Lymphocyte transfer in streptozotocin-induced diabetes: adhesion of donor cells to islet endothelium

MICHAEL ENGHOFER,1 JÖRG BOJUNGA,1 RALF LUDWIG,1 ANKE OLDBERG,1 AUGUST BERND,2 KLAUS HENNING USADEL,1 AND KLAUS KUSTERER1
Departments of 1Medicine I and 2Dermatology I, J ohann Wolfgang Goethe University, D-60590 Frankfurt am Main, Germany

Enghofer, Michael, J örg Bojunga, Ralf Ludwig, Anke Oldenburg, August Bernd, Klaus Henning Usadel, and Klaus Kusterer. Lymphocyte transfer in streptozotocin-induced diabetes: adhesion of donor cells to islet endothelium. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E928–E935, 1998.—The interaction between intravenously transferred lymphocytes derived from spleens of multiple low-dose streptozotocin-diabetic mice with islet, exocrine pancreatic, and gastric mucosal endothelium of nondiabetic recipient mice was investigated by in vivo microscopy. Donor lymphocytes were stained with acridine red in vitro. The adoptive transfer of these cells from diabetic donor animals resulted in significantly increased lymphocyte rolling (4.46 ± 1.32%, P < 0.05) and adhesion (3.86 ± 1.04%, P < 0.05) in islets of nondiabetic recipients that had been pretreated with a single subdiabetogenic dose of streptozotocin. No increased endothelial interaction was noted in nonpretreated recipients or in experiments with nondiabetic donors. Rolling (1.19 ± 0.61 to 2.71 ± 0.62%) and adhesion (0.61 ± 0.33 to 2.80 ± 0.97%) of donor lymphocytes were low in exocrine pancreatic and gastric mucosal control tissue. It is concluded that, in this animal model, lymphocytes from diabetic donors interact preferentially with recipient islet endothelium. However, additional stimulation of recipient islet endothelium by exogenous factors is necessary to enable transferred cells to adhere to pancreatic islets.

TYPE I DIABETES MELLITUS is preceded by progressive destruction of β-cells in islets of Langerhans by autoimmune mechanisms (2, 27). Histologically, this phase is characterized by lymphocytic infiltration of pancreatic islets (3, 15). The initial pathogenetic events leading to insulitis are still poorly understood. Several animal models have been developed that allow the investigation of islet pathology leading to autoimmune diabetes mellitus in vivo. One of these models uses the repeated administration of subdiabetogenic doses of the beta cytotoxic agent streptozotocin (STZ) in mice (21). This treatment results in a cellular immune attack against β-cells presumably made antigenic by low-dose STZ treatment (23). After 2–3 wk the animals develop overt diabetes (20). Main advantages of this experimental model are the high diabetes incidence and the invariant time course of histologic changes (20, 21, 24). Apart from publications from one institution (4), adoptive transfer of splenic lymphocytes from multiple low-dose diabetic donors to healthy recipients failed to induce overt diabetes in these mice (1, 10, 16, 17). However, lymphocytic infiltration of pancreatic islets was evident histologically in recipient mice after cell transfer (16, 17), and impaired insulin secretion capacity could be demonstrated in these animals (1). Several groups have been able to show preferential trapping of transferred lymphocytes in islets of recipient animals by use of radionuclide or fluorescent labeling of isolated donor lymphocytes in diabetes transfer experiments (1, 22, 34). In an attempt to study the interaction between circulating lymphocytes and islet endothelium in vivo, we applied a fluorescent staining technique for in vitro isolated lymphocytes that allows tracing of these cells in recipient animals after donor lymphocyte transfer (7). Experiments were performed to test the hypothesis that the interaction between donor lymphocytes and recipient islet vascular endothelium is increased in the transfer model of low-dose STZ-induced autoimmune insulitis. The effect of an injection of a single very small dose of STZ into recipient mice 1 day before the adoptive lymphocyte transfer on islet lymphocyte adhesion was studied in the same model.

METHODS
Experimental animals. Male C57BL/6 mice aged 6–10 wk were purchased from Charles River, Sulzfeld, Germany. The mice, weighing between 18 and 24 g, were kept in our laboratory animal facility according to National Institutes of Health standards. We chose C57BL/6 mice for our experiments because it has been shown that in transfer experiments of multiple low-dose STZ diabetes, this mouse strain shows a high rate of lymphocytic insulitis in recipient animals (17).

STZ treatment. Donor mice received daily intraperitoneal injections of 40 mg/kg body weight STZ (Sigma Chemical, Deisenhofen, Germany) on five consecutive days. All mice had free access to food and water during this time. STZ was given within 2 min after dissolution of STZ in 0.2 ml of sodium citrate buffer at 4°C. The animals in the control groups received five intraperitoneal injections of 0.2 ml of citrate buffer at 4°C. In the second part of the study, recipient mice received either a single very small dose of STZ (5 mg/kg body weight) diluted in 0.2 ml of buffer or 0.2 ml of pure sodium citrate buffer intraperitoneally 24 h before the lymphocyte transfer. It has been shown that this STZ pretreatment significantly decreases insulin secretion capacity in recipients in a transfer model of low-dose STZ diabetes in mice (1).

Lymphocyte preparation and staining. Twenty-one days after the first injection of STZ, the donor animals were checked for glucosuria with test strips (Diabur, Boehringer Mannheim, Germany). In case of a positive result, nonfasting blood glucose was measured by the hexokinase method (Clinical System 700 Analyzer; Beckman, Munich, Germany). Animals with nonfasting blood glucose values higher than 14 mmol/l were considered diabetic. The spleens of diabetic donor mice were homogenized immediately after splenectomy under aseptic conditions. The homogenate was collected in PBS (Sigma Chemical). Aliquots were layered on Ficoll Paque 400 (Pharmacia, Uppsala, Sweden) and centrifuged at 400 g...
for 30 min. The buffy coat containing the lymphocytes was collected and washed three times in PBS at 20°C. The cell pellet was resuspended in 10 ml of PBS containing 0.001% of the fluorochrome acidine red (Chroma, König, Germany) or 0.003% of the fluorescent dye rhodamine 6G (Sigma Chemical) for 10 min. The stained cells were harvested, washed once in PBS, and resuspended in 0.2 ml of PBS for intravenous infusion into recipient animals. Cell number and viability of every preparation were determined with a Neubauer chamber and the trypan blue exclusion test.

The labeling efficiency of this staining procedure was evaluated with a fluorescence-activated flow cyrometer (FACScan; Becton-Dickinson, Mountain View, CA). Single aliquots containing 10⁶ unstained cells were analyzed and compared with aliquots containing 10⁶ cells stained by acridine red or rhodamine 6G as a control fluorochrome. In two-dimensional forward and side-light scatterplots, the lymphocytes were gated, and the number of unstained cells, as well as the staining intensity of the individual lymphocytes in the gate, was estimated. The distribution of the fluorescence intensity of gated lymphocytes was determined in all three groups. To exclude a negative effect of the staining procedure on the expression of adhesion-modifying cell surface proteins, we additionally performed fluorescence-activated cell sorter (FACS) analyses on stained and unstained lymphocytes after incubation with monoclonal antibodies directed against lymphocyte function-associated antigen-1 (LFA-1; rat anti-mouse CD 11a: FITC, clone E21/7; Serotec, Oxford, England, UK) and against very late antigen-4 (rat anti-mouse VLA-4: FITC, clone PS/2, Serotec) for 10 min (n = 5 aliquots/group).

Lymphocyte proliferation assay. To quantify a possible negative effect of our staining technique on the capacity of isolated lymphocytes to proliferate on mitogenic stimulation, we carried out standard lymphocyte proliferation testing. Briefly, the isolated splenic cells in culture (RPMI medium; Gibco, Paisley, Scotland, UK; containing 10% fetal calf serum, 100 µ/ml penicillin G, and 100 mg/ml streptomycin) were incubated starting at an initial concentration of 10⁶ cells/well in triplicate wells of a 96-well microtiter plate. The cells were exposed to 5 µg/ml concanavalin A (Sigma Chemical) in 200 µl of culture medium, or to culture medium alone. After 72 h of culture (5% CO₂, 37°C) 1 µCi/well [3H]thymidine (NEN Research Products, Dreieich, Germany) was added and the cultures were incubated for 16 h. The cells were collected with an automatic cell harvester (Skatron, Sweden) on glass fiber filter paper. Incorporated radioactivity was measured with a liquid scintillation counter (Wallac 1409 LKB, Freiburg, Germany), and results were expressed as disintegrations per minute. The proliferation of unstained cells, of lymphocytes stained by acridine red, and of cells that had been incubated with the reference fluorochrome rhodamine 6G was tested (n = 4/group).

Adoptive lymphocyte transfer. On day 21 after the first of five daily STZ injections, diabetic donor mice were anesthetized and their spleens were removed under aseptic conditions. Donor splenic lymphocytes were isolated and stained with acridine red as described in Lymphocyte preparation and staining. The cells of one donor spleen were injected intravenously to stain the recipient animal's plasma. FITC-coupled dextran 150,000 (Sigma Chemical) were injected intravenously to stain the recipient animal's plasma. FITC-coupled dextran 150,000 (Sigma Chemical) were injected intravenously to stain the recipient animal's plasma.

Histology. Additional lymphocyte transfer experiments were performed to test the capacity of isolated and stained donor lymphocytes to transfer insulitis to healthy recipient mice in our model. Each recipient received an intraperitoneal injection of 1–3 × 10⁶ viable splenic lymphocytes derived from one donor mouse in 0.5 ml of PBS. Transfer experiments were performed from healthy to healthy animals (n = 5), from diabetic to healthy mice (n = 7), and from diabetic donors to nondiabetic recipients that had been given an intraperitoneal injection of 5 mg/kg STZ 24 h before the experiment (n = 6). Two weeks after the cell transfer, the recipient animals were killed. Their pancreata were fixed, embedded in paraffin, and prepared for light microscopic histology. In sections stained with hematoxylin and eosin, the percentage of recipient islets with lymphocytic infiltrates was determined in all three experimental groups. The evaluating investigator was unaware of the treatment that had been given.

In vivo microscopy of donor lymphocyte homing. The recipient animal was anesthetized intramuscularly with 5 g/kg urathan (Sigma Chemical). This anesthetic does not lead to relevant cardiovascular depression in rodents (11). A midline laparotomy was performed, and a catheter was inserted into the inferior vena cava. The dual loop was exteriorized and fixed to a thermocontrolled metal plate. The preparation was permanently bathed in physiological saline solution at 37°C. Five milligrams per kilogram of a 2.5% solution of FITC-coupled dextran 150,000 (Sigma Chemical) were injected intravenously to stain the recipient animal's plasma. The isolated and stained donor lymphocytes were infused intravenously over a 6-min period. The microcirculation of the exposed pancreas was observed via an epi-illumination microscope (MM-11; Nikon, Düsseldorf, Germany) equipped with a water immersion objective (CF Fluor 10/0.3 W; Nikon) and with two different filter sets. Filter set no. 1 (excitation filter 450–490 nm, barrier filter > 515 nm) was used to visualize FITC-marked structures, whereas with filter set no. 2 (excitation 515–560 nm, barrier filter > 580 nm), only the acridine red-marked donor cells were seen as fluorescent corpuscules moving across the video monitor (Fig. 1B). The microcirculation was recorded on videotape with a CCD video camera (Piper FK 6990-1Q, Schwerte, Germany) attached to the microscope and a video cassette recorder (Panasonic AG-7355 S-VHS) for later off-line evaluation. With this microscopy setup, the final magnification on the video monitor was 1,133-fold. Using the filter set no. 1 configuration, an area of recipient pancreatic tissue was located, where an islet of Langerhans lay directly under the visceral serosa. A frame was chosen that showed the islet under investigation on one side and an area of exocrine pancreatic tissue of at least the same size on the other side (Fig. 1A). Video recordings were obtained with filter set no. 2 over a period of 1 h. After every 5 min, the configuration was switched to filter set no. 1 to control for focus and to enable later off-line analysis of capillary blood flow in the pancreatic microvascular bed.

Studies of donor lymphocyte adhesion to recipient vascular endothelium were performed in four groups (groups 1–4) in exocrine pancreatic tissue and in islets of Langerhans. Additionally, in a control group of five STZ-pretreated C57BL/6 mice (group 5), the interaction of stained splenic lymphocytes from low-dose STZ-diabetic donors with gastric mucosal capillaries, a microvascular bed that is easily accessible by in vivo epi-illumination microscopy, was compared with donor cell interaction with exocrine pancreatic capillaries in group 4. The same transfer protocol was used in all experiments.
Group 1 was composed of untreated donors and untreated recipients (n = 7); group 2, multiple low-dose STZ-diabetic donors and untreated recipients (n = 8); group 3, untreated donors and STZ-pretreated recipients (n = 9); group 4, multiple low-dose STZ-diabetic donors and STZ-pretreated recipients (n = 9); and group 5, multiple low-dose STZ-diabetic donors and STZ-pretreated recipients (n = 5).

The interaction of donor lymphocytes to gastric mucosal endothelium was investigated to ensure that exocrine pancreas is a representative control vascular bed for islets of
Langerhans in the transfer experiments described in Adoptively lymphocyte transfer.

Evaluation of in vivo microscopic data. Evaluation of the experiments was performed off-line by a "blinded" examiner reviewing the video tapes. Immediately before cell transfer, the video image of the filter set no. 1 configuration was frozen. A semilucent foil was attached to the video monitor. The islet under investigation was outlined on the foil with a pencil. The foil was removed, and the islet window was cut out. A window of exactly the same size and shape was cut out next to the islet so that an area of exocrine pancreatic tissue containing only capillaries, small intra-acinar collecting venules, and supplying arterioles was displayed after reattachment of the foil to the video monitor. In review of video recordings in filter set no. 2 configuration, the numbers of passing, rolling, and permanently adhering donor lymphocytes were counted in both the exocrine and the endocrine windows over a 1-h period after the start of the intravenous lymphocyte transfer. In group 5 the size of the gastric observation windows was matched to the size of the exocrine pancreatic observation windows in group 4. "Lymphocyte rolling" was defined as marked slowing of cell velocity compared with the average speed of freely passing donor lymphocytes. Lymphocyte adhesion was noted in case of a complete stop of motion for ≥5 s of a donor lymphocyte passing one of the observation windows. To make these data comparable between the individual experiments, rolling and adherence data were expressed as cells exhibiting these phenomena per total number of cells passing the respective observation window in 1 h. Because the interaction of blood cells and endothelium is dependent on shear forces acting on margined cells in the circulation, we used a semiquantitative method for the assessment of microvascular blood flow designed to detect marked changes of this parameter (18). Briefly, by use of the filter set no. 1 recordings, every 5 min the flow in the microvessels under investigation was assigned to one of the following five scaled categories: 0, permanent stasis of blood flow; 1, slow flow with intermittent stops; 2, continuous but slow flow; 3, fast flow, individual cells discernible; and 4, very fast blood flow, no individual cells visible.

Statistical analysis. FACS data were compared by t-test. Because in all other experiments sample size was <10 and not all data sets were normally distributed, Kruskal-Wallis ANOVA on ranks was used to detect significant differences between the groups. Dunn's test as a multiple comparison method was applied to the results of these analyses to correct for the different sizes of the samples. Differences in lymphocyte rolling and adhesion between gastric mucosal and exocrine pancreatic observation windows and between islet and exocrine pancreatic observation windows were compared with the Mann-Whitney rank-sum test. The chi-square test was used to evaluate the histological data. All statistical operations were performed on a personal computer with the software program SigmaStat (Jandel Scientific, Erkrath, Germany). Data are expressed as means ± SE.

RESULTS

Lymphocyte preparation and staining. The mean yield of splenic donor lymphocytes in all five experimental groups was 2.18 ± 0.17 × 10^7 cells per donor spleen. Donor lymphocyte counts after the in vitro isolation and staining procedure were 1.54 ± 0.26 cells/spleen in group 1, 2.27 ± 0.22 cells/spleen in group 2, 2.66 ± 0.52 cells/spleen in group 3, 1.78 ± 0.14 cells/spleen in group 4, and 2.55 ± 0.30 cells/spleen in group 5. The number of transferred donor lymphocytes was not significantly different among the five groups. Trypan blue testing revealed a mean overall viability of 93 ± 5% of the cells stained by acridine red. Flow cytometry showed that the pool of isolated splenic cells contained only a small fraction of larger cells. These were identified under the microscope as monocytes and neutrophils. Because most corpuscles outside the gate were small cell fragments, the purity of the lymphocyte suspension was estimated to be >90%. After gating the FACS signal for lymphocytes, it could be shown that 100% of these cells were stained by acridine red, whereas very few lymphocytes had not been stained in the rhodamine group. The fluorescence intensity of lymphocytes stained by acridine red was higher and in a smaller range than that of cells stained by rhodamine 6G. Flow cytometry after preincubation of isolated lymphocytes with monoclonal antibodies directed against cell surface integrins showed that, in the lymphocyte gate, all acridine-stained and all unstained control cells were positive for the two integrins tested. Evaluation of mean channel fluorescence (MCF) revealed no statistically significant differences between stained and unstained lymphocytes, indicating that there was at least no dramatic decrease in the expression of LFA-1 and VLA-4 after staining the cells with acridine red. MCF of acridine-stained lymphocytes was 56.25 ± 4.44 (17.34 ± 0.30) compared with 57.76 ± 3.01 [17.90 ± 0.57, not significant (NS)] in experiments with unstained cells after incubation with anti-LFA-1 (or anti-VLA-4).

Lymphocyte proliferation assay. The separated and stained donor lymphocytes proliferated readily after mitogenic stimulation with concanavalin A. However, in vitro proliferation was significantly higher in unstained control lymphocytes. Incorporation of tritium thymidine after 72 h of incubation with concanavalin A was increased from 112 ± 18 to 6,125 ± 235 dpm in the unstained control group, from 106 ± 73 to 1,404 ± 143 dpm in the acridine red group (P < 0.05 vs. control group), and from 43 ± 9 to 374 ± 83 dpm in the rhodamine 6G group (P < 0.05 vs. both other groups). Hence, lymphocyte proliferation after incubation with concanavalin A was inhibited by rhodamine 6G more than by acridine red. Furthermore, in contrast to acridine red, rhodamine 6G led to a decrease of thymidine incorporation in unstimulated donor lymphocytes after 72 h (43 ± 9 vs. 106 ± 73 dpm, P < 0.05).

Histology. Investigation of a total of 435 islets in the three groups of recipient animals showed lymphocytic insulitis in 8% of islets in the control group, in 42% of islets after transfer of lymphocytes from diabetic donors (P < 0.001 vs. control), and in 51% of islets in STZ-pretreated recipients (P < 0.001 vs. control, NS vs. other group). The lymphocyte infiltrates were located in the islet periphery, often only at one islet pole. The cell transfer did not lead to overt diabetes in the recipient animals. Fasting blood glucose of recipient mice was between 4.66 ± 0.13 and 5.04 ± 0.57 mmol/l in the three groups 2 wk after lymphocyte transfer (NS).
In vivo microscopy of donor lymphocyte homing. Within 1 min after the start of the infusion of stained donor lymphocytes, the first cells became visible on the video screen as brightly fluorescent corpuscles tracking the course of pancreatic or gastric mucosal microvessels. Most of these cells moved with the same speed as the surrounding erythrocytes, which could be demonstrated by switching between the two filter sets of the microscope. After all donor cells had been infused, the number of passing fluorescent lymphocytes per window and time stayed constant in all groups during the 1-h observation period. In every experiment a variable proportion of perfusing donor cells passed the islet windows, whereas in untreated (diabetic donors, group 4) of all counted donor lymphocytes the number of cells was 392.0 6 73.0 lymphocytes in group 3, and 252.4 6 71.0 lymphocytes in group 4. In STZ-pretreated recipients, the lymphocyte flux was significantly higher in the islet windows compared with the exocrine pancreatic tissue. The scaled values were between 2 and 3 in this control vascular bed. Blood flow did not change over the 1-h observation period in all five groups.

Donor lymphocyte flux. In exocrine pancreatic tissue, mean lymphocyte flux over 1 h was 356.0 6 115.5 counted lymphocytes per window in group 1, 219.0 6 31.5 lymphocytes in group 2, 200.0 6 33.3 lymphocytes in group 3, and 103.8 6 29.8 lymphocytes in group 4. In islets of Langerhans, donor lymphocyte flux over 1 h was 392.0 6 248.2 counted donor cells per window in group 1, 356.8 6 64.1 lymphocytes in group 2, 466.2 6 73.0 lymphocytes in group 3, and 252.4 6 71.0 lymphocytes in group 4. In STZ-pretreated recipients, the lymphocyte flux was significantly higher in the islet windows compared with the exocrine pancreatic windows (P < 0.01 in group 3 and P < 0.05 in group 4). No significant differences of lymphocyte flux between endocrine and exocrine windows were detected in the untreated recipients in groups 1 and 2. In the groups in which recipients had been pretreated by STZ, 69.49 6 2.90% (nondiabetic donors, group 3) vs. 72.63 6 3.38% (diabetic donors, group 4) of all counted donor lymphocytes passed the islet windows, whereas in untreated recipients, only 42.29 6 6.12% (nondiabetic donors, group 1) vs. 60.08 6 6.16% (diabetic donors, group 2) of cells were counted in endocrine windows. There was no significant correlation between the amount of transferred donor lymphocytes and the number of cells perfusing the two observation windows in groups 1–4 (r² = 0.17).

Interaction of donor lymphocytes with gastric mucosa. In group 5, mean donor lymphocyte adhesion to gastric mucosal endothelium was 2.57 6 1.90% of all visible donor cells in the observation window. In this vascular bed, mean donor lymphocyte rolling was 1.19 6 0.61% of donor cells perfusing the area of interest. Neither donor lymphocyte adhesion nor donor lymphocyte rolling was statistically different in gastric mucosa (group 5) compared with exocrine pancreatic tissue (group 4), where adhesion was 2.80 6 0.97% and rolling was 1.55 6 0.59% of perfusing cells (Fig. 2). Donor lymphocyte flux was 103.8 6 29.8 cells/h in the exocrine pancreatic observation windows (group 4) and 196.8 6 83.3 cells/h in the gastric mucosal observation windows (group 5, NS).

Donor lymphocyte rolling. These data are presented in Fig. 3. In untreated recipient mice, donor lymphocyte rolling was low and did not significantly differ between endocrine and exocrine tissue, irrespective of whether the donors were diabetic or not. In this part of the study, lymphocyte rolling in the 1st h after cell transfer was 1.74 6 0.38% of perfusing donor cells in the exocrine window and 1.85 6 0.63% in the endocrine window if...
lymphocytes of untreated, nondiabetic donor mice had been transferred and $1.55 \pm 0.59\%$ (exocrine tissue, NS) vs. $1.65 \pm 0.57\%$ (islets, NS) in windows of recipients that had been infused with cells from diabetic donors. However, lymphocyte rolling was significantly enhanced in islets of STZ-pretreated recipients if lymphocytes from diabetic donors had been transferred ($4.46 \pm 1.32\%, P < 0.05$ vs. lymphocyte rolling in islets of untreated recipients that had received cells from nondiabetic donors). This constellation did not lead to increased lymphocyte rolling in exocrine tissue ($1.36 \pm 0.53\%,$ NS). In STZ-pretreated recipients, rolling of lymphocytes from nondiabetic donors was essentially unchanged ($2.71 \pm 0.62\%$ in exocrine, NS, and $2.78 \pm 0.72\%$ in endocrine tissue, NS).

Donor lymphocyte adhesion. Lymphocytes from nondiabetic donors showed very low adhesion to exocrine or endocrine pancreatic endothelium of untreated recipients ($0.96 \pm 0.40\%$ vs. $0.77 \pm 0.46\%$ of perfusing cells). The same was true for lymphocytes from diabetic donors, where lymphocyte adhesion was $0.61 \pm 0.33\%$ in exocrine tissue (NS) and $1.37 \pm 0.57\%$ of passing cells in islets (NS). In STZ-pretreated animals donor lymphocyte adhesion was generally higher in all four groups (Fig. 4). In these experiments adhesion of lymphocytes from nondiabetic donor mice was $2.17 \pm 0.83\%$ in the acinar pancreas (NS) and $1.31 \pm 0.36\%$ of perfusing cells in islets of Langerhans (NS). Enhanced donor lymphocyte adhesion was most marked in pancreatic islets if lymphocytes from STZ-diabetic donors had been transferred to STZ-pretreated recipient mice ($3.86 \pm 1.04\%$ of passing cells, $P < 0.05$ vs. lymphocyte adhesion in islets of untreated recipients that had received cells from nondiabetic donors). In the exocrine pancreas of pretreated recipients, $2.80 \pm 0.97\%$ of these lymphocytes firmly adhered to microvascular endothelium (NS).

**DISCUSSION**

One of the initial steps in the pathogenesis of autoimmune insulin-dependent diabetes mellitus is the adhesion of circulating cells of the immune system to islet endothelium (12, 13, 15, 32). Recruitment of these cells leads to insulitis with progressive β-cell destruction (9, 12, 27). In severely affected islets the infiltrate is mainly composed of lymphocytes in all species that have been investigated so far (20). The few studies that have addressed the homing of transferred islet reactive lymphocytes of diabetic donors to recipient islets of Langerhans in animal experiments used in vitro cell labeling to locate these lymphocytes histologically in different tissues after transfer (1, 22, 34). This technique, however, does not measure the nature and strength of the interaction between transferred cells and the endothelium of target tissues. Furthermore, the dynamic time course of this phenomenon and islet blood flow cannot be investigated simultaneously in such experiments. We therefore developed an in vivo microscopic method that enables the examiner to directly observe the behavior of transferred donor cells in one recipient islet of Langerhans and in the exocrine pancreatic acini nearby (7). In the model of adoptive lymphocyte transfer in multiple low-dose STZ diabetes mellitus in C57BL/6 mice, we demonstrate that splenic lymphocytes from diabetic donor mice preferentially adhere to islet endothelium if the recipient animals have been pretreated with a single very low dose of STZ 1 day before the cell transfer. Although insulitis could be demonstrated in the recipients, it was not possible to transfer overt diabetes in our transfer model, regardless of whether the recipient mice had been pretreated with STZ.

The isolation of splenic lymphocytes by Ficoll gradient centrifugation resulted in a highly enriched lymphocyte preparation, as could be demonstrated by analysis of light scatter in a flow cytometer and by microscopic examination of isolated cells in the Neubauer chamber. The cell suspension contained only few smaller cell fragments or bigger monocytes and neutrophils. FACS analysis showed that acridine red stained 100% of the isolated cells, whereas staining by rhodamine 6G resulted in incomplete cell staining. The fluorescent staining with acridine red did not significantly reduce the viability of donor cells, but it impaired these lymphocytes' ability to proliferate on mitogenic stimulation. This represents a known problem associated with fluorescent labeling of lymphocytes (30). However, staining with acridine red still permitted a 13.2-fold increase in [3H]thymidine incorporation after stimulation with concanavalin A, whereas rhodamine 6G-stained cells could be stimulated only 8.7-fold, and proliferation of unstimulated lymphocytes was compromised by this fluorochrome. Lymphocytes labeled with acridine red showed a preserved ability to adhere to endothelium in vivo. On the basis of these results, we conclude that, for lymphocyte transfer studies, acridine red is a better fluorescent marker than rhodamine 6G, which has
been widely used to stain leukocytes in microcirculation studies (5).

The advantages of our in vivo technique to track tagged donor lymphocytes in recipient tissue after cell transfer are accompanied by several problems that need to be addressed. The experimental setup allows the examination of only one of the many islets in the animal under study. Furthermore, the exposure to epi-illumination might artificially activate islet endothelium by light toxicity (25), thereby nonspecifically increasing islet lymphocyte adhesion. This problem was managed by investigating the nearby exocrine pancreatic tissue, which was exposed to exactly the same experimental conditions, as an endogenous control vascular bed. Because significantly increased donor lymphocyte rolling and adhesion could only be demonstrated in one group and only in islet tissue, a relevant impact of experimental influences on the interaction between donor cells and endothelium can be excluded. The same is true for possible changes in shear stress on interacting donor lymphocytes. The semiquantitatively estimated blood flow velocity was not significantly altered over the 1-h observation period in any group. This excludes at least marked changes in shear stress in our experiments. There were no significant differences in donor lymphocyte interaction with recipient vascular endothelium between exocrine pancreas and gastric mucosa. Hence, acinar pancreatic tissue can be regarded as a representative control vascular bed for lymphocyte adhesion studies in islets of Langerhans.

Rolling as well as endothelial adhesion of lymphocytes from diabetic donors was increased significantly only in islets. This indicates that the pool of transferred splenic lymphocytes of multiple low-dose STZ diabetic mice contains islet-specific cells and therefore underlines the immunologic pathogenesis of this diabetes model (14, 23, 29). Lymphocytes from nondiabetic donors did not show this phenomenon. It is therefore concluded that it is the induction of diabetes mellitus by STZ that leads to the presence of islet-specific lymphocytes in the reticuloendothelial system of this mouse species.

Interestingly, the interaction of transferred cells and recipient islet endothelium is significantly enhanced only if the recipient animal has been pretreated with a single very low dose of STZ. Because this dose was given 24 h before the lymphocyte transfer, the homing of donor lymphocytes to islets certainly is not a result of an interaction of STZ-reactive subpopulations with STZ itself, which is known to accumulate in islets of Langerhans (31). The very unstable STZ molecule has a half-life of few minutes in vivo (26). Thus islet endothelium appears to be activated either directly by STZ or, more likely, by inflammatory mediators released from β-cells damaged by the cytotoxic effect of STZ or by cytokines released from lymphocytes under the influence of STZ (6, 14). The pretreatment of recipient animals with STZ led to significantly increased donor lymphocyte flux in pancreatic islets. This shunt of lymphocytes from exocrine to islet tissue might be the result of a release of chemoattractant substances from islets of Langerhans under the influence of STZ. Obviously, increased delivery of lymphocytes to islet microvessels and enhanced interaction of these circulating immune cells with islet endothelium both play a role in the pathogenesis of lymphocytic infiltration of islets in the transfer model of multiple low-dose STZ diabetes mellitus. The need to pretreat recipients with STZ to produce enhanced islet adhesion of transferred lymphocytes from diabetic donors in this model illustrates that not only islet-reactive immune cells have to be present but also that there are environmental factors involved in the initiation of autoimmune insulinitis (19). Finally, another important factor in diabetogenesis, genetic background, is operative in the low-dose STZ model (28). Kiesel et al. (17) could demonstrate that insulinitis in multiple low-dose STZ diabetes mellitus is transferable in C57BL/6 mice but not in BALB/c or in other mouse strains.

In conclusion, the described experimental model for the investigation of islet lymphocyte homing has many features also observed in human autoimmune diabetes mellitus: the requirement of a permissive genetic background, triggering environmental factors, and the generation and action of islet-reactive lymphocytes. The presumed mechanism by which transferred lymphocytes from diabetic donors interact preferentially with islet endothelium of STZ-pretreated recipient mice is the induction of vascular adhesion molecules or an activation of these adhesion-promoting surface proteins on both cell types involved (8, 32). Thus investigation of this model with in vivo microscopy is a tool to gain insights in the initial steps leading to lymphocytic insulinitis. This approach will help to further characterize the interactions between circulating islet-reactive immune cells and islet endothelium in autoimmune diabetes mellitus, i.e., by investigating the effect of blocking monoclonal antibodies against cell adhesion molecules (33) or by other adhesion-modifying substances, such as soluble adhesion molecules.

The authors thank Sandra Diehl and Manfred Stegmüller for valuable technical assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to K. Kusterer (DFG KU 622/4–1).

Address for reprint requests: M. Enghofer, Dept. of Medicine I, J. W. Goethe Univ, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany.

Received 31 October 1997; accepted in final form 5 February 1998.

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