ANTIBODIES AGAINST CALCITONIN IN EXPERIMENTAL DIABETES MELLITUS

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In the last few years evidence of the role of calcium and calcitonin in the pathogenesis of diabetes mellitis has been obtained. In diabetic coma, on account of increased calcitonin secretion by C-cells of the thyroid gland, hypocalcemia and hypophosphatemia develop [6, 10]. In the stage of compensation of diabetes the calcium and phosphorus levels in the blood return to normal.

Calcitonin has a hyperglycemic effect, it participates in enzymic breakdown of the liver glycogen, and also inhibits insulin secretion by pancreatic β-cells and prevents the passage of glucose from the blood stream into the tissues [3, 8, 9]. Increased secretory activity of thyroid cells is observed not only in young and middle-aged patients with severe "wasting" diabetes, but also in experimental alloxan diabetes [1]. Calcitonin also possesses antigenic properties [2, 4]. This is confirmed by the fact that antibodies have been found in the blood on repeated injection of calcitonin. The facts described above suggest that calcitonin plays an important role in immunologic reactions in diabetes.

The aim of this investigation was a dynamic study of antibodies against calcitonin in experimental alloxan diabetes.

EXPERIMENTAL METHOD

Serum of rats with experimental alloxan diabetes was used as the test material. To produce alloxan diabetes the alloxan was first recrystallized, then injected into animals deprived of food for 2 days (male rats weighing up to 300 g) in the form of a 2% solution, into the caudal vein in a dose of 4 mg alloxan/100 g body weight. The development of diabetes was verified 7-10 days later by the raised blood sugar.

The blood glucose concentration was determined by the color reaction with ortho-toluidine. A mixture of sera from 70 control rats served as the control. In the experiment 40 sera from experimental rats with alloxan diabetes were used. The sera of the experimental rats were divided into three groups depending on the blood sugar level.

In experimental group 1 the blood sugar level in the rats after receiving alloxan varied from 4 to 7.6 mmole/ml (control limits). In the rats of experimental group 2 the sugar level varied from 10 to 17 mmole/liter. In the animals of group 3 marked hyperglycemia was observed: Their blood sugar level varied from 18 to 30 mmole/liter.

Meanwhile antibodies against calcitonin were determined in these same sera by immunoenzyme analysis by the enzyme-labeled antibodies test (ELAT) [5, 7]. Calcitonin isolated from normal rat thyroid glands was used as the antigen.

The conjugate for the test was prepared from the total globulin fraction isolated by means of ammonium sulfate from rabbit sera (N. F. Gameleya Institute of Epidemiology of Microbiology) against rat γ-globulins.

Protein was conjugated with the enzyme (horseradish peroxodase, brand "A," from Biokhimreaktiv, USSR) by periodate oxidation [7]. The reaction was carried out in polystyrene plates for immunologic reactions, made by the Leningrad "Medpolimer" Factory, in accordance with the following scheme. The plates were sensitized with a solution of calcitonin made up in 0.1 M

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TABLE 1. Relationship between Blood Sugar Level and Titer of Antibodies against Calcitonin in Experimental Diabetes Mellitus

Sugar concentration, mM	Optical density, relative units	Sugar concentration, mM	Optical density, relative units	Sugar concentration, mM	Optical density, relative units
6,9	0,29	16,8	0,36	21,6	0,32
4,2	0,17	10,4	0,57	18,7	0,44
4,8	0,18	13,1	0,45	18,7	0,45
4,2	0,15	12,6	0,40	19,2	0,60
5,0	0,17	13,4	0,39	22,8	0.70
4,2	0,17	17,0	0,50	21,6	0,60
7.6	0,24	16,8	0,36	30,5	0,84
7,2	0,24	11,7	0,40	23,2	0,60
4,0	0.15	16,8	0,46	28,2	0,70
4,6	0,24	17,4	0,34	28,6	0,74
6,0	0,17	17,5	0,40		
6,7	0,39	15,0	0,41		
7,6	0,39	14,2	0,21		
		13,4	0,89		
		16,3	0,62		
		13,9	0,62		
Mean 5,6	0,22	14,8	0,46	23,3	0,6
$n=13$	$n=13$	$n=16$	$n=16$	$n=10$	$n=10$

Legend: n) Number of experiments.

carbonate–bicarbonate buffer, pH 9.6, in a concentration of 350 μg/ml. Into each well of the plate 0.2 ml of the solution was added and the plate was kept overnight at 4°C. The plate was then carefully washed under a jet of tap water. All the wells were filled for 1.5-2 min with tap water containing 0.05% Tween-20 and again washed under a jet of water.

Into each well was poured 0.1 ml of phosphate-salt buffer, pH 7.2, with 0.5% Tween-20, and 0.1 ml of the test experimental and control sera with dilutions of 1:100 to 1:6400 was titrated on them. The plate was incubated for 1 h at 30°C. It was then washed as described above, and the conjugate was added to all the wells in a dose of 0.2 ml in a dilution of 1:1000 in phosphate–salt buffer with Tween. After incubation at 37°C (1 h) the plate was again washed. The substrate used was 5-aminosalicyclic acid [7].

The reaction was read 1 h after addition of the substrate on a spectrophotometer with vertical beam paths (Minireader R-590) at a wavelength of 490 nm. When the results were analyzed on the plate, the density of the mixture of sera from the control animals was subtracted from the optical density of the rat sera.

The titer of antibodies against calcitonin was judged from the difference thus obtained. A value of 0.3 for optical density in the experiment corresponded to an antibody titer of 1:6400. Alloxan and insulin served as the control for the antigen. For this purpose the plates were sensitized with alloxan solution made up in 0.1 M carbonate–bicarbonate buffer, pH 9.6, in a concentration of 133-400 μg/ml. Into each well 0.2 ml of alloxan solution was introduced.

The plate was sensitized with insulin solution as follows: insulin in a concentration of 40 IU/ml was diluted 1:20, 1:40, 1:80, and 1:160 with 0.1 M carbonate–bicarbonate buffer. Into each well of the plate 0.2 ml of insulin solution was added. After sensitization of the plates with insulin and alloxan the ELAT tests was carried out as described above, i.e., experimental and control sera, conjugate, and substrate were added successively to the wells step by step, with compulsory washing with tap water containing Tween, after which the results of the test were read.

EXPERIMENTAL RESULTS

No antibodies against alloxan and insulin were found. The experimental results are given in Table 1. In rats of experimental group 1, despite injection of alloxan, the blood sugar level remained low, on average at 5.6 mM, i.e., about the same as in the control. The opti-

cal density, from which the titer of antibodies against calcitonin was judged, was about 0.22, i.e., it was increased by very little above the control.

In the rats of experimental group 2, with a high blood sugar level (14-15 mM) the titer of antibodies against calcitonin was raised. The optical density was increased to about twice that found in animals of group 1 (mean 0.46).

In the animals of experimental group 3, which had high hyperglycemia (23 mM), the titer was about 3 times higher than that in the rats of experimental group 1.

Consequently, antibodies against calcitonin appear in the blood in alloxan diabetes. The presence of antibodies against calcitonin was observed only if the blood sugar level was high. The increase in the blood sugar was observed to depend on the level of antibodies against calcitonin. In the stage of latent diabetes, i.e., after injection of alloxan but the blood sugar level still remained low, no antibodies against calcitonin could be found. The presence of antibodies against calcitonin only when hyperglycemia is present, and also the possibility that calcitonin may participate directly in the production of hyperglycemia [5, 6, 8], suggest that the appearance of autoantibodies against calcitonin is a pathogenic factor in the development of hyperglycemia in alloxan diabetes.

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