Production and characterization of a monoclonal islet cell surface autoantibody from the BB rat

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Summary. Islet cell surface autoantibodies are present in the serum of the spontaneously diabetic BB rat. The availability in large quantities of such autoantibodies should help us understand their significance *in vivo*. Fusions between BB rat lymphocytes and rat myeloma cells were screened by cellular enzyme linked immunosorbent assay and indirect immunofluorescence on rat living cells. They resulted in a stable hybridoma, called IC₂, secreting a monoclonal immunoglobulin M specific for the surface of rat islet cells. This monoclonal

antibody was found to bind to the surface of 56% normal rat islet cells and 72% rat insulinoma cells. Protease treatment of rat islet cells resulted in a subsequent 72–100% binding inhibition of IC₂ to the surface of these cells, suggesting that IC₂ specific antigen is a protein.

Key words: BB rat, rat monoclonal islet cell surface autoantibody.

The spontaneously diabetic BB rat currently represents, along with the NOD mouse, the best available animal model for Type 1 (insulin-dependent) diabetes (1,2). Though the immune system appears more impaired in the BB rat than in Type 1 diabetic man, its involvement in the pathogenesis of B cell destruction is well established in both cases [3–5]. One of the main arguments in favor of such a role is the presence of circulating antipancreatic autoantibodies.

Such autoantibodies have been found in most Type 1 diabetic syndromes, whether spontaneous or virus-induced [6–8]. Islet cell surface antibodies (ICSA) have been shown to precede the onset of diabetes in the BB rat [9]. The presence of ICSA has been correlated with cytotoxicity for B cells *in vitro* in man and the BB rat [10, 11]. Therefore, if antipancreatic autoantibodies are involved in the pathogenesis of B cell destruction, ICSA must be considered as the best candidates.

To obtain monoclonal autoantibodies specific for the surface of islet cells, we have hybridized lymphocytes from diabetic BB rats with rat myeloma cells. This report describes the generation and preliminary characterization of one stable rat hybridoma which produces a monoclonal immunoglobulin M specific for the surface of rat islet cells.

Materials and methods

Inbred LOU rats were obtained from the National Institutes of Health (Dr. C. Hansen, Bethesda, MD, USA) and diabetes-prone BB rats were kindly provided by Dr. Pierre Thibert (Ottawa, ON, Canada). Outbred Wistar rats were purchased from Charles River Laboratories, (St. Constant, QC, Canada). Diabetic BB rats were used for fusions within 48 h after the onset of overt diabetes [1].

Two different rat myeloma cell lines were used; both originated from the LOU/C rat strain. The Y. 3 Ag 1.2.3 (provided by Dr. C. Milstein, Cambridge, UK) spontaneously secretes a kappa chain [12], whereas the IR 983 F is a non-secreting cell line (provided by Dr. H. Bazin, Louvain, Belgium) [13]. Four lines of other cells and several types of primary cultures were used to test for specificity of antibodies produced. RIN5F cells (NEDH rat insulinoma cell line) were supplied by Dr. A. Lernmark (Gentofte, Denmark). C58 (NT)D-I-G is a Wistar Furth rat T lymphoma cell line (provided by Dr. R. Hyman, Salk Institute, La Jolla, CA, USA). BRL is a Buffalo rat liver cell line (obtained by Dr. A. Bertolotti, CNRS, France). HIT-T15 is a hamster insulinoma cell line (kindly provided by Dr. R. Santerre, Eli Lilly, Indianapolis, IN, USA). A primary culture of Wistar rat fibroblasts was initiated from a preparation of newborn Wistar rat pancreatic islets as described by Brundstedt [14]. Normal Wistar rat hepatocytes were isolated by collagenase digestion [15]. Wistar rat peritoneal macrophages were obtained by a standard method [13]. Normal rat islet cells were prepared as described previously [9].

Y.3 Ag 1.2.3, IR 983 F and C58 cells were cultured in DMEM medium containing 10% foetal calf serum (FCS) and 60 mg/l gentamycin. RIN5F cells, BRL cells and fibroblasts were grown in RPMI 1640 with 10% FCS and 60 mg/l gentamycin, while HIT-T15 cells were grown in Ham's-F12 medium supplemented with 15% horse serum and 2.5% foetal calf serum. Culture media, foetal calf serum, horse serum and other supplements were purchased from Gibco Laboratories (Burlington, ON, Canada).

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Table 1. Results of the cellular enzyme linked immunosorbent assay

	RIN5F cells	Normal islet cells	Hepatocytes	BRL Cells	C58 (NT) D-1-G Cells	Fibro- blasts
Monoclonal antibody IC ₂	0.278	0.241	0.035	0.003	0.027	0.034
Control Monoclonal Antibody	0.021	0.011	0.044	0.000	0.004	0.000

Supernatants of IC₂ and control monoclonal immunoglobulin M were tested on the same number of six types of live rat cells. The values represent the mean optical density of duplicate evaluations. Blanking was obtained by replacing the primary antibody with buffer alone

Rat myeloma cells were used for fusion after a week of exponential growth. They were mixed with the BB rat lymphocytes (isolated by teasing of spleen and lymph nodes) in a 1:5 ratio and fused in the presence of polyethylene glycol as previously described [12, 13]. Cells were then plated in 24 and 96 well plates at a concentration of 10⁶ cells/ml in DMEM, 20% FCS, 2% HAT on a feeder layer of Wistar rat peritoneal cells or erythrocytes. Screening of the supernatants was performed 15 to 25 days after the fusion. Cloning of hybridomas was performed by two successive limiting dilutions. Several fusions involved BB rat lymphocytes which had been prestimulated in vitro for 24 h with lipopolysaccharide (LPS, 5 µg/ml) (Difco Laboratories, St. Laurent, QC, Canada). After cloning, hybridomas were grafted first subcutaneously and then intraperitoneally (for ascites production) into histocompatible (LOU/M × BB)F₁ animals.

The heavy and light chain isotype of the monoclonal antibodies (MAB) was determined by Ouchterlony's technique using specific antisera kindly provided by Dr. H. Bazin as previously described [13]. The mouse monoclonal antibody MARK-1, specific for rat kappa chain, was used to purify monoclonal antibodies with kappa light chain (more than 95% of rat Ig have kappa chains) by affinity chromatography as previously described [16].

Screening of hybridoma supernatants

Culture supernatants were screened by an indirect immunofluorescence (IF) and a cellular enzyme linked immunosorbent assay (CELISA) for antibodies reactive with the surface of living RIN5F cells. For IF, RIN5F cells grown as monolayers were detached with Dulbecco phosphate buffered saline without calcium and magnesium (D-PBS, CMF). After two washes in RPMI 1640, 5% FCS 10⁶ cells were incubated for 30 min at 4°C in 100 µl of supernatant. After two rinses, the cells were incubated for 30 min at 4°C with 100 µl of affinity purified goat antibody to rat IgM and IgG conjugated with fluorescein (Cappel Laboratories, Malvern, PA, USA). After washing, the cells were resuspended in medium with 0.1% NaN₃ and examined for surface fluorescence with a Leitz microscope.

For the CELISA, RIN5F cells were detached with D-PBS, CMF and distributed into a flexible, V-shaped, polyvinyl-chloride, 96-well microtitration plate (Dynatech Laboratories Inc., Alexandria, VA, USA) at a concentration of 2.5 × 10⁵ cells per well. The plate was centrifuged for 5 min (2000 rpm, 4°C) and the supernatant discarded. The plate was then vortexed to resuspend the cell pellet and 100 µl of supernatant was added to each well (each supernatant was tested in duplicate). After a 90-min incubation at 4°C, the cells were washed three times and resuspended in 100 µl of affinity purified sheep antibody to rat IgM and IgG conjugated with peroxidase (Cappel Laboratories, Malvern, PA, USA). After another 90 min incubation, the cells were washed five times and then transferred to another microtitration plate to avoid any non-specific binding of the different reagents to the plas-

tic. After the fifth wash, cells were resuspended in 200 µl of D-PBS, CMF. The PVC plate was put on top of a rigid, flat bottomed 96-well microtitration plate (Nunc Laboratories, Kamstrup, Denmark). The bottom of each upper well was then punched with an 18-gauge needle with trocar in place (Becton Dickinson & Co., Rutherford, NJ, USA) and the plates were centrifuged on top of each other for five min (2000 rpm, 4°C). The supernatant was flicked out of the rigid plate. The cells then were resuspended in 100 µl of a o-phenylenediamine (Sigma Laboratories, St. Louis, MO, USA) solution (2 mg/ml in phosphate-citrate buffer, pH5 with 0.1% H₂O₂). The enzymatic reaction was allowed to proceed for 15 min at room temperature and then was stopped by the addition of HCl, 4 N. The absorbance was read at 492 nm in an automatic ELISA reader (Flow Laboratories, Mississauga, ON, Canada). The intraassay and interassay variabilities were found to be 12% and 17% respectively.

Assessment of islet cell surface specificity

When monoclonal antibodies were found to react with the surface of RIN5F cells in both IF and CELISA assays, their specificity for rat islet cell surface determinants was assessed using the same tests on different types of rat living cell suspensions. Controls were normal rat affinity-purified immunoglobulins (Calbiochem-Behring, La Jolla, CA, USA) or other rat MABs of the same isotype, but different specificities and used at the same concentration. The monoclonal antibodies were also tested by IF on 4 µm unfixed cryostat sections of rat pancreas, thyroid, brain, stomach, kidney, thymus, adrenal gland and liver using the same reagents and incubation times as for cell suspensions. In addition, adsorption tests on different types of cells were carried out: equivalent volumes of supernatant and cells were mixed for 60 min at 37°C and subsequently for 30 min at 4°C, then separated and tested against RIN5F cells as described.

To characterize the biochemical nature of the target antigen on the surface of islet cells, dispersed RIN5F cells (10⁶) were incubated at 37°C for 15 min with 500 µg enzyme/ml D-PBS, CMF for pronase (pH 7.5), trypsin (pH 7.5), ficin (pH 6.0), chymotrypsin (pH 7.5) or for 60 min with protease-, aldolase-, and lecithinase-free neuraminidase (1 U/ml, pH 6.0) as described in [17]. Proteolytic enzymes were purchased from Boehringer Mannheim (Indianapolis, IN, USA), neuraminidase from Calbiochem-Behring Corp. (San Diego, CA, USA). Control incubations were carried out with buffer alone. After washing, monoclonal antibody binding to RIN5F cells was assessed by CELISA as described above.

Statistical analysis

Statistical analysis of the results (Wilcoxon's test) was performed with a statistical analysis package (Statpac, Walonick Associates, Minneapolis, Minn, USA) on an IBM personal computer.

Results

Twenty-four fusions have been made with either IR 983 F or Y.3 Ag 1.2.3 rat myeloma cells. The percentage of wells with growing hybridomas was high (>80%). However, most of the secreted antibodies which gave a positive reaction during the screening on RIN5F cells were found to be nonspecific for a particular cell type. Some of them were shown to react with cytoskeletal antigens as assessed by specific ELISA (P. Poussier, unpublished data). One fusion resulted in a stable hybridoma producing a monoclonal antibody specific for the surface of rat islet cells. The fusion involved Y.3 Ag 1.2.3 cells and LPS-prestimulated BB lymphocytes. This

Fig. 1 A and B. Indirect immunofluorescence staining of normal rat islet cells incubated with IC₂ (1:50 dilution of ascites fluid) followed by staining with FITC conjugated goat antirat IgM. **A** Phase contrast photomicrograph of three islet cells. **B** Fluorescence photomicrograph of the same field as in **A** showing the surface staining pattern on two of the islet cells ($\times 400$)

Fig. 2. Binding of IC₂ to the surface of RIN5F cells after exposure of these cells to different enzymes. Results are expressed on the Y-axis as the percentage of control absorbance values (assessed in cellular enzyme linked immunosorbent assay) observed after binding of IC₂ to enzyme exposed cells

monoclonal antibody, called IC₂, was shown to be an IgM with kappa light chains. Table 1 illustrates the results obtained in the CELISA with IC₂ and a control monoclonal antibody on different types of rat living cells. A positive response was observed only when IC₂ was tested on RIN5F or normal rat islet cells. In contrast, there was no difference between IC₂ and negative control when C58, BRL, fibroblasts or hepatocytes were used as target cells. When tested by CELISA on RIN5F cells, the optical density obtained after adsorption of IC₂ on RIN5F cells was 0.015 vs 0.315 without prior adsorption. This response was not significantly different from that of the control monoclonal antibody. In contrast, prior adsorption of IC₂ on BRL cells decreased minimally the OD (0.244 vs 0.298), which is not significantly different from unadsorbed IC₂. Thus IC₂ reacted only with RIN5F and normal rat islet cells in the CELISA. The results were confirmed by indirect immunofluorescence on rat living cell suspensions (Fig. 1): it bound to the surface of 72 $\pm$ 27% of RIN5F and 56 $\pm$ 5% of normal rat islet cells (mean $\pm$ SD, $n=6$). In contrast, it did not react with the surface of the other rat cells (i.e. erythrocytes, fibroblasts, hepatocytes, macro-

phages, C58 or BRL cells). However, when tested on HIT-T15 cells (hamster insulinoma cells) IC₂ was found to label the surface of 95 $\pm$ 3% of the cells.

Neuraminidase treatment of RIN5F cells failed to affect binding of IC₂ to the surface of these cells (Fig. 2). In contrast, treatment of RIN5F cells by proteolytic enzymes reduced subsequent IC₂ binding by 72–100%. These results suggest that IC₂ specific islet cell surface antigen is a protein.

Discussion

The spontaneously diabetic BB rat shows circulating ICSA as well as autoantibodies specific for multiple organs [1, 9]. This study illustrates that hybridization of diabetic BB rat lymphocytes with rat myeloma cells can result in a stable hybridoma secreting a monoclonal autoantibody binding to the surface of rat islet cells. Indeed, among the different rat cells which were used in IF and CELISA to assess the specificity of IC₂, only rat islet cells (either normal or tumoural) were found to carry on their surface an epitope bound by IC₂. Furthermore, binding of IC₂ to the surface of rat islet cells was abrogated by its prior adsorption on RIN5F cells, but not on other cells. The cross reactivity of IC₂ with the surface of normal rat islet cells and rat insulinoma cells confirms similar results observed with circulating anti-pancreatic autoantibodies in the BB rat [9]. Similarly, Yokono et al. [18] have produced a monoclonal islet cell surface autoantibody (3A4) in the NOD mouse which binds to the surface of normal mouse and rat islet cells, as well as hamster insulinoma cells. The lack of species specificity appears to be a common characteristic of 3A4 and IC₂ since both bind to the surface of hamster insulinoma cells.

Human and mouse monoclonal antibodies reacting with the cytoplasm of islet cells have been obtained [6, 19]. On the other hand, immunization of mice with rat islet cells or chicken embryo retina cells resulted in mouse monoclonal antibodies specific for ganglioside antigens present on the surface of islet cells [17, 20]. These mouse monoclonal antibodies gave an ICSA-like

pattern when tested on cell suspensions, but an ICA-like pattern when tested on pancreatic sections. This observation has been extended to question the duality of ICA and ICSA which are found in Type 1 diabetic syndromes [21]. IC₂ which binds to the surface of a relatively high percentage of normal rat islet cells, failed to label these cells when tested by indirect IF on cryostat sections of rat pancreas even at high concentrations (data not shown). It also failed to label cryostat sections of other rat organs. We do not have a satisfactory explanation for this observation, except that cell surface antigens are probably less numerous on cryostat sections than on cell suspensions. It might also suggest that IC₂ is specific for an islet cell surface antigen different from those bound by the ganglioside specific mouse monoclonal antibodies. One of these mouse monoclonal antibodies, R2D6, binds to the surface of normal, but not tumoural rat islet cells. R2D6 binding is inhibited by neuraminidase treatment of rat islet cells, but remains unaffected by protease treatment of these cells. In contrast, IC₂ reacts with the surface of both normal and tumoural rat islet cells, and its binding is almost completely abrogated by protease treatment of these cells. In contrast to ICSA, ICA have not been reported in the serum of the BB rat. Their apparent absence, as well as the failure of IC₂ to react with islet cells on cryostat sections, seems unlikely to be related to a technical difficulty since many laboratories have tried to find them. On the other hand, the NOD mouse monoclonal antibody 3A4 has not been reported to bind to pancreatic sections either, suggesting that its specificity could be similar to that of IC₂ especially when one considers that both react with the surface of normal rat islet cells and hamster insulinoma cells.

The availability of monoclonal islet cell surface autoantibodies such as IC₂ or 3A4 in animal models of Type 1 diabetes should be helpful in the understanding of antipancreatic autoimmunity particularly when they, as in the case of IC₂, have isotypes expected to react with the complement system [22].

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