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

MABEL ARATA, LIDIA BRUNO, CLAUDIA PASTORALE, FABIOLA PAGLIERO, AND JUAN CARLOS BASABE[1]

Centro de Investigaciones Endocrinológicas, Hospital de Niños Ricardo Gutiérrez, Gallo 1360, (1425), Buenos Aires, Argentina

Etiological agents of autoimmune processes that have been made nonvirulent by several treatments, i.e., mitomycin C (Mit C), can be used as a vaccine to protect against disease. In this work we studied the effects of splenocytes from diabetic mice on animals that had been injected with modified splenocytes (Mit C-treated splenocytes from multiple low-dose streptozotocin [mld-sz] mice) 15 days before. Splenocytes from mld-sz diabetic donors altered i.p. glucose tolerance and the first peak of insulin secretion pattern when injected into normal singeneic recipients. These effects can be prevented partially (one injection in a vaccine form) or completely (two injections with a 15-day interval) by a previous injection of Mit C-treated mononuclear splenocytes (MS) from mld-sz mice. The fact that control splenocytes previously treated with Mit C were not able to achieve similar results indicates that donor splenocytes have to be diabetic to prevent the disease. On the other hand, Mit C-treated diabetic MS were not effective in preventing the alterations in glucose tolerance and in the pattern of insulin secretion when injected into athymic mice. This suggests that the preventive effect of Mit C-treated diabetic MS injection also implies an active role of the T cells from the recipient mice. Mit C-treated diabetic splenocytes are preferentially trapped by the pancreas and the lymph nodes from recipient mice. Our results show that the impairment in glucose tolerance and in the insulin secretion pattern produced by diabetic splenocyte transfer can be prevented by one or two previous injections of Mit C-modified diabetic splenocytes.

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Key words: diabetic prevention; diabetes immunomodulation; vaccination; mononuclear cell transfer; insulin secretion patterns

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[1] To whom requests for reprints should be addressed at Centro de Investigaciones Endocrinologicas, Hospital de Niños R. Gutiérrez, Gallo 1330, (1425) Buenos Aires, Argentina. E-mail: diabexp@cedie.guti.gov.ar

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There is strong evidence that suggests that insulin-dependent diabetes (IDDM) is the consequence of the destruction of insulin-producing β-cells mediated by components of the immune system (1–5). The generation of aggressive T cells is not fully understood, but apparently helper T cells play a pivotal role in the activation of the autoimmune response (2, 6–8). The mechanisms underlying the development and maintenance of tolerance are believed to involve one or more of the following: clonal deletion, clonal anergy or active post-thymic suppressor mechanisms through T suppressor cells, and idiotypic networks (8–11).

The hypothesis that some imbalance between aggressive and suppressor mechanisms may be present in autoimmune diabetes as a predisposing factor seems to indicate that immunomodulation strategies leading to increase suppressor mechanisms could have a preventive effect (7–10). However, interventions at this point may be too late to preserve important β-cell function (β-cells represent 10%–30% of total mass). Therefore, the best alternative would be to immunomodulate during the preclinical period, before the vast majority of β-cells have been destroyed (10, 12–14). During the preclinical period, the detection of immunologic aggression markers and of metabolic abnormalities (first-phase insulin secretion) are highly predictive of the risk of developing IDDM (1).

There have been several attempts to block the immune aggressive process and to protect β-cells during the preclinical phase. Reich *et al.* (8, 10) have shown the possibility of using attenuated autoimmune T lymphocytes as cellular vaccines to prevent or reverse autoimmune diseases. Likewise, either an inoculum of T cell clones below the threshold to trigger the disease or irradiated, pressure-, or chemically treated (e.g., glutaraldehyde, mitomycin C [Mit c]) T cells have been used as vaccines (9, 14, 16), especially in rheumatoid arthritis and multiple sclerosis.

Studies on animal models of IDDM such as the Bio Breeding (BB) rat, the non-obese diabetic (NOD) mice, multiple low-dose streptozotocin (mld-sz), and athymic mice injected with splenocytes from IDDM patients have hinted at possible approaches in humans (18–20). The dif-

ferences between BB rats, NOD mice, and mld-sz have already been mentioned (19). BB rats and NOD mice have a strong genetic background that has little or no influence on mld-sz mice. Although siblings and first degree relatives of a proband are individuals genetically more exposed to risk, most new cases of type 1 diabetes are sporadic and occur in families with no previous history of diabetes. Therefore, a screening limited to the two former groups will not completely recognize all the individuals at risk. Consequently, this fact strongly supports the use of the mld-sz model (1, 6).

We recently reported an experimental model whereby abnormal glucose tolerance and impaired insulin secretion were induced by mononuclear splenocytes (MS) from mld-sz diabetic syngeneic donors in healthy naive recipient mice (19). In the present study we explore the β-cell function using the same experimental model with previously injected Mit C-treated diabetic MS.

Materials and Methods

Animals. Male C57BL/6J and congenitally athymic BALB/c (*nu/nu*, *+/nu*) mice, 8 to 12 weeks old, were obtained from Departamento de Radiología, Comisión Nacional de Energía Atómica (Buenos Aires, Argentina). The animals were maintained according to the NIH *Guidelines for the Care and Use of Laboratory Animals.*

Diabetes Induction in MS Donors. Induction of diabetes by mld-sz (Upjohn Company, Kalamazoo, MI). Sz was dissolved in 0.1 M citrate buffer (pH 4.5) and injected into nonfasting mice, i.p., within 5 min of dissolution. Each mouse received 40 mg of sz per kilogram of body wt in a 0.1-ml volume during 5 consecutive days. Controls were injected i.p. with 0.1 ml of citrate buffer per day. Fifteen days after the last injection, plasma glucose was determined, the mice were sacrificed, and their spleens were used for the transfer experiments.

Transfer Procedures. Transfer experiments were performed according to Buschard *et al.* (21). Spleens from control and diabetic donors were aseptically removed, pooled, and homogenized using soft mechanical disruption in cold sterile saline solution. MS were obtained using a Ficoll-Hypaque gradient (Ficollpaque, Pharmacia, Uppsala, Sweden) and were washed with sterile saline solution. Viability was assayed by the Trypan blue exclusion test (22) and 5×10^7 viable MS from diabetic (mld-sz) or control (mld-citrate) pools were injected i.p. into normal syngeneic mice using a 0.2-ml sterile saline solution.

In some experiments, prior to transfer, MS were incubated with Mit C (Sigma, St. Louis, MO, 1 μg/10^6 cells/ml, 20 min at 37°C in a 95% humidified air and 5% CO_2 atmosphere) to block DNA duplication and cell replication. After washing three times with sterile saline solution, 5×10^7 viable MS were injected i.p. into syngeneic mice in 0.1-ml sterile saline solution (control or diabetic Mit C-treated × 1). Fifteen days later they were injected with 5×10^7 viable MS obtained as in Buschard *et al.* (21).

In another group, 5×10^7 viable MS (control or diabetic Mit C-treated ~2) were injected twice, with a 15-day interval. Fifteen days later they received the last injection, 5×10^7 viable MS obtained as in Buschard *et al.* (21).

Another experimental group was treated using the procedure described in Buschard *et al.* (21) and the paragraph beginning with "In some experiments," but with athymic receptor mice.

Glucose Measurement and Intraperitoneal Glucose Tolerance Test. Plasma glucose levels were determined in recipient mice prior to splenocyte injection, and then again after 8 and 16 days. Blood samples were collected from the retroorbital venous plexus using microcapillary heparinized tubes. Plasma glucose concentration was assayed by the glucose oxidase method (Glycemia Enzymatic kit, Wiener La, Argentina) using a Beckman DB-G Spectrophotometer (Beckman Instruments, Fullerton, CA).

Animals were fasted for 12 hr and a glucose tolerance test was performed between 0800 hr and 1200 hr. Blood samples were taken at 0, 30, 60, and 120 min after an injection of 2 mg of glucose per gram of body wt.

MS Trapping in C57BL/6J Recipient Mice. Radiochromate was used due to its unique characteristic that once bound to the cell, its efficiency for rebinding is markedly decreased (23).

To study MS homing in recipient mice (24), spleens from C57BL/6J control and diabetic donors, with or without previous Mit C treatment were removed and MS was isolated as described above. The erythrocytes were hemolized with 0.83% NH_4Cl (Mallinkrodt, Chemical Works, St. Louis, MO), for 8 min at 37°C. MS were washed three times with a RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco, Cleveland, OH), 100 ng/ml sz and 100 units per milliliter Penicillin (Gibco, Grand Island, NY) and were then incubated with 25 μCi of $Na_2{}^{51}CrO_4/10^8$ cell per milliliter (Comisión Nacional de Energía Atómica) for 30 min at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. After incubation, MS were washed three times with fresh medium and the concentration was adjusted to 1×10^8 viable cells per milliliter. Each mouse received an i.v. dose of 1×10^7 cells in 0.1 ml of the medium. Recipient mice were sacrificed 24 hr after injection, their organs were removed, and ^{51}Cr activity was measured in blood cells (obtained from 0.1 ml of whole blood), plasma (0.1 ml), one kidney, the liver, the spleen, one lung, the pancreas, and the lymph nodes (mesenteric, inguinal, and axillar). Results were expressed for each organ as the percentage of $Na_2{}^{51}CrO_4$ uptake = (cpm organ/cpm total) × 100.

MS from mld-sz-induced diabetic mice were labeled with $Na_2{}^{51}CrO_4$ and were lysed by freezing thawing in distilled water to measure the rate of "free" ^{51}Cr uptake. After centrifugation, 0.2 ml of the supernatant was injected into C57Bl/6J mice. In all cases, the recipients were sacrificed 24 hr after injection, their organs were removed, and $Na_2{}^{51}CrO_4$ activity was measured as described.

Perifusion of Pancreas Slices. The techniques used were described by Burr *et al.* (25). Thin slices from the whole pancreas of a single mouse were used in each perifusion. 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 to 7.40. Perifusion flux was 1.8 to 2.2 ml/min. Proteolytic effect on hormone secretion was avoided by adding 1000 KIU Trasylol/ml (Bayer, Buenos Aires, Argentina) to the buffer and by collecting the samples on 0.25 M ethylene-diamine-tetraacetic acid (EDTA, Mallinckrodt, St. Louis, MO) in tubes at 4°C, which were immediately frozen at –20°C, until insulin determination by radioimmunoassay (RIA). 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 was measured between 3 and 7 min.

Insulin RIA. Insulin was determined using the method of Hebert *et al.* (26). Pork monoiodine ^{125}Insulin with high specific activity was obtained from Comisión Nacional de Energía Atómica. Rat standard insulin was obtained from the Novo Research Laboratories (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 for determinations within the range of 5.0 to 800 μU/tube . The insulin assay sensitivity was 0.5 μU/ml; the intraassay coefficient of variation (CV) was 8.2%, 6.6%, and 5.1% for 1 to 5, 5 to 10, and 10 to 50 μU insulin/ml determination ranges, respectively; the interassay CV was 6.6%, 4.9%, and 5.2% for the given ranges.

Statistical Analysis. Results were expressed as mean ± SEM. To evaluate insulin secretion in perifused pancreatic slices, results were expressed as microunits per minute secreted at Min 4 of perifusion (maximum peak) and as areas under the secretion curve. Statistical analysis of the data was performed by the two-tailed Student's t test for unpaired samples and by one-way analysis of variance (ANOVA) (27). Scheffe's test for multiple comparisons between individual groups was used. $P < 0.05$ was considered statistically significant.

Results

Effect of mld-Citrate (Controls) or mld-sz (Diabetic) Injections on Syngeneic Mice. Diabetic MS donors (mld-sz-injected mice) showed significant hyperglycemia 15 days after the last injection (539 ± 12 mg/dl vs 158 ± 9 mg/dl for the control group; $P < 0.001$, $n = 25$ in each group). Insulin secreted by the same groups is shown in Table IA. These groups (1 and 2) showing insulin secreted from mld-sz diabetic mice were only included to compare the degree of disease transferred.

Effect of MS Transfer (without Any Previous Injection) on Recipient Mice. In syngeneic recipient mice, glycemia was measured 15 days after transfer of MS from control and mld-sz diabetic donors. MS injection did not significantly change basal glycemia levels in either group (153 ± 12 mg/dl vs 142 ± 10 mg/dl for control MS and 156 ± 13 mg/dl vs 150 ± 9 mg/dl for diabetic MS, $n = 6$ in all groups). Table IB shows the insulin secreted by perifused pancreas slices (stimulated by 16.5 mM glucose)

Table I. Insulin Secretion from Perifused Pancreas Slices Stimulated by 16.5 mM Glucose

A: Group	Injected with	Min 4	First phase
1	mld-citrate	328.00 ± 17.26 $n = 6$	998.00 ± 26.12 $n = 6$
2	mld-sz	36.18 ± 4.27[a] $n = 6$	227.00 ± 13.25[a] $n = 6$
B: Group	**MS injection**	**Min 4**	**First phase**
3	Control (mld-citrate)	343.00 ± 8.19 $n = 6$	1048.00 ± 30.00 $n = 6$
4	Diabetic (mld-sz)	228.80 ± 7.23[b] $n = 6$	717.00 ± 12.60[b] $n = 6$
5	Control (Mit C-treated)	350.00 ± 8.64 $n = 6$	985.60 ± 45.58 $n = 6$
6	Diabetic (mld-sz) (Mit C-treated)	344.00 ± 9.66 $n = 6$	947.60 ± 19.09 $n = 6$

Note. C57BL/6J recipient mice transferred with control or diabetic (mld-sz) MS. Perifused was performed 15 days after the last low-dose citrate (control) or mld-sz (diabetic) injection. Insulin secretion is expressed as microunits per minute per 100 mg of wet tissue; at Min 4 (maximum peak) or as area under the first peak (microunits per minute per 100 mg of w.t.); data are expressed as mean ± SEM. Groups 1 and 2 correspond to insulin secretion from MS donor mice (injected with mld-sz or mld-citrate).

[a] $P < 0.01$.

[b] $P < 0.01$. Both compared with controls (groups 1 and 3, respectively).

from groups of mice transferred with MS from control and diabetic donors. Mice transferred with control or control plus Mit C do not show changes in the amount of insulin secreted (Table IB, groups 3 and 5). Mice transferred with MS from diabetic (mld-sz) mice do not show modified glucose levels, but do show a significant impairment of insulin secretion (Table IB, group 4). However, previous incubation with Mit C abolished this inhibitory effect (Table IB, group 6).

Figure 1 was included to show the wavelike forms of stimulated insulin secretion by perifused pancreatic slices and by perifused isolated islet in experimental conditions similar to groups 3 and 4 of Table IB.

Effect of MS Transfer on Recipients Previously Injected with Mit C-Treated MS from Control or Diabetic Mice. Table II shows that after an i.p. glucose tolerance test, MS from diabetic (mld-sz-injected) donors induced significant hyperglycemia 120 min after glucose injection, both on Days 8 and 16 (group 2). The same results can be observed when MS from diabetic donors were transferred 15 days after a previous injection of Mit C treatment control MS (group 5).

When recipient mice are transferred with MS from diabetic donors previously injected with Mit C-treated diabetic MS, there are two possible outcomes: hyperglycemia on Days 8 and 16 after the injection (group 4a, $n = 6$) or normoglycemia for the same days (group 4b, $n = 6$).

However, all 12 recipient mice previously injected twice, with a 15-day interval, with Mit C-treated diabetic MS, transferred with MS from diabetic donors, remained normoglycemic (group 8). Table III shows that previous injection (one or two) of control Mit C-treated MS did not modify the inhibitory effect of diabetic MS transfer (groups 5 and 7).

One injection of Mit C-treated diabetic MS, performed 15 days before the transfer of diabetic MS, could not prevent this inhibitory effect of the diabetic MS on the insulin secreted by 6 out of 12 recipient mice (group 4a), whereas it was observed on the other six recipients (group 4b) On the other hand, two injections of Mit C-treated diabetic MS (with a 15-day interval and the last injection performed 15 days before the transfer of diabetic MS) prevented the inhibitory effect of these diabetics cells on the insulin secreted by all recipient mice (group 8).

Table IV shows that one or two previous injections of Mit C-treated diabetic MS failed to prevent insulin secretion impairment observed in athymic recipient mice transferred with diabetic MS (Table IV, groups 4b and 8).

MS Trapping in Recipient Mice. Control, diabetic, and Mit C-treated diabetic MS were labeled with $Na2^{51}CrO_4$ and injected as previously indicated. Table V shows MS trapping in recipients 24 hr after cell injection. $Na_2{}^{51}CrO_4$ uptake was significantly increased in the pancreas from mice injected with either diabetic or Mit C-treated diabetic MS when compared with controls ($P < 0.05$). $Na_2{}^{51}CrO_4$ uptake was also increased in lymph nodes, but only in mice injected with Mit C-treated diabetic MS ($P < 0.05$). In the remaining orgâns, including plasma and blood cells, no significant differences among the groups studied were found. $Na_2{}^{51}CrO_4$ distribution in mice injected with lysed MS is also shown in Table V. The difference in the pattern of ^{51}Cr distribution observed in these animals indicates that $Na_2{}^{51}CrO_4$ activity in the above described groups was carried out by nondamaged viable MS.

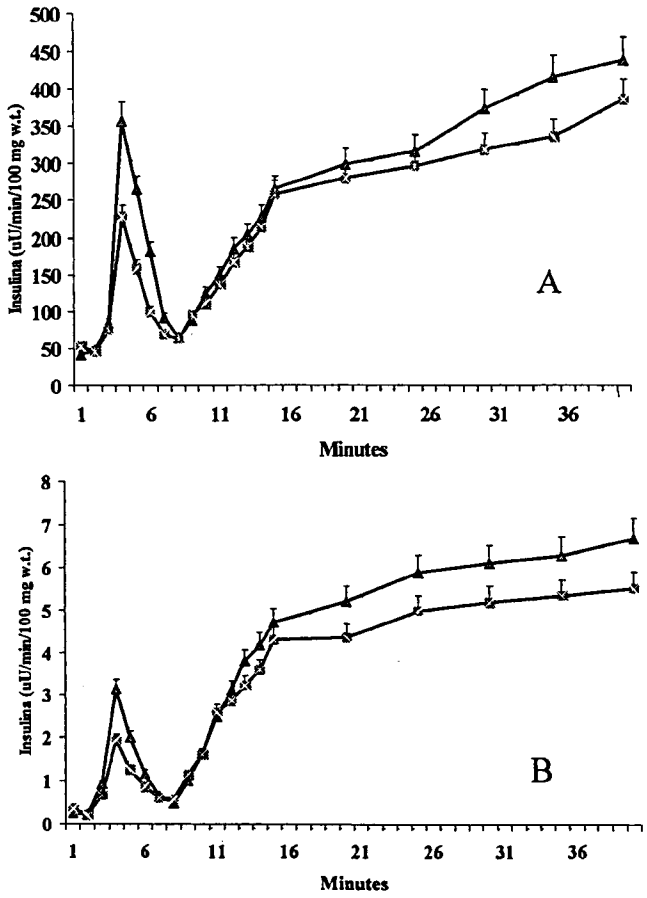

Figure 1. Insulin secretion patterns of perifused pancreatic slices (A) or islets (B) from groups 3 (▲) and 4 (+) in Table I.

Discussion

A physiological therapy for an autoimmune disease should restore the regulatory mechanisms of this natural immune network (10, 28, 29). The loss of tolerance to self antigens produces an aggressive autoimmune reaction probably related to a failure of the adequate regulatory (suppressor) control elements (1–3, 5). From a general perspective, the effect of T cell vaccination could be described as strengthening connections (8, 10, 28, 29), leading to suppressor mechanisms. There is evidence that etiological agents of autoimmune diseases suitably attenuated by various treatments, i.e., irradiation or chemicals (Mit C), can be used as effective vaccines (9, 10).

Our results showing that administration of control or diabetic Mit C-treated splenocytes did not modify the insu-

Table II. Intraperitoneal Glucose Tolerance Test

Group	Infected with Mit C-treated MS from	Transfer donor (MS from)	Intraperitoneal glucose tolerance test			
			On Day 8		On Day 16	
			Basal	120 min	Basal	120 min
1	—	Control (*)	150 ± 15 $n = 6$	146 ± 8 $n = 6$	136 ± 10 $n = 6$	145 ± 9 $n = 6$
2	—	Diabetic mld-sz diab (**)	148 ± 12 $n = 6$	240 ± 10[a] $n = 6$	142 ± 8 $n = 6$	266 ± 12[a] $n = 6$
3	Control	Control	145 ± 11 $n = 6$	139 ± 8 $n = 6$	140 ± 8 $n = 6$	150 ± 10 $n = 6$
4						
a	Diabetic	Diabetic	150 ± 12 $n = 6$	252 ± 10[b] $n = 6$	138 ± 7 $n = 6$	261 ± 11[b] $n = 6$
b	Diabetic	Diabetic	147 ± 9 $n = 6$	156 ± 6 $n = 6$	147 ± 12 $n = 6$	163 ± 9 $n = 6$
5	Control	Diabetic	139 ± 12 $n = 6$	244 ± 9[c] $n = 6$	145 ± 10 $n = 6$	272 ± 13[c] $n = 6$
6	Diabetic	Control	143 ± 14 $n = 6$	147 ± 8 $n = 6$	144 ± 11 $n = 6$	148 ± 9 $n = 6$
7	Control (twice with a 15-day interval)	Diabetic	153 ± 12 $n = 6$	258 ± 8[d] $n = 6$	165 ± 9 $n = 6$	275 ± 6[d] $n = 6$
8	Diabetic (twice with a 15-day interval)	Diabetic	162 ± 10 $n = 12$	168 ± 12 $n = 12$	153 ± 11 $n = 12$	174 ± 12 $n = 12$

Note. Blood samples were obtained 0, 30, 60, and 120 min after injection of 2 mg/g body wt. Results are expressed as milligrams per decaliter mean ± SEM. There are no differences in glucose values obtained at 0, 30, or 60 min when compared among the groups studied.
(*) Control, MS from citrate injected donors; (**) diabetic, mld-sz injected donor mice.
[a] $P < 0.05$ compared with basal and group 1.
[b] $P < 0.05$ compared with basal and group 3.
[c] $P < 0.05$ compared with basal and group 6.
[d] $P < 0.05$ compared with basal.

Table III. Insulin Secretion from Perifused Pancreas Slices Stimulated by 16.5 mM Glucose

Group	Mit C-treated MS from	MS injected	Min 4	First phase
3	Control × 1 →	Control	344.60 ± 4.77 $n = 5$	995.00 ± 24.37 $n = 5$
5	Control × 1 →	Diabetic	251.00 ± 5.76[a] $n = 10$	752.70 ± 23.22[a] $n = 9$
4				
a	Diabetic × 1 →	Diabetic	239.00 ± 9.99 $n = 6$	821.60 ± 21.31 $n = 6$
b	Diabetic × 1 →	Diabetic	328.00 ± 8.68[b] $n = 6$	1023.00 ± 18.95[b] $n = 6$
6	Control × 2 →	Control	343.67 ± 13.51 $n = 6$	950.33 ± 42.57 $n = 6$
7	Control × 2 →	Diabetic	245.60 ± 7.24[c] $n = 5$	732.80 ± 17.60[c] $n = 5$
8	Diabetic × 2 →	Diabetic	349.00 ± 21.00 $n = 6$	951.00 ± 33.30 $n = 6$

Note. C57BL/6J recipient mice transferred with control or diabetic MS previously injected once (×1) or twice (×2) with C-treated control or diabetic MS. Pancreas slices were perifused 15 days after the last treatment. Insulin expression and data as referred in Table I. We have used the same group number as in Table II.
[a] $P < 0.01$ compared with group 2.
[b] $P < 0.01$ compared with group 4a.
[c] $P < 0.01$ compared with groups 6 and 8.

lin secreted patterns strongly support the fact that Mit C treatment *per se* does not induce any effect.

MS from mld-sz diabetic donors induce abnormal i.p. glucose tolerance and a diminished first phase of glucose-induced insulin secretion when injected into normal recipients (19). Additionally, we show that the administration of Mit C-treated diabetic MS (one injection) can partially avoid these effects. The fact that one vaccination failed to prevent these effects in some mice is not easily explained. We are tempted to speculate that one vaccination seems to

Table IV. Insulin Secreted by Perifused Pancreatic Slices from Athymic BALB/c (*nu/nu*) Recipient Mice Transferred with Control or Diabetic MS from C57BL/6J Donor Mice

Group	Mit C-treated MS from	MS injected	Min 4	First phase
1	—	Control	336.00 ± 9.00 *n* = 4	958.75 ± 24.77 *n* = 4
2	—	Diabetic	228.00 ± 10.55[a] *n* = 4	823.75 ± 20.54[a] *n* = 4
3	Control × 1 →	Control	366.00 ± 12.00 *n* = 6	950.16 ± 31.86 *n* = 6
4b	Diabetic × 1 →	Diabetic	271.00 ± 8.12[b] *n* = 6	817.50 ± 23.48[b] *n* = 6
6	Control × 2 →	Control	352.00 ± 15.00 *n* = 6	969.65 ± 26.82 *n* = 6
8	Diabetic × 2 →	Diabetic	274.00 ± 8.55[c] *n* = 6	805.52 ± 20.66[c] *n* = 6

Note. We have used the same group number as in Table II and III, but in an athymic BALB/c (*nu/nu*) recipient mice.
[a], [b] and [c] $P < 0.05$ compared with controls (groups 1, 3, and 6, respectively).

Table V. Splenocytes Homing in C57BL/6J Mice

Organ	$Na_2{}^{51}CrO_4$ uptake Ms to be labeled were obtained from			
	Control	Diabetic	Diabetic (Mit C-treated)	Lysated MS supernatant[a]
Blood cells	3.64 ± 0.33	3.81 ± 0.48	3.52 ± 0.32	0.19 ± 0.07
Plasma (0.1 ml)	0.47 ± 0.14	0.59 ± 0.12	0.52 ± 0.09	5.68 ± 0.24
Kidney	8.27 ± 1.15	7.08 ± 0.96	7.06 ± 1.16	31.00 ± 2.36
Liver	23.83 ± 2.17	25.03 ± 2.67	23.15 ± 2.50	45.30 ± 3.07
Spleen	45.76 ± 3.18	44.24 ± 3.00	43.34 ± 3.75	7.19 ± 1.52
Pancreas	2.18 ± 0.23	4.25 ± 0.66[b]	4.08 ± 0.55[b]	0.24 ± 0.08
Lung	1.60 ± 0.61	1.59 ± 0.25	1.83 ± 0.28	0.69 ± 0.05
Lymph nodes	3.46 ± 0.55	3.70 ± 0.57	6.92 ± 0.96[b]	0.90 ± 0.12
Thymus	0.39 ± 0.09	0.46 ± 0.08	0.52 ± 0.2	0.23 ± 0.04
Carcass	10.00 ± 1.86	9.25 ± 0.23	9.06 ± 1.18	8.58 ± 0.46

Note. Results are expressed as the percentage of $Na_2{}^{51}CrO_4$ uptake (cpm $Na_2{}^{51}CrO_4$ organ/cpm $Na_2{}^{51}CrO_4$ total measurement × 100). Number of recipient mice, 20 in each group.
[a] $P < 0.05$ when compared with control splenocytes.
[b] Counts per minute statistically different in every organ when compared with any of the other groups.

be insufficient to block the aggressivness of transferred cells, which was achieved by two vaccines.

The immune reaction seems to be directed against altered structures of islet cells, which would be generated during sz treatment. This indicates that the pool of transferred MS of mld-sz diabetic mice contains islet-specific cells and, therefore, underlines the immunologic pathogenesis of this diabetes model. Lymphocytes from nondiabetic donors did not show this phenomenon, implying that it is the induction of diabetes by sz that leads to the presence of islet-specific lymphocytes in the reticuloendothelial system of this mouse species.

These results agree with previous reports showing that autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), thyroiditis, adjuvant arthritis, collagen II arthritis, and experimental autoimmune neuritis can be prevented or treated by administering autoimmune T cells specific for the target antigens under circumstances in which T cells were rendered avirulent (9, 10, 16, 17).

Although the existence of suppressor cells has been a matter of debate (6), studies on prevention of autoimmune diseases with attenuated T lymphocytes strongly suggest a regulatory role for some suppressor mechanisms or networks (3, 5, 10, 11). Results obtained by Lohse *et al.* (9) and by Gearon *et al.* (10) clearly showed that animals vaccinated with activated T cell clones may develop responses to clone-specific markers (anti-idiotypic) as well as a minor response to activation markers (antiergotypic).

Furthermore, CD4 T cells comprise two subsets: Th1 cells (responsible for IL2 and INFγ) and Th2 cells (responsible for IL4 and IL10) in opposition to each other by reciprocal downregulation (11, 29–31). The correlation between cytokine profiles suggested that Th1 $CD4^+$ cells are linked to aggression, whereas Th2 $CD4^+$ cells are associated with protection from diabetes (7).

When Mit C-treated diabetic MS are injected into athymic mice, prevention of the effect of transferred diabetic MS on glucose tolerance and insulin secretion cannot be

achieved. This entails that the preventive effect of Mit C-treated diabetic MS injection implies an active role of the T cells from the recipient mice.

The precise mechanism by which oral immunization induces tolerance still remains uncertain and important questions are unanswered (6, 7). Since we used intraperitoneal and not intravenous administration route in our experiments, the possibility that vaccines could be acting on intestinal lymph nodes and even inducing Th2 cannot be ruled out.

We observed that MS from mld-sz diabetic donors show a specific homing towards recipient pancreas, which might indicate an early stage in the immune aggression phenomenon. However, Mit C-treated diabetic MS, with a clear protective effect, also show a specific homing towards recipient pancreas (can this be an early stage in the protective process?). Mit C-treated diabetic MS and nondiabetic MS are also preferentially trapped by the recipient's lymph nodes. Whether this implicates the existence of a proliferation and maturation phase in the lymphoid organs before initiating a protective process in the pancreatic islets or the extent and speed with which mononuclear cells of the recipients are recruited in the pancreas is not clarified by our experiments and deserves further studies.

Kiesel (15) transferred experimental autoimmune insulitis using spleen cells in mice. Insulitis in recipient and in donor mice had similar characteristics. This finding supports the suggestion that lymphocytes react with similar pancreatic structures in both donor and recipient animals. Specific cellular immune reactions against islet cells are involved because lymphocytic infiltration was found only in the islet of Langerhans and not in the kidney and liver of recipient mice (15). In an experiment showing that the interaction between donor lymphocytes and recipients' islet vascular endothelium is increased in the transfer model of mld-sz, Enghofer (14) found lymphocytic infiltration of pancreatic islet in recipient mice. Histological studies to show the recipient pancreases after transfer of mld-sz MS with or without previous vaccination deserve further studies that are now in progress.

Complete Freund's adjuvant (CFA) also prevents recurrent diabetes in NOD mice (32), and vaccination with Bacillus Calmette-Guerin (BCG) does the same. Stimulation with CFA or BCG might induce a generalized unspecific activation of protective components of the immune system, thus debilitating the immune attack on β-cells. The major obstacle in studying an autoimmune disease has been the inability to identify the target cell antigen. A fundamental question is how to establish the original autoantigen(s) and T cell clones responsible for initiating the autoimmune response. In this context, attenuated diabetic MS might trigger one of the most important features of the immune system, i.e., specificity.

Reich (8) and Gearon (10) have demonstrated that NOD mouse islets contain effector cells capable of damaging pancreatic β-cells as well as cells that regulate. The development of IDDM depends on the balance between these two opposing forces. We may shift this immunoregulatory balance to favor suppression and impede β-cell function impairment, as performed in this study by repeated injections of Mit C-treated diabetic MS (8, 10).

Without considering which protection mechanism(s) is involved, our results suggest that an impairment in glucose tolerance and in insulin secretion pattern induced by diabetic MS transfer can be mitigated or prevented by the injection of specific attenuated MS.

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