Effect of Media Conditioned by Concanavalin A Activated Spleen Cells on Pancreatic Islet Cells

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Abstract. The effect of media conditioned by concanavalin A-activated spleen cells (C-sup) on insulin release and its islet cell cytotoxicity were studied. In a functional study, C-sup significantly inhibited both basal insulin release and glucose-stimulated insulin release. Morphologically, C-sup had a destructive effect on isolated islets after 72 h incubation. Islet cell cytotoxicity was shown by lactate dehydrogenase (LDH) release assay after 5 days incubation with C-sup in a dose-dependent manner. These results suggest that acceleration of the onset of diabetes in young diabetes prone (DP) Bio-Breeding/Worcester (BB/W) rats following the injection of C-sup may depend on the suppressive and cytotoxic effects of C-sup on pancreatic islet cells.

THE BB rat develops diabetes similar to human insulin-dependent diabetes mellitus (IDDM) [1, 2]. Approximately 50% of DP rats spontaneously develop diabetes between 60 and 120 days of age, while less than 0.5% of DP rats become diabetic before 60 days of age. An autoimmune pathogenesis of BB rat diabetes is thought to be due to the infiltration of mononuclear cells into the islets of Langerhans (insulitis) [3] and to the presence of circulating autoantibodies to pancreatic islets [4]. The disease is prevented by immunosuppression [5], thymectomy [1], bone marrow allografts [6, 7] and lymphocyte transfusion [8]. Concanavalin A (Con A) activated spleen cells from acutely diabetic BB rats adoptively transfer diabetes to young naive DP rats and non-diabetic Wistar Furth rats [9]. Furthermore, C-sup from acutely diabetic DP or non-diabetic rats accelerates the appearance of diabetes in young DP or diabetes resistant (DR) rats [10].

To investigate the mechanism of this acceleration, we studied the cytotoxic effect of C-sup on pancreatic islets and the effect of C-sup on insulin release in vitro. Our results suggest that the direct actions of C-sup on pancreatic islets play an important role in accelerating the onset of diabetes.

Materials and Methods

Preparation of C-sup

Spleens from Lewis rats (Charles River Laboratories, Osaka) were removed aseptically, teased with a cell sieve and washed 3 times in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd. Tokyo) and then suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah), 100 U/ml penicillin, 100 µg/ml streptomycin (M. A. Bioproduct, Inc., Walkersville, MD) and 2 mmol/l L-glutamine (complete medium) containing 5 µg/ml concanavalin A (BioMakor, Rehovot) and incubated in a humidified incubator at 37°C in 95% air and 5% CO₂ for 72 h. The supernatant was collected and stored at −20°C.

IL-1, TNFα and IFN-γ assay of C-sup
IL-1 assay: C3H/HeJ mice thymocytes were suspended in RPMI 1640 medium containing 10% fetal calf serum (FCS), antibiotics, $1 \times 10^{-4}$ M of 2-ME and 10 µg/ml of PHA (DIFCO laboratories, Detroit, MI) and seeded in a 96-well, flat bottomed microculture plate (1×10^6/well). Serially diluted samples were added to each well in triplicate. After 72 h incubation at 37°C, 5% CO₂, the cells were then pulsed for 24 h with 1 µCi/well [³H] thymidine (New England Nuclear, Boston, MA). Incorporation in the presence of samples was compared with that in the presence of dilutions of a human IL-1α standard (Dainippon Pharmaceuticals Ltd., Osaka). TNFα assay: TNFα-sensitive LM cells were suspended in MEM-Earle’s BSS containing 1% FCS, 1 mg/well actinomycin D and seeded in a 96-well, flat-bottomed microculture plate (5×10^4/well). Serially diluted samples were then added to each well in triplicate. After 48 h culture at 37°C, 5% CO₂, the cells were fixed with 25% glutaraldehyde and then stained with 0.05% methylene blue for 5 min. The dye from stained cells was extracted by adding 0.33N HCl and the dye concentration was measured with a Titertec Multiskan MCC/340 (Flow Laboratories Inc.). The sample titer was compared with that of a human TNFα standard (Dainippon Pharmaceuticals Ltd., Osaka).

IFN-γ assay: IFN-γ activity was determined by a cytopathic effect inhibitory assay performed in 96-well plates with L-929 cells as indicator cells and vesicular stomatitis virus as the challenge virus. The international reference MuIFN-γ was used to calibrate the assay.

Islet isolation and culture

Islets were isolated from male Wistar rats (Charles River Laboratories, Osaka) weighing 180–220g by the collagenase digestion method. Two hundred to 300 islets were maintained freely floating in complete medium for 2 days. After 2 days preculture, 50 islets in each group were transferred to new culture dishes and maintained for the 72 h experimental period. In the control group, islets were cultured in 5 ml of complete medium containing 5 µg/ml Con A. Islets allocated to the experimental group were cultured with C-sup (5-, 10- and 20-fold dilution with complete medium). Immediately after the 72 h culture, the islets were morphologically observed under a dissecting microscope. The islets which were cultured with 20-fold diluted C-sup for 72 h showed very subtle morphological changes, so these islets were also used for the insulin release study.

Insulin release and insulin concentration of islet

Insulin release was studied in quadruplicated groups of 10 islets. During the first hour of incubation, islets were cultured in Krebs Ringer Bicarbonate (KRB) solution containing 5.6 mM glucose, 20 mM Hepes (Wako, Tokyo), 0.5% bovine serum albumin (Seikagaku Kogyo Co., Tokyo) and 2.5 mM CaCl₂. The medium was then gently removed and replaced by another KRB solution containing 16.7 mM glucose and incubation was continued for a further 1 h. The insulin concentration in the medium was measured by radioimmunoassay (Dainabot Co., Ltd, Tokyo). Islets used for the insulin release experiment were ultrasonically disrupted in acid/ethanol for the assay of the insulin concentration in the islets.

Islet cells in monolayer culture and cytotoxicity assay

The isolated islets described above were dissociated into single cells by incubation at 37°C for 10 min in Ca²⁺ and Mg²⁺ free Hanks’ balanced salt solution supplemented with 0.5mg/ml trypsin and 0.2 mg/ml EDTA (Nissui, Tokyo). Dissociated cells were resuspended in complete medium and 2×10⁴ cells were seeded in each well in a 96 well, flat-bottomed microculture plate (Nunc, Roskilde, Denmark) and incubated for 24 h. The cells were gently washed and incubated again for 120 h with several concentrations of C-sup, 100U/ml human recombinant IL-1α, 100U/ml human recombinant TNFα, (provided by Dainippon Pharmaceutical Co., Osaka) and 1000U/ml mouse recombinant IFN-γ (provided by Shionogii Research Laboratories, Osaka) (150 µl/well in triplicate). An aliquot (100µl) of medium was collected from each well and the released LDH activity was measured by the Lactate Dehydrogenase C II-Test (Wako, Osaka). The total input of islet cell LDH was determined after ultrasonic disruption of the cells. The determination of total input was done at the beginning of the assay period in separated wells. The LDH activity released in the absence of an effector agent (spontaneous release) was less than 30% of total input. Specific cytotoxicity was calculated as a percentage by means of following formula [11].
Specific lysis (%) = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total input} - \text{Spontaneous release}} \times 100

Statistical analysis

Parametric data are presented as means ± SEM. The significant difference between groups was determined by unpaired Student’s t-test.

Results

IL-1, TNF-α and IFN-γ levels in C-sup

Pooled C-sup which was used in these experiments contained 500U/ml IL-1, 10U/ml TNF-α and 75U/ml IFN-γ.

Morphological Study

After 72 h of incubation with C-sup (5-, 10- and 20-fold dilution), the outline of the islets became blurred and they shrank to 50% of their initial size at maximum. These destructive effects of C-sup were demonstrated in a dose-dependent manner (Fig. 1).

Effect of C-sup on insulin release

(1) Effect of C-sup on basal insulin release.

Samples were taken from the medium which was used in the 72 h culture with or without C-sup (20-fold dilution) and the insulin level was measured. Insulin release from islets which were cultured with C-sup was significantly lower than from the control (P<0.01).

(2) Effect of C-sup on glucose stimulated insulin release.

Basal insulin release at 5.6mM glucose in KRB solution was slightly lower in the precultured group with C-sup (20-fold dilution) than in the control group but the difference was not significant. Glucose-stimulated insulin release at 16.7mM glucose was significantly lower in the precultured group than in the control group (P<0.05). Total insulin release from precultured islets was 84% of that from control islets. The insulin concentration in precultured islets also decreased significantly in comparison with control islets (P<0.05). These data suggest that C-sup might inhibit not only insulin release but also insulin synthesis (Fig. 2).

Cytotoxic effect of C-sup, IL-1α, TNFα and IFN-γ on monolayer islet cell culture of islet cells

C-sup showed a significant dose-dependent cytotoxic effect on monolayer islet cells. Concentrations of 100U/ml of IL-1α and 100U/ml of TNFα also showed signs of a strong cytotoxic effect on the islet cells, although 1000U/ml of IFN-γ failed to lyse the islet cells. Although 5-fold diluted C-sup contains 100U/ml IL-1, C-sup showed 21% greater cytotoxicity than IL-1α. Even though the greatest cytotoxic effect was due to IL-1, this difference suggests to us the synergistic effect of IL-1 and TNFα, IFN-γ or other cytokines (Fig. 3).

Discussion

It has already been reported that several kinds of lymphocytes are responsible for the destruction
Fig. 2. Effect of C-sup on insulin secretion from isolated islets and on insulin concentration in the islets. Total insulin release during 72 h incubation with or without 20-fold diluted C-sup (left), basal and glucose-stimulated insulin release after incubation with or without C-sup (center), insulin concentration in the islet after the insulin secretion test (right). Values are means ± SEM from 4 experiments, each performed in quadruplicate. The significant difference between groups was determined by Student's t-test. *P<0.05, **P<0.01 vs. control

Fig. 3. Cytotoxic effects on rat islet cells of C-sup (5-, 10- and 20-fold dilution), IL-1α (100 U/ml), IFN-γ (10³ U/ml), TNFa (100 U/ml) and Con A control medium (1 µg/ml). Islet cell monolayers were incubated with each medium for 5 days. Means ± SEM for percent specific lysis of LDH release assay in 4 triplicate experiments. The significant differences between the groups were determined by Student's t-test. *P<0.02, **P<0.01 vs. Con A control
*P<0.05, **P<0.01 vs. ×20 C-sup
The text reads:

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of pancreatic islet cells. First, natural killer (NK) cells from acutely diabetic DP BB/W rats showed signs of a cytotoxic effect on the rat islet cells regardless of the major histocompatibility complex (MHC) [12], and macrophages or peritoneal exudate cells from BB/W rats also had a cytotoxic effect on the rat islet cells or rat insulinoma cell line [13]. Second, the cytotoxic T cells from NOD mouse destroyed mouse islet cells in a MHC restricted manner [14]. In addition to these cell mediated cytotoxicities, it was also reported that crude IL-1 and several kinds of recombinant cytokines affect the function or mortality of rat and mouse islets [15–17], and also combined treatment with IFN-γ, TNF, lymphotoxin and IL-1 results in a synergistic cytotoxic effect on islet cells [18, 19].

Adoptive transfer of diabetes was successfully performed by the transfusion of Con A-activated spleen cells from acutely diabetic DP BB/W rats to young (less than 60 day old) DP rats or immunosuppressed non-diabetic Wistar Furth (WF) rats [20]. It was therefore assumed that cell-mediated cytotoxicity is important in inducing autoimmune diabetes. In addition to these reports, it has also been reported that C-sup accelerated the onset of diabetes in young DP rats and induced diabetes in young DR BB/W rats but failed to induce diabetes in other non-diabetic WF rats. Since 1% of DR rats become spontaneously diabetic and the depletion of RT6-positive lymphocytes could induce diabetes in 50% of DR rats [21], it was suggested that an inactive effector cell population may be present in DR rats. From these observations it was concluded that the activation of the native effector cell population in DP or DR rats by C-sup is more likely to be the mechanism that kills pancreatic islet cells than the direct cytotoxic effect of C-sup. However, it is difficult to maintain a certain concentration of C-sup in pancreatic islets, because the injection was done once a day and a high concentration bolus injection of C-sup could have a fatal side effect. Consequently, the direct effect of C-sup on pancreatic islets following in vivo administration still remains unclear.

In this experiment, we found cytotoxic activity of C-sup against islet cell monolayers, and IL-1 accounted for the most of this activity. Furthermore, IL-1 seemed to have a synergistic cytotoxic effect with other lymphokines on pancreatic islet cells. Although Con A itself is a lymphotrophic factor, unconditioned medium which contained only Con A failed to lyse the target cells. Therefore, the cytotoxicity of C-sup cannot be explained by Con A itself.

Since our present study clearly showed that C-sup showed remarkable islet cell cytotoxicity in vitro, we believe that C-sup could play an important role in accelerating the onset of diabetes when combined with the cytotoxicity of native effector cells in vivo.

Moreover, we also found that C-sup had an inhibitory effect on basal insulin release and glucose-stimulated insulin release. The inhibitory effect of C-sup was found not only in insulin release but also in insulin synthesis. These effects could be an another factor which contributes to the acceleration of the onset of diabetes.

In summary, we conclude that the direct effect of C-sup, which has a high titer of IL-1 activity, may play an important role in accelerating the onset of diabetes in young DP or DR rats.

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**References**


