Preventive effect of Ninjin-to, a Kampo formula on autoimmune diabetes in mice induced by multiple low doses of streptozotocin

Takao KOBAYASHI,*[a] Qing-Hua SONG,[a] Tie HONG,[a] Hajime KITAMURA[b] and Jong-Chol CYONG[a]

[a] *Department of Bioregulatory Function, Graduate School of Medicine, the University of Tokyo*
[b] *Department of Pathology, Yokohama Minami Kyosai Hospital*

(*Received February 22, 1999. Accepted April 21, 1999.*)

Abstract

We investigated the effects of 6 Kampo formulae, Mao-to (MOT ; 麻黄湯), Shimbu-to (SBT ; 真武湯), Ninjin-to (NJT ; 人参湯), Shigyaku-san (SGS ; 四逆散), Keishi-to (KST ; 桂枝湯) and Shimotsu-to (SMT ; 四物湯) on autoimmune diabetes in C57BL/KsJ mice induced by multiple low doses of streptozotocin (40 mg/kg/day for 5 daily doses). Among the 6 Kampo formulae, only NJT, given as drinking water (1.0 g/kg), significantly prevented hyperglycemia in MLDSTZ-induced diabetic mice. NJT-treatment also inhibited the increase in water and food intake and decrease in body growth induced by the disease. No effect of NJT on insulitis was seen. The proportion of IFNγ-producing cells in splenic CD4$^+$ cells was significantly increased by the development of MLDSTZ-induced diabetes and decreased by NJT-treatment. Thus the protective effects of NJT on MLDSTZ-induced diabetes may be due to regulation of IFNγ production.

Key words Kampo formulae, Ninjin-to, Autoimmune diabetes, Insulin-dependent diabetes mellitus, streptozotocin, T lymphocytes, IFNγ.

Abbreviations IDDM, insulin-dependent diabetes mellitus ; KST, Keishi-to (Gui-Zhi-Tang), 桂枝湯 ; MOT, Mao-to (Ma-Huang-Tang), 麻黄湯 ; MLDSTZ, multiple low doses of streptozotocin ; NJT, Ninjin-to (Ren-Shen-Tang), 人参湯 ; PBS, phosphate buffered saline ; SBT, Shimbu-to (Zhen-Wu-Tang), 真武湯 ; SGS, Shigyaku-san (Si-Ni-San), 四逆散 ; SMT, Shimotsu-to (Si-Wu-Tang), 四物湯 ; STZ, streptozotocin.

Introduction

Insulin-dependent diabetes mellitus (IDDM) is characterized by the progressive and selective destruction of B cells in the islets of Langerhans[1] and is believed to occur as the result of a T cell-mediated autoimmune reaction.[2,3] The islets of Langerhans of diabetic patients and animals are heavily infiltrated with mononuclear cells, most of which are T cells and macrophages.[4] Infiltrated T cells include both CD4$^+$ and CD8$^+$ cells and predominantly produce Th1 (Tc1)-type cytokine, such as IFNγ, IL-2[5,6] and TNF-α.[7] The role of these cytokines in the development of diabetes may be due either to a direct toxic effect on B cells[8] or to their function in regulating the differentiation and function of T cells.

Streptozotocin (STZ) is widely used experimentally to induce diabetes in animals.[9] A single high dose (150-250 mg/kg) of STZ rapidly induces hyperglycemia in all strains of mice due to its direct toxic effects on B cells. On the other hand, multiple low doses of STZ (MLDSTZ), injected at a dosage of 40 mg/kg/day in 5 daily doses, induces T cell-mediated autoimmune diabetes in certain strains of mice.[10,11]

We have focused on the 6 Kampo formulae, Mao-to, Shimbu-to, Ninjin-to, Shigyaku-san, Keishi-to and Shimotsu-to, which differ in their uses and contain

*〒113-8655 東京都文京区本郷7-3-1
東京大学医学部生体防御機能学講座 小林崇雄
7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

relatively fewer kinds of herbs, and have compared the pharmacological activity of these 6 formulae to examine the specificity of the actions of Kampo formulae.[12-14] In these studies, we have shown that Ninjin-to and Shigyaku-san improve the abnormalities in intrathymic T lymphocytes in the models for climacteric disorder (ovariectomized mice) and autoimmune diseases (MRL/Mp-*lpr*/*lpr* mice).[12] On the other hand, we have also revealed that Mao-to shows an antihyperglycemic effect on diabetes in mice induced by a single high dose of STZ.[13,14] In this study, we investigated the effects of the 6 Kampo formulae on MLDSTZ-induced autoimmune diabetic mice.

Materials and Methods

Animals and Treatment : C57BL/KsJ-+*m*/+*m* Jcl (H-2K^d) male mice were obtained from Clea Japan, Inc. (Tokyo, Japan) and used at 8 weeks of age. The diabetic controls and the experimental mice received intraperitonial injections of STZ (Sigma) at a dose of 40 mg/kg in citrate buffer, pH 4.5, in 5 daily doses.[10,11] The normal and diabetic control mice were provided with tap water *ad libitum* throughout the experiment. The experimental mice were given extracts (kindly provided by Tsumura, Co.,Tokyo, Japan) of Kampo formulae (Table I) of Mao-to (MOT, TJ-27), Shimbu-to (SBT, TJ-30), Ninjin-to (NJT, TJ-32), Shigyaku-san (SGS, TJ-35), Keishi-to (KST, TJ-45) and Shimotsu-to (SMT, TJ-71) at a concentration of 0.5 % in drinking water, beginning on the day of the first dose of STZ.

Blood glucose levels : Whole blood samples were collected from tail veins at 9 : 00-10 : 00 on days 0, 7, 14, 21 and 28 of treatment. On bleeding, the blood glucose levels were immediately determined by the glucose dehydrogenase method on a portable glucometer (Advantege ; Yamanouchi, Tokyo, Japan).

Water and food intake : Water and food intake was estimated from the differences in the weight of water bottles and food cases after 2-3 consecutive days in each cage.

Insulitis : On day 29 of treatment, mice were killed by decapitation under light ether anesthesia and the pancreas was removed. Pancreatic tissue in 10 % buffed formaline was embedded in paraffin, sliced at 6 μm and stained with hematoxylin and eosin. The inflammatory lesions in the islets were graded by J.C. Cyong without knowledge of the identity of the sample. Insulitis was graded as follows : 0, no intraislet cellular infiltrates ; 1, few intraislet mononuclear cells but with preservation of islet architecture ; 2, copious intraislet inflammatory cells with or without loss of islet architecture.

Antibodies : Antibodies used in this study were hamster anti-mouse CD3-ε IgG (145-2C11), hamster anti-mouse CD28 IgG (37.51), Çy-Chrome (CC)-conjugated rat anti-mouse CD4 IgG2a (RM4-5), fluorescein isothiocyanate (FITC)-conjugated rat anti-

Table I Prescription of Kampo formulae

Formulae	Herbs contained	Ratio(g)
Mao-to(TJ-27)	Glycyrrhiza Root	1.5
Formula ephedrae	Apricot Kernel	5.0
lot# : 240027010	Cinnamon Bark	4.0
	Ephedra Herb	5.0
Shimbu-to(TJ-30)	Peony Root	3.0
Formula	Ginger Rhizome	1.5
divinitatis nigrae	Atractylodes Lancea Rhizome	3.0
lot# : 250030010	Hoelen	4.0
	Heat Processed Aconite Tuber Powder	0.5
Ninjin-to(TJ-32)	Atractylodes Lancea Rhizome	3.0
Formula ginseng	Glycyrrhiza Root	3.0
lot# : 920032001PO	Ginseng Root	3.0
	Dried Ginger Rhizome	3.0
Shigyaku-san(TJ-35)	Bupleurum Root	5.0
Pulvis quadri-frigorium	Peony Root	4.0
lot# : 250035010	Immature Orange	2.0
	Glycyrrhiza Root	1.5
Keishi-to(TJ-45)	Peony Root	4.0
Formula cinnamomi	Ginger Rhizome	1.5
lot# : 230045010	Cinnamon Bark	4.0
	Glycyrrhiza Root	2.0
	Jujube Fruit	4.0
Shimotsu-to(TJ-71)	Peony Root	3.0
Formula quadri-	Rehmannia Root	3.0
medicamentorum	Japanese Angelica Root	3.0
lot# : 250071020	Cnidium Rhizome	3.0

mouse IFN γ IgG1 (XMG1.2) and R-phycoerythrin (PE)-conjugated rat anti-mouse IL-4 IgG2b (BVD4-1D11) monoclonal antibodies (Pharmingen, San Diego, CA, USA).

Cytokine expression of stimulated CD4$^+$ cells : Spleen cells (2×10^6/ml) were stimulated for 24 h with anti-CD3 and anti-CD28 antibodies (2 and 2 μg/ml) and were cultured for the final 5 h in a medium containing 3 μM monensin (Sigma). They were stained with 0.5 μg of CC-conjugated anti-CD4, fixed, permeabilized and subsequently stained with 0.1 μg of FITC-conjugated anti-IFNγ and PE-conjugated anti-IL-4 antibodies by using a cell fixation/ permeabilization kit (Cytofix/Cytoperm® Pharmingen) according to the manufactures' instructions. Fluorescence-activated cells were analyzed by an EPICS XL flow cytometer (Coulter Cytometry Co., Hialeah, FL, USA). A fluorescence histogram of at least 5,000 counts was collected for each sample.

Results

Blood glucose levels (Fig. 1a-f)

In the controls, blood glucose levels gradually increased and hyperglycemia developed 2 weeks after the first dose of STZ. The NJT-treated group was significantly lower than the controls in blood glucose levels on days 14 and 21 (Fig. 1c). The level was also significantly lower in the SGS- and KST-treated groups on day 14 of treatment (Fig. 1d,e).

Water and food intake

Before the onset of diabetes (day 0-6), the water intake was little different among all groups except the MOT-treated group, in which it was lower than the controls. The water intake in the diabetic controls increased with the development of hyperglycemia and appeared to be higher than in the normal controls (Fig. 2a). NJT-treatment clearly inhibited this increase in the water intake. The water intake in the MOT-treated group was much lower than in the diabetic controls throughout the experiment, and lower than in the normal controls until 3 weeks of treatment. Doses (g/kg/day) of Kampo medicines calculated from water intake were MOT 0.8, SBT 2.0, NJT 1.0, SGS 2.1, KST 1.8 and SMT 1.8. Food intake generally showed a trend similar to water intake (Fig. 2b).

Body weight change (Fig. 3a)

Body weight in the diabetic controls did not increase throughout the experiment and was significantly lower on days 14, 21 and 28 of treatment than in the normal controls, in which an increase in weight

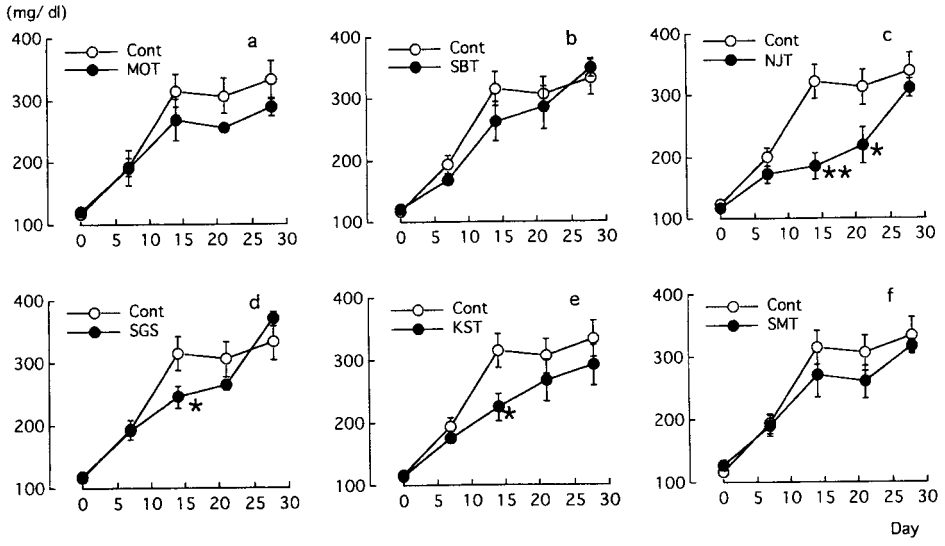

Fig. 1 Effects of Kampo formulae on blood glucose levels in autoimmune diabetic mice induced by multiple low doses of streptozotocin (MLDSTZ) (Mean ± S.E.M.). *or **Significantly different from the diabetic control group at $p<0.05$ or 0.01, respectively. Cont : Diabetic control, MOT : Mao-to, 麻黄湯 (a), SBT : Shimbu-to, 真武湯 (b), NJT : Ninjin-to, 人参湯 (c), SGS : Shigyaku-san, 四逆散 (d), KST : Keishi-to, 桂枝湯 (e), SMT : Shimotsu-to, 四物湯 (f).

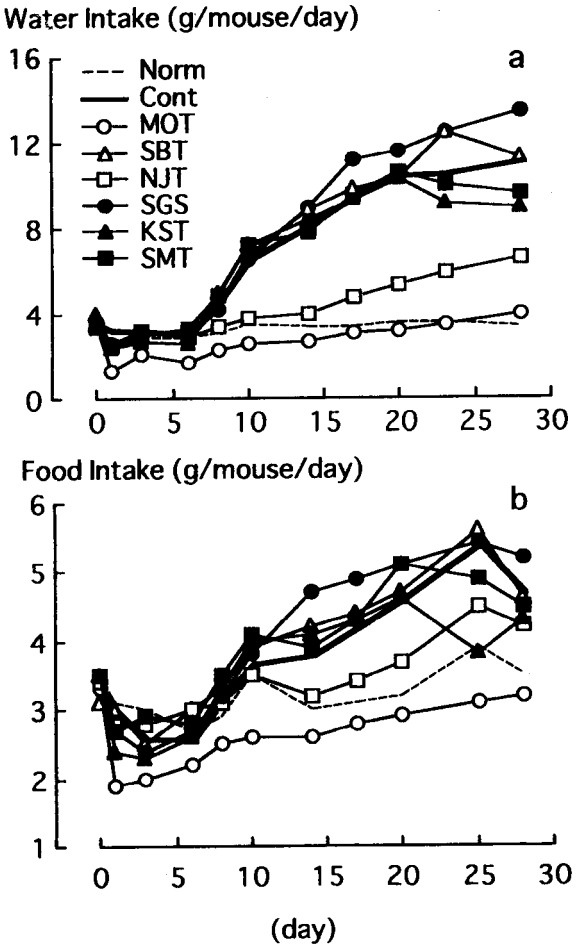

Fig. 2 Effects of Kampo formulae on water (a) and food (b) intake in MLDSTZ-induced diabetic mice. Norm: Normal control, Cont: Diabetic control, MOT: Mao-to, 麻黄湯, SBT: Shimbu-to, 真武湯, NJT: Ninjin-to, 人参湯, SGS: Shigyaku-san, 四逆散, KST: Keishi-to, 桂枝湯, SMT: Shimotsu-to, 四物湯.

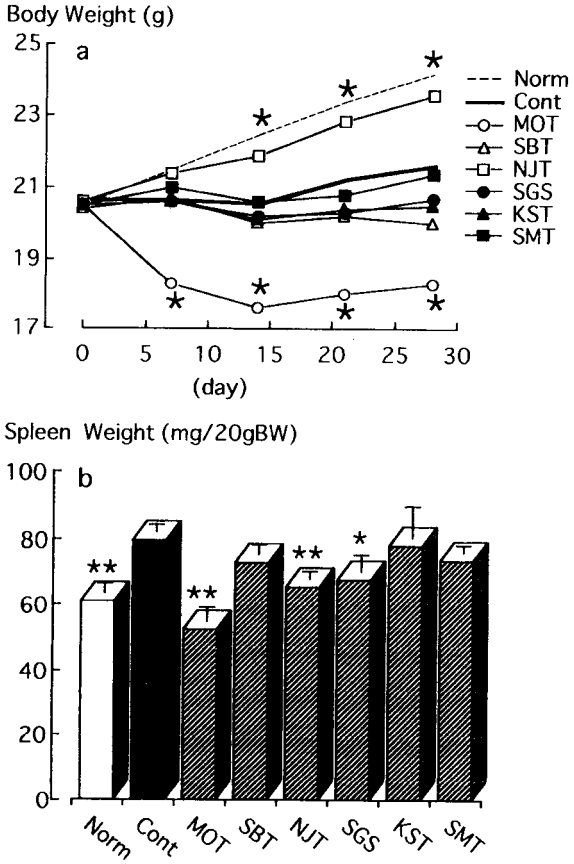

Fig. 3 Effects of Kampo formulae on weights of body (a) and spleen (b) in MLDSTZ-induced diabetic mice. *or **Significantly different from the diabetic control group at $p<0.05$ or 0.01, respectively. Norm: Normal control, Cont: Diabetic control, MOT: Mao-to, 麻黄湯, SBT: Shimbu-to, 真武湯, NJT: Ninjin-to, 人参湯, SGS: Shigyaku-san, 四逆散, KST: Keishi-to, 桂枝湯, SMT: Shimotsu-to, 四物湯.

was observed. Compared to the diabetic controls, body weight in the NJT-treated group appeared to increase and showed similar levels to the normal controls. However, the difference in body weight between the diabetic controls and the NJT-treated group was not statistically significant. On the other hand, MOT-treated mice showed siginificantly lower body weight than the diabetic controls on days 7, 14, 21 and 28 of treatment.

Spleen weight (Fig. 3b)

The spleen weight of diabetic controls was significantly higher than that of normal controls, while in the MOT-, NJT- and SGS-treated groups, significant decreases in spleen weight were observed, compared to the diabetic controls.

Insulitis (Table II)

The effect of NJT, which was regarded as the most effective of the Kampo formulae in the present study for the inhibition of development of MLDSTZ-induced diabetes, on insulitis was determiced. However, the scores for insulitis in the NJT-treated group did not differ from those of the diabetic controls.

Cytokine expression of stimulated CD4⁺ cells (Fig.4)

In the diabetic controls, IFNγ^+IL-4$^-$cells were significantly increased, compared to the normal controls. In the MOT-, NJT- and SGS-treated groups, significant decreases in IFNγ^+IL-4$^-$cells were observed. On the other hand, IFNγ^-IL-4$^+$cells did not change significnatly in MLDSTZ-induced diabetes,

Table II Effects of Kampo formulae on development of insulitis in MLDSTZ-induced diabetic mice.

Group	No.of estimates		Grade of Insulitis[a]		
	Mice	Islets	0	1	2
Normal Control	6	51	100 % (51/51)	0 % (0/51)	0 % (0/51)
Diabetic Control	6	45	0 % (0/45)	56 % (25/45)	44 % (20/45)
NJT-treatment	6	39	0 % (0/39)	56 % (22/39)	44 % (17/39)

[a]Insulitis was graded as follows: 0, no intraislet cellular infiltrates ; 1, few intraislet mononuclear cells but with preservation of islet architecture ; 2, copious intraislet inflammatory cells with or without loss of islet architecture. The data are presented as percentages (number of islets in each category/total No. of islets estimated).

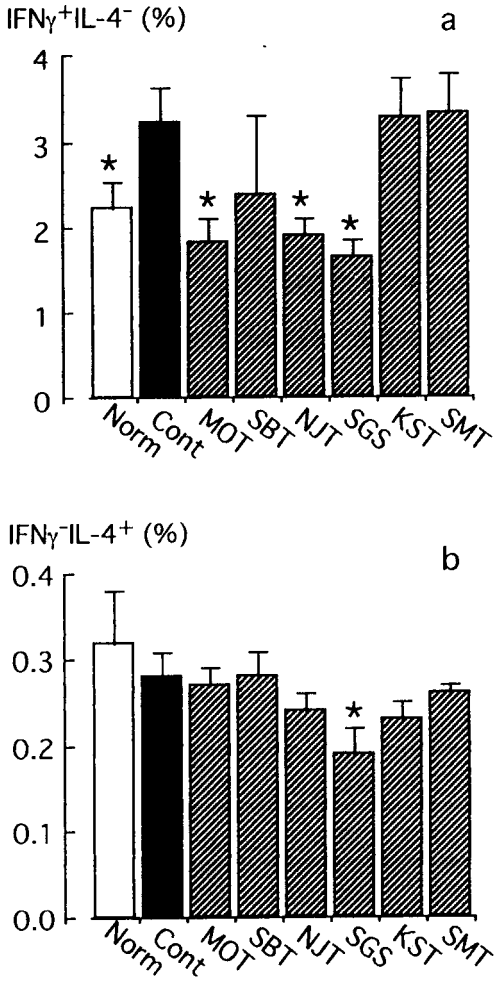

Fig. 4 Effects of Kampo formulae on IFNγ and IL-4 production of CD4+ T cells stimulated by anti-CD3/CD28 antibody in MLDSTZ-induced autoimmune diabetic mice (Mean ± S.E.M.). *Significantly different from the control at $p<0.05$. Norm: Normal control, Cont : Diabetic control, MOT : Mao-to, 麻黄湯, SBT : Shimbu-to, 真武湯, NJT: Ninjin-to, 人参湯, SGS: Shigyaku-san, 四逆散, KST : Keishi-to, 桂枝湯, SMT : Shimotsu-to, 四物湯.

while IFNγ-IL-4+cells in the SGS-treated group were significantly lower than in the controls.

Discussion

In this study, we have shown that the ingestion of NJT can prevent or delay the progression of hyperglycemia in MLDSTZ-induced diabetic mice. Blood glucose levels in NJT-treated group were significantly lower than the diabetic controls on days 14 and 21, however, the difference was not observed on day 28. Thus, the effect of NJT was not enough to inhibit the disease completely. In NJT-treated mice, though the increases in water and food intake were inhibited, body growth was enhanced in comparison with the diabetic controls and was almost the same as that of normal animals. These data show that the ingestion of NJT inhibited the pathological changes elicited by MLDSTZ-induced diabetes by preventing the development of the disease. On the other hand, MOT, inhibiting the water and food intake, also caused a more severe inhibition of body growth than that seen in the diabetic controls and showed little antihyperglycemic effect. Thus the effects of MOT on water and food intake are not specific for the prevention of diabetes and are clearly different from those of NJT. We previously found that the ingestion of MOT was followed by a marked suppression of hyperglycemia in mice with diabetes induced by a single high dose (200 mg/kg) of STZ, while NJT had no effect.[13,14] Thus it is suggested that NJT inhibits the process of development of progression of the disease, rather than the

hyperglycemia itself. We have also showed that NJT reduces the abnormalities in intrathymic T lymphocytes in the models for climacteric disorder (ovariectomized mice) and autoimmune diseases (MRL/Mp-*lpr*/*lpr* mice).[12] Since NJT may possess some immunoregulatory effects, it is possible that NJT inhibits MLDSTZ-induced diabetes by suppressing the process of autoimmune reaction.

STZ is a chemical believed to be selectively toxic to the pancreatic B-cells.[9] However, we have observed that a single high dose (200 mg/kg) of STZ induced a marked involution of lymphoid organs, such as the thymus and spleen (unpublished). On the other hand, enlargement of lymphoid organs was observed in many autoimmune model animals. In the present study, MLDSTZ (40 mg/kg/day×5)-induced diabetic mice were rather higher in spleen weight than normal mice. Further, in the NJT-treated group, significant decreases in spleen weight were observed. Thus these data may show that MLDSTZ-induced diabetes was mediated by the immune system and was inhibited by NJT with some immunoregulatory effects.

The islets of Langerhans of diabetic patient and animals are heavily infiltrated with mononuclear cells, most of which are T cells and macrophages (insulitis).[4] In this study, insulitis was also recognized after hyperglycemia had developed, but no effect of NJT on this was observed. The sequential study of NOD mice has shown that this infiltrate appears several weeks before the onset of diabetes.[15] The effect of NJT on insulitis might have been clarified if insulitis had been determined at an earlier phase, before hyperglycemia had developed. It has recently been demonstrated that the administration of recombinant IL-4 (rIL-4) prevents autoimmune diabetes but enhances pancreatic insulitis in NOD mice.[16] The authors concluded that the administration of rIL-4 might facilitate the development of Th2-like autoreactive T cells in the islets in NOD mice. Thus it was thought that NJT did not affect the infiltration but the function of the lymphocytes.

Mouse $CD4^+$ T cells can be divided into two categories on the basis of their production of IFNγ and IL-4[17,18] : Th1 cells secrete IFNγ and IL-2, whereas Th2 cells, in contrast, secrete cytokines such as IL-4 and IL-10. In autoimmune diabetes, islet-directed autoimmune reactions are thought to involve a Th1 rather than Th2 response,[8] because treatment with anti-IFNγ monoclonal antibody[19,20] and systemic administration of IL-4[16,21] and IL-10[22] have been found to prevent diabetes in NOD mice. IFNγ also enhances autoimmune, MLDSTZ-induced diabetes in A/J mice.[23,24] Moreover, monoclonal antibody against IFNγ, but not against IL-4, prevents hyperglycemia and insulitis in MLDSTZ-induced diabetes in C57BL/KsJ mice.[25] These data have been suggested that the diabetogenic autoimmune process might be aborted by inducing a shift in the relevant autoimmune T cell activity from the Th1 to the Th2 type.[26] In the present study, it was revealed that the proportion of IFNγ-producing cells in the $CD4^+$ cells was significantly increased by the development of MLDSTZ-induced diabetes and decreased by NJT-treatment, which prevented the disease. These data suggest that the protective effects of NJT on MLDSTZ-induced diabetes may be due to regulation of IFNγ production. However, significant decreases in IFNγ^+ cells were also observed in the MOT- and SGS-treated groups, in which development of the disease was not inhibited. In the SGS-treated group, not only IFNγ^+IL-4^-cells, but also IFNγ^-IL-4^+cells were decreased, but the effects of MOT on these parameters were similar to those of NJT. Thus not all of the mechanisms can be explained in this way.

In conclusion, present data suggest that NJT can prevent autoimmune diabetes in mice induced by MLDSTZ and that the protective effects of NJT may be due to regulation of IFNγ production, at least in part.

和文抄録

C57BL/KsJ マウスに streptozotocin を少量漸回投与 (40 mg/kg/day, 1日1回連続5日間) することによって誘発される自己免疫糖尿病に対する麻黄湯，真武湯，人参湯，四逆散，桂枝湯および四物湯の影響を検討した。6種の漢方方剤のうち人参湯 (1.0g/kg，飲水として自由摂取) のみが高血糖の発症を有意に抑制した。人参湯は自己免疫糖尿病の発症にともなう摂餌・摂水量の増加および体重の減少を抑制したが，膵島炎に対する影響は認められなかった。脾臓 $CD4^+$ 中の IFNγ 産生細胞の割合は糖尿対照群で正常群よりも有意に高かったが，人参湯

の投与により有意に減少した。人参湯はIFNγの産生を調節することにより，自己免疫糖尿病を予防する可能性が示唆された。

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