

PROTEASE TREATMENT DELAYS DIABETES ONSET IN DIABETES-PRONE NONOBESE DIABETIC (NOD) MICE

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Summary: *It has recently been demonstrated that proteolytic enzyme treatment modulates certain immune-mediated diseases. We have, therefore, studied the effect of administration of a protease mixture in the NOD mouse, an elegant animal model for autoimmune insulin-dependent diabetes mellitus (IDDM). Female NOD mice were fed proteolytic enzymes from age 6 weeks to 10 weeks, within the subclinical phase of IDDM. Once a week animals received intragastrically 1 mg Phlogenzym® (n=10 mice) or 0.5 mg Phlogenzym® (n=10) in 0.5 ml saline or saline only (n=10). Mice were followed for development of IDDM up to week 23. At week 21, all control animals were diabetic, whereas 25% of the treated mice were still normoglycemic at the end of the observation period. No significant appearance of autoantibodies against either isoform of the important islet cell antigen glutamic acid decarboxylase (GAD), GAD65 and GAD67, was observed in the mouse sera as determined by a highly sensitive radioimmunoassay. The histopathological examination of pancreatic islets showed signs of insulinitis in all mice with a tendency of milder insulinitis in the protease-treated groups.*

Introduction

Insulin-dependent diabetes mellitus (IDDM) results from the autoimmune destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. To this point in time, IDDM has not been prevented or cured in humans. The current therapy is to replace the insulin deficiency by injections several times a day. IDDM is a chronic disease with a dramatic, daily impact on affected individuals and the

risk of late complications such as kidney failure, blindness, heart disease, and neuropathy. In spite of extensive research, the exact pathoimmunological mechanisms leading to IDDM still remain to be elucidated. Disease initiation and progression, which are almost impossible to follow in humans, can be investigated in valuable animal models of spontaneous diabetes like the BioBreeding rat or the nonobese diabetic (NOD) mouse (1, 2). Using these models, evidence has been obtained showing that antigen-specific and nonspecific compartments of the immune system play an important role in IDDM etiology. The NOD mouse was developed in Japan in the 1970s in the course of a breeding program to establish a cataract-prone subline from noninbred iCR mice. The inbred NOD mouse strain is not cataract-prone and

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shares clinical, immunological, histopathological, and genetic features with human IDDM. In the NOD mouse, insulinitis, *i.e.*, the infiltration of leukocytes into pancreatic islets, precedes overt IDDM. This correlates to the fact that insulinitis is seen in humans who died soon after the onset of IDDM (3, 4).

Intravenous or oral application of proteolytic enzymes has been shown to modulate immune-mediated diseases including transplant rejection, as well as allograft arteriosclerosis and multiple sclerosis in animal models (5). Phlogenzym® therapy is basically free of side effects (meteorismus was observed in some cases where Phlogenzym® was taken in high doses) and, if given orally, noninvasive. We, therefore, orally administered Phlogenzym® to prediabetic NOD mice well after the onset of insulinitis and then followed the course of the disease. Interestingly, we found delayed IDDM onset in the treated animals compared to sham-fed control mice.

Material and Methods

Animals. The female NOD mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained behind a barrier under clean conventional housing conditions.

Intragastric feedings. The protease mixture (Phlogenzym®, Mucos Pharma, Munich, Germany) was dissolved in saline and was fed intragastrically at 2 mg/ml ($n=10$ animals) and at 1 mg/ml ($n=10$ animals). Control animals ($n=10$) were fed the saline vehicle only. Mice received 0.5 ml 1 x weekly for 5 consecutive weeks starting at 6 weeks of age.

Assessment of diabetes. Mice were tested weekly for glucosuria using test strips (Diabur-Test 5000®, Boehringer Mannheim, Mannheim, Germany). Animals with elevated urinary glucose levels (>14 mmol glucose/l urine) were retested the following

day. Glucosuric mice were then bled to confirm glycemia by using a glucose analyzer and, when again positive (>11 mmol/l, Glucometer elite®, Bayer Diagnostics, Munich), considered diabetic. Serum was collected at diagnosis of IDDM or at the end of the observation period at age 23 weeks.

Detection of GAD-specific antibodies. The GAD-radioimmunoassays were carried out with slight modifications as recently described (6). Briefly, recombinant human ^{35}S -GAD65 and ^{35}S -GAD67 were produced in a coupled transcription/translation system (Promega, Madison, WI, USA). Expression plasmids containing the cDNAs of rGAD65 or rGAD67 were used as templates for transcription. Labeled proteins were separated from unincorporated ^{35}S -methionine on Sephadex G25 (Pharmacia, Uppsala, Sweden). 5 μl of mouse serum was incubated in duplicates with 15,000 cpm of radioactive protein at 4 °C overnight. Protein A Sepharose was added and after 1 h antibody-bound GAD was separated from unbound GAD by washing in membrane-bottom microtiter wells (Millipore, Eschborn, Germany). Counts per minute (cpm) were determined in a beta counter. Experimental cutoffs were calculated as mean values of control mice plus 3 standard deviations (SD).

Immunohistopathological analysis. Pancreata from diabetic mice were fixed in 4% buffered formalin, embedded in paraffin, sectioned at 2-5 μm and stained with hematoxylin/eosin. At least six sections, 100 μm apart, were analyzed. Coded slides were examined without knowledge of the treatment regime under a light microscope.

Results

As shown in Fig. 1, the diabetes incidence between the various treatment groups started to separate from

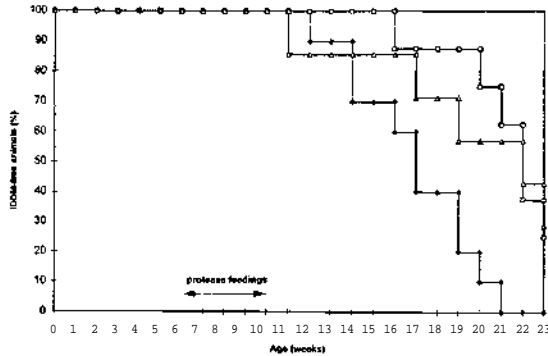


Fig. 1 Effect of protease treatment on IDDM in female NOD mice. Animals were treated from age 6 weeks to 10 weeks. Experimental groups (n=10): 1 mg Phlogenzym (A); 0.5 mg Phlogenzym (D); vehicle only (controls) (◆).

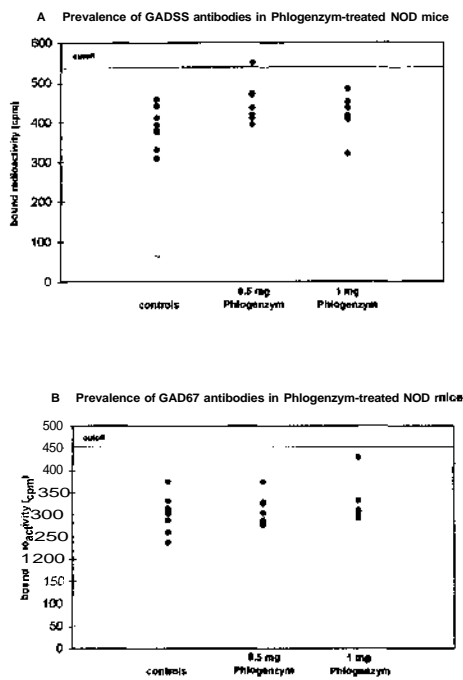


Fig. 2 A, B Serum levels of specific anti-GAD-antibodies determined by radioimmunoassay; (A) anti-GAD65-antibodies and (B) anti-GAD67-antibodies in controls, 0.5 mg Phlogenzym and 1 mg Phlogenzym treated NOD mice.

week 14. At week 16, 60% of the nontreated animals compared to 88% (0.5 mg Phlogenzym[®]) and 86% (1mg Phlogenzym[®]) of the enzyme treated groups remained IDDM-free. Three weeks later, only two (20%) control mice were still normoglycemic compared to 57% (1 mg Phlogenzym[®]) and 87% (0.5 mg Phlogenzym[®]) of the enzyme treated groups. The last control animals showed clinical signs of IDDM at week 21. At the end of the observation period at week 23 still about 25% (0.5 mg) and 43% (1 mg) of the Phlogenzym[®]-fed mice remained disease free. At this point in time the difference between the treated group (1 mg) and the control group was statistically significant ($p=0.01$, chi-square-test). Between the two treatment groups, 0.5 and 1 mg Phlogenzym[®] per feeding, no significant difference was observed.

Histological examination of the hematoxylin/eosin stained pancreas sections revealed no significant difference. When we compared pancreata of animals which became diabetic in the same week (week 16, control animal and animal of 0.5 mg Phlogenzym[®] group; week 20, control animal and animal of 0.5 mg Phlogenzym[®] group), insulinitis seemed to be milder in the treated animals.

Only one mouse showed very low levels of GAD65-A (0.5 mg Phlogenzym[®] group). All other animals were negative for antibodies against either isoform of the IDDM-associated antigen glutamic acid decarboxylase (GAD), GAD65 and GAD67, as found by radioimmunoassay (Fig. 2).

Discussion

In the human situation, it is assumed that prior to clinically overt IDDM, insulinitis coincides with the appearance of serological islet cell antibodies (ICA) and other autoimmune markers like antibodies against GAD65, tyrosinphosphatase IA2 or insulin. By investigating these diabetes-associated antibody patterns, people at high risk for progressing to IDDM

can be identified (7-11). At this prediabetic stage, it would be desirable to start an IDDM prevention therapy. To simulate this specific situation, we chose to start treating the NOD mice at age 6 weeks when insulinitis had already developed and beta cell destruction had begun. We found that by protease feedings IDDM onset could be delayed in NOD mice even after aggressive insulinitis was established. Since IDDM is known to be the consequence of an autoimmune destruction of the pancreatic beta cells, immune-mediated pathophysiological pathways may have been modulated. Both immune and nonimmune mechanisms may be involved. Loss of tolerance towards the beta-cell protein GAD has been shown to play an important role in the induction of IDDM in NOD mice (12, 13); in numerous studies the modulation of autoimmune responses against GAD was able to inhibit diabetes in female NOD mice (14-16). We found that protease treatment did not induce an humoral response against either isoform of the diabetes-associated antigen GAD. Lehmann *et al.* (17) and Haynes B.F. *et al.* (18) have shown that Phlogenzym® affects T-cell activation by changes in expression of surface molecules. Modulation of the activation threshold for autoreactive T-cells, which are the main players in the destructive insulinitis, may play a role in deceleration of IDDM onset in the studied mice.

The lack of significant differences in insulinitis of the various treatment groups may be due to the fact that pancreata could only be obtained after onset of IDDM. A possible explanation is that at IDDM onset most of the islets are already destroyed and therefore at this point differences in the time course of beta-cell destruction can no longer be detected.

In summary, we have shown for the first time that protease administration delays IDDM onset in an animal model for autoimmune diabetes, the NOD mouse. Further studies are required to elucidate in detail the mode of action of protease therapy in IDDM.

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