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Acceleration of diabetes in young NOD mice with peritoneal macrophages

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Abstract

To elucidate the roles of macrophages in the pathogenesis of NOD murine diabetes, peritoneal macrophages from NOD mice were injected into young NOD mice. We used 12 to 20 week-old NOD mice of both sexes as donors, and sex-matched 2-week-old NOD mice as recipients. Cyclophosphamide (CY), 200 mg/kg, was intraperitoneally injected into the donors. Two weeks later, peritoneal exudate cells (PEC) were collected from the diabetic donors. Macrophage-rich fractions (MRF) were collected by adherence. Then PEC($5–8 \times 10^6$) or MRF($3–7 \times 10^6$) were transferred, intraperitoneally, to the recipients. Two weeks later, some of the recipients were killed in order to perform immunofluorescent analysis of splenocytes and to assess pancreatic histology. Mac 1 positive splenocytes were increased in PEC- and in MRF-injected recipient mice. Insulitis was seen in PEC- and MRF-injected mice, but not in controls. Some of the recipients were injected with CY, 200 mg/kg, intraperitoneally, at two weeks post cell transfer. Two weeks after CY injection, the animals were examined for the presence of diabetes. The incidences of diabetes were 67% in PEC-injected mice, 40% in the MRF-injected group, and 3% in the controls. These results suggest that peritoneal macrophages accelerate the disease process in NOD mice.

Keywords: Insulin-dependent diabetes mellitus; Etiology; Macrophage; Non-obese diabetic mouse; Insulitis

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1. Introduction

Type 1 diabetes (insulin-dependent diabetes mellitus; IDDM) is considered to be an autoimmune disease which results in the selective destruction of pancreatic beta cells. One animal model for inherited autoimmune diabetes is the non-obese diabetic (NOD) mouse, which displays many of the characteristics of human IDDM [1,2]. In this mouse, lymphocytic infiltration of pancreatic islets, termed 'insulitis', is initially observed at 5 to 6 weeks of age and overt diabetes develops after 12 weeks of age. A T lymphocyte-dependent autoimmune mechanism is thought to be responsible for pancreatic beta cell destruction in NOD mice [3,4]. In addition to the involvement of T lymphocytes, macrophages participate in the development of insulitis in this animal model. Inflammatory macrophages can be detected at both peri- and intra-islet locations at early points in the disease process [5]. Administration of silica, which is selectively toxic to macrophages, prevents the development of diabetes in NOD mice [6,7]. Furthermore, transfer of diabetes in NOD mice can be prevented by blockade of the adhesion-promoting receptor on macrophages [8]. Recently, we reported that peritoneal exudate cells from CY-injected NOD mice are cytotoxic against tumor cell lines and cultured pancreatic beta cell lines in vitro [9]. However, the roles of macrophages in the anti-beta cell autoimmune response have not yet been defined. To elucidate the contribution of macrophages to the pathogenesis of NOD murine diabetes, peritoneal macrophages from NOD mice with overt diabetes (donor) were injected into young NOD mice (recipient).

2. Materials and methods

2.1. Mice

Our NOD mouse colony was produced from a breeding stock obtained from Clea Japan Inc. (Tokyo, Japan). In our colony, diabetic mice are observed after 14 weeks of age in females and after 20 weeks of age in males. Incidences of diabetes at 24 weeks of age are about 60% in females and 20% in males. ICR mice were purchased from Clea Japan Inc. Mice (2 to 20 week-old, both sexes) used in this study were maintained under specific pathogen free conditions.

2.2. Collection of cells from donor mice

Cyclophosphamide (CY; Shionogi, Osaka, Japan), 200 mg/kg, was injected intraperitoneally into donor mice. Twelve to 20 week-old mice of both sexes were used as donor mice. Two weeks after CY injection, diabetic mice were killed and peritoneal exudate cells (PEC) were aseptically collected by washing the peritoneal cavity with 5 ml of RPMI 1640 containing 1% fetal calf serum (FCS) (medium), and washed twice in medium and suspended in 0.2 ml of medium. Mice with glucosuria and blood glucose levels > 250 mg/dl were considered diabetic. We used no inducers of any kind to collect PEC. Macrophage-rich fractions (MRF) were obtained from PEC by utilizing an adherence technique [10]. Collected PEC were washed twice in medium, and adjusted to 1×10^6/ml. Five ml of the suspended cells were added to FCS-coated 60 mm plastic dishes (tissue culture dish, Corning, New York, USA), and incubated for 15 min at 37°C in 5% CO_2. After incubation, dishes were washed 5 times in medium to remove non-adherent cells. Then cooled EDTA in PBS containing 5% FCS was added to the dishes, and the cells were further incubated at 4°C for 40 min. After incubation, cells were removed by vigorous pipetting. Following three more washes in medium, MRF were suspended in 0.2 ml of medium. Some PEC samples were irradiated with a 2000 rad X-ray (Lineac) dose in order to prevent T cell proliferation (2000 rad PEC). These cells were also suspended in 0.2 ml of medium.

2.3. Donor mice cell populations

Portions of the collected PEC and MRF (1×10^6/ml) from donor mice were incubated with fluorescence-conjugated monoclonal antibodies specific for either Thy1.2 (Becton-Dickinson, California, USA) or B220 (RA3 3A1) Ag for 20 min at 4°C.

Another portion of the collected cells was incubated with anti-Mac1 (Boehringer Mannheim, Mannheim, Germany) monoclonal antibodies for 20 min at 4°C. After washing three times in PBS, biotinylated anti-rat IgG (Boehringer Mannheim, Mannheim, Germany) was added to the cell pellets for a further 20 min of incubation at 4°C. After three additional washings in PBS, PE-streptoavidin (Biomeda, California, USA) was added and incubation was continued for an additional 20 min at 4°C. For

dual staining of anti-Mac1 and anti-Ia (class II; I-A^d), after washing three more times in PBS, anti-Ia monoclonal antibodies were added for a further 20 min of incubation at 4°C. After three additional washings in PBS, fluorescence-conjugated anti-mouse IgG (Boehringer Mannheim, Mannheim, Germany) was added and the incubation proceeded for an additional 20 min at 4°C.

For dual staining of anti-L3T4 and anti-Lyt2, collected cells were put into antibody mixture (fluorescence-conjugated anti-Lyt2 and biotinylated anti-L3T4, Becton-Dickinson, California, USA). After 20 min of incubation at 4°C, cells were washed three times in PBS. PE-streptoavidin (Biomeda, California, USA) was added and cells were incubated at 4°C for an additional 20 min.

All of these stained cells were washed three times in PBS prior to analysis by flowcytometry (FACScan, Becton-Dickinson, California, USA).

Anti-Ia monoclonal antibodies were the generous gift of Dr. N. Shinohara (Mitsubishi Kasei Inst. Life Sci.).

2.4. Cell transfer and induction of insulitis and diabetes

Five to 8 $\times$ 10^6 PEC or 3–7 $\times$ 10^6 MRF in 0.2 ml of medium were transferred intraperitoneally into sex-matched 2 week-old NOD mice (recipient). Fourteen recipients were given PEC, 10 MRF, and 10 2000 rad PEC. Only 0.2 ml of medium was injected into the control mice. We used 18 females and 25 males, all two weeks old, as control mice (total: 43 mice). PEC from CY-treated ICR mice (CY, 200 mg/kg, was injected into ICR mice two weeks before killing) were also injected into 2 week-old NOD mice (n = 8).

Fourteen recipient mice (5 PEC-injected mice, 5 MRF-injected mice, and 4 control mice) were killed at two weeks post cell transfer (or medium injection), and immunofluorescent analysis of splenocytes and assessment of pancreatic histology (grading of insulitis) were done. The monoclonal antibodies and flowcytometry mentioned above were used for immunofluorescent analysis. Harvested pancreatic samples were fixed with 10% formaldehyde and embedded in paraffin. More than 5 slices were taken from each sample, and these sections were stained with hematoxylin-eosin. All of the islets on the slides were counted. Insulitis was graded as follows: 0, no evidence of insulitis; 1, mononuclear cells occupy 1–25% of a single islet (peri-insulitis); 2, 26–50% (moderate insulitis); 3, 51–100% (extensive insulitis). The degree of insulitis was determined by a pathologist who was unaware of the protocol for this experiment.

Moreover, 11 recipient mice (5 2000 rad PEC-injected mice and 6 control mice) were also killed at two weeks post cell transfer for lymphocyte proliferation assay of splenocytes from recipients against islet cell line (MIN6N-9a [9]) and for cytotoxicity assay of PEC from recipients against MIN6N-9a.

For lymphocyte proliferation assay, erythrocyte-depleted splenocytes from recipients, 1 $\times$ 10^6 cells/ml, were incubated in triplicate for 72 h (37°C, 5% CO_2) in 200 μl of culture medium (RPMI 1640 supplemented with 10% FCS, penicillin 1 $\times$ 10^6 μl, streptomycin 100 mg/l, sodium pyruvate 100 mg/l, L-glutamate 2 mmol/l, 2-mercaptoethanol 4 μl/l, MEM non-essential amino acid solution (100 $\times$) 10 ml/l, $NaHCO_3$ 2 g/l and HEPES 2.38 g/l) in microtiter wells in the presence or absence of antigens (MIN6N-9a; 1 $\times$ 10^5/ml), or control ovalbumin (100 μg/ml). One μCi [^{3}H]thymidine was added to each well for the final 12 h. The cells were collected with a cell-harvester. The results were computed as the stimulation index, the ratio of the mean test counts/min obtained in the presence of antigen to the mean background counts/min obtained in the absence of antigen.

For cytotoxicity assay, PEC from recipients, 2 $\times$ 10^6 cells/ml, were incubated in triplicate for 40 h (37°C, 5% CO_2) in 200 μl of culture medium in microtiter wells with MIN6N-9a (2 $\times$ 10^5/ml). One μCi [^{3}H]thymidine was added to each well for the final 18 h. The cells were also collected with a cell-harvester. The results were computed as the ratio of the mean test counts/min obtained in the presence of PEC to the mean background counts/min obtained in the absence of PEC.

CY 200 mg/kg was injected intraperitoneally into the remaining recipient mice at two weeks after cell transfer (or medium injection). Diabetes and pancreatic histology (grading of insulitis) were assessed at two weeks post CY injection. Mice with glucosuria and a blood glucose level > 250 mg/dl were considered diabetic. We checked urinary glucose with Tes-tape (Eli Lilly, Indiana, USA), and

blood sugar levels by a glucose oxidation method. In this experiment, glucose levels of all mice with glucosuria were > 600 mg/dl. Staining of pancreatic specimens and grading of insulitis were done by the same procedures as described above.

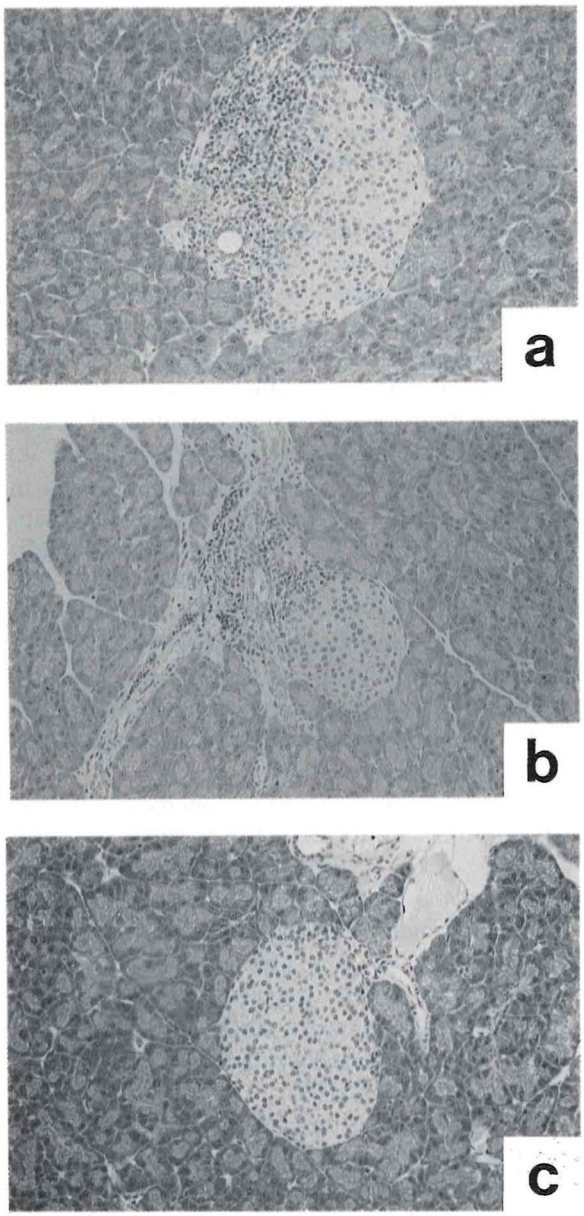

Fig. 1. Pancreatic histology at two weeks post cell injection (200×): insulitis can be observed in PEC- and in MRF-injected mice but not in control mice. a: PEC-injected group; b:MRF-injected group; c: medium-injected group.

2.5. Statistical analysis

Data are presented as mean ± S.D. Statistical analysis was performed with the unpaired Student's *t*-test to compare the mean value, and Fisher's exact test for comparison between groups.

3. Results

3.1. Cell populations of donor mice

Peritoneal exudate cell populations of donor mice ($n = 5$): $Mac1^+$ cells comprised 63.7 ± 3.2% (Ia^+Mac1^+ cells 61.6 ± 3.2%), $Thy1.2^+$ cells 13.1 ± 3.1%, $L3T4^+$ cells 10.2 ± 2.1%, $Lyt2^+$ cells 3.2 ± 0.8%, and $B220^+$ cells 0.8 ± 1.2% of PEC.

Macrophage-rich fraction cell populations of donor mice ($n = 3$): $Mac1^+$ cells comprised 82.9 ± 11.1% and $Thy1.2^+$ cells 16.8 ± 9.7%.

3.2. Induction of insulitis and diabetes in recipient mice

Two weeks after cell transfer, 14 mice were killed and pancreatic histology was assessed, and splenocyte populations were analysed by an immunofluorescent technique. Insulitis was observed in PEC- and MRF-injected mice at two weeks post cell injection, but not in control mice (Fig. 1, Table 1). The results of immunofluorescent analysis of splenocytes are shown in Table 2. Proportions of $Mac1^+$, $L3T4^+$ and $Lyt2^+$ splenocytes to total splenocytes are indicated.

'Ia^+Mac1^+' indicates $Mac1^+$ splenocytes expressing MHC class II molecules. Ia^+ and total $Mac1^+$ cells were significantly increased in MRF-injected mice as compared to control mice ($P < 0.05$; unpaired Student's *t*-test).

Splenocytes from 2000 rad PEC-injected mice showed a significantly stronger response to MIN6N-

Table 1
Pancreatic histology of recipient mice at two weeks post cell injection

Injected cells	Grading of insulitis			
	0	1	2	3
PEC* ($n = 3$)	23	7	2	0
Macrophage-rich fractions ($n = 3$)	30	1	0	0
Medium only ($n = 4$)	33	0	0	0

*All of the mice in this group showed insulitis.

Table 2
Immunofluorescence analysis of splenocytes from recipient mice

Injected cells	Ia$^+$Mac 1 (%)	Total Mac 1 (%)	L3T4 (%)	Lyt2 (%)
PEC ($n = 5$)	14.3 ± 6.1	16.7 ± 6.2	26.8 ± 2.8	11.6 ± 0.7
Macrophage-rich fractions ($n = 5$)	10.0 ± 1.7]*	13.2 ± 2.3]*	19.0 ± 2.6]**	9.1 ± 1.0
Medium only ($n = 4$)	7.0 ± 1.1]	9.2 ± 1.6]	27.1 ± 1.0]	10.6 ± 0.6

The results are expressed as mean ± S.D. of percentages per individual.
*$P < 0.05$, **$P < 0.01$ (unpaired Student's t-test)

9a than did those from controls (stimulation index (S.I.): 1.41 ± 0.76 vs. 0.79 ± 0.41, $P < 0.01$; Fig. 2). There were no significant differences between the two groups in response to ovalbumin (S.I.: 1.10 ± 0.67 vs. 1.10 ± 0.68).

PEC from 2000 rad PEC-injected mice significantly suppressed uptake of [^{3}H]thymidine more than did those from controls (0.289 ± 0.066 vs. 0.618 ± 0.123, $P < 0.01$; Fig. 3), i.e. PEC from 2000 rad PEC-injected mice were more cytotoxic than those from controls.

Two weeks after cell injection, CY 200 mg/kg was administered intraperitoneally to the remaining mice. Table 3 shows the incidences of overt diabetes at two weeks post CY treatment. The incidences of diabetes were 67% in PEC-injected mice and 40% in MRF-injected mice, whereas only 3% of control mice developed diabetes ($P < 0.01$, $P < 0.05$; Fisher's exact test). None of the mice given PEC from ICR mice developed diabetes ($P < 0.01$). Of the five mice given 2000 rad PEC in order to prevent T cell proliferation, two also developed diabetes ($P < 0.05$). At two weeks post CY, insulitis was more severe in PEC- and MRF-injected mice than in control mice (Table 4).

There was no difference between the sexes in the recipients in this system.

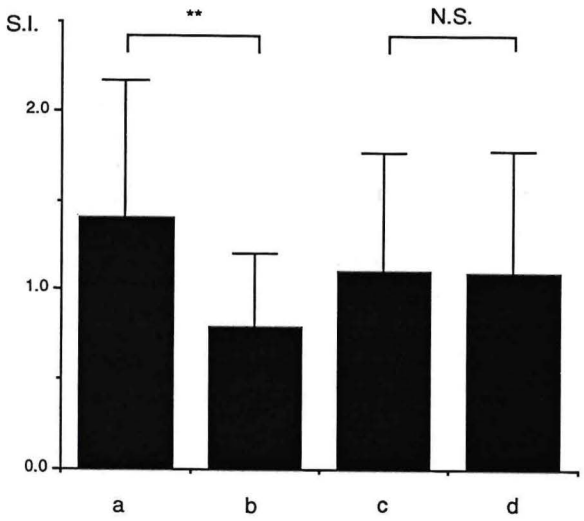

Fig. 2. Lymphocyte proliferation assay of splenocytes from recipients against MIN6N-9a at two weeks post transfer. a: MIN6N-9a + splenocytes from 2000 rad PEC-injected mice ($n = 5$); b: MIN6N-9a + splenocytes from medium-injected mice (controls, $n = 6$); c: ovalbumin + splenocytes from 2000 rad PEC-injected mice ($n = 5$); d: ovalbumin + splenocytes from medium-injected mice ($n = 6$). S.I., stimulation index. **$P < 0.01$ (Student's t-test).

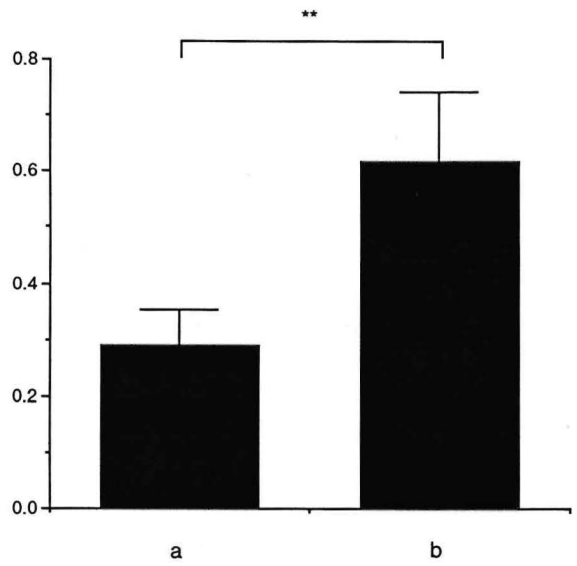

Fig. 3. Cytotoxicity assay of PEC from recipients against MIN6N-9a at two weeks post transfer. a: 2000 rad PEC-injected mice ($n = 5$); b: medium-injected mice (controls, $n = 6$). **$P < 0.01$ (Student's t-test).

Table 3
Incidences of overt diabetes in recipient mice

Injected cells	Incidences of overt diabetes after CY treatment
PEC	6/9(66.7%)
2000 rad PEC	2/5(40.0%)
Macrophage-rich fractions	2/5(40.0%)
Medium only	1/33 (3.0%)
PEC from ICR mice	0/8(0%)

*$P < 0.05$, **$P < 0.01$ (Fisher's exact test)

4. Discussion

Direct evidence for autoimmunity has been provided by cell transfer experiments in animal models. There have also been many reports of cell transfer experiments in NOD mice [3–5,11,12], but most of these have related mainly to the transfer of T cells. In this study, to elucidate the roles of macrophages in IDDM, we collected peritoneal macophages from diabetic donor mice and transferred them into young recipient mice, and found that peritoneal macrophages can accelerate the disease process in NOD mice.

In our preliminary studies, mice were observed for 6 months after cell transfer. Forty percent of PEC-injected mice (all male) became diabetic but this was observed after 12 weeks of age (20% in controls; all male). Thus, we could not distinguish whether our results were due to the effects of macrophage transfer or only the natural course of diabetes in NOD mice. In a previous report [13], diabetes could not be induced in 3-week-old mice by CY, and even at 5 weeks of age, the incidence of diabetes induced by CY was less than 10%. Insulitis is considered to occur naturally at 5 to 6 weeks of age in NOD mice.Therefore, even with CY treatment, young NOD mice, below 4 weeks of age, do not become diabetic. We thus chose to induce diabetes with CY two weeks after cell transfer (at 4 weeks of age). We assumed that if macrophages from diabetic donors accelerate the occurrence of insulitis, a difference in the incidence of diabetes would be observable between the transferred group and controls. Using this experimental protocol, we found that PEC from spontaneous diabetic and 'pre-diabetic' (18 week-old) female NOD mice induced diabetes in two of four recipients. First we collected PEC from spontaneous diabetic mice, but we could not get adequate numbers of cells. We also used CY to induce diabetes in donors in order to collect sufficient numbers of cells at one time. Finally, peritoneal macrophages from CY-induced diabetic donors were transferred into young recipients, then CY was injected at two weeks post cell transfer, and the incidence of diabetes was assessed at two weeks after CY treatment.

The incidences of overt diabetes in PEC-injected mice was slightly higher than in MRF-injected mice. The MRF took several hours to collect. In contrast, 'total PEC' could be injected soon after collection. Thus, we speculate that differences between the two are due to cell viability.

Generally, in cell transfer experiments, the most important challenge is the purification of the cell population. First, we used 'total PEC', and then macrophages collected by an adherence technique. Although we tried to obtain pure macrophages, T

Table 4
Pancreatic histology of recipient mice at two weeks post CY treatment

Injected cells	Grading of insulitis			
	0	1	2	3
PEC ($n = 4$)	7 (27%)*	9 (35%)	8 (31%)	2 (8%)
Macrophage-rich fractions ($n = 5$)	20 (49%)	12 (29%)	7 (17%)	2 (5%)
Medium only ($n = 13$)	52 (60%)	28 (33%)	5 (6%)	1 (1%)

*The percentages of the islets of each grade of insulitis are shown in the parentheses.

cell contamination accounted for about 15% of the cell population. We therefore attempted to deplete T cells by using anti-Thy1.2 + C′ (data not shown). Even with this treatment, 7% of PEC were Thy1.2 positive, and it was difficult to collect sufficient numbers of cells for transfer. On the basis of these results, we decided to use 2000 rad irradiation to suppress proliferation of T cells in PEC. Even in the irradiated PEC-injected group, an acceleration in the development of diabetes was observed, indicating that macrophages can accelerate the disease process in NOD mice. As mentioned above, administration of silica [6,7] or blockade of the adhesion-promoting receptor on macrophages [8] prevents diabetes in NOD mice. These observations can not be attributed to T cells alone.

What are the mechanisms underlying these observations? In the SJL mouse, a model of delayed-type hypersensitivity (DTH), adherent, radiation resistant, Ia^+Mac1^+ cells were transferred from DTH responders to non-responders [14]. In this experiment, macrophages acted as antigen-presenting cells (APC) and activated DTH effector T cells. In our study also, transferred donor macrophages might have acted as APC in the recipient immune system. Fourteen mice were killed at 2 weeks after cell transfer, i.e. at 4 weeks of age. In control mice, we observed no insulitis. But in PEC- or MRF-injected mice, insulitis was evident. Furthermore, $Mac1^+$ splenocytes (especially Ia^+Mac1^+ splenocytes, i.e. activated macrophages) were significantly increased at two weeks after cell transfer. Moreover, splenocytes from 2000 rad PEC-injected mice showed significantly stronger response against MIN6N-9a than did those from controls. Based on these results, we speculate that injected macrophages act as APC, inducing insulitis. Macrophages produce cytokines, such as TNF-α and IL-1. Local production of cytokines by macrophages might affect the expression of adhesion molecule VCAM-1 on the vascular endothelium, thereby facilitating mononuclear cell migration [15].

Another possibility is macrophage cytotoxicity. In this experiment, PEC from 2000 rad PEC-injected mice were significantly more cytotoxic to MIN6N-9a than those from controls. Recently, we reported that peritoneal exudate cells and splenic adherent cells from CY-treated NOD mice were found to be much more cytotoxic to both pancreatic beta cell lines and tumor cell lines than cells from non-CY-treated mice in vitro [9]. Peritoneal macrophages from CY-treated diabetic donor mice may thus be presumed to be cytotoxic to recipient islets. Macrophages produce soluble mediators, such as cytokines [16–18], oxygen free radicals [19–21], nitric oxide [9,22,23], serine protease [21] and eicosanoids [25], all of which are speculated to have cytotoxic activity against beta cells. Further research is required to determine which is responsible for macrophage cytotoxicity.

In conclusion, the antigen presenting function and cytotoxic action of macrophages might accelerate the disease process in NOD mice. Our data suggest that not only T cells but also macrophages are important in the IDDM disease process.

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