Originals

B cell-adherent splenocytes precede the onset of diabetes in low-dose streptozotocin-treated mice

B. Feve, J. P. Segain, B. Charbonnel and P. Sai

Laboratoire d'immunologie du diabète, Faculté de Médecine, Nantes, France

Summary. Splenocytes from low-dose ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) streptozotocin-treated mice were tested for their binding ability to rat insulinoma (RINm-5F) cells in a rosette-forming cell assay, before and during the onset of diabetes. They displayed a higher ($p < 0.0001$) RIN-adherence than control splenocytes. Such an enhanced binding of splenocytes from diabetic mice was observed on another B-cell (HIT cell) line, but not on non-B cells (particularly on exocrine pancreatic cells, endocrine cells or natural killer-target cells), suggesting that the increased RIN-binding is B-cell specific. This B-cell specificity was also suggested by the use of increasing splenocytes/RIN ratios showing a saturation of RIN-binding in streptozotocin-treated mice. Depletion of lymphocyte subsets revealed that supernumerary RIN-adherent splenocytes from diabetic mice were mainly T lymphocytes, involving both L3T4^+ and Lyt2^+ cells. Overall, the increased splenocyte-RIN binding was concomitant with the occurrence of islet destruction, but preceded the onset of hyperglycaemia

by five days and even the islet immune infiltration. An increased number of RIN-binding splenocytes was also found in mice treated with $33 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of streptozotocin, displaying insulinitis but not hyperglycaemia. This phenomenon was not found with splenocytes from mice displaying a “toxic” diabetes induced by a single high dose of streptozotocin. No correlation could thus be found between numbers of RIN-binding splenocytes and blood glucose levels, indicating that this phenomenon was not due to metabolic disturbances. These data describe a new marker of cellular immunity in this animal model. This marker is detectable before the clinical onset of diabetes and is more closely linked to insulinitis than to hyperglycaemia.

Key words: Streptozotocin, Type 1 (insulin-dependent) diabetes, RIN cell, cellular immunity, rosette-forming cell assay.

Diabetes induced in mice by multiple low-doses of streptozotocin (SZ) [1] depends on immune pathogenetical components [2], and thus is a model used for the study of human Type 1 (insulin-dependent) diabetes. Cell-mediated immunity seems to be of major importance in this animal model [2], while the role of humoral immunity appears minor since B lymphocyte-depleted mice retained their susceptibility to the disease [3]. This role of cellular immunity is established *in vivo* by the prevention of SZ-induced diabetes following injections of monoclonal antibodies directed against lymphocyte differentiation antigens [4, 5]. These studies have pointed out the implication of T-lymphocytes bearing either L3T4 or Lyt2 antigens, which have also been characterized in “insulinitis” [4, 6]. Moreover, the presence of an anti-B cell specific cellular immunity is suggested in SZ-induced diabetes by *in vitro* cytotoxicity [7, 8] and *in vivo* pancreatic “homing” [9] experiments.

We have recently reported that anti-B-cell cell-mediated immunity could be detected, in a “rosette-forming

cell” assay, by an increased B-cell specific binding of circulating T-lymphocytes during human recent-onset Type 1 diabetes [10]. This phenomenon was not detected with lymphocytes from patients with Type 2 (non-insulin dependent) diabetes or with other autoimmune diseases. It was specifically blocked by B-cell membrane extracts, by preabsorption of lymphocytes with B cells, or by anti-CD3 monoclonal antibodies [11]. These previous studies were performed with human patients. In the present study, in order to establish the presence of such a marker in an animal model of Type 1 diabetes, we have evaluated the B-cell binding of splenocytes from SZ-treated mice and the T-cell subsets involved. Furthermore, the B-cell specificity of the phenomenon was confirmed using a panel of non-B cells (especially exocrine pancreatic tumoral cells, endocrine cells, natural killer (NK)-target cells). Overall, in regard to the importance of the pre-diabetic period, evidence for the early occurrence of this marker was investigated before the onset of hyperglycaemia and concomitantly evaluated with the develop-

ment of insulinitis. We did not deal with this problem in our previous work with human patients [10, 11] who were only investigated for this marker after the clinical onset of diabetes.

Materials and methods

Animals

CD-1 male mice (Charles River France, Saint-Aubin-les-Elbeuf, France) were obtained at 40 days of age, randomly distributed and housed under conventional conditions. They received laboratory chow and water *ad libitum*.

Induction of diabetes

Streptozotocin (SZ) (Upjohn Laboratories, Paris, France) was dissolved in citrate buffer (pH 4.5), and immediately injected *i.p.* at doses of either 40 or 33 mg·kg⁻¹·day⁻¹, between 09.00 and 11.00 hours, during five consecutive days, for the induction of "autoimmune" diabetes or insulinitis [1]. The day of the first injection was designated as day 1. Toxic diabetes was induced by the injection of a single dose (165 mg/kg) of SZ. Control mice were treated by the citrate buffer only.

Determination of serum glucose levels

Blood samples of non-fasted mice were drawn from the retro-orbital vein plexus at the following times: day 1 (just before the first SZ injection), day 8 (72 h after the last SZ administration) and every 2 days thereafter, until the end of the study (day 15). Serum glucose levels were determined by the glucose-oxidase method.

Histological examination of pancreata

Pancreata were removed in 10% formalin, embedded in paraffin, and 5 µm sections were obtained. Every 10th section was stained with haematoxylin-eosin or aldehyde-fuchsin to assess the incidence of inflammatory changes and the degree of islet destruction. Slides were evaluated and scored double blind. The degree of insulinitis was graded as: (–), no infiltration; (+), changes with lymphocytes restricted to 10% of at least 50 islets of the same pancreas; (++) , infiltration around and within at least 30% of the islets; (+++) , infiltration within at least 60% of the islets. The degree of islet destruction was similarly graded double blind as: (–), no destruction; (+), destruction restricted to 10% of at least 50 islets of the same pancreas; (++) , destruction of at least 30% of the islets; (+++) , destruction of at least 60% of the islets. At least 5 sections were examined at varying depths for each pancreas. Results are presented as mean intensity of insulinitis or degree of islet destruction in 7 pancreases for each group.

Splenocyte isolation

Spleens were removed and floated in a Hanks' balanced salt solution (HBSS) (Seromed, Berlin, FRG) with 2% fetal calf serum (FCS) (Flow Laboratories, Irvine, UK). Splenocytes were flushed from the parenchyma by the injection of 30 ml of the above HBSS-FCS. Erythrocytes were then lysed by ammonium chloride (0.17 mol/l) for 10 min at 4 °C. This cell suspension was then washed twice and resuspended in RPMI 1640 (Gibco laboratories, Grand

Island, NY, USA) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml). All experiments were performed with cells from spleens of individual mice, unless otherwise specified.

Cell lines

The rat insulinoma RINm-5F [12], the human neuroblastoma SK-N-MC [13], melanoma SK-MEL-1 C32 [14], exocrine pancreatic carcinoma CAPAN-1 [15], ovarian carcinoma OVCAR-3 [16], breast carcinoma MCF7 [17] and the rat colon carcinoma TS cell lines [18] were maintained as monolayers in RPMI-10% FCS in plastic tissue culture flasks (Falcon Labware, div., Becton Dickinson, Oxnard, Calif., USA). The hamster B-cell subclone HIT 15 [19] was grown in Ham's F12 medium (Gibco, Paisley, Scotland, UK) with 10% FCS and 1% glutamine. When confluent, cells were detached and dissociated with trypsin (0.05%)–EDTA (0.02%) (Seromed) at 37 °C, washed, resuspended in RPMI-10% FCS, and incubated for 2 h at 37 °C before experiments. The human chronic myelogenous leukemia K562 [20], rat myeloma IR983F [21] and murine lymphoma YAC-1 cell lines were maintained in suspension culture in RPMI-10% FCS. All cell lines were grown at 37 °C in a humidified atmosphere of air: CO₂ (95%:5%). RIN cells were used at culture passages less than 75, when more than 10% of these cells were still stained with A2B5 monoclonal antibody (SERA-Lab, Sussex, UK) in an indirect immunofluorescence (IF) assay (data not shown).

Rosette-forming cell (RFC) assay

The numeration of splenocytes binding to target cells was performed with a method slightly modified from that previously described for human blood lymphocytes [10]. Briefly, 1.5 × 10⁶ splenocytes and 2 × 10⁵ irradiated (3000 rads) target cells (B or non B) were co-incubated in 1 ml of RPMI-10% FCS into 5 ml polypropylene culture tubes (Sarstedt, Rommelsdorf, Numbrecht, FRG) at 37 °C in a 5% CO₂ atmosphere for 48 h. After a careful homogenization, aliquots of the cell mixture were deposited into a Malassez counting chamber. Splenocytes bound and not bound to target cells were simultaneously counted under light microscopy. The whole grid area was scanned. Results are transformed into the total number of target-adherent splenocytes among the total number of splenocytes initially seeded in each tube. Each mouse was tested in triplicate (intra-assay variability always less than 10%). Control and SZ-treated mice were always tested simultaneously in each experiment. In some experiments, increasing numbers of splenocytes were incubated with a constant number (2 × 10⁵) of RIN cells (splenocytes/RIN ratios: 1 : 1, 3 : 1, 7.5 : 1, 10 : 1, 20 : 1), before the enumeration of cytoadherent spleen cells.

To assess the effects of a T cell mitogen, splenocytes from SZ-treated or control mice were seeded for 2 days into round-bottomed 96-well plates in RPMI-10% FCS containing concanavalin A (ConA) (Sigma, St. Louis, Mo, USA) at a final concentration of 2 µg/ml. Splenocytes were then washed, counted, and assayed for their binding ability to RIN cells in the RFC assay as described above.

To test the possibility that the RIN-binding ability of splenocytes is FC-receptor/antibody-mediated, heat-aggregated (63 °C; 30 min) mouse IgG (20 µg/ml) were added to splenocytes 1 h before the addition of RIN cells in the RFC assay.

RFC assay after depletion of T cell subsets

Splenocytes from control and SZ-treated mice were depleted from macrophages by incubation on plastic Petri dishes (Nunc, Rostkilde, Denmark, 15 × 10⁶/dish) for 2 h at 37 °C in RPMI-10% FCS, and the loss of adherent spleen cells was similar in SZ-treated and control

Fig. 1. RIN-adherence of splenocytes from streptozotocin (SZ) ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated (○) or from control (●) mice. Each point represents an individual mouse. Vertical bars denote means $\pm$ SEM. Asterisk indicates a statistical difference ($p < 0.0001$) between the SZ-diabetic and control mice

mice. Cells non-adherent to plastic were then counted, incubated for 45 min at 4°C with monoclonal antibodies (Dr S. Ezine, Paris, France) directed against either Thy1-2 (marker of mature T cells, produced by clone 4.221), L3T4 (conventional marker of "helper" T lymphocytes, produced by clone 172.4), or Lyt2 (conventional marker of "suppressor/cytotoxic" T lymphocytes, produced by clone 3.155), then washed twice and further incubated for 45 min at 37°C with rabbit complement (diluted 1 : 2) (CTS, Nantes, France). After three washes, 1.5×10^6 viable lymphocytes were finally incubated for 48 h with 2×10^5 RIN cells in the RFC assay. Cytotoxicity of complement alone did not exceed 5%. Depletion efficiency was assessed by indirect IF and was similar between the control and SZ-treated groups.

Statistical analysis

All data are presented as mean values $\pm$ SEM. The statistical significance of the differences between the experimental groups was evaluated using the Student's *t*-test or the non parametric Mann-Whitney U test when $n < 5$. $p < 0.05$ was considered as significant. The least-squares linear regression was used for correlation analysis.

Results

RIN-adherence of splenocytes from low-dose SZ-treated mice

The number of RIN-adherent splenocytes was significantly higher in SZ ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated mice ($n = 14$) analysed by the 10th day after the first injection of SZ than in control ($n = 15$) mice (respectively, $13\,728 \pm 622$ vs 8581 ± 415 ; $p < 0.0001$) (Fig. 1). This significant difference between SZ-treated and control mice was detectable from 24 h of co-incubation and persisted after 48 h and 72 h (data not shown).

When increasing splenocytes/RIN ratios in the RFC assay, the percent of RIN-binding splenocytes was significantly ($p < 0.01$) higher in SZ-treated than in control mice only at lower splenocytes/RIN ratios (1.5% vs 0.9% at 1 : 1; 1.4% vs 0.6% at 3 : 1; and 0.9% vs 0.6% at 7.5 : 1). At

Fig. 2. Incubation of RIN cells (2×10^5) with increasing numbers of splenocytes from control mice (●) ($n = 4$) and streptozotocin (SZ) ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated mice (○) ($n = 4$). Asterisks denote statistical differences between the SZ-diabetic and control mice, * $p < 0.01$

higher ratios (10 : 1, 20 : 1), the percent of RIN-binding splenocytes fell in SZ-treated mice to that of control splenocytes which remained constant (Fig. 2).

Target-cell specificity of cytoadherence

Simultaneous with the above difference in the RIN-binding ability, the number of splenocytes adherent to B-HIT-cells was similarly higher in SZ-treated mice than in control mice (Fig. 3). By contrast, no statistical difference was observed between SZ-treated and control mice in the number of splenocytes adhering to a panel of 9 non-B cell lines (Fig. 3). In particular, no difference was observed on exocrine pancreatic CAPAN cells, on cells of neuroectodermic origin (melanoma SK-MEL-1-C32, neuroblastoma SK-N-MC), or on NK-target cells (YAC-1, K562).

RIN-binding splenocytes precede the onset of diabetes and of insulinitis

The number of RIN-adherent splenocytes of SZ-injected mice was not different from that of control animals (Fig. 4) when examined by the 2nd day of SZ ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treatment when overt insulinitis had not yet been observed. This number increased significantly ($p < 0.01$) above control values as early as the day of final SZ-administration when no hyperglycaemia was found. At this time, only a mild islet destruction (+) was detectable (Fig. 4). This rise in the number of RIN-binding lymphocytes by the 5th and the 10th day after the final SZ-injection concomitantly with the occurrence of insulinitis and then with the onset of hyperglycaemia (Fig. 4).

Correlation between the number of RIN-adherent splenocytes and the onset of diabetes or glycaemic levels

No correlation could be found between the number of RIN-binding lymphocytes and glycaemia in SZ ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated mice examined at day 10, some mice even displaying a high number of RIN-binding spleno-

Fig. 3. Target-cell specificity. Splenocytes from streptozotocin (SZ; $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated (▨) and control (□) mice were incubated simultaneously with B- or non B-cells (2×10^5); $n = 3$ for SZ-injected or control mice on all cell lines, except for RIN cells (SZ: $n = 13$, controls: $n = 14$) and rat colon carcinoma (TS) cells (SZ: $n = 7$, controls: $n = 7$). Asterisks denote statistical differences between the SZ-treated and control mice, * $p < 0.01$

Insulinitis	-	-	+	++	+++
Islet destruction	-	+	+	++	+++

Fig. 4. Comparative timing of RIN-splenocyte adherence, insulinitis, islet destruction, and hyperglycaemia. The RIN-binding ability of splenocytes from streptozotocin (SZ; $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated (○—○) and from control mice (●—●) was evaluated concomitantly with the determination of glycaemia (□—□ and ■—■). Asterisks denote statistical differences between SZ-treated and control mice: * $p < 0.01$. Simultaneously, the pancreata were scored for insulinitis and islet destruction as mentioned in the table below the figure. Fourteen animals were included at each point

cytes without any hyperglycaemia (Fig. 5). Moreover, injections of lower doses ($33 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) of SZ led to a significantly ($p < 0.01$) increased number of RIN-binding splenocytes by 5 days after the last SZ injection (Fig. 5), while these animals remained normoglycaemic ($9.0 \pm 0.6 \text{ mmol/l}$, $n = 26$) but while insulinitis was noted with an incidence of 50%. Conversely, the number of RIN-adherent splenocytes remained at control levels in mice displaying a single "toxic" dose (165 mg/kg) SZ-induced diabetes without insulinitis (Fig. 5). Consequently, when low-dose (40 and $33 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$) and high-dose SZ-treated mice were simultaneously analysed with control mice (Fig. 5), numbers of RIN-adherent splenocytes did not correlate with blood glucose levels.

Fig. 5. Correlation between glycaemia and numbers of RIN-adherent splenocytes in control mice (●), mice treated with $40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (○) or $33 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (△) of streptozotocin (SZ), and mice injected with a single high dose (165 mg/kg) of SZ (■). Determinations were performed five days after the last low dose SZ-injection, and three days after the single high dose of SZ

Fig. 6. Number of RIN-binding splenocytes after depletion of lymphocyte subsets using monoclonal antibodies and complement. Pools of 2 spleens from control and streptozotocin (SZ)-treated mice were tested either using total lymphocytes (□), T-depleted (Thy 1–2^o) lymphocytes (▨), "helper"-depleted (L3T4^o) cells (▤) or "cytotoxic/suppressor"-depleted (Lyt2^o) cells (▥). The figure is representative of two experiments

RFC-assay after depletion of lymphocyte subsets, after T cell stimulation, or after blocking of the FC-receptor

After depletion of T-lymphocytes by monoclonal antibodies directed against the Thy 1–2 antigen, the number of RIN-adherent splenocytes from SZ ($40 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$)-treated mice was reduced by 42.2%, falling to control levels (Fig. 6). Figure 6 also shows that depletion of either L3T4⁺ or Lyt2⁺ subsets reduced ($p < 0.01$) the RIN-binding ability of splenocytes from SZ-treated mice (-26.5% and -31.5% , respectively).

Con A-stimulated splenocytes from SZ-treated mice displayed a higher ($p < 0.01$) RIN-binding ability ($14\,968 \pm 819$) than control splenocytes stimulated ($10\,970 \pm 689$) or not (9076 ± 628) by the T cell mitogen ($n = 4$).

In the presence of aggregated IgG, the number of RIN-adherent splenocytes was still higher ($p < 0.01$) in SZ-treated mice ($13\,158 \pm 741$; $n = 4$) than in control mice (8500 ± 574 ; $n = 4$).

Discussion

We have demonstrated *in vitro* that the increased B-cell adherence of lymphocytes, previously described in human Type 1 diabetes [10, 22], is also found during low-dose SZ-induced diabetes in mice. Overall, this phenomenon precedes the clinical onset of diabetes, and even the overt insulinitis. It seems more closely linked to insulinitis than to hyperglycaemia. Furthermore, we suggest that this cytoadherence may be B-cell specific and involves L3T4⁺ and Lyt2⁺ T lymphocyte subsets.

This RIN-binding ability of splenocytes from SZ-treated mice appears B-cell-specific, since a similar increase was observed on another B cell line (HIT cells), but not on 9 non-B cell lines. Particularly, this marker was not detected on exocrine pancreatic cells or on NK-target cells. Experiments with increasing splenocytes/RIN ratios confirm that the phenomenon may be specific for splenocytes from SZ-treated mice. Indeed, it was only detectable at lower splenocyte/RIN ratios, and not at higher ratios when "specific target receptors" on RIN-cells may have been "hidden" by a high number of splenocytes non-specific for B cells. During the same experiments, the % of control splenocytes involved in RIN-conjugates remained nearly constant thoroughly the ratioscale, probably because no B-cell specific lymphocytes were present. Splenocytes from SZ-injected mice binding specifically to B cells may represent, as in human diabetic patients [10], a marker of cell-mediated immunity, since they appear to be mainly composed of T-lymphocytes, as suggested by depletion experiments of lymphocyte subsets. These T-cells include both L3T4⁺ (CD4⁺) and Lyt2⁺ (CD8⁺) lymphocytes. One can note that only CD4⁺ (and not CD8⁺) were demonstrated in the increased B-cell binding of lymphocytes from human Type 1 diabetic patients [10, 11]. The reason for this discrepancy is not clear, but any comparison between blood lymphocytes (in human patients) and spleen cells (in SZ-treated mice) should be cautious since spleen lymphocytes can have different qualitative and quantitative features from those in peripheral blood, even in a given species. On the other hand, these results are consistent with the immunocytochemical characterization of numerous L3T4⁺ and Lyt2⁺ cells in SZ-induced insulinitis [4, 6]. The major pathogenetical role of these two lymphocyte subsets is also emphasized by immune intervention trials showing prevention of SZ-induced diabetes by monoclonal antibodies directed against these differentiation antigens [4]. Direct activation of the T cell compartment is thought to mediate at least part of the diabetogenic effect of low dose SZ since this treatment induces T cell stimulation and the secretion of lymphokines, particularly IFN- γ , and systemic MHC induction [23]. However, these direct effects of SZ on T cells are not likely to be sufficient to explain the increased B-cell adherence of lymphocytes from low-dose treated mice. Firstly, because *in vitro* activation of T cells by Con A was not sufficient, in our hands, to increase the RIN-binding ability of splenocytes from control mice to the level observed in low-dose SZ-treated mice. Secondly, because if this increased binding ability was only due to the direct effect of SZ on lymphocytes, it would probably be detectable whatever the target cells (B or non-B). In that respect, we

emphasize the fact that the adherence of splenocytes from SZ-treated mice was not increased on 9 non-B cell lines. These latter results suggest that the increased B-cell binding of lymphocytes from SZ-injected mice also involved a specific recognition of B-cell molecules.

If this hypothesis is correct, then the recognition of B-cell antigens by T-lymphocytes takes place in a xenogenic context, thus raising the problem of the apparent absence of restriction by MHC molecules, even if there is increasing evidence that this dogma is too absolute. Indeed, non-MHC-restricted T cells have now been described [24–27]. In regard to autoimmune diseases, CD8⁺ T cells from Type 1 diabetic patients specifically inhibited insulin release from xenogeneic islet cells [28], and non-MHC-restricted tissue-specific T cells recognized autologous oligodendrocytes in mice [29].

Alternatively, the B-cell specific adherence observed in our study may be due to B-cell adhesion molecules for lymphocytes. Such molecules, called "addressins" may be involved in selective homing or migration [30]. One can note that the expression of intercellular adhesion molecule has been preliminarily documented in human pancreatic islets [31].

On the other hand, the fact that aggregated IgG did not abolish the increased adhesion of splenocytes from SZ-treated mice to B cells suggests that this binding is not FC-receptor/antibody mediated.

Nevertheless, the prevailing fact of the present study remains that RIN-specific binding became detectable 5 days before the occurrence of hyperglycaemia induced by SZ (40 mg · kg⁻¹ · day⁻¹), and is, thus, an early dysfunction during the prediabetic stage in this animal model. This phenomenon also slightly precedes the overt insulinitis, although it is impossible to compare it with the earlier "single cell insulinitis" [32]. This early implication of cellular immunity may occur closer to the time of detection of islet cell surface antibodies, and before hyperglycaemia in SZ-treated mice [33]. Our preliminary data similarly suggest the presence of RIN-specific binding lymphocytes in some humans at high risk for Type 1 diabetes [34].

No RIN-specific binding was found with splenocytes from mice becoming diabetic following a single toxic-dose of SZ (165 mg/kg), when no insulinitis but only a B-cell necrosis is observed. Conversely, using the subdiabetogenic SZ-dose of 33 mg · kg⁻¹ · day⁻¹ unable to induce hyperglycaemia, the RIN-specific adherence of MNC was detectable while insulinitis was frequently present. All these data put together suggest that the increased RIN-adherence of splenocytes is rather correlated with the presence of insulinitis than with hyperglycaemia.

In summary, we have demonstrated a new marker of cellular immunity in low-dose SZ-induced diabetes. It seems B-cell specific and is rather linked to the insulinitis than to the hyperglycaemia. Overall, this marker precedes the onset of clinical diabetes and is, thus, an early dysfunction during the prediabetic period in this animal model of Type 1 diabetes.

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References

1. Like AA, Rossini AA (1976) Streptozotocin-induced pancreatic insulinitis: new model of diabetes mellitus. *Science* 193: 415–417
2. Kolb H (1987) Mouse models of insulin-dependent diabetes. Low-dose streptozotocin-induced diabetes and nonobese diabetic (NOD) mice. *Diabetes Metab Rev* 3: 751–778
3. Blue MC, Shin SI (1984) Diabetes induction by subdiabetogenic doses of streptozotocin in BALBc/BOM mice: non involvement of host B-lymphocyte functions. *Diabetes* 33: 105–110
4. Kantwerk G, Cobbold S, Waldmann H, Kolb H (1987) L3T4⁺ and Lyt2⁺ cells are both involved in the generation of low-dose streptozotocin-induced diabetes. *Clin Exp Immunol* 70: 585–592
5. Oschilewski M, Schwab E, Kiesel U, Opitz U, Kolb-Bachofen V, Stunkel K, Kolb H (1986) Administration of silica or monoclonal antibody to Thy-1 prevents low-dose streptozotocin induced diabetes in mice. *Immunol Lett* 12: 289–295
6. Herold KC, Montag AG, Fitch FW (1984) Treatment with anti-T lymphocyte antibodies prevents induction of insulinitis in mice given multiple doses of streptozotocin. *Diabetes* 36: 796–800
7. McEvoy RC, Andersson J, Sandler S, Hellerström C (1984) Multiple low-dose streptozotocin-induced diabetes in the mouse. Evidence for stimulation of a cytotoxic cellular immune response against an insulin-producing beta cell line. *J Clin Invest* 74: 715–722
8. McEvoy RC, Thomas NM, Hellerström C, Ginsberg-Fellner F, Moran TM (1987) Multiple low-dose streptozotocin-induced diabetes in the mouse: further evidence for involvement of an anti-B cell cytotoxic cellular auto-immune response. *Diabetologia* 30: 232–238
9. Braun M, Arata M, D'Alessandri VG, Lopez O, Borca MV, Basabe JC (1986) Lodging in the pancreas of lymphocytes from normal and diabetic mice. Sixth International Congress of Immunology, Toronto, N° 1.46.9 (Abstract)
10. Lang F, Maugeudre D, Houssaint E, Charbonnel B, Sai P (1987) Cytoadherence of lymphocytes from type 1 diabetics to insulin-secreting cells: a marker of anti- β cell cellular immunity. *Diabetes* 36: 1356–1364
11. Segain JP, Valentin A, Bardet S, Feve B, Sevestre H, Houssaint E, Charbonnel B, Sai P (1989) In vitro relationship of CD4 cells from Type 1 diabetic patients and xenogeneic B-cell membranes. *Diabetes* 38: 634–640
12. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Weir GC, Lauris V (1980) Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet-cell tumour. *Proc Natl Acad Sci (USA)* 77: 3519–3523
13. Biedler JL, Helson L, Spengler BA (1973) Morphology and growth, tumorigenicity and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 33: 2643–2652
14. Oettgen HF, Aoki T, Old LJ (1968) Suspension culture of a pigment-producing cell line derived from a human malignant melanoma. *J Natl Cancer Inst* 41: 827–831
15. Fogh J, Wright WC, Loveless JD (1977) Absence of HeLa cell contamination in 169 cell lines derived from human tumors. *J Natl Cancer Inst* 58: 209–214
16. Hamilton TC, Young RC, McKoy WM, Grotzinger KR, Green JA, Chu EW, Whang Peng J, Rogan AM, Green WR, Ozols RF (1983) Characterization of a human ovarian carcinoma cell line (NIH: OVCAR-3) with androgen and estrogen receptors. *Cancer Res* 43: 5379–5389
17. Soule HD, Vazquez J, Long A (1973) A human cell line from a pleural effusion derived from a breast carcinoma. *J Natl Cancer Inst* 51: 1409–1416
18. Martin F, Caignard A, Jeannin JF, Leclerc A, Martin M (1983) Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. *Int J Cancer* 32: 623–627
19. Santerre RF, Cook RA, Grisel RMRD, Sharp JD, Schmidt RJ, Williams DC, Wilson CP (1981) Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic β -cells. *Proc Natl Acad Sci (USA)* 78: 4339–4343
20. Klein E, Ben-Bassat H, Neumann H, Ralph P, Zeuthen J, Polliack A, Vanky F (1976) Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. *Int J Cancer* 18: 421–424
21. Bazin H (1982) Production of rat monoclonal antibodies with the LOU rat non secreting IR983F myeloma cell line. In: Peters H (ed) *Protides of the biological fluids*. 29th colloquium 1981. Pergamon Press, Oxford, New York, p 615
22. Huang SW, Maclaren NK (1976) Insulin-dependent diabetes: a disease of autoaggression. *Science* 192: 64–66
23. Cockfield S, Ramassar V, Hurmson J, Halloran PF (1989) Multiple low dose streptozotocin induces systemic MHC expression in mice by triggering T cells to release IFN- γ . *J Immunol* 142: 1120–1128
24. Wakabayashi S, Taniguchi M, Tokuhisa T, Tomioka H, Okamoto S (1981) Cytotoxic T lymphocytes induced by syngenic mouse melanoma cells recognize human melanomas. *Nature* 294: 748–750
25. Moingeon P, Nowill A, Courtois A, Azzarone B, Motte P, Ythier A, Buhuon C, Hercend TA (1985) Target structure for a series of human cloned natural killer cell lines is recognized by both anti-TNKTar and 4F2 monoclonal antibodies. *J Immunol* 134: 2930–2934
26. Lanier LL, Phillips JH (1986) Evidence for three types of human cytotoxic lymphocyte. *Immunol Today* 7: 132–134
27. Altmann DM, Lider O, Douek DC, Cohen IR (1987) Activation of specific T cell lines by the antigens avidin and myelin basic protein in the absence of antigen-presenting cells. *Eur J Immunol* 17: 1635–1640
28. Boitard C, Chatenoud LM, Debray-Sachs M (1982) In vitro inhibition of pancreatic β cell function by lymphocytes from diabetics with associated autoimmune diseases: a T cell phenomenon. *J Immunol* 129: 2529–2531
29. Jewtougoff V, Bach MA (1988) Non-MHC restricted tissue-specific T cells recognizing autologous oligodendrocytes in the normal SJL/J mouse. *J Autoimmunity* 1: 433–444
30. Nakache M, Lakey Berg E, Streeter PR, Butcher EC (1989) The mucosal vascular addressin is a tissue-specific endothelial adhesion molecule for circulating lymphocytes. *Nature* 337: 179–181
31. Vives M, Badenas J, Foz M, Pujol-Borrel R (1989) Expression of Intercellular adhesion Molecule-1 (ICAM-1) in human islets. *Diabetes* 38 [Suppl 2]: 186 (Abstract)
32. Kolb-Bachofen V, Kolb H (1984) "Single cell infiltration" of pancreatic islets: an alternative concept of B islet-cell destruction. *Diabetologia* 27: 279 (Abstract)
33. Itoh M, Funauchi M, Sato K, Kisamori S, Fukuma N, Hirooka Y, Nihei N (1984) Abnormal lymphocyte function precedes hyperglycaemia in mice treated with multiple doses of streptozotocin. *Diabetologia* 27: 109–112
34. Bardet S, Rohmer V, Maugeudre D, Segain JP, Gallois Y, Stetieh H, Marre M, Allannic H, Charbonnel B, Sai P (1989) Lymphocytes circulants adherant in vitro à des cellules insulinosecretrices: marqueur d'immunité cellulaire chez des sujets à risque de diabète de type 1. In: "Diabète et Métabolisme", Proc Congr Association de langue française pour l'étude du diabète et des maladies métaboliques. Masson Ed, Montpellier, VI, 22 (Abstract)

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Dr. P. Sai
Laboratoire d'immunologie du diabète
Faculté de Médecine (3ème étage)
1 rue Gaston Veil
F-44035 Nantes
France