T-cell Tolerance Following Bacterial Glutamic Acid Decarboxylase (GAD) Feeding in Streptozotocin-induced Diabetes

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ABSTRACT

Background: Autoimmune type 1 diabetes mellitus is caused by T-cell mediated immune destruction of the insulin-producing β-cell in pancreatic islets of Langerhans. Specificity of the auto-antibodies and of the auto-reactive T-cells has been investigated, in which several auto-antigens were proposed. Objective: To determine whether glutamic acid decarboxylase (GAD) feeding would induce oral tolerance of either T-cell or B-cell compartment in streptozotocin (STZ) diabetic rats. Methods: Rats in the experimental group were fed 2 mg/kg of GAD (extracted from Escherichia coli) 14 days before intra-peritoneal injections of streptozotocin (30 mg/kg body weight for 5 consecutive days). Two control groups were considered: diabetic control group, which underwent STZ injections without receiving GAD, and normal control group. Systemic response was compared between the three groups. T-cells response was assessed by a proliferation assay of spleen cells and those of the B-cells by enzyme-linked immunosorbent assay (ELISA) for anti-GAD specific antibodies in serum. Results: Compared with the diabetic control group, a significant reduction was observed only in the proliferative response of spleen cells, but not in the level of anti-GAD antibody. Conclusion: GAD feeding induces systemic T-cell tolerance in STZ-induced diabetes.

Keywords: Escherichia Coli, Glutamic Acid Decarboxylase, Streptozotocin, Oral Tolerance

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INTRODUCTION

Type 1 diabetes is a heterogeneous disorder, resulting in most cases from autoimmune mediated destruction of pancreatic beta cells, leading to an absolute deficiency of insulin production. The rate of destruction is quite variable, being rapid in some individuals and slow in others (1). In humans and in non-obese diabetic (NOD) mice, circulating glutamic acid decarboxylase (GAD) auto-antibodies (2) and GAD auto-reactive T-cells (3) have been characterized before the onset of diabetes, suggesting that GAD is one of the auto-antigens implicated in type 1 diabetes.

Feeding antigen can cause an antigen-specific reduction in many types of immunoresponses, including antibody production, delayed type hypersensitivity, and T-cell proliferation. This approach, known as oral tolerance, has been examined as a potential treatment for autoimmune disease and has been reported to be beneficial in some cases (4). Whereas oral tolerance therapy may be both simple and effective, the requirement of large amounts of protein will limit clinical testing of auto-antigens, which are difficult to produce. The use of plants as an expression system or bioreactor for the production of mammalian antigenic proteins for clinical use offers several advantages, including high production capacity with near unlimited scale up (5).

Yazdchi et al. (6) showed that both the extent of lymphocytic infiltration of the pancreatic islet cells and the severity of diabetes were reduced significantly by oral administration of GAD isolated from *Escherichia Coli* (*E.Coli*) to rats before intraperitoneal injections of streptozotocin (STZ). We sought to determine whether GAD feeding of rats induces oral tolerance of either T-cell or B-cell compartment.

MATERIALS AND METHODS

Isolation of GAD from *E.coli*. Glutamic acid decarboxylase (GAD) was obtained from *E.Coli* strain 8739 (Scientific and Industrial Organization, Karaj, Iran) according to the modified method of Yang and Metzler (7). Purity of the isolated enzyme was evaluated using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) in the presence of standard GAD of *E.Coli* (Sigma, USA). Enzyme activity was determined using a spectrophotometric assay according to Passonneau and Lowry method (8).

Determination of Endotoxin in Isolated GAD. Endotoxin contamination was determined using Limulus Ameobocyte Lysate (LAL) gelatin test according to the manufacturer recommendations (Biowittaker). In brief, 0.25 ml of appropriate test solution was added to the lysate vial. After gentle mixing, vial was incubated for 60 minutes at 37°C. At the end of this period, gelation was determined by carefully inverting the test vial. Positive and negative controls were included in each test (9).

Rats. Sprague-Dawley female rats, weighted 180-250 gr, were purchased from the Animal Research Center, Shiraz. They were housed under conventional conditions.

Experimental Design. A total of 15 rats were used (5 rats in each group). Rats in the experimental group were fed 2 mg of bacterial GAD twice weekly for 2 weeks, 14 days before STZ injection. Feeding was performed through a syringe fitted with a ball-type feeding needle. These rats, together with those in the diabetic control group, received intra-peritoneal injections of STZ (Zanosar, Pharmacia & Upjohn, USA) 30 mg/kg for 5 days consecutively (10, 11). Normal control group received intra-
peritoneal injections of the dissolving agent (NaCl 0.9%) for 5 consecutive days. Blood samples were collected at 14-day intervals from tail vein. 48 hours before sacrificing, rats were immunized orally with 2 mg GAD. Serum glucose levels were determined using an enzymatic procedure (Pars Azmoon, Iran). Hyperglycemia was defined as the serum glucose level exceeding 200 mg/dl (12).

**Histopathology.** Rats were killed by ether, pancreas gland was excised and processed for conventional histological studies after fixation in 10% formaldehyde.

**GAD Autoantibody Assay.** Isolated GAD (5μg/well) was bound to 96-well plates (Nunc, Denmark) with 0.2 M NaHCO3 at PH of 9.0 and was incubated overnight at 4°C. Wells were blocked with 3% Bovine Serum Albumin (BSA) in Phosphate Buffer Solution (PBS) for 1 hour, then washed three times with PBS-tween. Rats' sera were added (50μl of 1/800 dilution) and incubated for 1hour at 25°C. Washed plates were incubated with Fab fragments of anti-rat immunoglobulin conjugated with alkaline phosphatase (50μl of 1/1000 dilution) (Boehringer Mann Heim Biochemica) for 30 minutes at 37°C. The test was developed with paranitrophenyl phosphate (P-NPP) substrate. Absorbance was detected at 405 nm using ELISA reader.

**T-cell Proliferation Assay.** Splenocytes were cultured (200000 cells/well) in 96-well cell culture plates (Nunc, Denmark) in RPMI 1640 medium (Rose Well Park Memorial Institute) and supplemented with 10% Fetal Calf Serum (FCS). Triplicate wells were filled with extracted GAD (10μg/well) and phytohaemagglutinin (PHA) at 1/20 final dilution (GIBBCO BRL) as positive control or medium alone as negative control. Plates were incubated at 37°C with 5% CO2 for 3 days. Thereafter, wells were pulsed with [3H]-thymidine at 1μci/well for 15-20 hours and harvested on filter papers. Count per minute (cpm) was determined using β-counter. Results were expressed as a stimulation index (SI), showing the ratio of the mean Ag-stimulated cpm divided by the mean unstimulated cpm.

**Statistical Analysis.** Non-parametric tests were used. Comparison between groups was performed using Man-Whitney U-test. P-values less than 0.05 were considered significant. Data are expressed as mean ± SD.

**RESULTS**

**GAD Extraction.** SDS-PAGE confirmed the identity and purity of the GAD extracted from *E.Coli* using the LMW calibration solution and standard GAD (Figure 1). Gel clot LAL test detected contamination of the extracted GAD with endotoxin.

**Treatment with Bacterial GAD.** To investigate the capability of GAD in inducing oral tolerance, the severity of diabetes and the lymphocyte infiltration rate of the langerhans islands were evaluated.

**Insulitis.** Oral administration of GAD fourteen days before STZ injection in the experimental group induced a decrease in pathologic manifestations of insulitis compared with the diabetic control group. Eighty percent of the diabetic rats had severe lobular tissue infiltration with lymphocytes and mast cells and 20% had moderate peri-lobular tissue infiltration with the same cells. On the other hand, only 20% of the experimental rats had moderate peri-lobular tissue infiltration with lymphocytes and mast cells.
Figure 1. SDS-PAGE analysis of the extracted GAD compared with standard GAD and LMW calibration. An aliquot of the extracted enzyme was applied on 10% SDS-PAGE. Lane 1: extracted enzyme, Lane 2: standard GAD, Lane 3: LMW calibration solution (size marker).

Severity of Diabetes. Blood glucose levels are shown in figure 2. Blood glucose levels higher than 200 mg/dl were considered as hyperglycemia (overt diabetes). Normal control group (group 1) showed normal blood glucose levels (126.6 ±15.2 mg/dl) and STZ injections induced a severe hyperglycemia (323 ±63.7 mg/dl) in diabetic control rats (group 2), differences were statistically significant (P=0.008). Experimental rats (group 3) also had normal blood glucose levels (161.6±15.4 mg/dl).

Figure 2. Effects of orally administered GAD on blood glucose levels determined in individual rats at 14-day intervals. Results are mean for each group. Group 1: normal control group (126.6 ±15.2 mg/dl); group 2: diabetic control group (323 ±63.7 mg/dl); group 3: experimental group in which rats received bacterial GAD fourteen days before STZ injections (161.6 ±15.4 mg/dl).
**Serum Anti-GAD Antibody.** Anti-GAD antibodies in normal and diabetic control groups and in experimental rats were measured; the means of optical densities (OD) are depicted in figure 3. There was a significant difference (P<0.05) between diabetic control (1.61±0.41) and normal control (0.59±0.07) groups. However, the means of OD in the GAD-fed group decreased moderately compared with diabetic rats and the difference remained insignificant (1.27±0.22 vs. 1.61±0.41).

**Figure 3.** Effects of oral administration of GAD on Anti-GAD antibody level. Group 1: normal rats (0.59±0.07); group 2: diabetic rats (1.61±0.41); group 3: experimental group which received GAD fourteen days before STZ injections (1.27±0.22). Anti-GAD antibodies were determined using ELISA method.

**T-cell Proliferation Assay.** Anti-GAD T-cell proliferation was assessed in both control and experimental rats and results are shown in figure 4. SI in the diabetic control group was significantly higher than the normal control group (P<0.05). Interestingly, SI for the GAD-fed group was significantly reduced (P<0.05) in comparison with diabetic rats (2.3±0.7 vs 5.16±1.8, P<0.05).

**Figure 4.** Effect of oral administration of GAD on lymphocyte proliferation test (LTT). Proliferative response of spleen cells in individual rats to PHA, GAD, and media were measured. Columns represent the stimulation indices ± SD of [3H]-thymidine uptake in each group after in vitro challenge with bacterial GAD. Group 1: normal rats (2.4±1.6); group 2: diabetic rats (5.16±1.8); group 3: experimental group that were fed GAD fourteen days before STZ injections (2.3±0.7).
DISCUSSION

It is postulated that GAD65 is a key initial target antigen in NOD mice (13). We investigated whether it is possible to administer extracted bacterial GAD in a mode that would induce oral tolerance in streptozotocin-induced diabetes. Severity of diabetes and the lymphocyte infiltration rate of the langerhans islands were evaluated. Our results revealed that oral administration of bacterial GAD for 14 days before STZ injections reduces the severity of diabetes and insulitis.

Results of the present study demonstrate that oral tolerance can be induced by administration of oral GAD in streptozotocin–diabetic rats. However tolerance was limited to T-cell compartment. In fact, although Anti-GAD antibody in experimental group was decreased slightly, it was not significant in comparison with those of diabetic rats (P<0.05). Considering the profound influence of dose, frequency of administration, and type of antigen on oral tolerance induction in experimental animals (14,15), it is possible that by changing one or more of these parameters, tolerance might occur in B-cells as well. This is confirmed by experiments showing that T-cell tolerance could be induced after feeding low doses of antigen to animals (2), while B-cells generally can be tolerized by feeding larger amounts of antigen (14-16).

Moreover, sensitivity to tolerance induction varies among different T-cell subsets (17), there seems to be a gradient of sensitivity to tolerance induction, with Th1>Th2>B cells. GAD-specific T cell proliferation mainly involves T helper (Th1) function (18) and therefore, this was significantly inhibited by GAD feeding. In the current study, administration of GAD in experimental group reduced T-cell proliferation; however, Th2 and B-cells were not tolerized. Therefore, Th1 reduction in T-cell proliferation in GAD treated group is a result of absence in Th1 proliferation. In line with these findings, Husby et al. also reported a significant reduction in keyhole limpet hemocyanin (KLH)-specific T-cell proliferation (though not that of B-cells) will occur after ingestion of KLH (17). The outcome of the LAL test showed that the bacterial GAD extracted from E.coli contains endotoxin. The quantity of endotoxin and its exact influence on the induction of oral tolerance by GAD feeding was not investigated in this study.

One possibility is that the protein antigens given orally require mucosal adjuvants to effectively activate both T-cells and B-cells. Brix et al. (19) have shown that lipopolysaccharide (LPS) contamination of an aqueous protein solution does not affect oral tolerance induction, whereas LPS present in emulsion prevents oral tolerance induction toward the food protein. Sun et al. (20) suggest that mucosal adjuvants such as cholera toxin B-subunit (CTB) may enhance oral tolerance to co-administered antigens by targeting small amounts of protein antigens to specialized antigen-presenting cells of the gut-associated lymphoid tissue.

Despite the fact that in the present study a significant difference was observed only in the spleen cells proliferative response but not in the level of antibody production, yet signs of the disease failed to appear.

We can conclude that the induction of T-cell tolerance to ingested antigens may be of considerable importance in the amelioration of diseases with T-cell dominant effector mechanisms. Our results in the streptozotocin-induced model of diabetes raise the possibility that orally administered E.Coli GAD and the induction of T-cell tolerance could provide a new approach for the prevention of autoimmune diabetes in humans.
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