Effect of Modified Diabetic Splenocytes on Mice Injected with Multiple Low-Dose Streptozotocin

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This work reports the effects of a previous injection of mitomycin-modified splenocytes from multiple-low dose streptozotocin-treated mice (mld-sz) on autoimmune diabetes produced by mld-sz. Our work shows that a previous inoculation of modified mononuclear splenocytes from mld-sz mice prevents alterations in glycemia, in insulin secretion (IS) pattern from isolated perifused islets, and in mass of pancreatic islets. Immunohistochemistry showed an alteration in the number of beta, but not of alpha or delta cells. While a mononuclear intra-islet infiltration was observed in mld-sz mice, a predominantly polar or peri-islet infiltration was seen in vaccinated mice. Islet-associated mononuclear cells from mld-sz mice produced diabetes and induced a diminished IS when transferred to normal receptors. Those cells from previously vaccinated mld-sz mice had no effect when injected into normal receptors. In addition, they also inhibited the damage induced in normal receptors by the islet-associated mononuclear cells from mld-sz animals. Cellular death was also prevented by previous vaccination. Our results suggest that vaccination with modified splenocytes from mld-sz mice is capable of shifting the islet cells infiltration pattern from an aggressive one toward a protective one and thus preventing the β cell destruction observed in mld-sz mice.

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Key words: diabetes prevention; diabetes immunomodulation: vaccination; insulin secretion pattern; morphometric, Immunohistochemistry, and cellular death studies; Islet-associated mononuclear cell transfer

The notions of specific immune therapies will quite probably change as knowledge of immune and regeneration mechanisms become more complete. Recent advances in the prediction of type I diabetes have led to the first trials of disease prevention. Pilot trials using different approaches, mainly immunomodulation, are also under study (1). In this area, future developments will allow the design of immunotherapeutic agents that will be tailored to the correlates of protection for a particular disease (2). Vaccination using attenuated autoreactive lymphocytes has been utilized in several animal models of autoimmunity. Some clinical trials involving patients with rheumatoid arthritis and multiple sclerosis have been reported (3, 4).

Diabetes can be induced experimentally in mice by multiple low doses (mld) of streptozotocin (sz), which develops a delayed and/or progressive hyperglycemia, insulitis, and severe destruction of β cells (5, 6). Diabetes by injecting splenocytes of mld-sz-induced diabetic mice to normal recipients (5) and diabetes delayed or avoided by immunosuppressive agents (6) have been reported and strongly indicate an immunological mechanism. Kolb and Kröncke (7) proposed three pathogenic steps involved in the mechanism of action of sz: 1) direct toxicity of sz, 2) nonspecific islet inflammation, and 3) sensitization to autoreactive T cells (7). The purpose of this work was to study the effect of mitomycin C (mit C)-treated diabetic splenocytes (as a vaccine) on mld-sz injected mice.

Materials and Methods

Animals. Male C57Bl/6N, 8- to 12-week-old mice were obtained from Departamento de Radiología, Comisión Nacional de Energía Atómica (Buenos Aires, Argentina). The animals were maintained according to the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals.*

Diabetes Induction *mld-sz mice.* Streptozotocin (Sigma, St. Louis, MO,) was dissolved in 0.1 ml of citrate buffer, pH 4.5, and was injected i.p. into nonfasted mice within 5 min of dissolution. Each mouse received 40 mg sz/kg body wt in a 0.1-ml volume during five consecutive days. Controls were injected i.p. with 0.1 ml of citrate buffer/day. Fifteen days after the last injection, plasma glucose was determined, mice were sacrificed, and their pancreas' were studied.

mld-sz mice previously injected with mit C-treated diabetic mononuclear splenocytes (from mld-sz diabetic mice). In some experiments, MNS were incubated with mit C prior to transfer (Sigma, 1 μg/10^6 cells/ml for 20

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min at 37°C in a 95% humidified air and 5% CO_2 atmosphere). After washing three times with sterile saline solution, 5×10^7 viable modified mononuclear splenocytes were injected i.p. into syngeneic mice in 0.1 ml of sterile saline solution (control or diabetic mit C-treated ×1). Fifteen days later, mld-sz were administered in the same manner.

Splenocytes from control and diabetic mice were obtained and incubated with mit C as previously described. These splenocytes (5×10^7 mit C-treated MNS from both groups) were then injected twice, 15 days apart, into normal syngeneic mice. Fifteen days after the last injection, mld-sz were administered in the same manner.

Transfer of islets-associated mononuclear cells (IAMNC) from mice with mld-sz with or without previous MNS injections. Islets from both groups were isolated according to the method of Lacy and Kostianovsky (8). Islets were obtained using a Ficoll-Hypaque gradient (Ficollpaque, Pharmacia, Uppsala, Sweden) and they were then washed three times with sterile saline solution. These islets (20–25/well) were cultured for 7 days with 10 U/ml human recombinant interleukin 2 (rIL 2) in Click's Eagle's Ham's Amino Acids (EHAA) medium supplemented with 5% fetal calf serum (FCS). The autoreactive MNS that grew out of the islets were maintained on the following schedule: Day 0, the cells were placed into Click's medium supplemented with 5% FCS; Day 2, mit C-treated syngeneic spleen cells were added to the cultures; and Days 4–21, the cells were maintained in Click's FCS supplemented medium with the addition of 10 U/ml rIL2. Cell viability was estimated by the Trypan blue exclusion test (9) and only cell suspensions having at least 90% viable cells were used. Groups of 1×10^5 MNS were transferred to syngeneic mice according to Buschard *et al.* (10). Fifteen days later, mice were sacrificed and their islets were perifused as described later. Six groups of mononuclear cells were injected to syngeneic mice, i.e., 1) 10^5 splenocytes from citrate mice, 2) 10^5 IAMNC from mld-sz diabetic mice, 3) IAMNC from mld-sz previously treated with mit C (×2), 4) a mixture of 10^5 IAMNC from Groups 2 and 3, 5) a mixture of 10^5 IAMNC from Group 2 plus the same amount of Group 1, and 6) 5×10^5 splenocytes from mld-sz mice.

Glucose Determination. Blood was collected from the retrorbital venous plexus by using microcapillary heparinized tubes. Plasma glucose concentration was assayed by the glucose-oxidase method (glycemia enzymatic kit; Wiener Laboratories, Rosario, Argentina) using a DB-G Spectrophotometer (Beckman Instruments, Fullerton, CA). Values of glucose over 200 mg/dl were considered hyperglycemic.

Perifusion of Pancreas Slices and of Isolated Islets. The technique used was that described by Burr *et al.* (11). Thin slices from the whole pancreas of a single mouse were used in each perifusion. Sections of pancreas weighing approximately 100 mg were dissected free of fat, connective tissue, and visible ganglia, and were immediately placed in oxygenated buffer for further mincing into pieces approximately 1 mm in diameter using stainless steel scissors. Krebs-Ringer bicarbonate buffer supplemented with 1 g/dl bovine albumin (Fraction V; Sigma) and 3.3 mM glucose was used as the perifusion buffer. The buffer was gassed with 95% O_2 and 5% CO_2, and the pH was kept constant at 7.38–7.40. Perifusion flux was 1.8–2.2 ml/min. The pancreas slices were placed into perifusion chambers (1.3 cm in diameter), buffer was passed from reservoir flask to the inverted perifusion chambers, and the pancreatic slices were retained within the chambers by Millipore filter covers (5 μm;). The samples were collected after an initial 15-min recovery period. Samples obtained at 0 and 1 min were used for baseline determinations. A stimulus of 16.5 mM glucose was added between 2 and 40 min. The first phase of insulin secretion (IS) was measured between 3 and 7 min and the second phase was measured between 10 and 40 min. Proteolytic effect on hormone secretion was avoided by adding 1000 KIU Trasylol/ml (Bayer, Buenos Aires, Argentina) to the buffer and then collecting the samples on 0.25 M EDTA (Mallinckrodt, St. Louis, MO) in tubes at 4°C, which were immediately frozen at –20° C until insulin determination by radioimmunoassay (RIA).

Islets of Langerhans were obtained from collagenase-treated (Sigma) mice pancreas by the method of Lacy and Kostianovsky (8). Groups of 20–30 islets from a single animal were used. The methodology was similar to the perifusion previously described and flux was 0.8–1 ml/min.

Insulin RIA. Insulin was determined using the method of Herbert *et al.* (12). Pork monoiodine ^{125} I-insulin with high specific activity was obtained from Comisión Nacional de Energía Atómica. Rat standard insulin was obtained from Novo Research Laboratories (Baagsvaerd, Denmark). The anti-pork insulin antibody proved to be sufficiently "nonspecific" as to allow pork-labeled insulin to be displaced by rat or mouse insulin. The method allows determination within 5.0–800 uU/ml tube range. The insulin assay sensitivity was 0.5 uU/ml; the intraassay coefficient of variation (CV) was 8.2%, 6.6%, and 5.1% for 1–5, 5–10, and 10–50 uU insulin/ml determination ranges, respectively. The interassay CV was 6.6%, 4.9%, and 5.2% for the given ranges.

Histological Studies. Mice were sacrificed by cervical dislocation and pancreases were removed and dissected free of adipose tissue, lymph nodes, and great vessels in a solution of buffer at 4°C and under a stereoscope microscope. The pancreases were then cut into two halves, one containing the head and the body and the other containing the tail. Both halves were weighed, and one half was embedded in paraffin and prepared as follows: five to six consecutive 3-μm-thick sections were cut and mounted in different slides. After sectioning the block and leaving a 300-μm space, another five to six consecutive 3-μm-thick section was cut and mounted on the previous slides. This was repeated five to six times, thus each slide had five to six different sections, each separated by a 300-μm interval that allowed the identification of 10–25 different islets. One

slide served for morphological analysis and the other four or five slides served for immunohistochemical studies.

Morphometric analysis. Sections were stained with hematoxylin and eosin and were examined at a 250× magnification using a microscope with an ocular grid. All islets in each section were studied as described by Weibel (13). The percentage between exocrine and endocrine tissue was obtained by point counting on microscope slides. These percentages were then multiplied by pancreas weight, and the results were expressed as milligrams of islets per 100 mg of pancreas. Seven to 13 islets from each pancreas slides were examined in each animal and were classified as normal islets (no alteration in shape and/or infiltrating cells) or infiltrated islets (when mononuclear cells were observed inside or around them). Four degrees of severity were used to evaluate mononuclear cell infiltration, and a mean score from each observer was obtained: 0, no infiltration; 1, polar islet infiltration; 2, peri-islet infiltration; 3, intra-islet infiltration; and 4, extensive intra-islet infiltration.

Cellular death was a frequent finding (picnotic nuclei, homogeneous and eosinophilic cytoplasm, and apoptotic bodies). Four degrees of severity of cellular death were evaluated: 0, no cellular death; 1, microphocal cellular death (isolated groups of four to five dead cells); 2, partial cellular death (approximately 50% of the islet); and 3, total (the whole islet).

Immunohistochemical studies. Insulin and glucagon were colocalized on the same slide and somatostatin was localized on a different one. Insulin was localized using a monoclonal anti-insulin antibody, monoclonal (Sigma) with a 1:8000 dilution, followed by incubations with a biotinylated second antibody/streptavidin-peroxidase (Dako, Carpinteria, CA), and it was revealed with diaminobencidine (DAB). For glucagon localization, a monoclonal antibody (Sigma) with an optimal concentration of 1:8000 was used and it was revealed by a streptavidin-alkaline phosphatase complex followed by Fast Red (BioGenex, San Ramón, CA).

Somatostatin was localized on a second section from the same animal. A polyclonal anti-somatostatin antibody without dilution, followed by incubations with secondary antibody, streptavidin-peroxidase, and finally with the cromogen DAB, were used.

A point counting technique was used to quantify the different labels within each islet, and the results were expressed as percentages.

Statistical Analysis. Statistical analysis of the data was performed by the two-tailed Student's test for unpaired samples and by one-way analysis of variance (ANOVA) and Scheffe's test. All results are expressed as the mean ± SEM. To evaluate IS from perifused pancreatic slices or islets, we also integrated the areas under the stimulated IS curves. For pancreatic slices, the first secretory phase was integrated between Min 3 and 8 and for islets, between Min 3 and 7 of perifusion, respectively. Mann-Whitney test was used to compare insulitis and immunohistological scores, and P values less than 0.05 were considered statistically significant.

Results

Table I shows that injections of mld-sz caused a significant hyperglycemia 15 days after the last injection when compared with citrate (Groups 1 and 2). One or two injections of mit C-treated control MNS (from citrate) had no effect on citrate-injected animals (Groups 3 and 6) and were unable to inhibit the hyperglycemia in diabetic animals (Groups 4 and 7).). One injection of mit C treated diabetic MNS (from mld diabetic sz) partially inhibited the hyperglycemia of mld-sz injected mice (Group 5). When these cells were injected twice, they almost completely inhibited the hyperglycemia of mld-sz injected mice (Group 8).

Table II shows that mld-sz significantly inhibit IS from

Table I. Glycaemia Levels: Effect of Previous Injection of Mit C-Treated MNS from Citrate (control) and mld-sz Diabetic Mice on Recipients Injected with Citrate and mld-sz.

Group	Previous treatment (−15 day)	Injected with	Day 0	Day 15
1	—	Citrate (control)	153 ± 19	142 ± 16
2	—	mld-sz (diabetic)	146 ± 21	635 ± 59
3	Mit C-treated control MNS, ×1	Citrate (control)	152 ± 19	138 ± 12
4	Mit C-treated control MNS, ×1	mld-sz (diabetic)	143 ± 15	708 ± 63[a]
5	Mit C-treated diabetic MNS, ×1	mld-sz (diabetic)	150 ± 16	316 ± 20[abc]
6	Mit C-treated control MNS, ×2	Citrate (control)	159 ± 12	150 ± 12
7	Mit C-treated control MNS, ×2	mld-sz (diabetic)	143 ± 15	659 ± 50[a]
8	Mit C-treated diabetic MNS, ×2	mld-sz (diabetic)	138 ± 12	193 ± 20[ce]

Note. Glycemia levels from mice that were injected with citrate (controls) or with mld-sz (diabetics) at Days 0 and 15. Each group had received 15 days before no treatment (groups 1 and 2); one injection (groups 3 and 4) or two injections (groups 6 and 7) of mit C-treated control MNS; one (group 5) or two (group 8) injections of mit C-treated mld-sz MNS (one diabetic vaccination, group 5); two injections of mit C-treated mld-sz MNS (two, 15-day interval diabetic vaccination, group 8). Results are expressed as milligrams per decaliter, mean ± SEM, $n = 8$ in all cases.

[a] $P < 0.05$ compared with groups 1, 3, and 6, respectively.

[b] $P < 0.05$ compared with group 2, 4 and 7.

[c] P, ns compared with group 6.

[d] $P < 0.05$ compared with group 8.

[e] $P < 0.05$ compared with group 7.

Table II. Insulin Secretion from Perifused Pancreatic Slices

Group	Previous treatment	Injection	Min 4	Insulin secretion area under the 1st phase
1	—	Citrate (control)	300.00 ± 21.00 (6)	974.00 ± 37.70 (6)[a]
2	—	mld-sz (diabetic)	58.00 ± 5.40 (6)	232.66 ± 15.55 (6)
3	Mit C-treated control MNS, ×1	mld-sz	46.00 ± 9.99 (6)	225.26 ± 22.84 (6)
4	Mit C-treated diabetic MNS, ×1	mld-sz	259.00 ± 24.00 (8)[b]	694.80 ± 50.63 (8)[b]
5	Mit C-treated control MNS, ×2	mld-sz	63.40 ± 6.28 (5)	235.40 ± 20.35 (5)
6	Mit C-treated diabetic MNS, ×2	mld-sz	286.00 ± 9.04 (6)	857.00 ± 34.10 (6)[cd]

Note. Each group was injected 15 days before with nothing (groups 1 and 2), one or two injections of mit C-treated citrate MNS (groups 3 and 5), or one or two injections of mit C-treated mld-sz MNS (groups 4 and 6). Perifusion was performed 15 days after the last low-dose citrate (control) or sz (diabetic) injection. Insulin secretion stimulus: 16.5 mM glucose. Results are expressed as uU/min/100 mg w.t. at Min 4 (higher value of first peak) or as area under the first peak (uU/4 min/100 mg w.t.); data are expressed as mean ± SEM.

[a] $P < 0.001$ compared with group 2, 3, and 5.

[b] $P < 0.05$ compared with group 1 and <0.001 compared with group 2.

[c] $P < 0.05$ compared with group 1 and <0.001 compared with group 2.

[d] $P < 0.05$ compared with group 4. The same significance can be observed expressing the data on either way (4 min or area under the first peak) except for the difference indicated in d, which is not significant for the 4-min results.

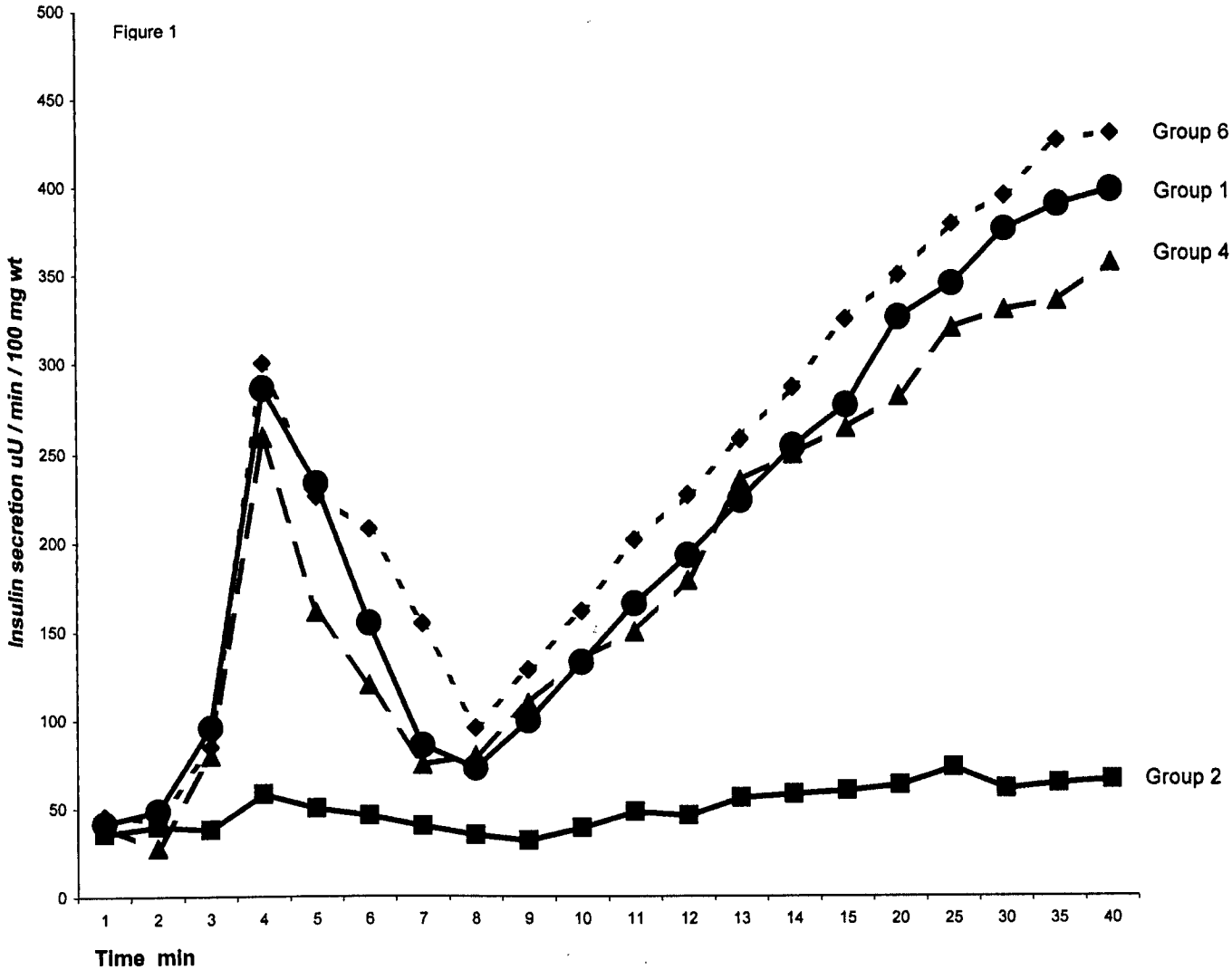

Figure 1. IS patterns from perifused pancreas slices stimulated with 16.5 mM glucose. Wavelike forms of stimulated pancreatic slices in experimental conditions similar to groups 1, 2, 4, and 6 of Table II.

perifused mice pancreas when compared with citrate (Groups 1 and 2). mit C-treated control MNS failed to restore IS from mld-sz (Groups 3 and 5). On the other hand, mit C-treated cells from diabetic animals injected once partially prevented the IS inhibition (Group 4) . When mit C-treated diabetic MNS were injected twice, they almost completely prevented the inhibitory effect of mld-sz on IS (Group 6). Figure 1 is included to show the wavelike form of stimulated IS by perifused pancreatic slices in experimental conditions similar to Groups 1, 2, 4, and 6 of Table II.

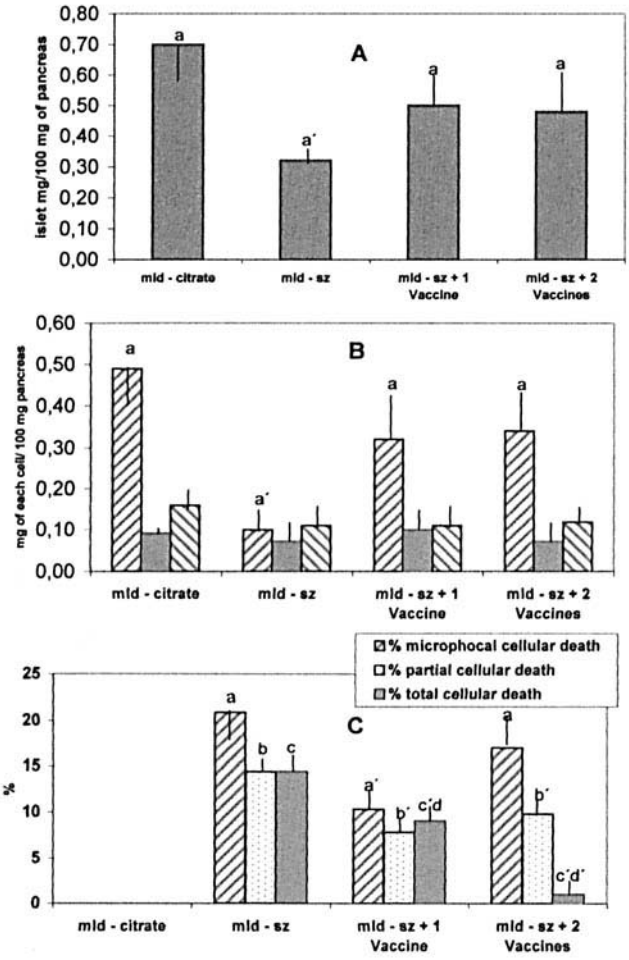

Figure 2. (A) Mg of islets/100 mg of pancreas from mice injected with citrate (control, group 1), with mld-sz (diabetic, group 2), and with mld-sz and one previous injection (group 3, one vaccine), or with two previous injection (group 4, two vaccine) of mit C-treated diabetic MNS. (B) In mld-citrate (control), mld-sz, mld-sz plus one vaccine, and in mld-sz plus two vaccines groups, the 1st column indicate cells containing insulin, the 2nd column indicate somatostatin and the 3rd column indicate glucagon. Results are expressed in milligrams of each cell type per 100 mg of pancreas, as mean ± SEM. (C) Cellular death expressed as the percentage of degree of severity. Three degrees of severity are indicated (1st column, microfocal; 2nd column, partial, and 3rd column, total), in control, mld-sz, mld-sz plus one vaccine, and mld-sz plus two vaccines. Results are expressed as mean ± SEM, $n = 6$ in all cases, excepts for mld-sz group in A and B where $n = 7$. In all of the figures, the same letters with or without apostrophes (a, a'; b, b'; and c, c') indicate significant difference ($P < 0.05$); the same letters (a, a') indicate that P is not significant.

Figure 2A shows that the endocrine pancreas diminished in mld-sz animals. This diminution is prevented by one or two previous injections of MNS. The mass of islets (milligrams of islets per 100 mg of pancreas) is 0.69 ± 0.08 in controls and 0.32 ± 0.04 in animals injected with mld-sz ($P < 0.01$). One or two injections of mit C-treated diabetic MNS increases the value to 0.50 ± 0.04 and to 0.49 ± 0.05, respectively (ns vs control). Histological analysis of the different groups shows marked differences: whereas in the group of pancreas from mld-sz mice an intra-islet infiltration is predominantly observed, the group previously vaccinated with MNS (one or two doses) shows polar or peri-islet infiltration (Fig. 3). This can also be observed in the insulitis score, which is lower in the mld-sz previously vaccinated mice (included in Fig. 3).

The volume of insulin-labeled cells dramatically diminished in mld-sz-treated animals. One or two previous injections of MNS prevented this diminution. Glucagon- or somatostatin-labeled cells did not show any change in experimental animals (Fig. 2B).

Figure 2C shows the degree of severity of cellular death, expressed as the percentage of islets with different degrees of cell death. Although there is no cell death in the pancreas from control animals, there is a marked increment in the group of mld-sz mice. This increment is partially prevented in both groups of mld-sz previously injected with either one or two doses of MNS. In particular, the percentage of islets with 100% cell death diminished or almost disappeared with two injections of MNS (Fig. 2C).

Table III shows the transfer of 10^5 IAMNC from mld-sz and mld-sz previously treated MNS (×2). A significant

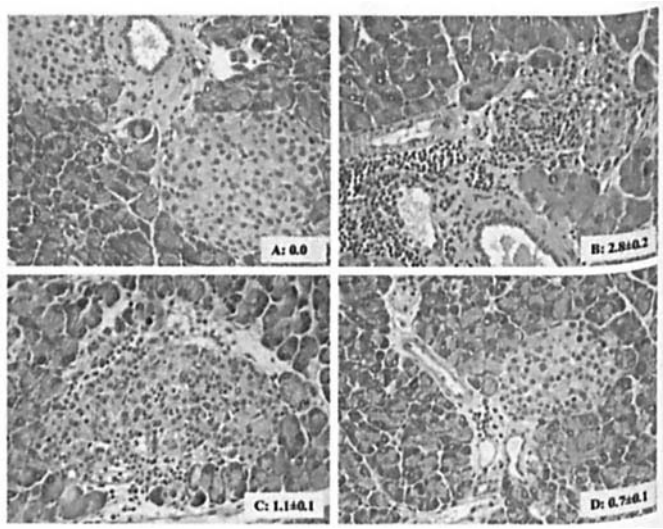

Figure 3. Degrees of severity of mononuclear cells pancreatic infiltration. Islets from control (A), mld-sz mice (B), and mld-sz previously treated with one (C) or two (D) injections of mit C-treated MNS. (H&E staining ×230). Insulitis scores (infiltrated islets) are included in each of the four subfigures. Results are expressed as means ± SEM, $n =$ 9 in each group; $P < 0.001$ comparing groups C and D with group B, and $P < 0.05$ when comparing groups C and D.

Table III. Insulin Secretion from Perifused Isolate Islets That Have Been Transferred with IAMNC from Control and from mld-sz Diabetic Mice

Group	Transferred with	Insulin secretion (min 4)	Area under the 1st phase
1	10^5 splenocytes from citrate	3.27 ± 0.94^{ab}	8.77 ± 0.32^{ef}
2	10^5 AIMNC from mld-sz	1.11 ± 0.03	4.00 ± 0.10
3	10^5 AIMNC from mld-sz plus vaccine x2	2.74 ± 0.88^{d}	7.47 ± 0.28^{h}
4	10^5 AIMNC from mld-sz plus 10^5 AIMNC from mld-sz plus vaccine x2	2.53 ± 0.12	7.09 ± 0.18
5	10^5 AIMNC from mld-sz plus 10^5 splenocytes from citrate	1.15 ± 0.22	4.19 ± 0.79
6	5×10^7 splenocytes from mld-sz	1.66 ± 0.11^{c}	5.09 ± 0.26^{g}

Note. Secretion stimulus: 16.5 mM glucose. Results are expressed as mean uU/islet/minute ± SEM. $n = 5$ in all cases.
[a] $P < 0.05$.
[b] $P < 0.01$ both compared with control group.
[c] $P < 0.05$ compared with group 5. Note that P is not significant when comparing groups 4 and 5 and in the area in groups 5 and 6.
Minute 4:
[a] $P < 0.05$ compared with group 3.
[b] $P < 0.01$ compared with groups 2 and 4.
[c] $P < 0.05$ compared with groups 2 and 5. and [d] P, not significant compared with group 4. Area under the 1st phase.
[e] $P < 0.05$ compared with groups 3 and 4.
[f] $P < 0.01$ compared with groups 2, 5, and 6.
[g] P, not significant compare with groups 2 and 5.
[h] P, not significant compared with group 4.

inhibition of IS pattern and hyperglycemia (336 ± 23.5 mg/dl) is observed after transferring only 10^5 IAMNC from mld-sz mice. IAMNC from vaccinated mld-sz not only fails to inhibit the IS pattern, but shows a first phase of IS almost similar to the control group (compare Groups 2 and 3 in Table III; glycemia, 158 ± 17.3 mg/dl). The first phase IS can almost be maintained when IAMNC from vaccinated mice is injected together with the same amount of IAMNC from mld-sz. The diabetogenic effect of IAMNC from mld-sz on IS is not modified by the addition of the same amount of splenocytes from citrate-injected mice (compare Groups 2 and 5). As a comparative group, mice transferred with 5×10^7 splenocytes from mld-sz also show significant alteration in IS (Group 6).

Discussion

Type I diabetes is an autoimmune disorder that can be transferred to normal singeneic recipient mice by autoreactive lymphocytes. This has led to the development of a strategy of disease prevention modifying this pathogenicity by means of a previous injection of inactivated lymphocytes, a process that has been termed lymphocyte vaccination (14, 15). Among these strategies of immunomodulation, the inactivation of autoreactive lymphocytes with drugs such as mit C and their subsequent application in a vaccine form is of particular interest.

Our results show that previous injections of diabetic splenocytes from mld-sz diabetic mice inactivated with mit C (injected twice, 15 days apart) prevent the alterations in glycemia and in the pattern of IS observed in mld-sz diabetic mice. These data are in agreement with previous results (15–18) that proposed the existence of a balance between islet specific lymphocytes capable of β cell destruction and other T cells capable of protecting β cells by preventing the potential of autoreactive lymphocytes (15).

Despite numerous studies, the mechanism(s) by which MNS mit C treated are effective is far from being clear. Two classes of suppressor cells that can inhibit autoimmune disease have been proposed. One is anti-idiotypic putatively specific for the receptor on the inducing autoreactive lymphocyte, and the other is anti-ergotypic putatively specific for the activated state (17). Differences between Th2 type cells with a pattern of cytokine production IL-4/IL-10 and Th1 with a pattern of cytokine IFN-γ have been postulated and extensively discussed (15, 24–28). Islets infiltrating lymphocytes in the NOD mice that received previous vaccine with modified splenocytes showed significantly reduced staining for IFN-γ compared with nonvaccinated NOD mice (15).

Our morphometric studies show that the parameters measured (islets per 100 mg of pancreas) were diminished in the mld-sz mice and were conserved in the animals treated with one or two vaccines. As expected, the immunohistochemical study confirmed that the alteration was present in insulin-secreting β cells, whereas glucagons- and somatostatin-secreting cells were not affected.

The most interesting results were those related to insulitis and cellular death. Two main patterns of cellular infiltration were observed and have also been confirmed by the insulitis score. Although a predominant pattern of intra-islet infiltration can be seen in the mld-sz mice, a significant tendency to the polar or peri-islet infiltration was observed in the vaccinated mice.

In a previous ultrastructural study of mld-sz diabetic mice, Papaccio *et al.* (19) observed that sporadic and sequential vasoconstriction and vasodilation may play a key role in the pre-infiltration state. In such a state, mononuclear cells and capillary vessels were considerably increased in number (19). Attraction of blood mononuclear cells into the islet capillaries and migration through capillary and venule

walls into the islets parenchyma came later. The pancreatic vessels of the NOD mouse show an abnormal infiltration with dendritic cells and macrophages, as well as an alteration of their function at the vessel walls (20). The presence of para- and peri-insulitis clearly shows that this accumulation around the islets is not harmful. Macrophages showing intra-insular infiltration may be fundamental for the onset of diabetes (20). Why these mononuclear cells invade the islets might thus become a key question in understanding β cell destruction in diabetes.

Mononuclear cells migration from blood into tissue is a highly complex process involving a cascade of adhesion and activation events. Some molecular adhesion interactions have been described in diabetes type I (21). Antibodies specific for some of these molecules have been used to delay, decrease, or prevent the incidence of insulitis and spontaneous diabetes (22, 23).

To see whether IAMNC from mld-sz and from vaccinated (×2) mice have different effects on β cells, a transfer experiment was designed. As few as 10^5 IAMNC from mld-sz mice were able to transfer diabetes and impair the pattern of IS. The same number of IAMNC from previously vaccinated (×2) mld-sz mice do not have the capacity to transfer the disease or to impair the first peak of IS. Moreover, when MNS from mld-sz mice were injected together with the same number of mld-sz vaccinated MNS, IS secretion was not affected and transfer was not possible. In other words, MNS from previously vaccinated mld-sz mice seem to have a protective effect.

Pancreatic cellular death can be achieved in two ways: necrosis and apoptosis. Islet-infiltrating cells are thought to damage β cells in autoimmune diabetes by producing several mediators such as free radicals, nitric oxide (NO), cytokines, FAS ligand, and perforin through an active and programmed process of apoptosis (29–31). This apoptosis can be modulated, and β cell hyperexpressing bcl 2 protein can be protected from cytokine-induced dysfunction and destruction. (32).

Strong inducible NO synthase (iNOS) expression was associated with destructive insulitis. Furthermore, the close correlation of iNOS and of IFN-γ expression with autoimmune diabetes development suggests a contribution of intra-islet NO production to β cell destruction (33). Sz is a diabetogenic agent that can elicit nonspecific inflammation of β cells with mononuclear cells infiltration. Sz contains a nitroso moiety that can liberate NO and can induce apoptosis of β cells. It has been postulated that apoptosis could be triggered, at least partially, by NO derived from sz and that this might be an important mechanism participating in sz-induced diabetes (34).

Although there was no cellular death in our control animals, a clear and significant number of dead cells could be observed in the mld-sz animals. The diabetic mice previously injected with one or two vaccines showed a significant diminution of cellular death. In mice injected with two vaccines, very few islets with total cell death were present.

These results seem to show that immunomodulation with modified diabetic cells has the capacity to shift the balance between aggressive and protective cells in favor of protection, and thus to avoid β cell damage and destruction. Vaccination appears to have the potential of a specific and safe strategy to be used for autoimmune diabetes therapy.

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