IMMUNOENZYME ASSAY DETECTION OF ISLET CELL SURFACE ANTIBODIES BY ELISA IN AUTOIMMUNE DESTRUCTION OF THE INSULAR APPARATUS

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One of the mechanisms of destruction of the β-cells of the islets of Langerhans in the pancreas, leading ultimately to the development of insulin-dependent (type 1) diabetes, is immune autoaggression with the participation of the T and B systems of immunity [8]. A reflection of this pathological process is the various immunological phenomena that are frequently detectable long before the first clinical manifestations of the disease have appeared. This is particularly true of the heterogeneous group of autoantibodies against pancreatic islet cells. Islet cell surface antibodies (ICSA) probably play a pathogenetic role, for they are capable of depressing *in vitro* glucose-stimulated insulin secretion [9], of inducing complement-dependent lysis of β-cells [4], and of participating in the various antibody-dependent cell-mediated reactions of destruction of the insular apparatus [3]. Improvement of methods of detection of ICSA may contribute to the further development of our ideas on autoimmune diabetogenesis, and also may facilitate the elaboration of measures for the early diagnosis and immunoprophylaxis of insulin-dependent diabetes, resulting in improved immunological monitoring after transplantation of β-cells.

The aim of this investigation was to study the possibility of using enzyme-linked immunosorbent assay (ELISA) for ICSA screening, and the results are given of the investigation of small groups of people with high risk of development of insulin-dependent diabetes and with various types of the manifest disease.

EXPERIMENTAL METHOD

Since ICSA are not species-specific but organ-specific [10], islet cells of newborn Wistar rats, readily available and isolated as described previously [1], were used as antigenic for their detection. Preparation of the microplates for cell culture (from Linbro, England and Dynatech, USA), and the ELISA itself were carried out by the modified scheme described in [11]. The basic buffer solution consisted of Hanks' solution with the addition of 0.1% bovine serum albumin (BSA, from Serva, West Germany), pH 7.2. The cells were fixed to the bottom of the wells ($2 \cdot 10^4$-$3 \cdot 10^4$ cells per well) and dried in air. Additional fixation was carried out with 0.1% glutaraldehyde (from Serva) in the cold. After saturation of nonspecific binding sites on the plate with Hanks' solution containing 4% BSA for 2 h at 37°C the cells were again dried at room temperature until required for use. Goat antibodies against human IgG, conjugated with peroxidase (type IV, from Sigma, USA), in a dilution of 1:400, were used as the second antibodies. The chromogenic substrate was a 0.08% solution of 5-aminosalicylic acid (from Merck, West Germany) pH 6.0 and a 0.05% solution of hydrogen peroxide in the ratio of 9:1. The cells were incubated with the sera for 2 h at 37°C, with the enzyme-conjugated antibodies for 1 h at room temperature, and with the substrate for 30 min at the same temperature, in light. Between the different stages the wells were washed three times with the basic buffer solution. Automatic measurements of optical density was carried out on the "Titertek-Multiscan" Instrument (Flow Laboratories, England) at a wavelength of 450 nm. The results were subjected to statistical analysis by Student's t test. Sera for detection of antibodies were obtained from various clinical deparments of the Institute of Experimental Endocrinology and Hormone Chemistry, and control sera were obtained from healthy blood donors at the blood transfusion station of the Central Institute of Hematology and Blood Transfusion, and they were kept until use at −18°C.

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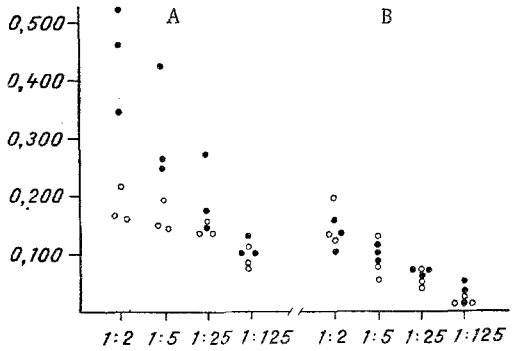

Fig. 1. Effect of serum protein concentration on specific binding of ICSA with various rat target cells. A) Islet cells, B) hepatocytes. Abscissa, degree of dilution of diabetic (filled circles) and normal (empty circles) sera; ordinate, optical density of solutions in wells of micropanels at at wavelengths of 450 nm.

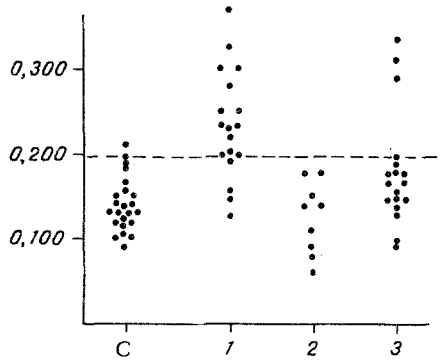

Fig. 2. ICSA in sera of subjects with differences in functional state of their insular apparatus. Abscissa, serial no. of group studied; ordinate, optical density of wavelength of 450 nm. Broken line shows upper limit of normal.

EXPERIMENTAL RESULTS

The concentration of immunoglobulins was found to have a significant effect on the magnitude and significance of differences between the positive and control sera during immunoenzyme assay of ICSA. The greatest differences were found with serum in a dilution of 1:2 (Fig. 1A). With an increase in dilution to 1:5-1:25 these differences were sharply reduced, and in a dilution of 1:125, optical density values corresponding to diabetic and normal sera completely overlapped. Later, during primary screening of antibodies, the sera were used in a dilution of 1:2.

The specificity of interaction of ICSA with the target cells was tested in a parallel experiment with rat hepatocytes. Unlike the islet cells, no difference between the diabetic and control sera were observed on liver cells whatever the dilutions used (Fig. 1B). This result is further confirmation of the organ-specificity, but not the species-specificity of ICSA and that the use of antigen-carrying rat cells in this test system is appropriate. To assess any possible nonspecific binding of human immunoglobulins with rat islet cells, such as is observed during radioimmunoassay of ICSA [7], the sera were absorbed before testing with freshly isolated rat splenocytes (10^8 cells in 1 ml of suspension) for 1 h at 37°C. Contrary to expectation, the values of optical density for normal sera (N-11) were increased from

0.125 ± 0.013 to 0.160 ± 0.014, and for diabetic (N-11) they were reduced 0.237 ± 0.014 to 0.010. Similar results also were obtained in the case of exhaustion of sera with an acetone powder of rat liver, evidence that these methods are ineffective and their use for immunoenzyme assay of ICSA cannot be recommended.

An important feature distinguishing this method is the ability of antibodies against insulin to interfere with ICSA [11], evidently on account of interaction with antigenic determinants of the hormone in the incomplete phase of secretion, present on the surface of fixed β-cells. To diminish the contaminating effect of antibodies against insulin (characteristic of patients with diabetes receiving insulin therapy) bovine insulin (from Serva) was added to the sera before testing. The final concentration of the hormone was 1-2 μg/ml, quite sufficient to neutralize antibodies to insulin during their immunoenzyme assay [5]. As a result the mean optical density for normal sera (N-11) was somewhat increased (from 0.075±0.011 to 0.096±0.014 $P > 0.5$), whereas for diabetic (N-11) it was reduced (from 0.215±0.012 to 0.198±0.012, $P > 0.5$). Differences between normal and diabetic sera, however, both in the absence of insulin and after its addition, remained significant ($P < 0.001$). Addition of the hormone to the incubation medium is evidently an easy and sufficiently effective solution to the problem of intereference between the two types of antibodies during determination of ICSA on fixed islet cells.

Taking into account the technical details described above, sera from 68 persons, subdivided on the basis of clinical and biochemical data and in accordance with the recommendations of WHO [13], into several groups were tested for the presence of ICSA. The control group consisted of a random sample of 23 clinically healthy blood donors with no family history of diabetes. The upper limit of normal was determined for optical density for the serum of this group (Fig. 2) as the arithmetic mean value plus three times the standard deviation, at a 1% level of significance. Group 1 consisted of 18 patients with insulin-dependent diabetes with a duration of not more than 5 years. ICSA found in about 60% of them (Fig. 2). According to the results of indirect immunofluorescence, the frequency of detection of these antibodies was maximal at the time the diagnosis was made (70-80%), but for patients with a duration of their illness of 5 years or more, it was about 30% [2]. Considering the heterogeneity of the group studied with respect to the duration of their diabetes, the results are in good agreement with those given in the literature. Group 2 consisted of 9 patients with insulin-independent (type 2) diabetes, which differs from the insulin-dependent type not only clinically, but also pathogenetically [13]. ICSA were found in none of them (Fig. 2). Group 3 (the risk group) included 18 healthy subjects whose closest relatives included patients with insulin-dependent diabetes. This group was heterogeneous with respect to the presence of ICSA (Fig. 2). Moreover, it was clearly divided into two subgroups ($P < 0.01$): 1, the larger subgroup, did not differ from the control ($P > 0.5$), whereas the other was closer to the group of type 1 manifest diabetes. Considering the long latent period of development of insulin-dependent diabetes [6, 12], the presence of an initial stage of autoimmune process in the endocrine apparatus of the pancreas can be postulated in the three serum-positive donors. This problem can be resolved conclusively by a prospective study of the risk group, involving functional, immunologic, and endocrinologic tests.

ELISA for ICSA is one of the most promising screening methods for the study of the immunologic aspects of the pathogenesis of insulin-dependent diabetes.

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PREPARATION AND STUDY OF MONOCLONAL ANTIBODIES TO SOME GANGLIOSIDES

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The role of gangiosides in tissue interaction, in differentiation of immunocompetent cells, and in interaction of these cells with tumor cells is increasingly engaging the attention of research workers. The particular interest in gangliosides is also due to the fact that they are characteristic antigens of certain malignant neoplasms. For instance, in colorectal and certain other carcinomas, an antigen bound with the tumor is sialosyl-lacto-N-fucopentaose II, and the corresponding antigen for small-cell carcinoma of the lung is fucosyl-GM_1 [8, 9]. Immunochemical methods are nowadays widely used to study the localization and functional role of gangliosides [1, 2, 6, 7, 10]. However, the polyclonal serum antibodies avialable for use do not enable gangliosides to be accurately differentiated, and it is therefore necessary to use monoclonal antibodies for this purpose.

The aim of this investigation was to obtain monoclonal antibodies against gangliosides by immunizing animals with human melanoma cells.

EXPERIMENTAL METHOD

BALB/c mice were immunized with cultured human melanoma cells of the Mewo strain by the following scheme: first immunization — subcutaneous injection of 2×10^6 melanoma cells in Freund's complete adjuvant (1:1), followed by intraperitoneal injections of increasing numbers of cells (10^7, 2×10^7, 3×10^7) at intervals of 4 weeks. The mice were decapitated 3 days after the fourth (last) immunization, the spleen removed, and a suspension of splenocytes prepared for hybridization. The splenocytes were fused with cells of a syngeneic non-secreting P3-X63 Ag 8 myeloma in the ratio of 7:1 with the aid of polyethylene glycol (PEG-4000, from Merck, West German) by the usual method.

Antibodies secreted by the hybridomas were tested by the direct sandwich ELISA method. Individual gangliosides, isolated from the brain of the rays *Raja clavata* and *Dasyatis pastinaca*, were used. The gangliosides were extracted by the method described previously [4], then subjected to alkaline hydrolysis and purified on columns with Sephadex G-25, using chloroform—methanol—water (60:30:4.5) as the solvent. Gangliosides GM_1, GM_2, GM_3, GD_{1a}, GD_2, GD_3, GT_1, GQ_{1c}, were isolated by preparative thin-layer chromatography on silica-gel KSK (chloroform—methanol—2.5 N NH_4OH or chloroform—methanol—water, in the ratio 60:35:9, was used as the solvent). The fractions were refractionated on plates to obtain purified (95-97%) individual gangliosides. A 96-well flat-bottomed polystyrene plate (from Flow Laboratories, England) was sensitized with a 2 mM solution of the gangliosides in 96% ethanol. A panel of the above-mentioned gangliosides was used. After removal of the ethanol by evaporation, a 1% solution of bovine serum albumin (BSA) in buffered physiological saline (BPS) was added to the wells, which were then washed twice with BPS containing 0.05% Tween-20 and 2 mg/liter of BSA (BPS-T). The supernantants of the hybridomas were introduced into the wells for 1 h at 37°C, after which the plate was washed four times with cold BPS-T. Rabbit antibodies against mouse immunoglo-

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