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Preventive effect of a new immunosuppressant FK-506 on insulitis and diabetes in non-obese diabetic mice

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Summary. We investigated the effect of an immunosuppressant FK-506 on histological change of islets, the onset of diabetes, and the change of spleen cell subsets in female non-obese diabetic mice. Mice administered intraperitoneally with FK-506 from 5 to 20 weeks of age showed marked suppression of mononuclear cell infiltration (insulitis) at 10 weeks of age. Among the subsets of the spleen cells, a significant decrease in the population of Thyl.2-positive T cells (pan-T), L3T4-positive T cells (mainly helper/inducer), and Lyt2-positive T cells (mainly suppressor/cytotoxic) was observed in FK-506-treated mice. Furthermore, glucose tolerance of the mice at 15 weeks of age was clearly improved. Cumulative incidence observed up to 40 weeks of age was 86% in control mice and 23% in FK-506-treated mice ($p < 0.01$). These data indicate that FK-506 has a preventive effect on insulitis and diabetes by the suppression of cell-mediated autoimmunity in non-obese diabetic mice.

Key words: Non-obese diabetic (NOD) mouse, FK-506, insulitis, Type 1 (insulin-dependent) diabetes mellitus, immunotherapy.

Many attempts have been made to introduce remission in newly diagnosed Type 1 (insulin-dependent) diabetes mellitus including immunosuppressive therapy. A novel immunosuppressant FK-506 which belongs to a macrolide family was isolated in 1984 from a strain of streptomyces [1]. In vitro, FK-506 inhibits the production of several lymphokines including interleukin 2 (IL-2) and interferon γ, the generation of cytotoxic T cells and the expression of IL-2 receptor [2], indicating that FK-506 has a suppressive effect mainly on T lymphocyte function. The non-obese diabetic (NOD) mouse is an animal model for Type 1 diabetes mellitus, and cell-mediated autoimmunity based on the genetic background is considered to play a major role in the pathogenesis [3]. In this study, we investigated the effect of FK-506 on insulitis, the onset of diabetes, and the change of splenic mononuclear cell subsets in NOD mice.

Materials and methods

Animals

The NOD/Shi mouse colony was produced from several mice originally purchased from Clea Japan Inc. (Osaka, Japan) and maintained in a specific pathogen free condition. Urinary sugar was tested with Tes-Tape (Eli Lilly & Company, Indianapolis, Ind., USA) twice a week, and the onset of diabetes was determined when glucosuria was detected on two consecutive tests. The cumulative incidence of our colony was 95% in females and 5% in males at 40 weeks of age.

Experimental protocol

A total of 39 female animals were divided into two groups ($n = 20$/control group, $n = 19$/experimental group). In the experimental group, FK-506 (crystalline powder originally made for intramuscular or subcutaneous administration Lot 119273K, gift from Fujisawa Pharmaceutical Co. Ltd. Osaka, Japan) was suspended in 154 mmol/l NaCl at a concentration of 200 μg/ml and kept at 4 °C. Immediately after thorough mixing by hand shaking at least 50 times, the drug was injected i.p. using a 1 ml syringe at a dose of 2.0 mg · kg^{-1} body weight every other day from 5 to 20 weeks of age. FK-506 prepared as above is structurally stable and pharmacologically as well as immunologically active for at least 3 months when stored at 4 °C. In the control group, only 154 mmol/l NaCl (0.01 ml · g^{-1} body weight) was injected in the same way as the experimental group. Five weeks after the start of injection (10 weeks of age), 6 animals from each group were killed by terminal anaesthesia, and pancreas, liver and kidney were removed for histological examination after perfusion fixation with Bouin's solution. Before fixation, spleens from 5 out of 6 animals in each group were removed for the analysis of mononuclear cell subsets. At 15 and 40 weeks of age, body weight was measured in each animal and intraperitoneal glucose tolerance test (IPGTT) (1.0 g · kg^{-1} body weight) was per-

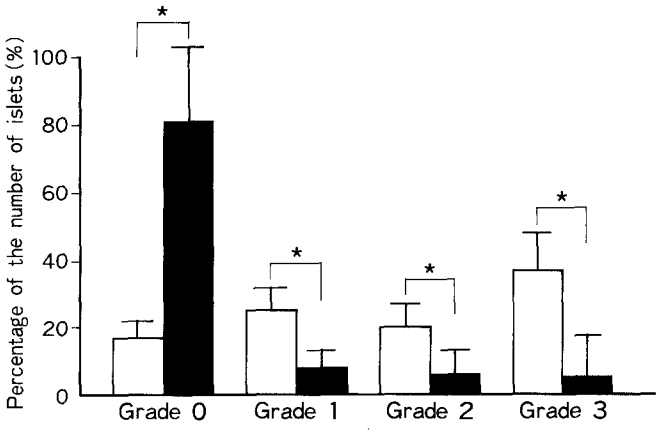

Fig. 1. Effect of FK-506 on the severity of insulitis in NOD mice. The degree of the severity is scored from grade 0 to grade 3 in each islet of control (□, $n = 6$) and FK-506-treated (■, $n = 6$) mice (10 weeks of age). Grade 0: no mononuclear cell infiltration; grade 1: infiltrating cells restricted to the periphery of the islet; grade 2: infiltrating cells occupying up to 50% of the islet area; grade 3: infiltrating cells occupying more than 50% of the islet area. Values are means ± SD. * $p < 0.01$

formed 15 h after starvation. Blood was obtained at 0 min (before load) and 60 min after glucose load, and plasma glucose was measured by Fuji Drichem system (Fuji Medical System Co., Ltd., Toyonaka, Japan). Cumulative incidence of overt diabetes was observed up to 40 weeks of age.

Histological examination

Paraffin-embedded sections stained with haematoxylin and eosin were examined. In the endocrine pancreas, 5 different sections approximately 500 μm distant from each other were prepared from each animal to evaluate the severity of insulitis. The degree of insulitis was scored as follows: grade 0, no mononuclear cell infiltration is observed; grade 1, infiltrating mononuclear cells were restricted to one pole of, or around, the islet and not seen within the islet; grade 2, mononuclear cells are present in and around the islet and occupied up to 50% of the islet area; grade 3, the infiltrated area exceeds 50% of the islet area. Fifty different islets were scored in each pancreas. For the evaluation of the drug toxicity, several sections of the liver and kidney as well as the pancreas of each animal were examined.

Preparation of spleen cell suspensions and mononuclear cell subset analysis

Spleen cell suspensions were prepared as described elsewhere [3] with minor modification. Antibodies used in this study were fluorescein isothiocyanate (FITC)-conjugated rat anti-Thyl.2 monoclonal antibody (MoAb) (Becton & Dickinson, Mountain View, Calif., USA), FITC-conjugated rat anti-Lyt2 MoAb (Becton & Dickinson), phycoerythrin-conjugated rat anti-L3T4 MoAb (Becton & Dickinson), and FITC-conjugated goat anti-mouse immunoglobulin (Cappel, Westchester, Pa, USA), for pan-T cells, cytotoxic/suppressor T cells, helper/inducer T cells, and B cells, respectively. To detect natural killer (NK) cells and macrophages, the indirect fluorescence method was applied. Antibodies were rabbit anti-asialo GM1 (Wako, Osaka, Japan) and FITC-conjugated anti-rabbit immunoglobulin (Miles, Elkhart, Ind., USA) for NK cells, and rat anti-macrophage MoAb (F4/80) (Serotec, Bicester, Oxfordshire, UK) and FITC-conjugated goat anti-rat IgG (Cappel), for macrophages. The samples were analysed on a FACScan (Becton & Dickinson).

Statistical analysis

The significance of difference between two groups was determined by analysis of variance and by Student's t test.

Results

Administration of FK-506 significantly suppressed the onset of diabetes, and cumulative incidence was 23% (3 of 13) in the FK-506-treated group and 78% (11 of 14) in the control group at 30 weeks of age ($p < 0.01$). At 40 weeks of age, the overall cumulative incidence was 23% (3 of 13) in the FK-506-treated group and 86% (12 of 14) in the control group ($p < 0.01$). Histological examination of the pancreas at 10 weeks of age revealed marked suppression of mononuclear cell infiltration in the FK-506-treated mice. In the control mice, about 40% of the islets showed severe insulitis (grade 3), and only 17% appeared intact, although degrees of insulitis varied in each animal. In contrast, about 80% of the islets in the FK-506-treated mice were intact (grade 0) (Fig. 1). Endocrine cells of islets with no infiltrate in the FK-506-treated mice showed no clear degenerative changes such as pyknosis and vacuolation. There were no morphological differences in the liver and kidney between the two groups, suggesting no apparent adverse effect of FK-506. In the population of splenic mononuclear cells, the FK-506-treated mice showed significant decreases in Thy1.2-positive T cells, L3T4-positive T cells, and Lyt2-positive T cells ($p < 0.01$) (Fig. 2). Furthermore, a decrease in L3T4/Lyt2 ratio was also observed (2.54 ± 0.20 vs 2.06 ± 0.28, $p < 0.05$, mean ± SD). The percentages of asialo GM1-positive cells or F4/80-positive cells were not different between the two groups, and the percentage of surface immunoglobulin (SIg)-posi-

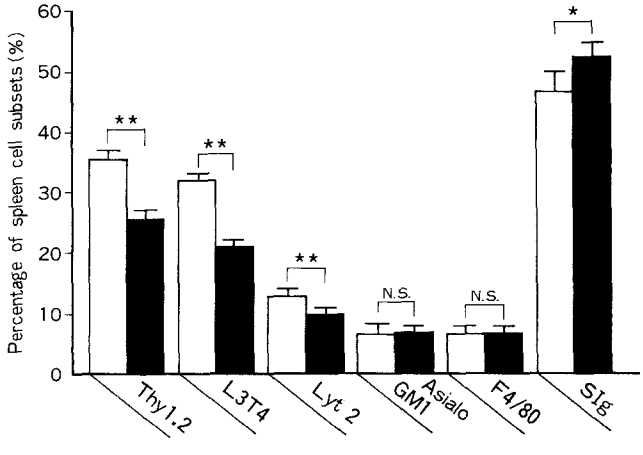

Fig. 2. Percentage of fluorescein-positive cells from spleen cells in control (□, $n = 5$), and FK-506-treated (■, $n = 5$) NOD mice (10 weeks of age). Thy 1.2: Thy 1.2-positive T (pan-T) cell, L3T4: L3T4-positive (helper/inducer) T cell, Lyt2: Lyt2-positive (suppressor/cytotoxic) T cell, Asialo GM1: asialo GM1-positive (NK) cell, F4/80: F4/80-positive cell (macrophage), SIg: surface immunoglobulin-positive (B) cell. Values are means ± SD. ** $p < 0.01$, * $p < 0.05$, NS = not significant

tive cells (B cell) was slightly but significantly elevated ($p < 0.05$) in the FK-506-treated mice.

There was no significant difference in the body weight between the FK-506-treated mice (21.1 ± 1.5 g) and the control NOD mice (22.1 ± 1.3 g) at 15 weeks of age. In the FK-506-treated mice, plasma glucose levels before and 60 min after glucose load were 3.77 ± 0.77 mmol/l and 4.49 ± 0.47 mmol/l, respectively, while in the control mice, they were 3.78 ± 1.06 mmol/l and 8.39 ± 2.25 mmol/l. Thus, plasma glucose was significantly lower in the FK-506-treated mice ($p < 0.01$) after 60 min of glucose load. At 40 weeks of age, plasma glucose levels before and 60 min after glucose load were 3.63 ± 0.92 mmol/l and 6.01 ± 1.33 mmol/l, respectively, in the FK-506-treated mice. The plasma glucose level of these mice 60 min after glucose load was significantly higher than that of 15-week-old FK-506-treated mice but significantly lower than that of 15-week-old control animals ($p < 0.01$).

Discussion

In this study, both the development of insulitis and the occurrence of spontaneous diabetes in NOD mice were significantly suppressed by the administration of FK-506. The results at 40 weeks of age clearly show that the preventive effect of FK-506 on diabetes is evident even 20 weeks after stopping the drug, although glucose tolerance slightly deteriorates. Accumulating data suggest that T cells play an important role in beta cell destruction in NOD mice, although almost all kinds of mononuclear cells including T cells, B cells, NK cells and macrophages are observed in the infiltrate in the islets of NOD mice. Among these cells, both L3T4-positive and Lyt2-positive cells appear to be essential for the process from the start of insulitis to the onset of diabetes as evidenced by in situ analysis of mononuclear cell subsets in the infiltrate, cell transfer experiment, and the successful isolation of T cell lines from the insulitis lesion [4]. These lines of evidence together with the result of the present study on the analysis of spleen cell subsets suggest that the inhibitory effect of FK-506 on both insulitis and the occurrence of diabetes may be ascribed to the suppression of the population of L3T4-positive cells and Lyt2-positive cells.

In conclusion, FK-506 has a preventive effect on insulitis and diabetes in NOD mice without apparently serious side-effect.

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