In vitro lymphocyte recognition of islet cells following in vivo priming with allogeneic murine pancreatic islets

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Abstract. Lymphocytes from patients with insulin-dependent diabetes have been shown to be sensitized to pancreatic tissue antigens. Mice immunized with homologous pancreatic islets have been found to develop glucose intolerance and insulin. Since lymphocytes may be involved in diabetogenesis, we wished to determine if lymph node cells from islet-immunized mice can recognize and respond to islet cells in vitro.

A.TL female mice were immunized with an emulsion of BALB/c islet homogenate and complete Freund's adjuvant (CFA); sham-treated A.TL mice were injected with adjuvant and water. Mice were sacrificed 7–8 days later and the draining lymph nodes were removed. The lymph node cells were co-cultured with freshly prepared irradiated BALB/c islet cell, which served as stimulator cells. The co-cultures were incubated for 24–26 h at 37°C, followed by a 16 h [3H]thymidine (Tdr) pulse.

A significant proliferative lymph node cells from islet-primed mice was induced during the in vitro stimulation with irradiated islet cells when compared with lymph node cells from sham-treated mice (P < 0.001). The response may be islet-cell-specific, since irradiated lymph node cells from BALB/c mice failed to elicit a proliferative response under the same culture conditions (P > 0.80).

It is suggested that pancreatic islet cells have an ability to stimulate proliferation of lymphocytes which recognize islet-cell-specific antigens. This lymphocyte recognition phenomenon may be relevant to our understanding of autoimmune mechanisms in insulin-dependent diabetes mellitus.

Autoimmune processes may be involved in the pathogenesis of insulin-dependent (Type 1) diabetes mellitus (IDD). The islets of Langerhans in patients with IDD have been found to be infiltrated with mononuclear cells (Gepts 1965; Gepts & DeMey 1978), and lymphocytes from patients with IDD have been shown to be sensitized to pancreatic antigens (Nerup et al. 1971; MacCuish et al. 1974). It has been suggested that the histopathologic changes in IDD are triggered by an immunologic response to foreign antigens or to the host's own antigen (Nerup et al. 1971; Irvine et al. 1976; Lendrum et al. 1976).

In laboratory animals a transient diabetic condition can be induced following immunization with homologous pancreatic islets (Nerup et al. 1974a; Egeberg et al. 1976; Kromann et al. 1979). This diabetic state includes inflammatory lesions of the pancreatic islets (insulitis), β-cell degranulation, a reduction in glucose tolerance, antibodies directed against the surface of islet cells, an immunological in vitro reactivity (leukocyte migration inhibition) similar to that of IDD patients (Nerup et al. 1974b), and a decrease in insulin release from isolated islets (Berggren & Lernmark 1977).

If IDD is indeed an autoimmune disorder, it should be possible to demonstrate lymphocytes...
specifically reactive against normal constituents of the individual. We therefore initiated studies to determine whether lymph node cells from mice immunized with homologous islets would proliferate during co-culture with dispersed islet cells.

**Materials and Methods**

**Animals**

Female BALB/c (H-2^d^) mice (Bomholtgaard, Ry, Denmark), 3–8 months of age, served as donors of islets for antigen and of islet cells for the stimulator cell population. Female A.TL (H-2^k^) mice, 3–5 months of age, served as recipients of the antigen and as the source of the lymphocytes for the responder cell population. The A.TL mice have been bred and maintained in our laboratory. All mice were housed in the same room and received food and water ad libitum until the day before sacrifice, at which time islet donors were fasted.

**Preparation of antigen and stimulator cells**

Pancreatic islets were isolated by collagenase digestion and Percoll density-gradient centrifugation (Steffes et al. 1981), and were individually selected under a stereomicroscope. The islets were maintained overnight at 4°C in RPMI-1640 (Flow Laboratories, Glasgow, Scotland) containing 2 g/l NaHCO_3_ and supplemented with 2 mmol/l glutamine, 10 mmol/l N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid (HEPES), 10 IU/ml penicillin, 100 µg/ml streptomycin, and 5% heat-inactivated horse serum (GIBCO Europe, Edinburgh, Scotland). The islets were washed by centrifugation in distilled water and the volume was adjusted to a final concentration of 10 islets/µl. An equal volume of CFA (Difco Laboratories, Detroit, Michigan) was added, and an emulsion prepared by sonication for 5–10 s. This antigen-CFA emulsion was injected sc into both hind footpads (~20 µl), allowing each mouse to receive a dose of ~500 islets. 'Sham-treated' mice received 100 µl of a 50% (v/v) emulsion of distilled water and CFA in an identical manner.

Stimulator cells were prepared by dissociating islets in RPMI containing 2 mmol/l 1,2-dit-(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid (EGTA) by gentle aspiration through a constricted blunt-ended Pasteur pipette. The resulting single cell suspension was washed once by centrifugation (50 x g, 10 min) and re-suspended in RPMI containing horse serum, as described above. The cells were adjusted to a concentration of 2.5 x 10^5 cells/ml and subjected to ~2500 rads of γ-irradiation. Lymph node stimulator cells were also prepared (see below) from BALB/c mice at a concentration of 2.5–5 x 10^5 cells/ml and irradiated.

**Preparation of lymph node cells**

The islet-primed and sham-treated animals were sacrificed by cervical dislocation 7–8 days after immunization. The 2 inguinal, 2–3 periaortic and 2 popliteal lymph nodes were removed aseptically (Alkan 1979), trimmed free of excess fat, and placed in Hank’s balanced salt solution (HBSS) (Flow) supplemented with 25 mmol/l HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2.5% horse serum. The lymph nodes were teased through sterile nylon gauze and cell clumps were eliminated by sedimentation of the cell suspension for 5 min on ice. The cells were washed twice by centrifugation (400 x g, 10 min) at 4°C in HBSS containing 2.5% horse serum, and once in the RPMI culture medium described above. The lymph node responder cells were adjusted to a cell concentration of 2 x 10^6 cells/ml. Eosin Y exclusion tests revealed a viability of >95%. In each experiment, 3–5 sham-treated and 2–5 islet-primed mice served as lymph node donors. The lymph node cells of each mouse were assayed separately.

**Determination of [³H]TdR incorporation**

The mixed cell reaction was performed in Nunclon (Nunc, Roskilde, Denmark) flat-bottom microtitre plates using 2 x 10^5 responder cells (A.TL lymph node cells) and either 2.5 x 10^4 irradiated BALB/c stimulator islet cells or 2.5–5 x 10^4 irradiated BALB/c stimulator lymph node cells in a total volume of 0.2 ml. Cultures were also performed with responder or stimulator cells alone with and without 20 µg/ml concanavalin A (Con A). The concentration of Con A in a system using horse serum should be ~10 times higher than in culture systems using foetal calf serum (Alkan 1979). The culture medium was RPMI with 5% horse serum, as described above.

After an initial 24–26 h incubation at 37°C in a humidified atmosphere of 5% CO_2_ in air, the cultures were pulsed with 1 µCi/well of [³H]methylthymidine ([³H]-TdR, specific activity 25 Ci/mmol, the Radiochemical Centre, Amersham, England), and incubated for another 16 h. At the end of the incubation period, 25 µl medium was removed from each well for subsequent insulin determinations. The incubation was stopped by addition of 50 µl of cold 50% (w/v) trichloroacetic acid (TCA) containing 6 mmol/l non-radioactive TdR. The contents of each well were gently mixed, and the plate left on ice for 15 min before centrifugation (100 x g, 10 min) at 4°C. After removing the supernatant, all wells were washed by centrifugation (100 x g, 10 min) 3 times with 200 µl cold 8.3% TCA containing 1 mmol/l non-radioactive TdR. The final precipitate was dissolved in 200 µl 1 N NaOH during an overnight incubation at room temperature. The contents of each well were finally transferred into scintillation vials containing 500 µl of 3 mol/l acetic acid and each well was rinsed twice with 250 µl of 3 mol/l acetic acid. All samples were counted.
in 10 ml of Aquasol-2 scintillation fluid (New England Nuclear, Boston, Massachusetts) in a Packard liquid scintillation spectrometer. The results were expressed as CPM.

In separate experiments (not shown), the incubation period was extended to 3–5 days without increased proliferation of the lymphocytes co-cultured with islet cells at the usual 1:8 stimulator-to-responder cell ratio. Unfortunately, at these extended incubation periods allogeneic responses (MLRs) were observed in lymphocyte-lymphocyte co-cultures; consequently, subsequent lymphocyte-islet cell co-cultures were carried out for only 40–42 h.

To determine if the proliferative response of lymphocytes co-cultured with islet cells was the result of the allogeneic nature of the stimulator cells, rather than islet cells per se, we performed experiments with several mice (n = 14) in which 2 × 10^6 responder lymphocytes were co-cultured with 2.5 or 5.0 × 10^6 irradiated allogeneic lymph node cells (a 1:8 or 1:4 ratio), and other experiments in which 8 × 10^5 responder lymphocytes were co-cultured with 1.0 or 2.0 × 10^6 irradiated allogeneic lymph node cells (also 1:8 or 1:4). Only at the higher plating density did we observe an allogeneic mixed lymphocyte response (MLR) within the 40–42 h incubation period. When plated at a density of 8 × 10^5 cells/well, lymph node cells from both islet-primed and sham-treated mice co-cultured with 1 × 10^5 irradiated allogeneic lymphocytes incorporated more than twice the amount of [3H]TdT incorporation by lymphocytes cultured alone; an increase in stimulator cells to 2 × 10^5 lymph node cells/well (1:4 ratio) resulted in [3H]TdT incorporation 5–6 times the amount of [3H]TdT found in wells containing lymphocytes cultured alone (data not shown). In marked contrast, however, no MLR was observed within 40–42 h when the plating density of the cells was kept a 2 × 10^5 responder cells/well (see Results). For this reason, all subsequent lymphocyte-islet cell co-cultures were plated with 2 × 10^5 responder cells/well.

**Insulin determinations**

The insulin released into the medium within the microtiter wells was assessed by radioimmunoassay. Free and antibody-bound hormone was separated by ethanol precipitation (Heding 1966), and crystalline rat insulin was used as standard.

**Statistical analyses**

Results are given as mean ± standard error of the mean (SE) for the number of experiments shown. Student’s t-test (two-tailed) was used to test differences between experimental and control incubations (Remington & Schork 1970). In lymphocyte-islet cell co-cultures, the statistical tests were performed after subtracting out the background radioactivity found in wells plated with irradiated stimulator islet cells alone. In lymphocyte-lymphocyte co-cultures, and in cultures of Con A-stimulated lymphocytes, the residual radioactivity found in wells plated with responder lymphocytes alone was subtracted from the [3H]TdT values.

**Reproducibility of the assay**

The number of replicate samples averaged 5/mouse in each experiment. In replicate samples of lymphocyte-islet cell co-cultures of an individual mouse, the coefficient of variation between wells was 17% for sham-treated and 16% for islet-primed mice. The coefficient of variation between mice was 12% for sham-treated and 13% for islet-primed mice. The coefficient of variation between all 17 mice tested in lymphocyte-islet cell co-cultures was 23% for sham-treated mice and 19% for islet-primed mice.

**Results**

At the end of the 40–42 h incubations, the TCA precipitation procedure used to separate cell-bound from free [3H]TdT left a residual mean
radioactivity of $2157 \pm 196$ CPM (n = 5 experiments) in the blank wells (containing medium only), $2543 \pm 104$ CPM (n = 34 mice) in wells with unstimulated responder lymph node cells alone, and $2155 \pm 337$ CPM (n = 4 experiments) in wells with irradiated islet cells alone (data uncorrected; mean $\pm$ SE). These values were not statistically different from one another.

The radioactivity data were corrected for control values as described in Methods. Responder lymph node cells from islet-primed mice co-cultured with allogeneic irradiated islet cells demonstrated a significant increase in [H]$^3$TdR incorporation (Fig. 1, column B). In contrast, responder lymph node cells from sham-treated mice failed to demonstrate a proliferative response (Fig. 1, column A). Student's t-test (two-tailed) confirmed that there was a significant difference ($998 \pm 114$ vs $399 \pm 110$ CPM; $P < 0.001$) in [H]$^3$TdR incorporation in lymphocytes from islet-primed mice compared to sham-treated mice in their response to islet cells in co-culture. Wells with lymph node cells from sham-treated mice co-cultured with islet cells contained no more [H]$^3$TdR than wells containing lymph node cells alone ($P > 0.20$).

As discussed in Methods, no allogeneic mixed lymphocyte response was detected within the 40–42 h incubation period when $2 \times 10^5$ responder lymphocytes were co-cultured with $2.5$ or $5 \times 10^4$ irradiated allogeneic lymph node cells (Fig. 1, column C).

Responder lymph node cells ($2 \times 10^5$ or $8 \times 10^5$ cells/well) were tested at 7–8, 14 and 21 days after in vivo priming. As shown in Table 1, when co-cultured with irradiated islet cells, responder lymphocytes from islet-primed mice consistently incorporated more [H]$^3$TdR than responder lymphocytes from sham-treated mice, at all periods tested and at stimulator-to-responder cell ratios varying between a high of 1:4 and a low of 1:32. Those experiments consisting of $2 \times 10^5$ lymph node cells/well, at a 1:8 ratio and utilizing mice immunized 7–8 days previously, constitute the experiments from which Fig. 1 was constructed.

Table 1.

Effects of in vivo priming time and stimulator/responder cell ratio on lymph node cell population.

<table>
<thead>
<tr>
<th>Lymph node cells/well</th>
<th>Stimulator/responder cell ratio</th>
<th>Days after priming</th>
<th>(n)$^2$</th>
<th>[H]$^3$TdR incorporation (CPM/well)$^1$</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymph node cells + BALB/c islet cells</td>
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<tr>
<td>$2 \times 10^5$</td>
<td>1:4</td>
<td>7</td>
<td>(3)</td>
<td>1315 ± 409</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2851 ± 515</td>
</tr>
<tr>
<td>$2 \times 10^5$</td>
<td>1:8</td>
<td>7–8</td>
<td>(17/13)*</td>
<td>399 ± 110</td>
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<td></td>
<td></td>
<td></td>
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<td>990 ± 114</td>
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<tr>
<td>$8 \times 10^5$</td>
<td>1:16</td>
<td>14</td>
<td>(3)</td>
<td>731 ± 44</td>
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<td>2981 ± 507</td>
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<tr>
<td>$8 \times 10^5$</td>
<td>1:32</td>
<td>14</td>
<td>(3)</td>
<td>531 ± 153</td>
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<td>2981 ± 507</td>
</tr>
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<td>$8 \times 10^5$</td>
<td>1:16</td>
<td>21</td>
<td>(2)</td>
<td>858 ± 220</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1495 ± 201</td>
</tr>
<tr>
<td>$8 \times 10^5$</td>
<td>1:32</td>
<td>21</td>
<td>(2)</td>
<td>79 ± 79</td>
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<td></td>
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<td>437 ± 209</td>
</tr>
</tbody>
</table>

1 All values are corrected for the radioactivity found in control wells; mean values $\pm$ SE for the number of mice shown.
2 n = the number of sham-treated or islet-primed mice in each experiment.
* Data pooled from 5 experiments, 17 mice, for islet cell studies; data pooled from 4 experiments, 13 and 12 mice, for Con A studies.
No significant difference was observed between responder A.TL lymph node cells from islet-primed and sham-treated mice in their ability to incorporate $[^3H]$TdR in response to 20 μg/ml Con A (Table 1). Con A-induced $[^3H]$TdR incorporation increased with increasing lymph node cell number. Con A had no effect on the incorporation of $[^3H]$TdR in either irradiated or non-irradiated BALB/c islet cells, nor in irradiated responder lymph node cells (data not shown).

Microtiter wells with $2.5 \times 10^4$ irradiated islet cells plus $2 \times 10^5$ responder lymph node cells from sham-treated mice contained $198 \pm 19$ ng insulin/well (mean ± SE, n = 10 mice). This was not significantly different from the insulin content ($199 \pm 20$ ng/well; n = 9 mice) in wells with islet cells and responder lymph node cells from islet-primed mice.

Discussion

Mice primed with homologous pancreatic islet cells develop transient glucose intolerance and other diabetes-like phenomena such as β-cell degranulation, some inflammatory lesions of the pancreatic islets, and islet-cell-surface antibodies (Nerup et al. 1974a; Egeberg et al. 1976; Kromann et al. 1979). We now demonstrate that lymph node cells from mice similarly immunized can recognize and proliferate in response to a second exposure to dispersed irradiated pancreatic islet cells. The secondary in vitro response was small, but statistically significant proliferation was detected.

In our assay, the responding cell population was a mixture of lymphocytes and accessory cells. An unknown which remains to be investigated is the determination of which functional population of lymphocytes is reflected in our proliferative assay. It is reasonable to assume that the responding cells are T-lymphocytes (Lee et al. 1979).

Our major concern to us in these studies was the release of insulin by the cultured β-cells, since insulin is known to augment the intermediary metabolism of insulin-receptor-bearing lymphocytes (Helderman & Edwards 1981). Resting lymphocytes apparently do not bear insulin receptors in vivo (Helderman & Strom 1977), and physiologic concentrations of insulin have no effect on the metabolism of unstimulated, resting lymphocytes (Helderman & Edwards 1981). In or system the release of insulin by the cultured β-cells was identical in the presence of lymphocytes from islet-primed and sham-treated mice. Thus, the released insulin might have enhanced the proliferation of the β-cell-reactive lymphocytes, but would not have affected the unstimulated lymphocytes, nor could it have been the initiating proliferative stimulus. It is also unlikely that insulin was the primary sensitizing agent during immunization.

The lymph node cells from islet-primed A.TL mice were not significantly reactive to BALB/c stimulator lymph node cells when the number of responding lymphocytes per well was $2 \times 10^5$, although a small MLR was noted at the higher plating density of $8 \times 10^5$ responder cells per well, with significantly enhanced MLR at a higher stimulator-to-responder cell ratio. The absence of an allogeneic response in our co-cultures might be explained by the low plating density and the low stimulatory-to-responder cell ratio (1:8 or 1:4), as well as the short incubation period (40–42 h). Conditions of mixed lymphocyte cultures, normally carried out at a 1:1 stimulator-to-responder cell ratio, could not be achieved with a sufficient number of cells, due to limited availability of islet cells. It is also of note that suppressor cells activated by alloantigens in vivo are known to release soluble factors that suppress the proliferative phase of mixed lymphocyte reactions (Engleman et al. 1978). This lack of detectable MLR suggests that lymph nodes isolated from islet-primed mice contain sensitized lymphocytes which proliferate in vitro in response to islet-cell-specific determinants only. Definitive evidence of islet-cell-specificity will come from investigation of islet-cell-induced lymphoproliferation in mice immunized with syngeneic islets; these studies are presently underway.

The possible presence of islet-cell-specific antigens is supported by recent experiments demonstrating that a heterologous islet cell antiserum detects a β-cell-specific cell surface glycoprotein (Dyrb erg et al. 1982).

Our observations are in accordance with recent studies of primary and secondary lymphoproliferative responses with islet cells and lymphocytes from dogs (Rabinovitch et al. 1981). The authors of those studies observed a primary proliferative response to islet cells. In vitro primed lymphocytes were found to proliferate in response to a second challenge with islet cells, but not with lymphocytes or hepatocytes, as stimulator cells. Taken together, these studies suggest the presence of lymphocytes able to recognize and proliferate in response to islet-cell-specific determinants.
Acknowledgments

This study was supported in part by the Juvenile diabetes Foundation and Vera og Carl Johan Michaelsens Legat. Dr. Scott is a Juvenile Diabetes Foundation Postdoctoral Research Fellow. We thank the Tissue Typing Laboratory at the University Hospital (Rigshospitalet), Copenhagen, for allowing us to use their X-ray machine.

References


Received on February 15th, 1983.