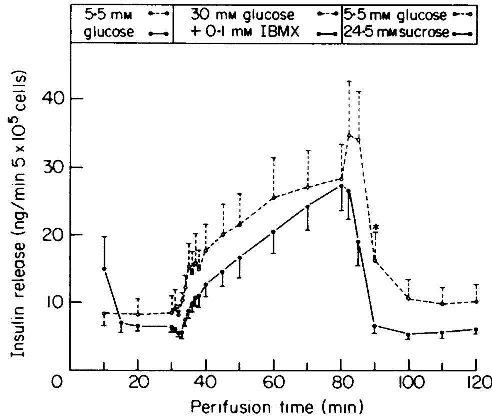

Fig. 1. Effects of glucose and IBMX on insulin release from rat islet cells, perifused on columns of Bio-Gel P-2 polyacrylamide beads. The number of cells added to the columns in experiments without sucrose (o——o) was $3{\cdot}6 \pm 0{\cdot}4 \times 10^5$ (mean ± s.e.m. for five experiments). The number of trypan-blue-negative cells amounted to $3{\cdot}5 \pm 0{\cdot}4 \times 10^5$. In the experiments with 24·5 mmol/l sucrose (•——•), the number of islet cells was $2{\cdot}4 \pm 0{\cdot}4 \times 10^5$ (mean ± s.e.m. for six experiments). The number of trypan-blue-negative cells was $2{\cdot}3 \pm 0{\cdot}4 \times 10^5$. The cells were perifused for 120 min at 37°C at a flow rate of 0·6 ml/min in Swim's medium with 40 g/l BSA, 2·5 mmol/l $CaCl_2$ and 0·25 mg/ml soybean trypsin inhibitor. Insulin release is expressed as ng per min per 5×10^5 trypan-blue-negative cells added to the columns at the start of the perifusion experiment. * Effect of sucrose $P < 0{\cdot}05$.

insulin release continued to rise throughout the entire period of stimulation. The switch from high glucose and IBMX back to 5·5 mmol/l D-glucose alone induced an 'off-response' within 2 min, which was not present if the sudden change in osmotic pressure was prevented by the addition of 24·5 mmol/l sucrose (Fig. 1). The secretory response of β cells perifused in the presence of 0·1 mmol/l IBMX throughout the experiment was biphasic after stimulation with 30 mmol/l D-glucose and monophasic after stimulation with 20 mmol/l L-arginine (Fig. 2). Following a period of 40 min at low D-glucose (5·5 mmol/l) and L-arginine (0·8 mmol/l) respectively, both in the absence or presence

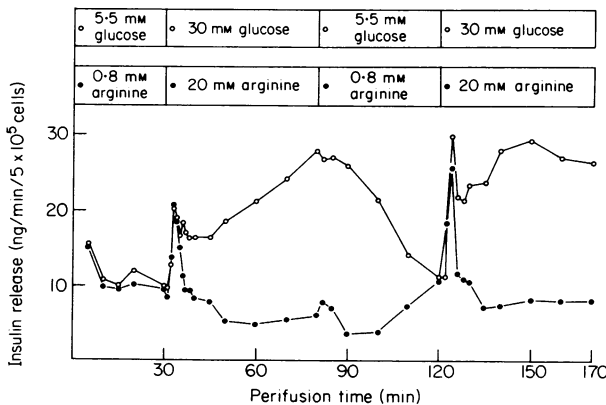

Fig. 2. Effects of glucose and arginine on insulin release from rat islet cells perifused on columns of Bio-Gel P-2 polyacrylamide beads. In one column, the cells were perifused as indicated by the bars with 5·5 followed by 30 mmol/l D-glucose (o——o) and with 24·5 mmol/l D-sucrose present during the period 80–120 min. In the other, 0·8 followed by 20 mmol/l L-arginine (•——•) were used as the stimulus with 19·2 mmol/l sucrose present during the period 80–120 min. All media were supplemented with 0·1 mmol/l IBMX. Mean of two different experiments with 5×10^5 cells perifused in each column.

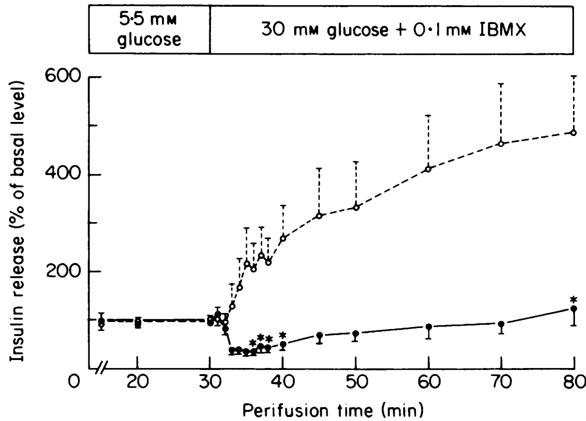

Fig. 3. Glucose-stimulated insulin release from rat islet cells exposed to islet cell antiserum and complement before perifusion on columns of Bio-Gel P-2 polyacrylamide beads. Suspensions of rat islet cells ($4{\cdot}8 \pm 0{\cdot}2 \times 10^5$ cells) were incubated at 37°C for 60 min in Swim's medium supplemented with 50% (v/v) of either normal rabbit serum (o——o) or islet cell antiserum (•——•). After 60 min, guinea-pig complement (10%, v/v) was added and the incubation continued for another 60 min. The islet cell suspensions ($3{\cdot}2 \pm 0{\cdot}3 \times 10^5$ and $2{\cdot}8 \pm 0{\cdot}3 \times 10^5$ cells from normal rabbit serum and islet cell antiserum respectively) were transferred to two perifusion chambers and perifused in parallel. The basal release was $9{\cdot}2 \pm 1{\cdot}4$ and $6{\cdot}8 \pm 1{\cdot}11$ ng/min and 5×10^5 cells ($P > 0{\cdot}05$) from cells exposed to antiserum and normal rabbit serum respectively. Mean ± s.e.m. for five different experiments. * Effect of islet cell antiserum $P < 0{\cdot}05$.

of sucrose to correct for change in osmotic pressure, a higher response was obtained when the cells were stimulated a second time (Fig. 2). IBMX was added to potentiate the effect of 30 mmol/l glucose; by comparing Figs 1 & 2 it can be noted that IBMX did not affect the basal release of insulin.

Effects of rat islet cell antiserum and complement

In a first series of experiments the effects of islet cell antiserum and complement on insulin release were tested by incubating the dispersed rat islet cells first for 60 min with 50% (v/v) of either normal rabbit serum or the rabbit anti-rat islet cell serum and then for an additional 60 min with guinea-pig complement at a final concentration of 10% (v/v). The cells were then mixed with the beads in two columns and perifused in parallel (Fig. 3). While there was at least a five-fold increase in insulin release in response to 30 mmol/l D-glucose and 0·1 mmol/l IBMX from cells previously exposed to normal serum, cells exposed to rabbit antiserum and complement did not secrete insulin at an increased rate, but rather showed a decrease to a level below basal.

The effect of the islet cell antiserum was therefore tested in a second series of experiments in which the cells were first prelabelled with radioactive chromium before treated with serum and complement (Fig. 4). The release of chromium during the subsequent perifusion was taken as a measure of cytotoxicity. The results shown in Fig. 4 indicate, however, that the rate of ^{51}Cr-release in response to 30 mmol/l D-glucose and 0·1 mmol/l IBMX was similar whether the cells had been exposed to antiserum or normal serum. The discrepancy in results compared to those shown in Fig. 3 lead us to measure insulin in all fractions (Fig. 4). The results demonstrate that 30 mmol/l D-glucose and 0·1 mmol/l IBMX had no effect on the release of insulin whether the cells had previously been exposed to antiserum or not.

The possibility that the pretreatment with chromium affected the ability of the β cells to release insulin was tested in a third series of experiments (Fig. 5). Islet cells were first incubated without or with 0·1 mmol/l non-radioactive Na_2CrO_4 before being added to the perifusion columns. The results (Fig. 5) indicate that there was no release of insulin in response to glucose and IBMX from cells exposed to Na_2CrO_4. However, following a pulse of complement during exposure to the islet cell antiserum, there was an immediate release of insulin from the β cells pre-exposed to Na_2CrO_4 as well as from control cells. Compared to the rate of insulin release from the control cells, chromium

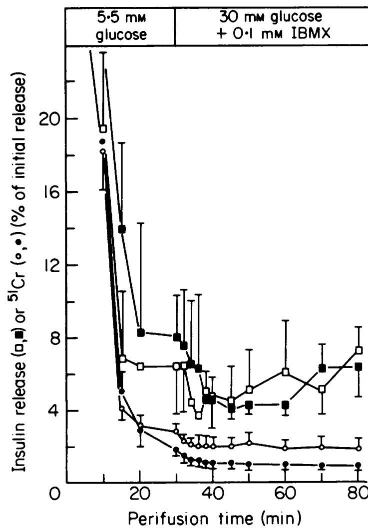

Fig. 4. Effects of glucose and IBMX on insulin (■, □) and ^{51}Cr-release (●, ○) from ^{51}Cr-labelled rat islet cells exposed to islet cell antiserum and complement before perifusion on a column of Bio-Gel P-2 polyacrylamide beads. Islet cells (10^6 cells/ml) were labelled with $Na_2{}^{51}CrO_4$ and washed by centrifugation before incubation at 37°C for 120 min, in one set with 50% (v/v) normal rabbit serum, in the other with 50% (v/v) islet cell antiserum. Guinea-pig complement (10% v/v) was added after 60 min of incubation. The cells were finally perifused as described in the legend to Fig. 3. Results are given as a percentage of initial release. Mean ± s.e.m. for three experiments with 3–5 × 10^5 cells and 1·09 ± 0·22 c.p.m. per cell of chromium radioactivity added to the columns.

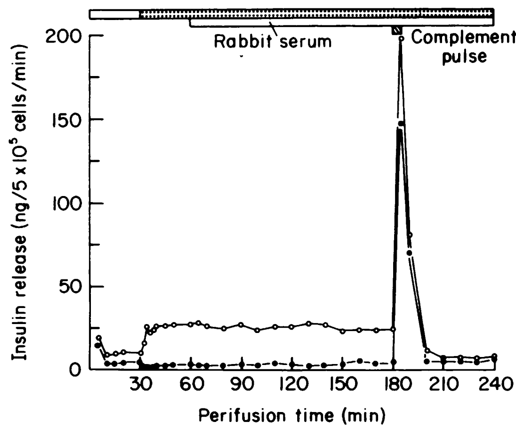

Fig. 5. Insulin release from rat islet cells pretreated without (○——○) or with (●——●) 0·1 mmol/l Na_2CrO_4 before perifusion on columns of Bio-Gel P-2 polyacrylamide beads. At 30 min, 30 mmol/l glucose and 0·1 mmol/l IBMX were added (*stippled bar*). Islet cell antiserum raised in rabbits (bar labelled *Rabbit serum*) was added after 60 min. At 180–185 min, 10% (v/v, final concentration) guinea-pig complement was also present. The results of a typical experiment are shown.

exposure suppressed insulin release 65% during the period 15–30 min, 88% during 30–180 min and 66% during 180–190 min.

DISCUSSION

The preparation of viable, dispersed pancreatic islet cells (Lernmark *et al.*, 1978, 1980; Lernmark,

1974; Kanatsuna *et al.*, 1981) permits studies to assess the direct effects on β-cell function of macromolecules, which would only react with superficially located cells if intact islets were used. In the present experiments we have demonstrated that the dynamics of insulin release can be studied using dispersed β cells in a perifusion system, with Bio-Gel polyacrylamide beads as the inert supporting matrix. The perifused β cells responded rapidly upon addition or removal of stimulators of insulin release with secretory patterns similar to those reported in the isolated perfused rat pancreas (Randle & Hales, 1974). It was also noted that the insulin release in response to high concentrations of glucose or arginine was greater when the same stimuli were used during a second period of stimulation following an intervening period of perifusion at low secretagogue concentrations. This phenomenon of glucose adaptation or 'memory' is present in the perfused pancreas as well as in isolated islets (Grodsky *et al.*, 1969; Lernmark, 1971; Grill *et al.*, 1979).

The dispersed β cells were sensitive to sudden changes in osmotic pressure (Fig. 1). A so-called 'off-response' following the termination of secretagogues was previously demonstrated in the perfused rat pancreas (Landgraf, Kotler-Brajtburg & Matschinsky, 1971; Pek, Santiago & Tai, 1978). The insulin 'off-response' may be explained by a reduction in osmolarity of the perifusion medium (Blackard *et al.*, 1975) or by some undefined metabolic events in the β cells (Pek *et al.*, 1978). When studying the dynamics of chromium release, a peak of radioactivity was also observed when the perifusion medium was changed from high to low glucose (data not shown). The temporary increase could be abolished by adding sucrose to correct for osmotic changes in the medium. Therefore, in perifusion of isolated rat β cells, the insulin 'off-response' seems to be induced by a shift in osmotic pressure. All these characteristics of insulin release by these cells encouraged us to conclude that this technique can be used to study the effect of various ligands reacting with cell surface components, such as islet cell surface antibodies.

Our previous study (Kanatsuna *et al.*, 1981) indicated that an islet cell surface antiserum and complement were able to induce a block in glucose-stimulated insulin release. The results shown in Fig. 3 corroborate our previous findings in a new preparation of antiserum. The cytotoxic effect did not seem to involve a complete lysis since cell number and viability were little affected. Thus it is concluded from this and our previous study (Kanatsuna *et al.*, 1981) that lesions are induced which result in a block of the stimulus–secretion pathway.

The release of ^{51}Cr from prelabelled cells is often used as a measure of cell viability (Dobersen *et al.*, 1980; Kanatsuna *et al.*, 1981; Lernmark, 1974; Soderstrum, Freedman & Lernmark, 1979). Perifusion of prelabelled rat islet cells with complement resulted in a dramatic and immediate release of ^{51}Cr radioactivity only from cells that were exposed to islet cell surface antibodies (Kanatsuna *et al.*, 1981). It was concluded that the complement-dependent cytotoxic reaction rapidly rendered the cells leaky. This release of ^{51}Cr occurred promptly and it is likely that in the experiments shown in Fig. 4 it had occurred already during the batch-type incubation preceding the perifusion. The inability of glucose to affect either insulin or chromium release is explained by the results in Fig. 5 which demonstrate that pretreatment with Na_2CrO_4 renders the β cells unable to release insulin in response to glucose. Chromium has also been reported to inhibit insulin release from intact rat pancreatic islets (Ghafghazi & MacDaniel, 1979). However, as shown in Fig. 5, islet cell surface antiserum and guinea-pig plasma were still able to cause a prompt release of insulin during the chromium-induced block of secretion. In cells not exposed to chromium the transient release of insulin was followed by a decreased rate of secretion.

These results indicate that complement-dependent cytotoxicity of islet cell surface antibodies involves different phenomena. Firstly, the cytotoxic reaction results in a transient release of insulin whether the physiological release mechanisms were blocked by chromium or not. Secondly, in cells not treated with chromium the cytotoxic reaction renders the β cells unable to release insulin in response to glucose. It is concluded that the cell-perifusion system allows us to investigate complement-dependent damage on either the physiological release of insulin or in terms of an immunological damage expressed as release of chromium or insulin from chromium-labelled cells. Although chromium release compares favourably with cytotoxic assays using supravital staining (Wigzell, 1965; Kromann *et al.*, 1980), the present results make it doubtful to what extent chromium release reflects β cell death.

The pathogenetic importance of complement-dependent cytotoxicity in insulin-dependent

diabetes remains controversial. Sera from diabetic patients appear capable of mediating a complement-dependent cytotoxic reaction against rat (Soderstrum *et al.*, 1979; Dobersen *et al.*, 1980) or human islet cells (Lernmark *et al.*, 1981). The presence of islet cell surface antibodies capable of mediating a complement-dependent cytotoxic reaction does not necessarily imply that these antibodies are actively 'diabetogenic' since they were present in a large proportion of first-degree relatives of diabetic probands (Dobersen *et al.*, 1980). The present technique, which should also be applicable to human β cells, will make it possible to test whether islet cell surface antibodies in serum from human diabetics will affect the physiological release of insulin or cause an immunological damage or both.

Provided that the macromolecules of the complement system can transverse the capillary barrier along with antibodies, the cytotoxic reaction is likely to be detrimental to the β cells and of pathogenetic importance.

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REFERENCES

Blackard, W.G., Kikuchi, M., Rabinovitch, A. & Renold, A.E. (1975) An effect of hyposmolarity on insulin release *in vitro*. *Am. J. Physiol.* **228,** 706.

Bottazzo, G.F., Florin-Christensen, A. & Doniach, D. (1974) Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet*, **ii,** 1279.

Del Prete, G.F., Betterle, C., Padovan, D., Erle, G., Toffolo, A. & Bersahi, G. (1977) Incidence and significance of islet-cell antibodies in different types of diabetes mellitus. *Diabetes*, **26,** 909.

Dobersen, M.J., Scharff, J.E., Ginsberg-Fellner, F. & Notkins, A.L. (1980) Cytotoxic autoantibodies to beta cells in the serum of patients with insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **303,** 1493.

Ghafghazi, T. & MacDaniel, M. (1979) Chromium inhibition of insulin secretion from isolated islets of Langerhans. *Diabetes*, **39,** 404 (abstract).

Grill, V., Adamson, U., Rundfeldt, M., Andersson, S. & Cerasi, E. (1979) Glucose memory of pancreatic B and A cells. Evidence for common time-dependent action of glucose on insulin and glucagon secretion in the perfused rat pancreas. *J. clin. Invest.* **64,** 700.

Grodsky, G.M., Curry, D., Landahl, H. & Bennett, L. (1969) Further studies on the dynamic aspects of insulin release *in vitro* with evidence for a two-compartmental storage system. *Acta Diabetol. Lat.* **6** (Suppl. 1), 554.

Irvine, W.J., MaCullum, C.J., Gray, R.S., Campbell, C.J., Duncan, L.J.P., Farquhar, J.W., Vaughan, H. & Morris, P.J. (1977) Pancreatic islet-cell antibodies in diabetes mellitus correlated with the duration and type of diabetes, coexistent autoimmune disease, and HLA type. *Diabetes*, **26,** 138.

Kanatsuna, T., Lernmark, Å., Rubenstein, A.H. & Steiner, D.F. (1981) Block in insulin release from column-perifused pancreatic β-cells induced by cell surface antibodies and complement. *Diabetes*. **30,** 231.

Kromann, H., Christy, M., Egeberg, J., Lernmark, Å., Nerup, J. & Richter-Olesen, H. (1980) Direct streptozotocin toxicity on dispersed mouse islet cells determined by ^{51}Cr-release. *Med. Biol.* **58,** 322.

Landgraf, R., Kotler-Brajtburg, J. & Matschinsky, F.M. (1971) Kinetics of insulin release from perifused rat pancreas caused by glucose, glucosamine, and galactose. *Proc. Natl. Acad. Sci. USA*, **63,** 536.

Lendrum, R., Walker, G. & Gamble, D.R. (1975) Islet-cell antibodies in juvenile diabetes mellitus of recent onset. *Lancet*, **i,** 880.

Lendrum, R., Walker, G., Cudworth, A.G., Theophanides, C., Pyke, D.A., Bloom, A. & Gamble, D.R. (1976) Islet-cell antibodies in diabetes mellitus. *Lancet*, **ii,** 1273.

Lernmark, Å. (1971) Isolated mouse islets as a model for studying insulin release. *Acta Diabetol. Lat.* **8,** 649.

Lernmark, Å. (1974) The preparation of, and studies on, free cell suspension from mouse pancreatic islets. *Diabetologia*, **10,** 431.

Lernmark, Å., Bækkeskov, S., Dyrberg, T. & Soderstrum, K. (1981) Islet cell antibodies and islet-cell specific antigens. *Acta Endocrinol. (Kbh).* (In press.)

Lernmark, Å., Freedman, Z.R., Hofmann, C., Rubenstein, A.H., Steiner, D.F., Jackson, R.L., Winter, R.J. & Traisman, H.S. (1978) Islet-cell-surface antibodies in juvenile diabetes mellitus. *N. Engl. J. Med.* **299,** 375.

Lernmark, Å., Hägglöf, B., Freedman, Z.R., Irvine, W.J., Ludvigsson, J. & Holmgren, G. (1981) A prospective analysis of antibodies reactive with pancreatic islet cells in insulin-dependent diabetic children. *Diabetologia.* **20,** 471.

Lernmark, Å., Kanatsuna, T., Patzelt, C., Diakoumis, K., Carroll, R., Rubenstein, A.H. & Steiner, D.F. (1980) Antibodies directed against the pancreatic islet cell plasma membrane. *Diabetologia*, **19,** 445.

Lowry, P.J. & McMartin (1974) Measurement of the dynamics of stimulation and inhibition of

steroid genesis in isolated rat adrenal cells by using column perfusion. *Biochem. J.* **142,** 287.

MacCuish, A.C., Jordan, J., Campbell, C.J., Duncan, L.J.P. & Irvine, W.J. (1974) Autoantibodies to islet-cells in insulin-dependent diabetics with coexistent autoimmune disease. *Lancet*, **ii,** 1529.

Morgan, C.R. & Lazarow, A. (1963) Immunoassay of insulin: two antibody system. Plasma levels of normal, subdiabetic and diabetic rats. *Diabetes*, **12,** 115.

Pek, S., Santiago, J.C. & Tai, T.-Y. (1978) L-leucine-induced secretion of glucagon and insulin, and the 'off-response' to L-leucine *in vitro*. I. Characterization of dynamics of secretion. *Endocrinology*, **103,** 1208.

Randle, P.J. & Hales, C.N. (1974) Insulin release mechanism. In *Handbook of Physiology* (ed. by D. F. Steiner and N. Freinkel), Vol. 1, Section 7. American Physiology Society, Washington, D.C.

Soderstrum, W.K., Freedman, Z.R. & Lernmark, Å. (1979) Complement-dependent cytotoxic islet cell surface antibodies in insulin-dependent diabetes. *Diabetes*, **28,** 397.

Wigzell, H. (1965) Quantitative titrations of mouse H-2 antibodies using ^{51}Cr-labelled target cells. *Transplantation*, **3,** 423.