Department of Physics, Chemistry and Biology

Final Thesis

Identification and characterization of molecular nature of the IC2 auto antigen on the pancreatic beta cell membrane.

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Preface

This project was initiated based on preliminary immuno HPTLC studied performed by Professor Steven Spitalnik at the Columbia University in New York. He described a surprising binding of the monoclonal autoantibody IC2 to sulfatides, even down to epitope level. The difficulties to understand this specificity to be beta-cell specific inspired us to do additional studies with glycolipids from pancreatic beta-cell lines.

The Master's thesis (From April 2008 to March 2009) is framed within a large research project to understand the molecular nature of the IC2 auto antigen on the pancreatic beta-cell plasma membrane. The project is funded by the International Juvenile Diabetes Research Foundation. Further funding is now requested from the American Institute of Health (R 24 grant). In this work we were trying to establish that IC2 reacts with Sulfatide, the preliminary study of Professor Steven Spitalnik, Columbia University, New York. The work is planning to continue in June-July, 2009, further collaboration with Dr. Jackues Protoukalian to complete the last immuno TLC part.

Copenhagen, 20th march, 2009 *Md. Golam Kafi Afrose Mia*

Content

1. Abstract

IC2, a well known monoclonal antibody, derived from newly diabetic BB rat and seems to be an important biomarker for non-invasive functional imaging of beta cells in vivo. It specially and uniquely binds with pancreatic beta cells as confirmed in some previous studies. RIN-5AH is a pancreatic beta cell, which reacts with IC2 is used here to identify and characterize the molecular nature of the IC2 auto antigen by using TLC and HPTLC following by immuno-staining. Actually, an unpublished work already had done by Spitalnik et al, 1991 with another rat pancreatic beta cell (RINm5F) extracted glycolipids. In this studies, the same work was done not only with glycolipids from various cell lines but also lipids extracted from purified plasma membrane is made to confirm or refuge that IC2 was found to bind with only the glycolipids containing galactose-3-sulfate. This highly unique observation can however hardly explain the unique beta cell surface specificity without involvement of other more beta cell specific antigenic structures. We are therefore also searching the protein part involved in the auto antigenic determinant. Analyzing the molecular nature of IC2 binding auto-antigen, will help to understand both the role it might plays in the pathogenesis of insulin dependant diabetes. It could also help to elucidate the etiology of diabetes and finally to be a new serum autoantibody biomarker.

2. List of Abbreviations

BSA- Bovine Serum Albumin

PBS-Phosphate Buffered Saline pH 7.4

TBS- Tris Buffered Saline

TID- Type-1 Diabetes

LPS- Lipo Poly Saccharides

BB rat- Bio Breeding rat

BCM- Beta Cell Mass

NKT- Natural killer T cells

SNARE- Soluble N-ethylmaleimide sensitive factor attachment protein receptor.

ATP- Adenosine Tri-phosphate

TLC- Thin Layer Chromatography

HPTLC- High Performance Thin Layer Chromatography

KCL- Potassium Chloride

3. Introduction

Diabetes mellitus is a metabolic disorder characterized by beta cell loss and insulin deficiency. Due to the hereditary disorder, blood glucose level rises abnormally which is also controlled by the complex interaction of multiple chemicals and hormones like insulin, which produced in the beta cells of the pancreas. Actually, Diabetes mellitus means a group of diseases that lead to high blood glucose levels due to abnormalities either in insulin secretion or its action (Rother KI, 2007). Insulin is the principal hormone for regulation of glucose metabolism as well as to convert sugars, starches and other foods into energy required for our life. It's deficiency or insensitivity of It's receptors play a vital role in all types of diabetes mellitus. Nowadays, diabetes has been treatable due to the availability of insulin but it is still incurable.

There are two common types of diabetes depending on insulin unavailability or its functional activities. In type-1 diabetes (TID), the pancreas is unable to produce insulin. Glucose from food increases the blood glucose level due to lack of insulin and cause health problem. Normally, insulin-producing beta cells are mainly destroyed by an autoimmune process (Saudek et al, 2008). Transplantation of pancreas or isolated islets have shown that type 1 diabetes can be reversed providing efficient immuno suppression to prevent both rejection and autoimmune destruction (Serup et al, 2001). Unlike type 1 diabetes, type 2 diabetes pancreas can produce insulin but it is unable to metabolise blood glucose, as a result blood glucose level increase. In both human and animals, auto immunity plays an important role in the pathogenesis of TID (Mandrup-Poulsen T & Nerup J, 1990 and Mordes et al, 1987). In case of TID, body has mistakenly identified and targets insulin producing beta cells as being foreign or non-self and destroyed it.

IC2, a unique beta-cell surface specific monoclonal autoantibody and stable hybridoma that raised from LPS–stimulated spleen cell of a non-immunised diabetic BB-rat (Sweet et al, 2004) and fulfilled both the specificity and affinity criteria required for in vivo imaging (Brogren CH et al, 1986). The aim of raising monoclonal auto antibodies from animal models and diabetic patients is to understand the role of these auto antibodies in the pathogenesis during diabetics, that already reviewed (Brogren and

Lernmark, 1982). Many other monoclonal antibodies and autoantibodies have been developed to understand autoimmune diseases but none of them is specific for beta cell surface (Madsen et al, 1990). IC2 seems to be the only useful marker for non-invasive functional imaging of native beta cells (Moore et al, 2001 & Hampe CS 2005). Therefore, it was chosen as a suitable candidate for the marker on the surface of beta cells to establish a pancreatic beta cell mass (BCM) quantification technique by Moore et, al (2001). The exact molecular feature of this auto antigen is still illusive and questioned by many researchers, which is apart from not only specific to insulin producing cells but also its specific binding location in the plasma membrane (Aaen K et al, 1990). It is also known that the binding of IC2 to beta-cells is trypsin sensitive but no standard immune precipitation methods has identified this protein-like auto antigen. According to Buschard et al (2005) Sulfatide can play role for both type 1 and type 2 diabetes which was isolated from the islet of Langerhans by Buschard et al (1989). It also isolated from leukocytes and Natural Killer T cells (NKT) which is non covalently bound (hydrogen-binding) to CD1d molecules. This Sulfatide CD1d complex regulates the autoimmune aggregation and insulitis in diabetic NOD- mice. As the expression of Sulfatide is directly related to the functional state of the beta-cell and its insulin producing capacity (Buschard et al, 1988 & Aaen et al, 1990), it is essential to study its molecular nature. Buschard et al, (2005) has speculated that Sulfatide epitope might involve in insulin secretion process which insist us to study this epitope on the pancreatic beta cell. For understanding its eventual impact on the insulin secretion where in insulin granules secretion is regulated by SNARE-proteins, and even the autoimmunity involved in the pathogenesis of diabetes (Brogren CH & Lernmark A 1982), it is important to study the molecular nature of the IC2 auto antigen on the pancreatic beta-cell plasma membrane. In this point of view we are trying to prove the hypothesis that, sulfatide loosely bound to an anchoring protein.

Sulfatide auto antibodies are found in several diseases, including type 1diabetes. Binding of IC2 to beta cells depends on two epitopes, one from galactose-3-sulfate group and another from the anchoring beta cell plasma membrane protein.

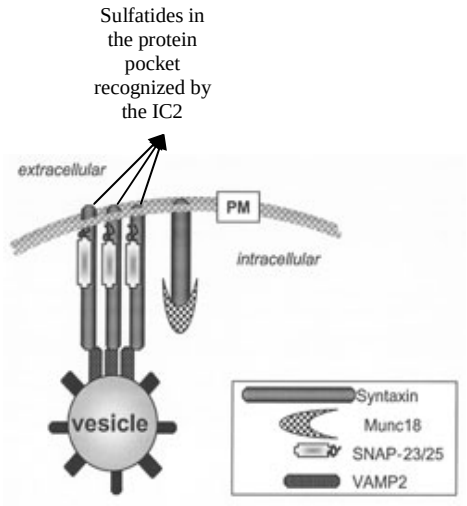


Figure 1- SNARE proteins and their function in insulin secretion also regulated by Sulfatide.

IC2 showed non-invasive in-vitro imaging to beta cells and the target molecules, "sulfatide" with beta cell surface anchoring protein present on islet cell. This epitope is present on the plasma membrane of pancreatic beta cells (Buschard K 1993b) and could be the relevant autoantigen in type 1 diabetes (Maria Blomqvist et al, 2003). Sulfatides control glucose-induced insulin secretion by activating ATP-sensitive K^+-(K_{ATP}) channels (Buschard K, et al, 2002). In addition, monomerization of insulin is also facilated by the sulfatide. It preserves insulin crystals and helps in proinsulin folding (Osterbye T et al. 2001). Scientists are still trying to establish monoclonal auto antibodies specificities to pancreatic beta cell imaging (Ladriere L, et al, 2001). Certainly, the sulfatide autoantigen is found on beta cell plasma membrane, forming complex structures which yet to be clarified. As it is easier to produce monoclonal antibody from rodent than human, mouse hybridoma technique has mostly been chosen to raise monoclonal autoantibody from diabetic mouse models (Moore et al, 2001). However, the BB rat shows similar insulin dependant diabetic syndromes in the aetiology of pancreatic beta cell destruction. Monoclonal autoantibodies derived from a newly diabetic BB rat by using hybridoma

technique, have been developed by other groups as well, but only the clone IC2 appear specific to beta cells.

SNARE protein complex in plasma membrane, exocytosize insulin secretary granule and dis-regulated insulin secretion. Insulin is released when the SNARE complex i.e. Syn 1A/ SNAP-25/ VAMP2 is expressed and very minor amount of this complex has been found in Diabetes (Ostenson CG, et al, 2006). In case of diabetic patient, dis-regulation of insulin secretion reduced (about 80%) Syn-1A protein and cognate SNARE proteins affect exocytosis and Ca^{2+}/ K^{+} ion channel activities. Glucose stimulation of beta cells increases surface binding of IC2 and also controlled the expression of the SNARE protein complex. Therefore, it is important to understand the molecular feature of the targeted autoantigenic epitope, which probably is linked with plasma membrane SNARE complex. Although IC2 autoantibody targets a galactose-3-sulphate containing sulfatide, its anchoring plasma membrane protein has not yet found.

In case of TID, BCM is decreased due to selective auto immune destruction of beta cells (Weir G, et al, 1990). Here we have done our experiments on both alfa and beta pancreatic plasma membrane to get the more clear and comparable picture on molecular nature of the IC2 beta-cell surface auto-antigen, which is important, perhaps also understand for the autoimmunity in diabetes as well as the pathogenic process in TID. Actually, we are trying to detect the exact composition of IC2 antigen on the beta cell surface, which is secreted with insulin in near junction of the plasma membrane, it is not clear whether degranulated beta cells would expressed enough of it to be detected by IC2 antibodies (Weir G et al, 1990).

Our hypothesis is to identify and characterize the IC2 auto-antigen by TLC, HPTLC and through immuno blotting of plasma membrane. Here, we are trying to determine whether IC2 specifically recognized using HPTLC immuno-TLC techniques according to the Spitalnik et al. (in preparation). Our aim 1) To verify his preliminary observation that sulfatide is the target epitope for IC2. 2) To identify sulfatide anchoring protein this is specific for beta cell.

4. Materials and Methods

3.1 Cells, culture media and their growth conditions – β cell: Rat insulinoma cell (RIN-5AH), α cell: Pancreatic T cell (α TC19), Rat mucosal mast cell (RBL cell), Rat pancreas cell (AR42J) and IC2 (auto antibodies) hybridoma cells derived from Rat myeloma Y3 cell were taken. RIN 5AH, α TC19, RBL and IC2 cell lines were cultured in RPMI 1640 medium containing 10% heat treated (56^0 C for 30 min) Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin (PS) and 0.1% 2-Mercaptoethanol (MeSH) according to Billestrup N & Martin JM (1985), Howard CJ, et al. (1993), J. Bertoglio, D. Gerlier & J.P. Gerard (1981) and Arinobu Tojo et al, (1987) respectively. Only AR42J cell line cultured in DMEM medium in the same conditions and 0.1% Plasmocin was added to protect mycoplasma in the media of IC2 hybridoma cells by following Brogren et al (1986). All cell lines were cultured in the incubator at 37^0C and 5% CO_2.

4.2 Cell counting and growth rate observation- The growth rate of different cell lines were observed daily by counting them in Hemocytometer chamber under light microscope by following Margaret Wolf et al, 2008.

4.3 Expansion of culture to NUNC cell factory – Cells were expand (using trypsin detachment) from small to medium to 10-12 large flasks (175 cm^2 each) and finally into the 10 layer (6300 cm^2) NUNC cell factory according to the open NUNC procedure of Margaret Wolf et al, 2008 , where the cells are grown for > 50% confluence before cell harvesting.

4.4 Cell harvesting- Different cell lines were harvested into 50 ml centrifuge tubes from the NUNC cell factory (using PBS EDTA detachment) for plasma membrane separation. Cells for lipid extraction were stored freezer after adding N_2 gas.

4.5 Lipid Extraction from cell pellets- Lipids from different cells were extracted by following the Spitalnik et al (Submitted) paper. Frozen cells were solubilized in glass centrifuge tube with 20 times of 1^{st} extraction solution; Chloroform: Methanol: Water

(4:8:1) by shaking for 15 min. Then remove the protein and other cellular parts by centrifuging at 2000 rpm for 10 min. The lower chloroform part containing lipids are washed with water and centrifuged for 5 min in the same speed to separate the lipid contains chloroform layer and dried under nitrogen flow.
Lipid pellets were solubilized in 2^{nd} (chloroform: methanol: water = 4:2:1) & 3^{rd} extraction solution (chloroform: methanol: water = 2:1:1) respectively and centrifuged in 2000 rpm for 10 min according to the Folch method. Lower chloroform part was taken and evaporated under nitrogen flow for 2^{nd} time to get pure dry lipid.

4.6 Plasma membrane Isolation- Cells from NUNC factory re-suspended in HM-buffer (0.25 M sucrose, 5mM Hepes, 0.5mM EDTA adjusted to pH 7.4) containing protease inhibitors cocktail tablets, complete tablet (50ml buffer/ tablet). To get plasma membrane, cells were disrupted in the "French press" machine under high pressure (580 psi corresponding to 40 bars), according to Brogren et al, 1979. The ice cold cell lysates solution (having plasma membrane ghost and the intact cell nuclei) were centrifuged at 2000 rpm (740 g) for 15 min. The nucleus free ghost cytoplasmic solution from the top was taken and the post nuclear supernatant mixed with 90% Percoll suspension (HM buffer with inhibitors were added to make 90% Percoll solution) to get a final 15% Percoll solution with supernatant (*Di Mario U & Dotta F, 1998)*. The plasma membrane layer (top) was separated from the granula fraction (bottom) by applying high speed (20,000 rpm) centrifugation for 35 min. Then the pure plasma membrane pellet was washed and isolated in the bottom by using high speed (25,000 g) ultracentrifugation for 1 hour from the plasma membrane containing solution (top layer). Plasma membrane pellet was stored at -20^0C to -80^0C after adding N_2 gas (to infinite lipid extraction).

4.7 Lipid from plasma membrane- More pure lipid also extracted from the plasma membrane pellets by following the same protocol that was applied to extract lipids from cell pellets.

4.8 Thin layer chromatography (TLC) and High performance TLC (HPTLC) - Various concentrations of cell lipids and plasma membrane lipids from five types of cells (RIN-5AH, RBL cell, α TC19, AR42 J and IC2) were applied in TLC and HPTLC plates. Here, Silica gel coated both AL_2OH (MERCK, Cat no-1055540001) & Glass plates (MERCK, Cat no-1137480001) were used as stationary phase. Different mobile phases like Chloroform: Methanol: Water (5:4:1, 3:4:1, 8:4:1 6:8:1, 1:5:1, 5:1:1, 2:5:1, 1:10:2) and 2-Methyl propanol: Ethanol: H_2O: 28% NH_4OH (100:67:67:2.3) were used for 40-45 min to get better separation.

4.9 Detection of TLC and HPTLC bands- To visualize separation bands, TLC and HPTLC plates were stained by different staining methods (Primulin, Orcinol, Thymol, Carbazole, Cu-acetate and Fluorescence) that are shown in the table-1. In all cases except Primulin and Fluorescence, heat treatment in high temperature was needed to detect bands. On the other hand for primulin and Fluorescence UV light at 350 nm is needed to visualize clear band.

Staining Methods	Chemical composition	Heat treatment	UV lamp	Stability
Primulin	5 mg primulin in 80 ml acetone + 20 ml H_2O	Not needed	350nm UV light	Stable
Orcinol	0.1gm orcinol in 40.7ml conc. HCL +1ml 1% iron III chloride in 100 ml 70% H_2SO_4	100^0C-120^0C for 15-30 min	Not needed	Stable
Carbazole	0.5 gm carbazole in 95ml ethanol and 5 ml 97% H_2SO_4	100^0C-120^0C for 15-30 min	Not needed	Not stable
Cu-acetate	3% Cu + 8% Phosphoric acid+ H_2O	150^0C-180^0C for 5-10 min	Not needed	Stable
Thymol	0.5 gm thymol in 95 ml ethanol and 5ml 97% H_2SO_4	100^0C-120^0C for 15-30 min	Not needed	Stable
Fluorescence procedure	10ml 2′7′dichlorofluroscence	Not needed	350nm UV light	Not stable

Table-1 Different staining methods that were performed to visualize both TLC and HPTLC separation bands.

4.10 Immuno blotting of TLC and HPTLC plates

4.10.1 Separation and Blocking

Dry plates were stained with 0.1% (poly-isobutylmethacrylate) + 0.1% (500 ml acetone+5mg poly isobutylmethacrylate) solutions for 90 seconds and dried under fume hood. They were incubated for 45 min in Tris-BSA (0.05 M Tris, 0.15 M NaCl, p^H 7.8 with 1% BSA and 0.1% NaN_3) after spraying with PBS containing 1% BSA (Bovine serum albumin).

4.10.2 Antibody treatment (will be done on the June-July, 2009)

This is the last part that I have a plan to do it on the June-July, 2009. Plates will be treated by the 1:40 dilution of primary monoclonal antibody IC2 for 1 hour. Here, we have a plan to use affinity purified rat monoclonal antibody *IA2F10* as a negative control and wash with PBS according to the unpublished work of Spitalnik et al,() . Then 2nd incubation for another 1 hour with 1:5000 dilution of rabbit anti rat IgM antiserum (ICN, Irvine, CA) in tris-BSA and overlaid with 2 × 106 CPM/ ml of 1251-labeled protein A (ICN;>30µCi/µg) for another 1 hour after washing with PBS will needed. At last plates will be washed dried and exposed to x-ray film overnight.

5. Results

5.1 Cell line and their growth curves-

To optimize the growth rate of different cell lines, growth curve were made from both hybridoma (IC2) and beta (RIN 5AH) cell lines. Here, Fig 2.1 and Fig 2.2 shows the growth curve of IC2 and RIN 5AH cells respectively.

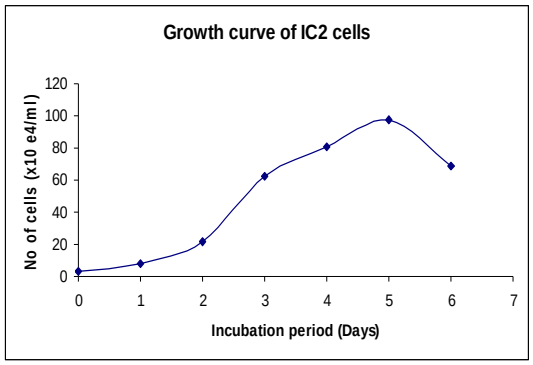


Fig- 2.1

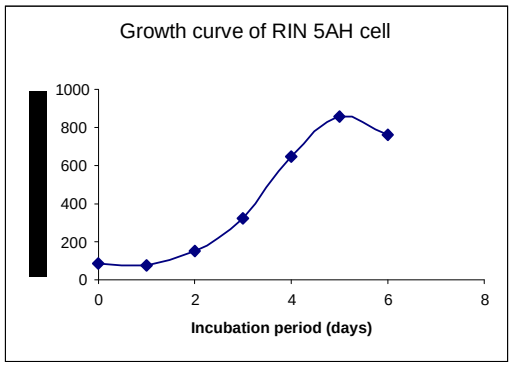


Fig- 2.2

Fig-2.1 Linear growth curve of IC2 hybridoma cell. The maximum cell growth is shown in the 5th day of incubation.

Fig- 2.2 Linear growth curve of RIN 5AH cell. The maximum cell growth is shown in the 5th day of incubation.

Although, the starting number of cells were not same for these two cell lines but their maximum growth were observed in the 5th days of their incubation and after that the number of cells were declined rapidly in both cases. Again, 3 times more growth rate was observed in case of IC2 cells than the RIN 5AH cells.

5.2 Extraction of lipids and plasma membrane from different cell lines-

Both lipids and plasma membranes were extracted from three different cell lines (RIN 5AH, α TC19 & RBL) to apply them in TLC and HPTLC experiments. In case of IC2 cell line only lipids were extracted and no plasma membrane isolation was done. On the other hand only plasma membrane was isolated from ARJ 24 cell lines. Table-2 shows the percentage of extracted lipids from different cell lines and the percentage of plasma membrane we extracted from different cells are shown in the Table-3.

cells	Mass of cell pellets	Mass of extracted lipids	Mass (%) of extracted lipids from cell pellet	Date of extraction
RIN 5AH	1.2 ml	125 µl	11.25	20-01-2009
α TC19	1.0 ml	120 µl	12.0	22-09-2008
RBL	750 µl	115 µl	15.3	13-01-2009
IC2	1.0 ml	125 µl	12.5	17-10-2008

Table-2 *The amount of extracted lipids from different cell lines.*

cells	Mass of cell pellets	Mass of extracted plasma membranes	Mass (%) of extracted plasma membranes from cell pellet	Date of extraction
RIN 5AH	5 ml	850 µl	17.0	17-11-2008
α TC19	4.5 ml	800 µl	17.77	10-12-2008
RBL	6.0 ml	1.3 ml	21.66	19-12-2008
ARJ 24	5.5 ml	1.2 ml	21.8	10-02-2009

Table-3 *The amount of extracted plasma membranes from different cell lines.*

Although lipids were extracted from different types of cell lines (alfa, beta & hybridoma) but it seems not so much differences in the percentage of lipids that we extracted. They were in between 11%-15% (table-2). On the other hand, same scenery is shown in case of plasma membrane extraction. 17%-22% (table-3) plasma membrane was isolated from different types of cells.

5.3 Thin layer chromatography (TLC) experiments

Glycolipids were isolated from different cell lines, separated by thin layer chromatography (TLC) as described in materials and methods. Separation bands on TLC plates, by applying different concentration of RIN 5AH extracted glycolipids are shown in the Fig-3. Denser band is observed in higher concentration lipids (lane-3, Fig- 3).

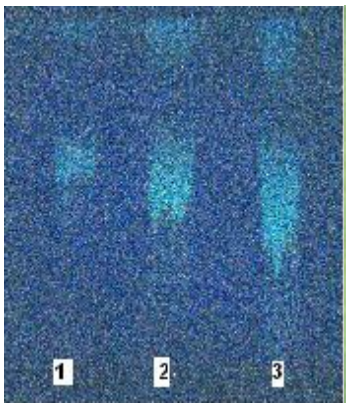


Figure -3 **Figure -4** **Figure- 5**

Fig-3 Three different bands (light blue) of RIN 5AH extracted lipids on the aluminium backed Silica gel TLC plate are shown after running with mobile solution (chloroform: methanol: 0.25% aqueous KCL 5:4:1). Here, 1 µl, 5 µl & 10 µl sample solution were added respectively in lane-1, lane-2 & lane-3 and TLC plate was stained with Cu-acetate procedure as described in the materials and methods. Denser band are shown in higher sample solution.

Fig-4 Separate bands (dark black) from RIN 5AH lipid (lane-1) and IC2 lipids (lane 2) are shown, by staining with carbasol procedure. Here, 10 µl of both two samples were added in the same TLC plate and was run with the same mobile phase as in the fig-3.

Fig-5 Separate bands (light yellow) from RIN 5AH lipid (lane-1) and IC2 lipids (lane 2) are shown, by staining with 2′7′- dichlorofluroscence procedure. Here, 5 µl of both two samples were added in the same TLC plate and was run with the same mobile phase as in the fig 3.

Separation bands of both IC2 (lane-1) and RIN 5AH (lane-2) extracted lipids are shown by staining with carbasol (Fig-4) and 2′7′- dichlorofluroscence (Fig-5) procedure. Here, glycolipids extracted from IC2 (applied in the lane-1 in both Fig-3 & Fig-4) used as control.

5.4 High Performance Thin Layer Chromatography (HPTLC) experiments

Glycolipids extracted from both IC2 (Fig-6) and RIN 5AH (Fig-7) cell pellets were separated by HPTLC procedure as described in the materials and methods part. In both cases different concentration of sample were added in different lanes and denser bands were observed in higher sample concentration (lane -4).

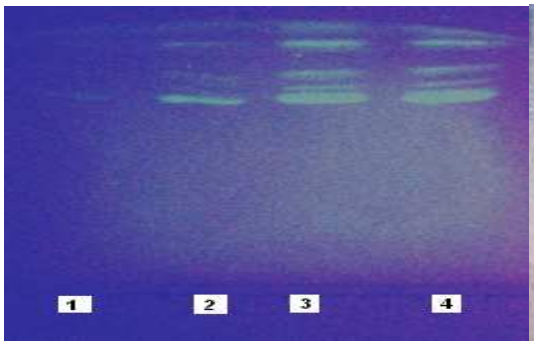


Fig- 6

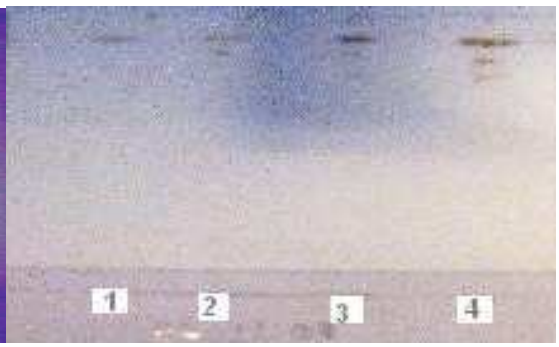


Fig- 7

Figure 6 – HPTLC experiments on IC2 lipids. Here, 1 µl, 5 µl, 10 µl and 15 µl samples were added in lane 1, 2, 3 and 4 respectively. Especial HPTLC glass plate (MERK) was used as stationary phase and chloroform: methanol: 0.25% aqueous KCL 5:4:1 mobile solution was used to separate the glycolipids. Denser bands observed in higher sample concentration (15 µl in lane 4) after staining with primulin spray.

Figure 7 – Same HPTLC experiments like Fig 6 was done with RIN 5AH extracted lipid. Here also denser bands are shown in higher sample concentration stained with Orchinol spray.

In both experiments same four different concentrations of sample applied on the same HPTLC plates and also run them in same kind of mobile solution (chloroform: methanol: 0.25% aqueous KCL 5:4:1). To visualize separated bands on HPTLC plate, Primulin spray was applied for Fig 6 and orcinol spray was applied in Fig 7. Here, IC2 extracted lipid (Fig-6) actually used as a control as TLC experiments. Different staining methods were carried out to visualize the separation bands of glycolipids. Fig 8 shows the bands that observed after primulin spray. On the other hand thymol solution was used to get the band in Fig 9. But in both Fig 8 and Fig 9 we used same mobile phase (chloroform: methanol: water 2:5:1) and HPTLC plates to separate the RIN 5AH glycolipids.

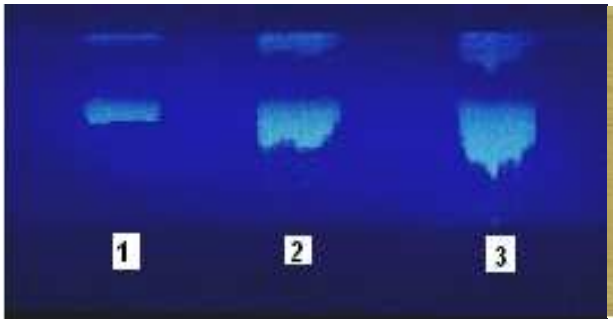


Fig-8

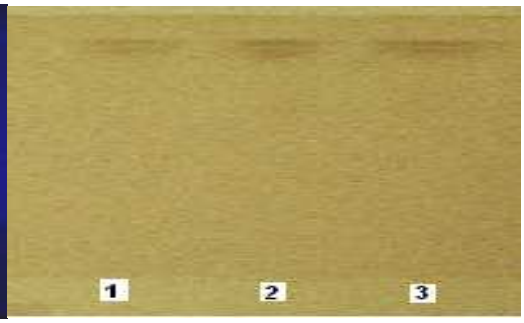
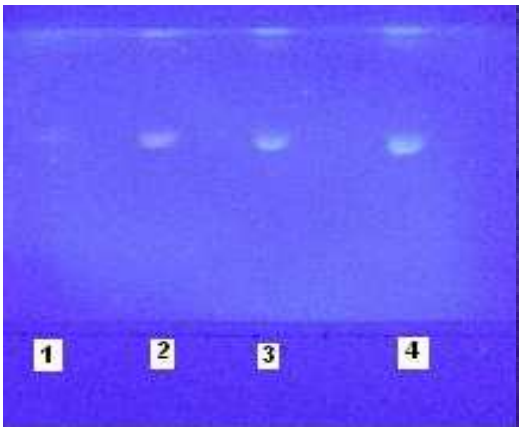


Fig-9

Fig- 8 HPTLC of RIN 5 AH lipids on the HPTLC plate using chloroform: methanol: water (2:5:1) as mobile phase and stained by primulin spray. 10 µl, 15 µl and 20 µl samples were applied in lane 1, 2 & 3 respectively.

Fig-9 HPTLC experiments on RIN 5AH lipids with same mobile phase as Fig 7. Here, 1 µl, 5 µl and 10 µl samples were added in lane 1, 2 & 3 and Thymol solution was used to stain the plate.



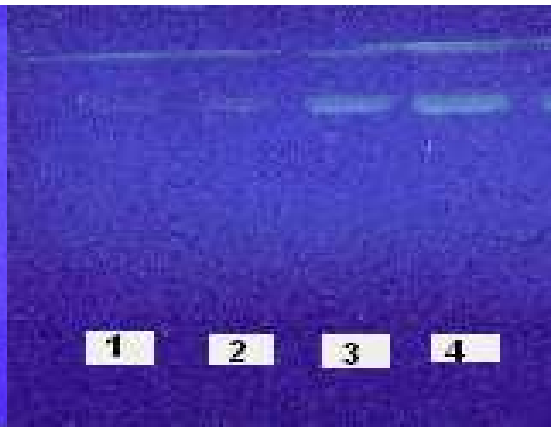


Fig-10 Fig-11

<u>Fig-10</u> HPTLC on RIN lipids using chloroform: methanol: water (1:6:2) and stained by primulin spray. Here we added 1 µl, 2 µl, 5 µl and 10 µl samples in lane 1, 2, 3&4 respectively on the HPTLC plate. Bands were separated by running with the mobile phase, chloroform: methanol: water (1:6:2) and visualized after staining with primulin spray.

<u>Fig-11</u> HPTLC on RIN lipids using chloroform: methanol: water (2:4:1) and stained by primulin spray. Here we added same sample on the HPTLC plate as Fig 10. Bands were separated by running with the mobile phase, chloroform: methanol: water (1:6:2) and visualized after staining with primulin spray were more clear.

Moreover, HPTLC experiments performed with different mobile phase to get the better separation bands of our glycolipids, extracted from RIN 5AH. Both Fig 10 and Fig 11 show the bands after staining with primulin spray. In Fig 10 we used chloroform: methanol: water (1:6:2) as our mobile phase, whereas chloroform: methanol: water (2:4:1) was used as mobile phase in case of Fig 11.

6. Discussion

Monoclonal antibody IC2, derived from diabetic BB rat, binds not only normal cells but also to their transformed counterparts (RIN 5AH cells), which is supported by Brogren CH et al, 1986 and Buschard K et al, 1988. By immunostaining the TLC and HPTLC plates this IC2 antibody should recognized glycolipids isolated from RIN 5AH cells (Spitalnik et al. unpublished). Although, we have done different normal staining of our TLC (Fig 3, 4 and 5) & HPTLC (Fig 6, 7, 8, 9, 10 and 11) plates and we have a plan to continue the important immunostaining part on the june-july, 2009. Cell lines and tissues used in the study of the cellular specificity of the monoclonal autoantibody IC2 from 1982-2009 are shown in the table 4. In this table both the work done by me during 2008-09 and the work that I have a plan to do in 2009 are shown. From this table it is also clear that various cell lines and tissues have been studied since a long period in order to understand the cellular specificity of this interesting monoclonal antibody, IC2. It is however, still unclear, what the exact target epitope is on beta cells, since this auto antibody specially binds with beta cells involved in diabetes.

Table 4: Cell lines and tissues used in the study of the cellular specificity of the monoclonal autoantibody IC2 from 1982-2009.

	Name	**Year of plasma membrane isolation**	**Origin of species**	**Cellular specificity**	**Glycolipids Extract**		**Analysis**					**Published and unpublished studies**
					From cells	From plasma membrane	IF ICC Confocal	Flow cytometry	ELISA cellular	ELISA plasma membrane	Immuno HPTLC	References
1	**RIN-5F**	1982	Rat	Panc. *β*cell				1981-83	1982	1982	1990	Brogren and Andersen 1982, Brogen et al 1986, Spitalnik et al.1990, unpublished
2	**RIN-14B**	1982	Rat	Panc. δcell				1981-83	1982	1982		Brogren and Andersen 1982
3	**Rat islet cells**		Rat	Panc. *β*cell				1982-86	1983			Brogren et al. 1986, Buschard et al. 1988, Aaen et al. 1990
4	**Mouse islet cells**		Mouse	Panc. *β*cell								Moore et al. 2001 (in vivo study), Buschard et al 1985 unpublished
5	**Human islet cells**		Human	Panc. *β*cell				2008				Brogren and Larsen 2008, unpublished
6	**C58(NT) D-I-G**		Rat	T lymphoma			1983		1983			Brogren et al. 1986
7	**BRL**		Rat	Liver cell			1983		1983			Brogren et al. 1986

8	**Hepatocytes**		Rat	Primany			1983		1983			Brogren et al. 1986
	Erythrocytes		Rat	Primary			1983					Brogren et al. 1986
9	**Fibroblast**		Rat	Primary			1983		1983			Brogren et al. 1986
10	**Macrophages**		Rat	Primary			1983					Brogren et al. 1986
11	**INS-1E**	2007	Rat	Panc. *β*cell			2009	2007				Brogren et al. 2006 unpublished
12	**RIN-5AH**	2007-08 Mia	Rat	Panc. *β*cell	2008 Mia	2008 Mia	2009	2007-09		2007-08	2009	Brogren et al. 2008, Brogren and Larsen 2007-09 unpublished , Brogren 2009 unpublished
13	**RBL-2H3**	2008 Mia	Rat	Mast cell	2008 Mia	2008 Mia		2008		2009		Brogren, Poncet unpublished
14	**AR42J**	2009 Mia	Rat	Panc. exocrine	2009 Mia	2009 Mia		2009		2009		Brogren et al. 2009, unpublished
15	**Rat-2**		Rat	Fibroblast								Brogren 1982, unpublished
16	**PC-12**		Rat	Fibroblast								Brogren 1982, unpublished
17	**RIN-1046-38**		Rat	Panc. *β*cell								Waiting
18	**RIN-56A**		Rat	Panc. αcell								Waiting
19	**RIN-1027-B2**		Rat	Panc. δcell								Waiting
20	**RIN-T3**		Rat	Panc. δcell								Waiting
21	**MSC 60**	2009	Mouse	Schwann cell								Baron van Evercooren et al., 2006 unpublished
22	**MIN-6**		Mouse	Rat βcell								Waiting
23	**alpha-TC 1-9**	2008 Mia	Mouse	Panc. αcell	2008 Mia	2008 Mia		2009	2009	2008-09		Brogren and Larsen 2008, unpublished
24	**Beta-TC-tet**	Will be done on 2009	Mouse	Panc. *β*cell								Waiting
25	**Alpha-TC1-6**	Will be done on 2009	Mouse	Panc. αcell	Will be done on 2009	Will be done on 2009						Waiting
26	**InR1G9**		Hamster	Panc. αcell								Waiting
27	**HIT-T15**		Hamster	Panc. *β*cell								Brogren et al. 1986
28	**BHK**		Hamster	Kidney cell								Waiting
29	**Human insulinoma**		Human	Insulinoma					1982			Brogren et al 1983 (lyophilized insulinoma)
30	**Rat insulinoma**		Rat	Insulinoma								Brogren and Lernmark 1982 (unpublished)
31	**Mouse insulinoma**		Mouse	Insulinoma								Waiting
32	**Hamster insulinoma**		Hamster	Insulinoma								Waiting
34	**9L gliasarcoma**		Rat	gliasarcoma								Moore et al. 2001 (in vivo study)

We are trying to re-establish and confirm the work of Spitalnik S et al, 1991 (unpublished) that shows that IC2 binds to a specific sulfatide glycolipids. Actually, we are working to get the same figure (Fig 12) that was made by Spitalnik S et al, 1991.

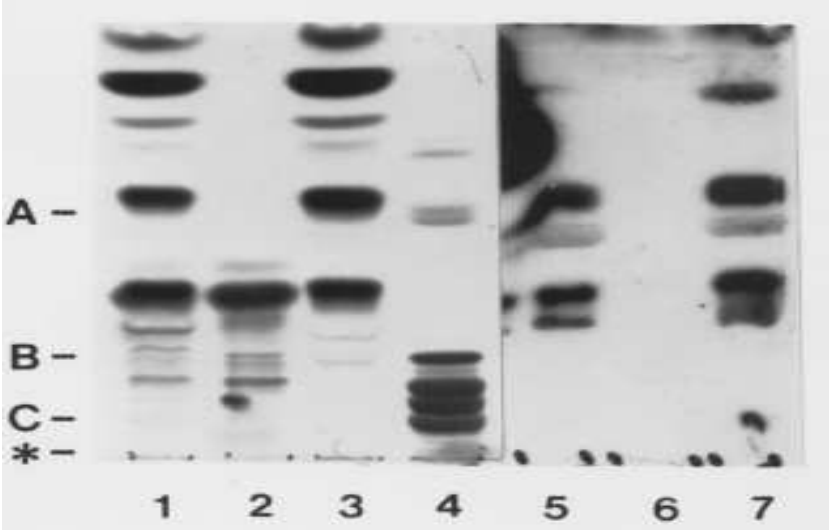


Fig-12 RINm5F glycolipids were isolated, separated by TLC. Bands are shown by applying orcinol staining (lane 1-4) and immunostaining by IC2 (lane 5-7). Here, the position of human Brain chromatographic Standards galactosyl Sulfatide, ganglioside GM-1b ganglioside GT-1b are indicate by A-C respectively. (*) denotes the origin. Lane 1 & 5 RINm5F total lipid extract. Lane 2 & 6 RINm5F Folch upper phase's lipids. Lane 3&7 RINm5F Folch lower phase's lipid. Lane 4 human Brain glycolipid. (Fig-1 Spitalnik et al, 1991)

The galactosyl Sulfatide of RIN glycolipids clearly binds with IC2 auto antibody (lane 5, Fig- 12), supported our hypothesis that sulfatide is the target epitope for IC2. According to the Spitalnik et al, (1991) sulfatides perhaps within the groove of the extracellular

anchoring protein (our hypothesis) are recognised by the IC2 autoantibody (Fig-1) this protein seems associated with t-SNARE proteins regulating the insulin secretion. The association is supposed to be no-covalent occurring the plasma membrane lipid rafts (our hypothesis).

Here, in our current studies we tried with different cell lines as already indicated in the materials and methods part. As we have done our experiment on glycolipids, we get sufficient purified plasma membrane glycolipids we had to expand our cell culture up to NUNC cell factory level. To understand the best growth, we have done growth curve experiments on both hybridoma (IC2) and beta cell line (RIN 5AH). By analyzing this growth curve experiments (Fig 2 & 3), we got maximum growth on the 5^{th} days of incubation and we incubated our cell lines for 5 days to get maximum cell pellets as well as maximum lipids and plasma membranes etc. Moreover, plasma membrane from cells will be extracted to get more pure lipid from plasma membrane and they were applied in TLC and HPTLC experiments to get more confirmed separation bands. To optimize better separation of our glycolipids, different mobile phases were tried. Chloroform: Methanol: 0.25% aqueous KCL 5:4:1 was the best one and better separation bands are shown in the Fig 6 and 7. Different staining methods also applied to visualize all sharp bands. We got better result on primulin spray (Fig 6,8,10 & 11) but orcinol spray also effective (Fig 7). By analyzing both TLC (Fig 3, 4 & 5) and HPTLC (Fig 6-11) exercise it is clear that HPTLC is far better than TLC that can be easily optimized by comparing the stained plates from both TLC and HPTLC separation. Though Spitalnik et al (1991 unpublished) had done their experiments on glycolipids extracted from cells but not from plasma membrane, then we have planned to study both of them because more pure lipids should be got from plasma membrane. Hopefully, more clear and comparable result could be obtained by applying the glycolipids from plasma membrane. Again, Spitalnik et al (1991) has done only the normal staining of human brain glycolipids and got light band of Sulfatide (lane 4, fig 12). Immuno staining of human brain glycolipids with IC2 auto antibody is still missing, which is important to support our hypothesis. Further experiments should be done to confirm his findings.

We already have done plasma membrane and lipid extraction from the AR42J cell lines that can be used as a negative cell line control with IC2, because this rat cell line had no IC2 binding properties, already analysed by flow cytometry (Brogren et al, unpublished). To prove the involvement of IC2 auto antigen in pathogenesis of TID, we are just behind the last immunobloting part of our TLC and HPTLC plates, that we have a plan to do on the June- July, 2009.

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