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DIABETES RESEARCH

THE SUPPRESSIVE EFFECT OF ANTI-ASIALO GM1 ANTIBODY ON LOW-DOSE STREPTOZOTOCIN-INDUCED DIABETES IN CD-1 MICE

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SUMMARY To elucidate the role of natural killer (NK) cells in the pathogenesis of diabetes in streptozotocin-induced diabetes, we examined whether treatment with anti-asialo GM1 antibody prevents the occurrence of diabetes in CD-1 mouse model. Anti-asialo GM1 antibody was injected intraperitoneally 2–3 times a week starting three days before the first streptozotocin injection. In controls, rabbit immunoglobulin or saline was injected instead of anti-asialo GM1 antibody. Three of twelve anti-asialo GM1 antibody-treated mice developed diabetes, however eight of eight (100%) rabbit immunoglobulin injected mice and 20 of 23 saline-injected mice developed diabetes. The incidence of diabetes in the anti-asialo GM1 antibody-injected group was significantly higher than in the two control groups ($p < 0.01$, $p < 0.01$, respectively). The NK-cell activities of spleen cells from anti-asialo GM1 antibody-treated mice were significantly lower than in control mice. Flowcytometry analysis demonstrated that anti-asialo GM1 antibody-positive cells had disappeared from spleens of anti-asialo GM1 antibody-injected mice but no suppression of T-lymphocytes could be demonstrated. These results suggest that NK cells play a role in the pathogenesis of streptozotocin-induced diabetes in CD-1 mice.

Key words: CD-1 mouse, streptozotocin, diabetes, NK cell, anti-asialo GM1 antibody

INTRODUCTION

LOW-DOSE STREPTOZOTOCIN-induced diabetes in mice is characterized by delayed but progressive hyperglycemia and mononuclear cell infiltration of the pancreatic islets leading to marked insulitis (1).

Immune mechanisms, especially T-cell-dependent immunity, participate in this type of diabetes since the administration of anti-lymphocyte serum with 3-O-methyl-D-glucose completely protects mice from the development of diabetes (2); T-cell deficient mice do not develop diabetes (3–5), treatment with monoclonal antibodies against L3T4 or Lyt2 T-lymphocyte subsets prevents insulitis and the development of diabetes (6), and insulitis can be transferred by spleen cell from streptozotocin-treated mice to normal athymic recipients (7). It has also been reported that macrophages may play a role in the pathogenesis of this type of diabetes (8). However, it was not evident whether NK cells exert a destructive effect on pancreatic beta cells. To clarify the role of NK cells in the pathogenesis of this type of diabetes, we administered anti-asialo GM1 antibody to streptozotocin-injected CD-1 mice and examined its effect on the development of diabetes.

MATERIALS AND METHODS

Animals and Reagents

Male CD-1 mice were obtained from Japan Charles River Inc. (Tokyo, Japan). The mice were kept under specific pathogen free conditions, and used at 9–10 weeks of age.

Streptozotocin was purchased from Sigma Co. Ltd. (St. Louis, USA). It was dissolved in 0·05 mol sodium citrate buffer, pH 4·5, 4°C and used within five minutes.

Anti-asialo GM1 antibody was purchased from Wako Pharma. Co.

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(Tokyo, Japan). One vial of anti-asialo GM1 antibody (10 mg protein) was dissolved in 3 ml of distilled water and 0·2 ml were injected intraperitoneally.

Induction of Diabetes

Mice were divided into three groups for streptozotocin treatment. All groups of mice received streptozotocin intraperitoneally at 40 mg/kg body weight for five consecutive days. In addition, anti-asialo GM1 antibody was injected intraperitoneally 2–3 times a week starting three days before the first streptozotocin injection up to 21 days after the first streptozotocin injection. In control group 1, rabbit immunoglobulin was injected intraperitoneally instead of anti-asialo GM1 antibody, and in control group 2, saline was injected as above.

Plasma Glucose Determination

Blood samples were collected from the retro-orbital vein plexus of non-fasting mice between 11 and 12 a.m., using capillary tubes. Plasma glucose values were determined by the glucose oxidase method and expressed in mg/dl. Individual mice were classified as diabetic when plasma glucose levels were over consistently above 250 mg/dl.

NK Activity Assay

Suspensions of YAC-1 cells were incubated at 37°C for 60 min in 1 ml RPMI 1640 with fetal bovine serum (FBS) and 0·05 mCi $Na_2\ ^{51}CrO_4$. After incubation, the cells were washed three times by centrifugation (450 rev for 20 min) in RPMI with FBS, and used for target cells. The suspensions of target cells were incubated at 37°C in 5% CO_2 for 5 h with effector cells. After incubation, released ^{51}Cr was counted by a well type gamma counter and specific release was calculated using the following formula:

$$\text{specific release} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100\%.$$

Spleen Cell Analysis

Ten days after the first streptozotocin injection, spleen cell analyses were performed. Spleen cells obtained from the mice were incubated with polyclonal anti-asialo GM1 for 20 min and were facilitated with goat anti-rabbit IgG. After washing three times, goat anti-rabbit IgG (Cartego, USA) was added to the cell pellets for a further 20 min of incubation on ice. After washing with PBS three times, the cells were fixed with 1% paraformaldehyde.

For dual staining of anti-L3T4 and anti-Lyt2, spleen cells were put into an antibody mixture (Fluorescence-conjugated anti-Lyt2 and biotinylated anti-L3T4, Becton Dickinson, California, USA). After 20 min of incubation on ice, cells were washed three times. PE-streptavidin (Biomeda, California, USA) was added and cells were incubated on ice for an additional 20 min. After washing with PBS three times, the cells were fixed with 1% paraformaldehyde. Profiles of stained cells were analyzed with FACSan (Becton Dickinson, California, USA). For each sample, data from 10,000 viable cells were collected.

Histological Examination

Histological analyses of pancreatic islets were performed 56 days after the first streptozotocin injection. Pancreata were removed, fixed in Bouin's solution, embedded in paraffin, then stained with hematoxilin–eosin, and examined by light microscopy.

Results

In the anti-asialo GM1 antibody-injected group, 3 of 12 mice developed diabetes (Figure 1). However, the rates of diabetes in the rabbit immunoglobulin-injected group and saline-injected group were 8/8 (100%) and 20/23 (87%), respectively (Figures 2, 3). The incidence of diabetes in anti-asialo GM1 antibody-treated mice was significantly lower than in the two control groups ($p < 0{\cdot}01$, $p < 0{\cdot}01$) respectively.

The NK-cell activities of spleen cells from streptozotocin-treated mice five days after the first streptozotocin injection are shown in Figure 4. Spleen cells from streptozotocin-injected mice without anti-asialo GM1 antibody showed a significantly higher specific lysis of YAC-1 cells than normal CD-1 mice ($p < 0{\cdot}05$). Spleen cells from streptozotocin-injected mice with anti-asialo GM1 antibody treatment exhibited a significant decrease in YAC-1 target cell lysis compared with streptozotocin-injected mice without anti-asialo GM1 antibody ($p < 0{\cdot}01$).

As to FACS analysis, anti-asialo GM1-positive cells disappeared completely in anti-asialo GM1 antibody-treated

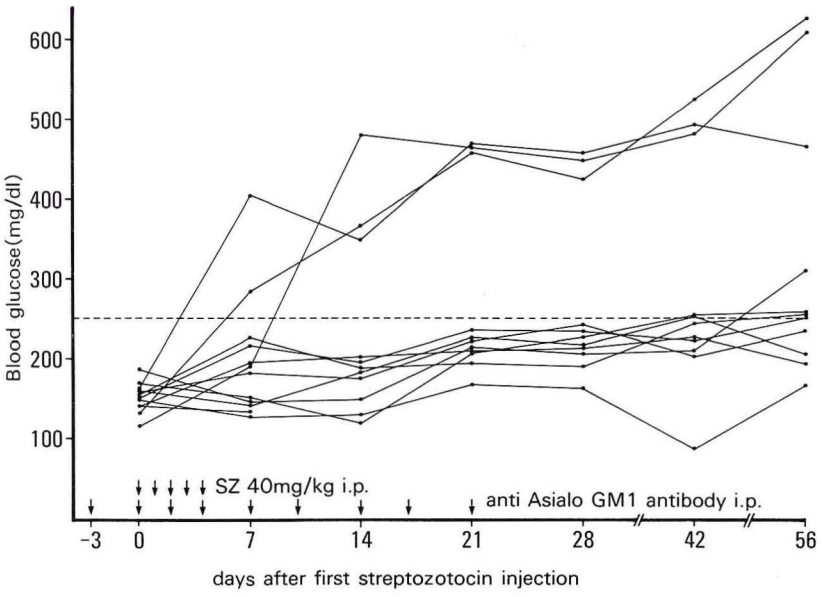

Figure 1. Blood glucose levels of low-dose streptozotocin-injected CD-1 mice with anti-asialo GM1 antibody treatment.

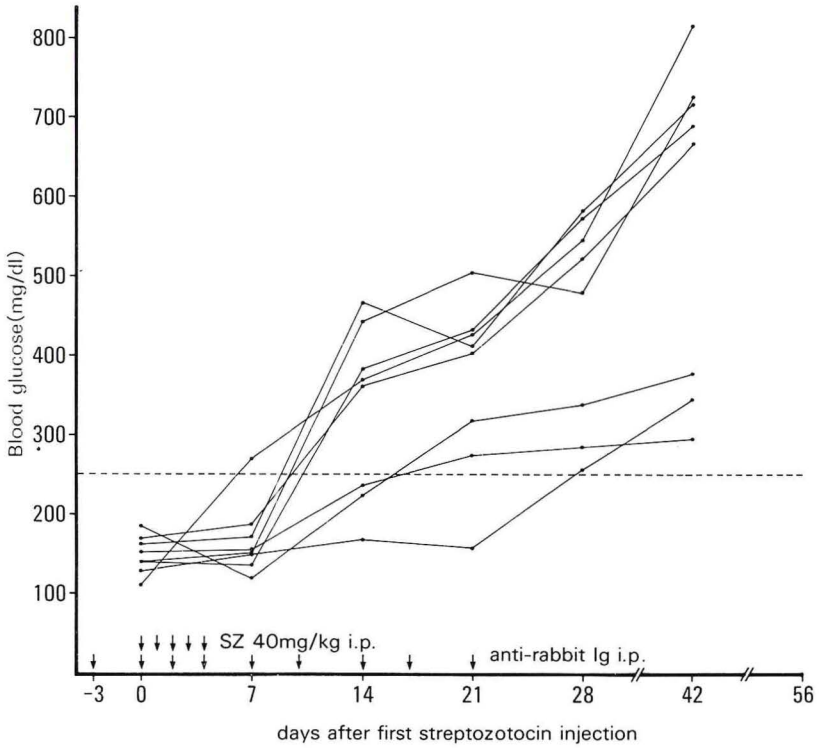

Figure 2. Blood glucose levels of low-dose streptozotocin-injected diabetic CD-1 mice with rabbit immunoglobulin treatment.

mice (Figure 5). However, there were no significant differences between the anti-asialo GM1 antibody-treated group and controls in terms of Lyt2- and L3T4-positive cells (Figure 6).

Insulitis was seen in all streptozotocin-treated groups of mice and there were no significant differences between anti-asialo GM1 antibody-treated mice and control groups in the degree of lymphocytic infiltration of islets though infiltration did appear to be lighter in anti-asialo GM1-treated mice than in control mice.

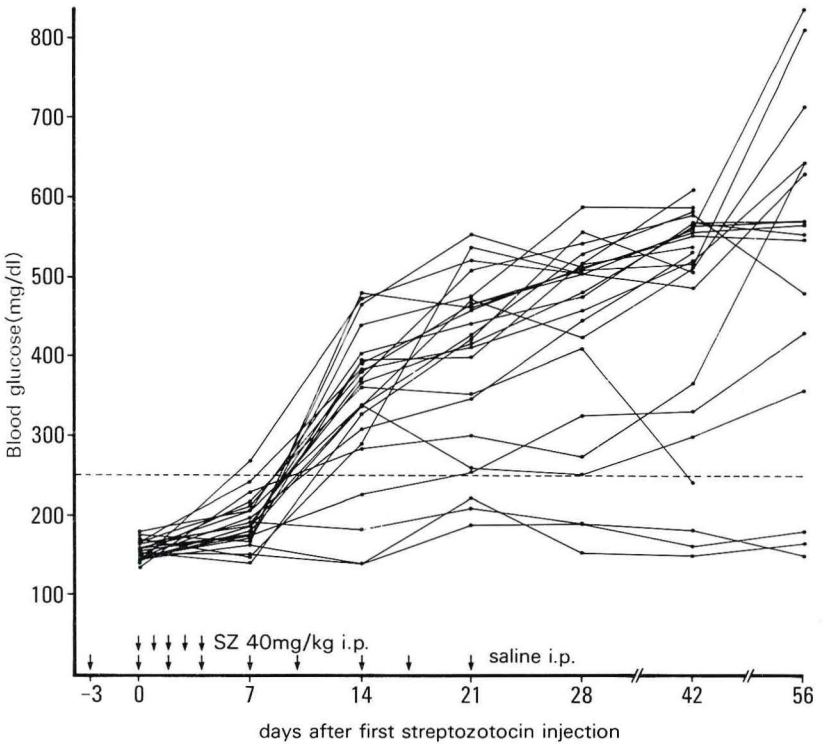

Figure 3. Blood glucose levels of low-dose streptozotocin-injected CD-1 mice with saline treatment.

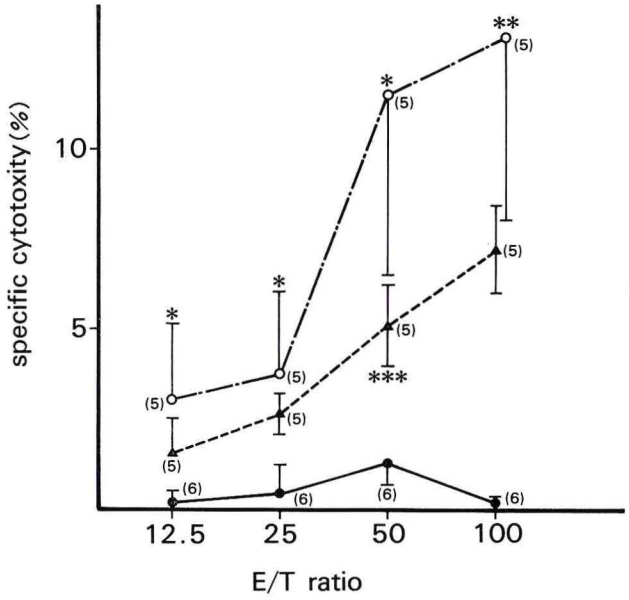

Figure 4. NK-cell activities of spleen cells from normal CD-1 mice. Streptozotocin-treated mice with and without anti-asialo GM1 antibody. ●——●: anti-asialo GM1- and streptozotocin-treated group; ○-·-·-○: saline- and streptozotocin-treated group; ▲--▲: normal CD-1 mice. Data express mean ± SD. * $p < 0.05$ compared with saline- and anti-asialo GM1 antibody-treated mice, ** $p < 0.01$ compared with saline- and streptozotocin-treated mice, *** $p < 0.05$ compared with normal CD-1 mice.

Discussion

Various immunological effector mechanisms have been proposed to play a role in the diabetogenic autoimmune response which initiates insulin-dependent diabetes mellitus. In streptozotocin-induced diabetes, T-cell mediated immunity and activated macrophages are regarded to play an important role in destroying pancreatic B cells (2–8). As to the role of NK cells in the pathogenesis of streptozotocin-induced diabetes, we have previously reported that NK-activity increased just before and after the onset of diabetes (9). In addition, the presence of NK cells in the insulitis lesion was demonstrated by Cossel *et al.* (10) but it was not clear whether the NK cells exerted a destructive effect on pancreatic beta cells. Therefore, we examined the effect of anti-asialo GM1 antibody therapy on autoimmune mechanisms in the development of streptozotocin-induced diabetes in CD-1 mice.

Our results demonstrated that anti-asialo GM1 antibody suppressed the development of streptozotocin-induced diabetes in CD-1 mice and that the NK-cell activity of spleen cells from anti-asialo GM1 antibody-treated mice and anti-asialo GM1-positive cells was significantly reduced. However, we could not find a significant reduction in the relative percentages of T-lymphocyte subsets. These results suggest that anti-asialo GM1 antibody-positive cells have a direct damaging effect on pancreatic B cells. In rodents, most anti-asialo GM1 antibody-reactive cells are NK cells whose activities are reduced by anti-asialo GM1 antibody injection (11, 12). Therefore, our results suggest that NK cells play an important role in the pathogenesis of the development of streptozotocin-induced diabetes in CD-1 mice. However, note that anti-asialo GM1 antibody is not a specific marker for NK cells. Because asialo GM1 expression has

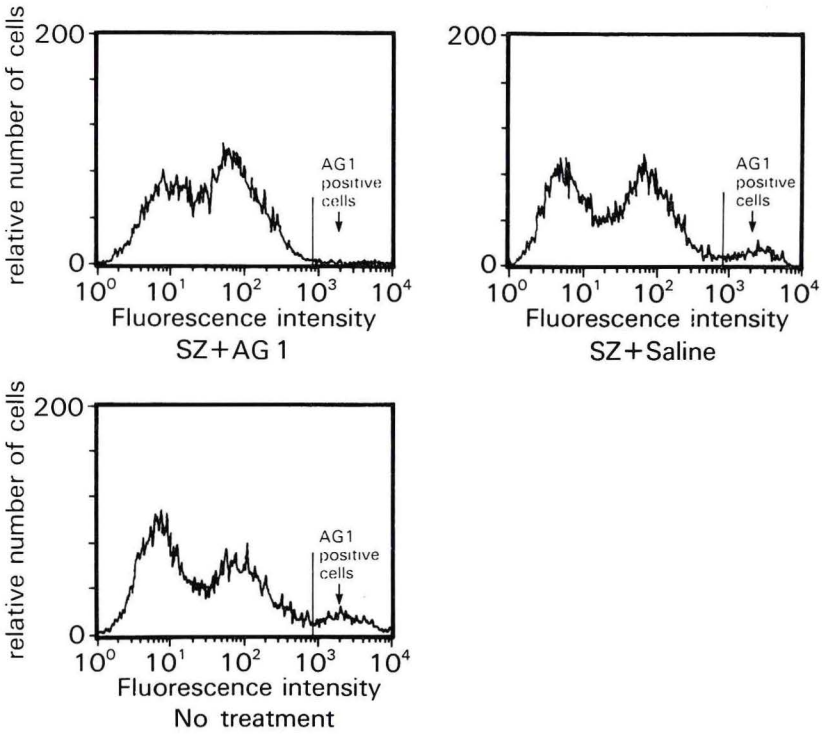

Figure 5. Flowcytometry analysis of spleen cells from streptozotocin-injected mice 10 days after the first streptozotocin injection. Anti-asialo GM1 antibody-positive cells had completely disappeared in anti-asialo GM1 antibody-treated mice.

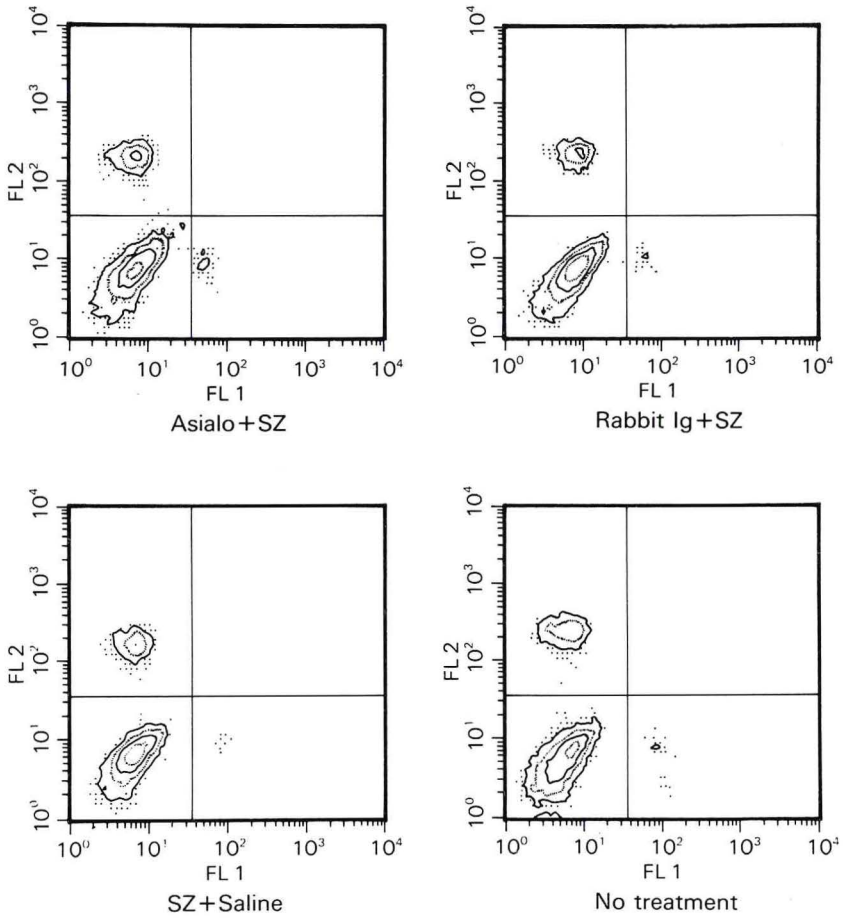

Figure 6. Flowcytometry analysis of spleen cells from streptozotocin-injected mice 10 days after the first streptozotocin injection. There were no significant differences between anti-asialo GM1 antibody-treated mice and control mice in terms of L3T4- and Lyt2-positive cells.

been found on activated peritoneal macrophages, thymocytes, BMCs, and alloimmune cytotoxic T-lymphocytes (11, 12), a direct effect on other immune effector cells can not be excluded. Further investigation of the role of NK cells in the pathogenesis of the development of streptozotocin-induced diabetes in CD-1 mice will be necessary.

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