IMMUNOLOGIC PREVENTION OF IDDM BY ORAL ADMINISTRATION OF GLUTAMIC ACID DECARBOXYLASE

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Abstract- - We have investigated the preventive effects of oral administration of isolated E.coli glutamic acid decarboxylase (GAD) in animal models. Based on our results, the blood glucose levels were reduced by oral administration of GAD to rats 14 days before intraperitoneal injections of streptozocin (40 mg/kg on five consecutive days). On the other hand, oral administration of GAD to rats before streptozocin treatment significantly (P< 0.05) reduced the levels of GAD - specific antibodies and improved the in vitro proliferative responses of splenocytes to Con A. These data demonstrate that oral GAD administration probably generates active cellular mechanisms that suppress the disease and raise the possibility of using E.coli GAD as a new means for the prevention of autoimmune diabetes.

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INTRODUCTION

Type I diabetes or insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that results from the destruction of insulin-producing β cells in the islets of Langerhans (1). Several autoantigens have been reported to become the target for immune recognition and the selective destruction of β cells (2). Antibodies against islet-associated antigens i.e. anti-islet cell antibodies, antibodies to insulin, carboxypeptidase - H, GAD_{65} and GAD_{67} (2,3) have been detected before the onset of IDDM in patients.

Glutamic acid decarboxylase (GAD) catalyses the synthesis of the inhibitory neurotransmitter gamma - aminobutyric acid (GABA). Two isoforms of GAD, GAD_{65} and GAD_{67}, have been detected in mammalian central nervous system (4). In rat and human pancreatic islets GAD_{65} predominates (5). It has been reported that perturbation of the GABA network and up-regulation of GAD in β cells is implicated in the pathogenesis of IDDM (6). Furthermore, it has been shown in a number of systems, that oral administration of an autoantigen can downregulate experimental autoimmune disease (7,8,9). Studies of rodent models have shown that the completion of β cell destruction can be considerably delayed or prevented by parenteral administration of GAD_{65} peptides (10), GAD_{67} (11) and nasal administration of GAD_{65} peptides (12). Oral tolerance as a means to prevent or treat diabetes is especially attractive because of its virtual lack of toxicity and its inherent clinical applicability (13).

Due to the structural homology between mammalian and bacterial GAD (14), we aimed to investigate the preventive effects of the isolated E.coli GAD in rats made diabetic with multiple low doses of streptozotocin (STZ)(15).

MATERIALS AND METHODS

Animals

Sprague-Dawley female rats, aged 150-200 days were housed under conventional conditions and were allowed free access to food and water.

STZ injections

STZ (Sigma, USA) 40mg/kg was dissolved in 0.05M sodium citrate buffer, pH = 4.5, just before use and injected intraperitoneally for five consecutive days. Control animals received only citrate buffer (15). The blood samples were collected at 14 days intervals from the retroorbital sinus at 7-9 a.m. under non-fasting conditions. The serum glucose levels were determined by Autoanalyser (Clinical System, Sweden). Hyperglycemia was defined as the serum glucose level exceeding 11.1 mM (15).

Oral administration of GAD

GAD was prepared from E.coli strain ATCC 8739 (16) (Scientific and Industrial Research Organization, Karaj, Iran), accodring to the method of Young and Metzler (17). Female rats were fed 1mg of bacterial GAD twice weekly for two weeks, before STZ injections. GAD was dissolved in 0.5ml of PBS, pH = 7.4 and administered orally through a syringe fitted with a ball - type feeding needle.

Antibody measurement

GAD-specific antibodies were detected by means of a Dot Immuno Binding Assay (DIBA)(18). Antigen solutions (1 mg/ml in TBS: 10 mM Tris-HCl containing 0.15 M NaCl, pH = 7.6) were dotted into each nitrocellulose disk (1 μl) in the wells of flat-bottomed 96-well plates (Nunc, Denmark) and allowed to dry at 37 °C for 30 minutes. The disks were then incubated in the blocking buffer containing 1% bovine serum albumin (BSA, fraction V, Fluka, Switzerland) in TBS at RT for 30 minute. After washing with 0.02% Tween 20 in TBS (TBS-Tween) together with gentle shaking, serial dilutions of serum (1:100, 1:200, ...) were added to wells and incubated at 37 ° C for 2 hrs. After washing, anti-rat Ig-AP, Fab fragments (Bohringer Mannheim Biochemica, Germany) diluted 1:1000 in TBS, was added to wells and incubated at 37 °C for 30 minutes. It was then washed and covered with colour development solution (BCIP-NBT, Sigma) and incubated in the dark. Positive reactions appeared as purple spots after 5 to 10 minute of incubation. The wells were then washed with dH_2O. The last well with a well-defined purple spot was regarded as positive.

Proliferation of splenocytes in response to concanavalin A (Con A)

Spleens of rats were removed in Rosewell Park Memorial Institute 1640 medium (RPMI 1640, Sigma, USA) and splenocytes were flushed. Erythrocytes were lysed by ammonium chloride (0.16 mol/lit, 5 min, 4 °C). The cell suspension was washed and resuspended in RPMI 1640. Viable nucleated cells were evaluated using trypan blue exclusion. Splenocytes were aliquoted into round-bottomed 96-well microtiter plates (Nunc, Denmark) at 5 × 10^5 in a final volume of 0.2 ml Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA), 10% FCS (Sigma, USA), 200mM L-glutamine (Sigma, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Con A (Sigma, USA) was added at a final concentration of 2.5 μg/ml. Plates were incubated at 37C in 5% CO_2 for 3 days and pulsed by adding 1 μCi H^3 - thymidine for the final 18 hrs. Cells were then harvested on filter papers. The harvested cells were counted using 2 ml of scintillation fluid. Data were obtained from the mean values of triplicate wells and results were expressed as the stimulation index (SI), i.e. mean count with Con A/ mean basal count × 100 (19).

Statistical analysis

The statistical significance of the results was evaluated by Student's t-test, $P < 0.05$ being considered significant. Other data are presented as mean values ± SD.

RESULTS

The severity of diabetes in rats administered GAD before STZ - injections was compared with that of healthy control and also diabetic rats (treated only with STZ). Ten rats were used in each group.

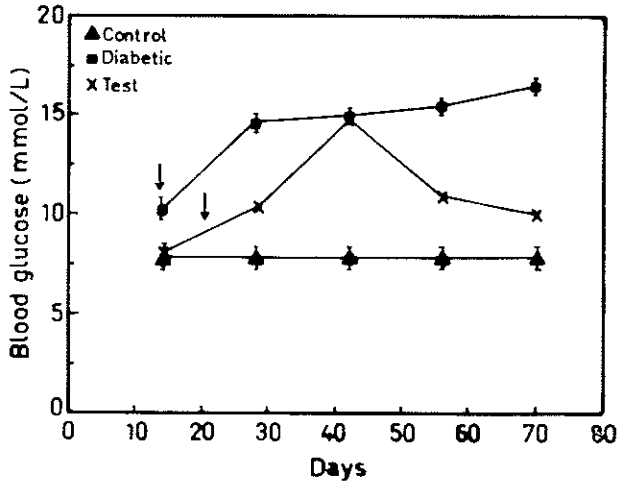

Fig. 1. Effects of orally administered GAD on blood glucose levels. Groups of 10 rats were administered GAD before STZ - injections. STZ injections are shown by arrows. Blood glucose levels were tested in individual rats at the indicated time intervals. Results are mean ± SD of each group.

The results of blood glucose measurement are shown in Figure 1. Control animals showed normal glycemic values, while STZ induces a notable hyperglycemia. There was significant difference ($P<0.05$) between the blood glucose levels in STZ treated diabetic rats (14.82 ± 0.11) and healthy control animals (7.86 ± 0.41) at day 28. Glycemia increased in diabetic rats up to 16.5 ± 0.13 at day 70. Oral administration of GAD, fourteen days before STZ-injections in test group animals, reduced blood glucose levels after 55 days of feeding by almost 27%.

Fourteen days after STZ - injections GAD - specific antibodies were determined using a DIBA and the results were represented as the Geometrical Mean of Reversed Titer (GMRT) ± SD in each group (Fig. 2). There was significant difference ($P<0.05$) between the levels of GAD-specific antibodies in diabetic and healthy control animals (3.41 ± 0.14 versus 2.06 ± 0.04). Oral administration of GAD before STZ - injections in test group animals caused a significant reduction ($P< 0.05$) in the levels of GAD specific antibodies (2.36 ± 0.22) in comparison with those of the diabetic rats (3.41 ± 0.14).

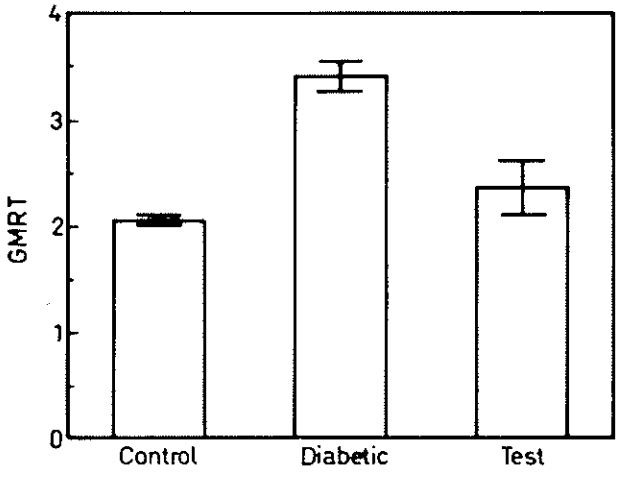

Fig. 2- Effect of oral administration of GAD on the levels of GAD - specific antibodies. Groups of 10 rats were administered GAD before STZ - injections. Fourteen days after STZ-injections, blood samples were taken from the rats and the individual sera were tested for antibody titers to E.coli GAD using a Dot-Immuno Binding Assay. Control rats had background levels of GAD antibodies. The data are presented as the geometrical mean of reveresed titer (GMRT) ± SD in each group.

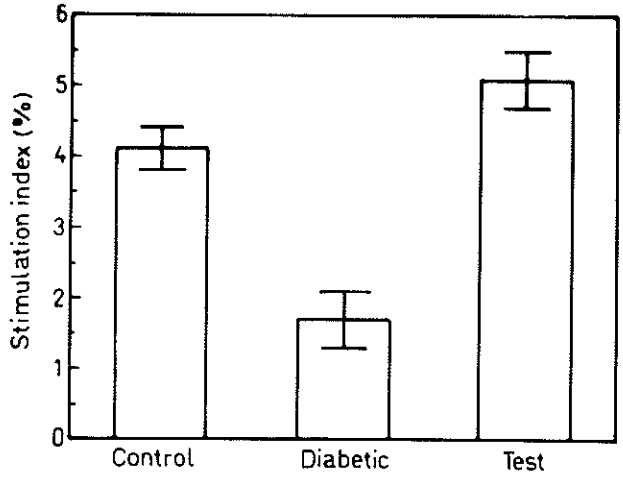

Fig. 3- Effects of oral administration of GAD on in vitro splenocyte responsiveness to Con A. Groups of 10 rats were administered GAD before STZ - injections. The proliferative responses of individual spleens were measured. Columns represent the mean stimulation indices ± SD of H^3 - thymidine uptakes in each group to in vitro challenge with Con A.

The in vitro proliferative responses of splenocytes to Con A in both control and experimental rats were compared. According to Fig. 3, the proliferative responses in diabetic rats were significantly reduced ($P<0.05$) in comparison with those of the control healthy rats (SIs = 1.7 ± 0.4 versus 4.1 ± 0.3). There was significant difference ($P<0.05$) between the in vitro proliferative responses to Con A in rats administered orally GAD before STZ - injections (SIs = 5.1 ± 0.4) in comparison with those of the diabetic rats (SIs = 1.7 ± 0.4).

DISCUSSION

It has been reported that GAD is a key autoantigen in the early stages of type 1 diabetes in human (2,3) and rodents (20). If autoreactivity to GAD is an early event in the pathogenesis of STZ induced diabetes, orally administered GAD should affect the disease process. Our study demonstrates that oral administration of E.coli GAD before STZ-injections reduces the severity of diabetes. This reduction was accompanied by low levels of blood glucose and GAD-specific antibodies and also improvement of in vitro proliferative responses of splenocytes to Con A in the treated animals. Our findings are in agreement with other investigations. Immunization of young NOD mice with human GAD_{65} (21,22), GAD_{67} (11) and GAD_{65} peptides (10) reduces the incidence of diabetes.

In this study, we have observed the reduction of GAD - specific antibodies in rats administered orally GAD before STZ - injections. Although, type I diabetes is considered as a T-cell mediated autoimmune disease, but it is possible to predict IDDM by analysis of autoantibody markers. Islet cell antibodies (ICAs) are highly predictive in this context and their performance can be enhanced by combined analysis with other autoantibody markers i.e. autoantibodies to insulin, GAD and IA-2 (23). We have also found that oral administration of GAD before STZ-injections improves the in vitro proliferative responses of splenocytes to Con A. Sai and his colleagues have reported that the in vitro proliferative responses of splenocytes to Con A were not different in GAD peptide 524-543 immunized mice and control NOD mice (10).

Based on our observations, it may be concluded that the E.coli GAD may delay or prevent the completion of β cell destruction in STZ - induced diabetes. Our results are in agreement with the theory presented by Zechel MA and his colleagues (24). According to their theory, only administration of an autoantigen before the onset of disease or in the early stages of disease, should affect the disease process.

The effect(s) of oral administration of GAD on the immune responses are probably due to the blockage of the autoimmune process through generation of active

suppression of diabetes and also clonal deletion / anergy. The suppressor cells generated during oral tolerance induction mediates what is known as bystander suppression.

Although our data clearly demonstrates amelioration of STZ - induced diabetes by oral administration of E.coli GAD, however the complete protection has not been achieved by this technique. It is certainly necessary to investigate the use of different delivery vehicles such as multiple emulsion system (25) and cholera toxin B subunit (26) or more frequent dosing schedules, in order to lead to a better prevention program in diabetes. Additionally, it may be neccessary to use more than one pancreatic antigen to achieve the goal.

One of the primary goals of immunotherapy in autoimmune disease is to find nontoxic therapies that can be administered before the onset or early in the course of disease. Our results in the STZ - induced model of diabetes raise the possibility that orally administered E.coli GAD could provide a new approach for the prevention and treatment of autoimmune diabetes in man.

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