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Human pancreatic islet cell specific 38 kilodalton autoantigen identified by cytomegalovirus-induced monoclonal islet cell autoantibody

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Summary. Our previous finding that about 15% of newly diagnosed patients with Type 1 (insulin-dependent) diabetes mellitus had human cytomegalovirus genome in their lymphocytes and islet cell autoantibodies in their sera, suggests that autoimmune Type 1 diabetes is associated with persistent cytomegalovirus infection under certain circumstances. This investigation was initiated to see if cytomegalovirus can induce islet cell autoantibodies and if the autoantibodies react with any specific islet protein(s). Monoclonal antibodies were generated after immunizing Balb/c mice with human cytomegalovirus. When these monoclonal antibodies were tested for the presence of islet cell antibodies, one (MCMVA-51) of 13 monoclonal antibodies reacted strongly with the islets. The titer of islet cell antibodies was 1:2000. When this monoclonal antibody was reacted with the proteins from the solubilized fraction of human pancreatic islets using the western immunoblotting technique, a band with a molecular weight of 38 kilodalton was detected. The 38 kilodalton band was not observed when the monoclonal antibody was reacted with the proteins prepared from pancreatic islet tissues of rats and mice or from other human organs including stomach, liver, spleen and brain, indicating that the 38 kilodalton protein is human islet cell-specific. It is concluded that human cytomegalovirus can induce islet cell antibodies that react with a 38 kilodalton human islet cell protein and that this protein component may represent islet cell-specific target antigens associated with persistent cytomegalovirus infection.

Key words: Cytomegalovirus, islet cell antibody, 38 kilodalton antigen, Autoimmunity.

Type 1 (insulin-dependent) diabetes mellitus is characterized by pancreatic Beta-cell destruction which leads to hypoinsulinaemia and hyperglycaemia. Genetic factors appear to be a prerequisite for the development of diabetes, but non-genetic factors such as environmental factors can also influence clinical expression of the disease. Viruses, as one of the non-genetic factors affecting the induction of diabetes, may act as primary injurious agents for pancreatic Beta cells or as inducing agents for autoimmunity in man and animals [1].

Recently, we reported that pancreatic Beta cell-specific expression of endogenous retrovirus is associated with the development of insulitis and diabetes in nonobese diabetic (NOD) mice which spontaneously develop an autoimmune-mediated diabetic syndrome [2]. In addition, congenital rubella syndrome provides one of the best pieces of evidence that viral infection can be associated with the subsequent development of autoimmune Type 1 diabetes in man [3]. Other evidence includes a case-report of a child with congenital cytomegalovirus (CMV) infection who became diabetic at the age of 13 months [4]. In another report, characteristic inclusion bodies (20/45 cases) were found in the islet cells of infants and children who died of disseminated CMV infections [5]. We recently reported that about 15% of newly diagnosed Type 1 diabetic patients had both CMV genome in their lymphocytes and islet cell autoantibodies in their sera, suggesting that persistent CMV infections may trigger autoimmune Type 1 diabetes under certain circumstances [6]. The present study was designed to test the hypothesis that CMV can induce islet cell autoantibodies (ICA) and, if so, to determine whether or not the autoantibodies are able to react with any specific proteins from detergent lysates of human islet cells.

Materials and methods

Cell fusion

Twelve-week-old BALB/c female mice obtained from the Jackson Laboratory (Bar Harbor, ME) were immunized intraperitoneally once a week for a period of 5 weeks with 5×10^6 $TICD_{50}$ of human cytomegalovirus (strain AD 169). Three to four days after the final virus injection, mouse splenocytes (10^8 cells) were fused with 8-aza-

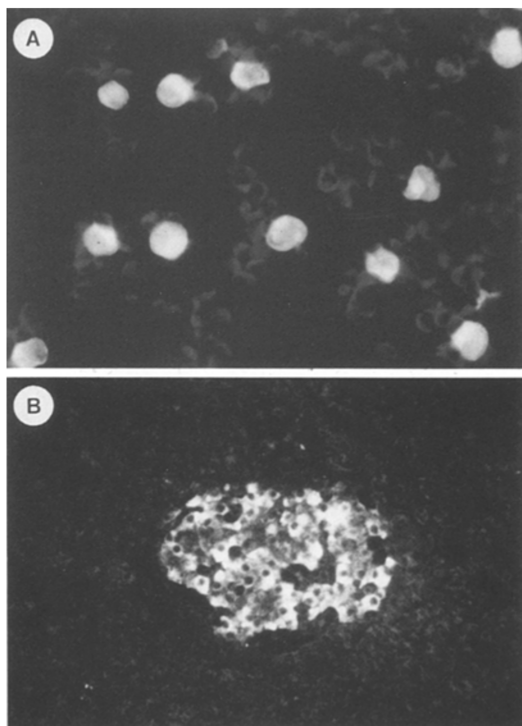

Fig. 1 A–B. Human lung fibroblasts (WI-38 cells) infected with cytomegalovirus and reacted with MCMVA-51 monoclonal antibody followed by FITC-labelled anti-mouse IgM staining. Several cells reveal viral antigen (**A**) (X400). Monoclonal antibody MCMVA-51 reacted with frozen section of normal human pancreas. Cytoplasmic staining of islet cells (**B**) (X200)

guanine-resistant nonimmunoglobulin-secreting mouse myeloma cells (sp-1) (10^7 cells) using 50% (weight/volume) polyethylene glycol 1000 according to the method described elsewhere [7]. Hybridomas were selected in HAT medium containing 10^{-4}M-hypoxanthine, 4×10^{-7}M-aminopterin, and 1.6×10^{-5}M-thymidine. Two to four weeks after seeding of the hybridomas, the supernatant fluids from the cultures were tested for immunoglobulin production. Hybridomas making antibodies against cytomegalovirus were cloned by the limiting dilution method [7]. Peritoneal-exudate cells from Balb/c mice were used as feeder cells for the cloning of hybridomas. Antibodies from culture supernatants were concentrated by precipitating immunoglobulins with 50% saturated ammonium sulfate. The immunoglobulin class was determined by the Ouchterlony method.

Detection of antibodies against cytomegalovirus

Supernatant fluids from hybridomas were screened for antibodies against CMV by the indirect immunofluorescence technique. Briefly, human lung fibroblasts (WI-38 cells), cultured on coverslips, were infected with CMV (strain AD169) at 10 multiplicities of infection. Five to seven days later, the cells were washed in phosphate buffered saline (PBS) and fixed with cold acetone for 10 min. Supernatant fluids from each of the hybridoma cultures were incubated with the cells for 40 min at room temperature and then washed in PBS. Fluorescein isothiocyanate (FITC)-labelled anti-mouse immunoglobulin (IgG and IgM) was added to the cells, incubated for 40 min at room temperature, and washed in PBS. The coverslips were mounted on clean slides with either Elvanol or a mixture of 50% glycerol and 50% PBS, and the cells were observed under an Olympus BH-2 fluorescence microscope. After screening for reaction against CMV, the positive supernatant fluids were screened further for reaction against other herpes viruses, ie, herpes simplex virus (HSV, Type 1 and 2) and Epstein-Barr viruses (EBV, strains EB-3 and HR-1). HSV were cultured on vero cells and EBV were cultured on human lymphoblastoid cells and were screened as described above.

Detection of autoantibodies against islet cells

For the first screening, only supernatant fluids that contained anti-CMV antibody were tested for autoantibodies against islets by indirect immunofluorescence on unfixed frozen sections of normal human pancreas from a cadaveric donor with blood-type "O". The frozen pancreatic sections were first incubated with the supernatant fluids from the hybridomas for 3 h at room temperature, washed, and then incubated with an appropriate dilution of FITC-conjugated anti-mouse IgG or IgM for 3 h at room temperature. Background staining was assessed by using monoclonal IgG or IgM antibodies that did not react with human tissues. For the second screening, 127 randomly selected supernatant fluids which did not contain anti-CMV antibody were also tested for islet cell autoantibodies. None of them contained such antibodies. Similarly, fresh frozen sections of pancreata from rat (Wistar-Furth) and mouse (SJL/J) were stained with MCMVA-51 to test for species-specificity.

Double-labelled immunofluorescent antibody staining

The fresh frozen sections of human pancreata (blood type "O") were fixed in cold acetone for 10 min and air-dried. The sections were incubated with the hybridoma supernatant which was positive for islet cell staining for 3 h at room temperature and then washed with PBS. The next incubation was with fluorescein isothiocyanate (FITC)-labelled antibody against mouse IgG or IgM, followed by washing in PBS. The sections were then incubated with antibody against human insulin or antibody against human glucagon (raised in guinea pig) (Incstar, Stillwater, Minn., USA) for 3 h at room temperature, washed in PBS, and incubated with tetramethyl rhodamine isothiocyanate (TRITC)-labelled anti-guinea pig IgG. Finally, the sections were washed in PBS, mounted with Elvanol, and examined for immunofluorescence with an Olympus BH-2 microscope. Double-stained cells were identified by examining the section first with a fluorescent filter, and then with a rhodamine filter.

SDS-PAGE and western immunoblotting

Fresh normal human pancreatic islets (Donor's HLA type: HLA A1, A26, B8, DR2/7) were obtained from Dr. R. Rajotte at the University of Alberta, Edmonton, Canada. The islets were lysed in 1% NP-40 solution by homogenization, and incubated for 60 min at 4 °C. After discarding the cell debris by centrifugation, the solubilized cellular protein was obtained by centrifugation at 100,000 *g* for 1 h at 4 °C. The protein concentration was measured by the Bio-Rad method (BioRad, Richmond, Calif., USA). The islet protein (20 μg/lane) was electrophoresed in 10% SDS-polyacrylamide gel (SDS-PAGE) for 5 h at 40 mA. The proteins were transferred onto nitrocellulose membrane electrophoretically in blotting buffer solution (25 mmol/l Tris, 192 mmol/l glycine, 20% methanol) overnight at 120 mA at 4 °C. The membrane was preincubated with 5% bovine serum albumin in PBS at room temperature for 3 h. The membrane was incubated with diluted monoclonal antibody (1:200 with PBS) for 5 h at room temperature with gentle agitation. The membrane was washed in PBS with 0.05% Tween-20 (PBS/Tween) at room temperature for 30 min, and incubated with the alkaline phosphatase-labelled anti-mouse IgG or IgM (1:200 diluted) at room temperature for 2 h. After extensive washing in PBS/Tween, the substrates [nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in Tris buffer] were added and colour development was ob-

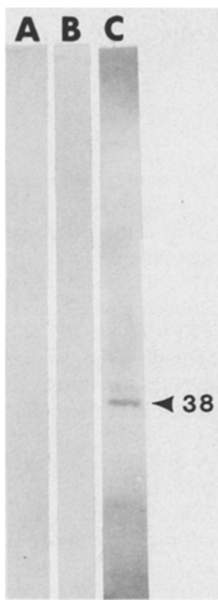

Fig. 2. Western immunoblotting of human islet cell-protein extracts with monoclonal antibodies including MCMVA-10 (Lane A), MCMVA-93 (Lane B), and MCMVA-51 (Lane C). Molecular weight marker is indicated at right. Lane A, MCMVA-10 and Lane B, MCMVA-93 failed to react with 38 kd islet protein but Lane C, MCMVA-51 reacted with 38 kilodalton islet cell protein

served. In addition to the islets, normal human stomach, lung, spleen, and brain tissues were obtained from a cadaveric donor. The proteins from these tissues were extracted and western immunoblotting was performed as described above.

Results

Thirteen monoclonal antibodies against CMV were obtained from 13 fusions after immunization with CMV (Fig. 1 A). These monoclonal antibodies did not react with other herpes viruses such as HSV-1, HSV-2, or EBV strains EB-3 and HR-1 indicating that these monoclonal antibodies are specific for CMV. When these 13 monoclonal antibodies, including MCMVA-10, 14, 25, 51, 57, 60, 66, 88, 93, 98, 110, 129, and 135, were tested for autoantibodies against islets by indirect immunofluorescence on frozen sections of normal human pancreas, only one of them, designated MCMVA-51, was found to be positive (Fig. 1 B). This monoclonal antibody had a titer of 1:2000 and an immunoglobulin class of IgM. MCMVA-51 was tested for reaction with pancreatic islets from rat and mouse. These tissues were not stained. In addition, MCMVA-51 reacted with insulin containing Beta cells but not with glucagon-containing alpha cells.

When all 13 monoclonal antibodies were screened on total detergent pancreatic islet lysates, using western immunoblotting techniques, only one of them (MCMVA-51) reacted with the protein of MW 38 kilodalton (kd) (Fig. 2). In contrast, none of the 13 monoclonal antibodies reacted with the 38 kd protein of the detergent lysates of stomach, lung, spleen, and brain.

Discussion

Patients with Type 1 diabetes have a high prevalence of autoantibodies against pancreatic islet cells at the time of diagnosis. However, what triggers the production of islet cell autoantibodies has yet to be discovered. In the present study, we have shown for the first time, that ICA, which reacts with a 38 kd human pancreatic islet cell-specific protein, can be induced by human CMV.

It has been suggested that viral infections might trigger an autoimmune response [8]. One of the possible ways that CMV could induce ICA is through molecular mimicry, defined as similar structures shared by molecules from dissimilar genes or by their protein products [8, 9]. An antigenic determinant on CMV might be similar to a determinant on the pancreatic islet cells. If this is the case, the monoclonal antibody raised against the antigenic determinant of the viral protein could react with the islet cells. When the MCMVA-51 was reacted with the solubilized cellular protein of human pancreatic islets using the western immunoblotting technique, a band with a molecular weight of 38 kd was detected. This 38 kd protein was not found in other human tissue extractions and MCMVA-51 did not react with rat or mouse 38 kd islet cell proteins, indicating that the 38 kd protein is human islet cell specific. The species-specific recognition of the monoclonal antibody (MCMVA-51) is probably due to the epitope of human CMV. A specific antigenic determinant of human CMV, which induces monoclonal antibody MCMVA-51, may have a certain amino acid sequence homology with human islet cell antigen, but not with mouse islet cell antigen. Thus, this monoclonal antibody, generated in the mouse, does not recognize mouse islets.

We have also examined the prevalence of anti-38 kd antibody in sera from 20 non-diabetic individuals and 46 Type 1 diabetic patients with or without CMV genomes. The antibody against 38 kd protein was not found in non-diabetic individuals. The prevalence of anti-38 kd antibody in the Type 1 diabetic patients with CMV genome (57.6%) was significantly higher than that in patients without CMV genome (20.0%), suggesting that CMV genome is somehow associated with the induction of anti-38 kd antibody in the patients (Pak, Cha, McArthur and Yoon, unpublished data). A possible explanation for association of CMV with the induction of an antibody is that persistent CMV infection in pancreatic islets might result in the expression of islet cell-specific 38 kd autoantigen, which subsequently induces antibodies against the protein. Another possibility is that some portion of CMV antigen expressed on the CMV-infected islet cells may have amino acid sequence homology with some portion of the 38 kd islet cell protein.

It was previously shown that amino acid sequence homology was found between viral (hepatitis B virus) protein and rabbit myelin basic protein (MBP) encephalitogenic protein [9]. Hepatitis B virus polymerase (HBVP) was found to share six consecutive amino acids with the encephalitogenic site of rabbit MBP. Rabbits given injections of a selected 8- or 10-amino acid peptide from HBVP produced antibody that reacted with the sequences of HBVP and also with native MBP. A recent study showed sequence homology and immunologic cross-reactivity of human CMV with the HLA-DRβ chain [9]. Whether or not CMV can really induce 38 kd autoantigen, and if CMV antigen has amino acid sequence homology with islet cell protein remain to be determined.

It was previously suggested that 64 kd and/or 38 kd protein components may represent islet cell-specific target antigens in Type 1 diabetes [10]. What might induce these islet cell-specific target antigens is, however, not known. In this study, we have shown that persistent CMV infection might be involved in the expression of Beta cell-specific 38 kd antigen, which cross-reacts with ICA, in Type 1 diabetes. In a previous study, a 38 kd component from HLA-DR3-positive donor islets was precipitated in a reaction with sera from Type 1 diabetic patients [10], and the authors suggested that further studies were needed to determine whether islet cells from DR3-positive individuals contain a unique antigen recognized by diabetic sera. In our study, we used donor islets from HLA-DR2/7-positive individuals; sera from Type 1 diabetic patients reacted with 38 kd islet protein, indicating that it may not be a HLA-DR3 unique antigen. However, the detection of different antigenic components with the same molecular weight cannot be excluded since two different techniques, immunoprecipitation and western blotting, were used.

Finally, a variety of aetiological factors may be involved in the pathogenesis of Type 1 diabetes. It appears that the disease process is likely to be genetically mediated but environmentally initiated and modulated. If viruses are one of the environmental factors involved in the pathogenesis of diabetes, we should consider at least two possibilities: one, that cytolytic infection of Beta cells results in the destruction of the cells without the induction of autoimmunity or may be a final insult leading to the clinical onset of diabetes in individuals with an already decreased Beta-cell mass resulting from an autoimmune process, and two, that persistent infection results in the triggering of an autoimmune process [9]. In the latter case, we have previously shown that persistent CMV infection might trigger Beta cell-specific autoimmune disease under certain circumstances [6]. In connection with that, we now report that human CMV can induce islet cell autoantibody which reacts with a 38 kd human pancreatic islet cell-specific protein.

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