Inhibition of insulin-specific autoreactive T-cells by sulphatide which is variably expressed in beta cells

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Abstract

Aims/hypothesis. Sulphatide and insulin are present in the secretory granules and at the surface of beta cells in islets of Langerhans. Insulin autoantibodies and T-cell reactivity against insulin exist during the development of Type I (insulin-dependent) diabetes during which active beta cells may be more vulnerable than passive. Our aims were to examine the presence of sulphatide in active and passive beta cells and to clarify whether sulphatide influences the direct autoimmunity against insulin.

Methods. We incubated rat islets in 2.8, 11.0 or 20.0 mmol/l glucose for 24 h and did an electron microscopic evaluation after labelling with a specific anti-sulphatide monoclonal antibody. The reactivity of an insulin-specific T-cell clone isolated from a patient with Type I diabetes, was examined using insulin or insulin B-chain (B11–27) peptide incubated together with sulphatide.

Results. We detected lower amounts of sulphatide per insulin secretory granule in active compared

with passive beta cells ($p = 0.003$). The presence of sulphatide in vitro at doses of 43–8.3 $\mu\text{mol/l}$ resulted in greatly reduced proliferation (median 3.4% of control value, $p = 0.0004$) of the insulin-specific T-cell clone. No inhibition was found using the precursor of sulphatide, galactosylceramide, or GM1. Sulphatide did not reduce non-specific proliferation (induced by phorbol myristate acetate plus anti-CD3) or specific proliferation induced by insulin peptide.

Conclusion/interpretation. These results imply that sulphatide possibly affect processing of the insulin molecule. Sulphatide which has been reported to interfere with phagosome-lysosome fusion, conceivably interacts with insulin. We hypothesize a (patho)physiological role of sulphatide, variably expressed in beta cells, by reducing the antigenicity of insulin. [Diabetologia (1999) 42: 1212–1218]

Keywords Type I diabetes mellitus, insulin, autoreactive T-cells, sulphatide, beta cells.

Sulphatide (3'-sulphogalactosylceramide) is a glycolipid biosynthesized from ceramide originating from sphingosine, to which a galactose substituted with sulphate is attached. Galactosylceramide (galcer) is the direct precursor without sulphate. Sulphatide is known to be present in both the central and pe-

ripheral nervous system as well as in kidney tubules and in islets of Langerhans [1, 2]. The physiological role of sulphatide is not fully known but an involvement in cation transport has been suggested as well as in cell-to-cell interactions and in intracellular events [3]. In nerves sulphatide is implicated in myelination [4].

Several possible patho-physiological functions of sulphatide have been described; sulphatide may play a part in adhesion as being a ligand to CD62/P-selectin [5]. Also, sulphatide has been shown to be a modulator of cytokine production from neutrophils [6], T-cells [7] and monocytes [7]. Sulphatide autoanti-

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Abbreviations: Gal-cer, galactosylceramide; PMA, phorbol myristate acetate.

bodies have been shown in association with diseases affecting organs where sulphatide is localized. Considering the nervous system such antibodies have been found in Guillain-Barré syndrome [8], in multiple sclerosis [9] and in peripheral nerve disease in AIDS [10]; in the islets, sulphatide antibodies are expressed in Type I (insulin-dependent) diabetes mellitus.

In islet cells, sulphatide is present in the secretory granules [2] together with insulin, which is an autoantigen in Type I diabetes giving rise to both anti-insulin autoantibodies [12] and T-cell autoreactivity against insulin [13]. The function of sulphatide in the granules is not known but its physical and chemical properties possibly imply that sulphatide is involved in vesicle transport, vesicle pH regulation [3], and/or the exocytotic process of insulin secretion. Increased beta-cell activity has been suggested as a diabetogenic risk factor [14] and beta cells at rest seem less likely to be attacked by the immune system as illustrated by prophylactic insulin treatment [15–17]; this possibly, however, also acts by tolerating for the insulin autoantigen [18].

The aim of our study was to compare the presence of sulphatide in secretory granules in active and passive beta cells and to explain whether sulphatide plays any part in the direct autoimmunity against insulin.

Materials and methods

Islet origin and preparation. Islet tissue was obtained from 32,8 to 10-week-old, male Lewis rats purchased from Møllegaard (Ll. Skensved, Denmark). The islets were isolated as described previously using a collagenase method [19]. Thereafter, the islets were divided into three portions and incubated at 2.8, 11.0 and 20.0 mmol/l glucose, respectively, in RPMI 1640 (Gibco, Paisley, UK) with 10% fetal calf serum and 1% Penicillin-Streptomycin (10000 IU/ml–10000 µg/ml, Gibco) for 24 h at 37°C. Beta cells in isolated intact islets are known to respond to increasing glucose concentration with increasing insulin secretion [20]. The Principles of laboratory animal care (NIH publication no. 85–23, revised 1985) were followed.

Electron microscopy. Immediately after the incubation, the islets were fixed for 1 h/30 min in a mixture of 2.5% paraformaldehyde and 0.2% glutaraldehyde. After washing in cacodylate buffer, pH 7.3, and dehydration in 70% alcohol, specimens were embedded in LR-white (Bio-Rad, Watford, UK). Ultra-sections were incubated for 2 h at 20°C with the monoclonal antibody, Sulph I [21], diluted 1:75. Sulph I is specifically directed against sulphatide [21]. The concentration of the primary antibody was based on dilution experiments showing no background labelling. After incubation for 1 h with a rabbit anti-mouse immunoglobulin (F261, Dako, Glostrup, Denmark) diluted 1:100 and absorbed with rat serum, sections were incubated for 1 h with goat anti-rabbit immunoglobulin conjugated with colloidal gold (10 nm, British Biocell, Cardiff, UK) diluted 1:100. Specimens were after washings postfixed in 2% glutaraldehyde for 10 min and stained with uranyl acetate/lead citrate before examination in a Philips 208 electron microscope. For each experiment, controls were prepared by omit-

ting the primary antibody as were sections treated with an irrelevant primary antibody. These controls yielded the expected negative results.

We did four independent experiments each using eight rats with isolation of islets and incubation with low, medium and high glucose concentrations. From each experiment photographs were taken of four different motifs of beta cells from four different islets, altogether giving 192 photographs with a magnification of 30000 each showing an area of 5100 × 6900 nm of beta cells. The photographs were evaluated blindly. Two photographs were excluded due to poor quality. All staining dots in each photograph were counted and localized either to the secretory granules or to cytosol. Also, the actual number of secretory granules was determined on each photograph. The four experiments gave consistent results.

T-cell proliferation assay. The T-cell proliferation assay was done as described previously [22]. Briefly, 10⁴ T-cells and 5 × 10⁴ irradiated (40 Gy) HLA-matched mononuclear cells (antigen-presenting cells) were cultured in 96-well flat bottom microtitre plates (Greiner, Nürtingen, Germany). The cells were cultured in triplicate in the presence of 43, 8.6, 4.3 or 0.9 µmol/l sulphatide alone or together with 4.3 or 0.9 µmol/l insulin for 3 days at 37°C in a humidified 5% CO₂–95% air mixture. We added [³H]thymidine (NEN DuPont, Boston, Mass., USA) in 50 µl RPMI 1640 at 20 mmol HEPES (Gibco) 18 hours before termination of the culture. The samples were collected on glass fibre filters and [³H]thymidine incorporation was determined by liquid scintillation counting. We investigated two T-cell clones; a T-cell clone responding to insulin [23] (see below) and a T-cell clone responding to insulin secretory granule proteins [22]. As antigens we used; human Actrapid 100 U ml⁻¹ (Novo Nordisk, Copenhagen, Denmark), synthetic peptide of amino acid 11–27 insulin B-chain [23] and RINm5 beta-cell insulinoma membranes [22], respectively. As stimuli we used; sulphatide, galactosylceramide (gal-cer) and ganglioside GM1 (see below). For non-specific stimulation we used phorbol myristate acetate (PMA) (1 ng/ml, Sigma, Zwijndrecht, The Netherlands) together with anti-CD3 (100 ng/ml OKT3, Ortho, Raritan, N. Y., USA).

Generation of insulin-specific T-cell clone. The human insulin-specific T-cell clone used in the study was derived from peripheral blood of a newly diagnosed patient with Type I diabetes and is described in detail elsewhere [23]. The peptide recognized by the clone was mapped to the insulin B-chain (B11–27). Functionally, the human insulin-specific CD4⁺ T-cells had a Th 1/0-like cytokine profile and were restricted by HLA-DR16.

Preparation of glycolipids. The following glycolipids were used; 3'-sulphogalactosylceramide (sulphatide), galactosylceramide (gal-cer) and ganglioside GM1 (Fig. 1). All three molecules have an amphiphilic character with a hydrophilic sugar chain and a hydrophobic part consisting of ceramide, i.e. sphingosine and fatty acid. Gal-cer represents a molecule that differs from sulphatide by the lack of a negatively charged sulphate group. The GM1 ganglioside has a negative charge like sulphatide but in this case expressed by sialic acid. All the glycolipids were isolated from pig brain as described earlier [24] and their structures analysed by fast atom bombardment mass spectrometry [25]. Before use the glycolipids were prepared as follows. Stock solutions of each glycolipid were made in chloroform/methanol/water (60:30:4.5 v/v/v) in concentrations of 1 mg/ml; the tube was sealed and after 1 h at room temperature the solution was kept in a refrigerator. The desired amount of µl (nmol of glycolipid) was transferred to

Fig. 1. Schematic drawing of glycolipids and related molecules discussed in the text. $\sim$ lines indicate carbon chains, $\square$ galactose, $\blacksquare$ glucose, $\circ$ galactosamine, ∇ *N*-acetylneuraminic acid (sialic acid)

glass tubes and evaporated at room temperature overnight. The dried glycolipids were re-dissolved in PBS, pH 7.1 and sonicated for 20 s using a Branson sonicator (Danbury, Conn., USA). Immediately after this insulin was added. After 3–5 days at 4°C the solutions were used.

Dynamic light scattering analyses. Samples of sulphatide, galcer or GM1 with or without insulin prepared as described above, were analysed by dynamic light scattering using a Zetasizer 5000 (Malvern Instruments, Malvern, UK). Particles of a diameter less than 10 nm were not detectable. In one experiment the samples were examined both before and after vigorously vortexing 30 s at 2500 rpm using a MS1 Minishaker (IKA-Works, Wilmington, N. C., USA).

Statistics. T-cell proliferation data are presented as means $\pm$ SD. The significance of difference was evaluated using unpaired Student's *t* test (significant level set at 0.05).

Results

Electron microscopy studies. The results of the electron microscopy studies are presented in Table 1. Beta cells contained sulphatide in their secretory granules (Fig. 2) and active beta cells had less sulphatide staining dots than beta cells at rest. This was due to a massive decrease of staining dots in the secretory granules (from median 97 to 38) whereas there was a slight increase in sulphatide staining dots in the cytosol. The number of insulin secretory granules was reduced in the active cells but to a lesser extent than the reduction of sulphatide staining dots in the granules. Thus, the number of sulphatide staining dots per insulin granule was lower in active than in passive beta cells (median 1.03 vs 0.77, $p = 0.003$).

Insulin-specific T-cell clone. Incubation of the insulin-specific T-cell clone with sulphatide (43 $\mu\text{mol/l}$) and insulin (4.3 $\mu\text{mol/l}$) resulted in greatly decreased T-cell proliferation in seven out of nine experiments (median 3.4%, range 0.9%–35.8%) compared with incubation with insulin alone (100.0%, $p = 0.0004$, Fig. 3A). When using a five times lower concentration of both sulphatide (8.6 $\mu\text{mol/l}$) and insulin (0.9 $\mu\text{mol/l}$) the decrease was less pronounced (6.7%) but still highly significant ($p = 0.0005$). When using ten times less sulphatide (i.e. equimolar range with insulin), there was no decrease measurable. Incubation of insulin with a similar high concentration of galcer as the highest sulphatide (43 $\mu\text{mol/l}$) only resulted in a slight decrease in cpm (80.8%, NS), whereas galcer concentration of 4.3 $\mu\text{mol/l}$ and high (43 $\mu\text{mol/l}$) or the lower concentrations of GM1 did not affect the T-cell response to insulin at all.

In contrast, stimulation of the T-cells with insulin B-chain peptide (B11–27) induces proliferation similar to insulin but does not require processing by the antigen presenting cells. Addition of sulphatide did not reduce the T-cell proliferation. Co-stimulation with sulphatide + insulin (Fig. 3B) did not decrease the proliferation for the low insulin concentration

Table 1. Counting results of anti-sulphatide mAb (Sulph I) labelled islet ultramicrographs

Glucose concentrations (24 h)	Insulin secretory granules (number per ultramicrograph)	Sulphatide staining dots present in: (number per ultramicrograph)			Sulphatide dots/insulin granules
		insulin granules	background cytosol	all	
2.8 mmol/l (<i>n</i> = 64)	97 (78–113)	97 (68–122)	64 (47–81)	153 (136–198)	1.03 (0.74–1.24)
11.0 mmol/l (<i>n</i> = 63)	86 (69–104) (<i>p</i> = 0.02)	62 (43–89) (<i>p</i> = 0.0002)	53 (38–75) (<i>Ns</i>)	112 (87–159) (<i>p</i> = 10^{-4})	0.71 (0.51–1.03) (<i>p</i> = 0.002)
20.0 mmol/l (<i>n</i> = 63)	51 (35–76) (<i>p</i> = 10^{-11} , 10^{-8})	38 (18–65) (<i>p</i> = 10^{-10} , 10^{-4})	79 (60–97) (<i>p</i> = 0.001, 10^{-5})	118 (93–156) (<i>p</i> = 10^{-4} , <i>Ns</i>)	0.77 (0.54–1.05) (<i>p</i> = 0.003, <i>Ns</i>)

Values are presented as median number and in brackets lower and upper quartile. *n* indicates the number of micrographs evaluated in each glucose concentration group. The *p* values

refer to comparison between 11 and 2.8 mmol/l glucose concentrations or between 20 and 2.8 or 11 mmol/l glucose concentrations, respectively

Fig. 2 A–C. Sections in original magnification $\times 12500$ of **A** an ultramicrograph of 2.8 mmol/l glucose incubation showing part of a beta cell with mitochondria (asterisks) and numerous secretory granules immunogold labelled for sulphatide (arrows). The surface of the cell with few microvilli is seen to the right **B** a micrograph of 11.0 mmol/l glucose incubation revealing parts of two beta cells with few microvilli on their surfaces. Profiles of rough endoplasmic reticulum (short arrows), few mitochondria (asterisks) and several secretory granules are seen in the cytoplasm. Many of the latter are unlabelled whereas some reveal immunogold labelling (long arrow) **C** a micrograph of 20.0 mmol/l glucose incubation with numerous mitochondria (asterisk), developed Golgi apparatus (thin arrow) and reduced numbers of secretory granules. Sulphatide labelling is seen (thick arrow)

sample (0.9 $\mu\text{mol/l}$). For the high insulin concentration (4.3 $\mu\text{mol/l}$) there was a minor, statistically non-significant decrease in proliferation at the 10:1 sulphatide:insulin molar ratio. Overall the changes were minimal compared with incubation with sulphatide + insulin alone which resulted in the expected reduction compared with insulin-induced T-cell proliferation [1311 ± 999 (5.3 %) vs 24889 ± 6091 (100.0 %), $p = 0.002$]. In the same experiment we stimulated the T-cells non-specifically with PMA and anti-CD3 during incubation with either sulphatide + insulin or insulin alone (Fig. 3B). The presence of sulphatide in this experiment even in high concentrations did not cause significant reduction of PMA plus anti-CD3 induced T-cell proliferation.

Secretory granule-specific T-cell clone. The proliferation of the T-cell clone specific to the insulinoma membrane protein [22] when incubated with RIN cell insulinoma membranes was unaffected by simultaneous incubation with gal-cer + insulin (82.8 %), GM1 + insulin (78.7 %) or with sulphatide alone (80.7 % of the glycolipid-free control). When simultaneous incubation was done, however, with the RIN cell membranes together with sulphatide + insulin a decrease was seen in T-cell proliferation [7866 ± 1873 (21.4 %) vs 36598 ± 3327 (100.0 %), $p = 0.0002$].

Dynamic light scattering analyses. To evaluate whether sulphatide and insulin molecules interact, we did dynamic light scattering analyses. The mean particle diameter size for sulphatide sonicated 20 s was smaller when insulin was present; for sulphatide $\pm$ insulin (43/4.3 $\mu\text{mol/l}$): 283 ± 22 vs 366 ± 40 nm, $n = 4$, $p = 0.03$. The mass of material in aggregated form calculated on basis of the relative number of particles and particle volume, was 2.3 ± 0.2 times higher in the samples with sulphatide alone than in the samples with sulphatide + insulin. Hence, the fraction of sulphatide not detectable by dynamic light scanning (molecular or aggregated form less than 10 nm in size) was substantially enhanced when insulin was present. Vigorous vortex procedure resulted in a particle size of 103.5 ± 3.9 % of the non-vortexed samples indicating that sizes of the aggregates were unaffected by this procedure. Gal-cer + insulin solution showed an average particle size of 2794 nm and gal-cer alone 9562 nm. In contrast, GM1 did not form detectable micelles either alone or together with insulin.

Discussion

This study has shown that sulphatide – but not its precursor (non-sulphated galactosylceramide) or another negatively charged glycolipid, GM1 – is able to inhibit insulin-induced proliferation of a specific T-cell

Fig. 3. A Proliferation of [3 H]thymidine incorporated insulin-specific T-cells incubated with sulphatide (■), gal-cer (▽), or GM1 (○) and insulin. Indicated are mean values ($\pm$ standard deviation) of triplicate values of a representative experiment. Each experiment was done at least three times. No inhibition of proliferation was observed of the insulin specific T-cell clone in response to IL-2. None of the glycolipids, either in the presence or absence of insulin, affected the proliferation of T-cells specific to imogen-38 or hsp65 in response to their antigen. The IC_{50} of insulin for the insulin-specific T-cell clone is 1.7 μ mol/l **B** Proliferation of [3 H]thymidine incorporated insulin-specific T-cells incubated with sulphatide + insulin. The T-cells were stimulated either with insulin B-chain (B11-27) peptide (◆) or non-specifically with PMA plus anti-CD3 (▼). Indicated are mean values ($\pm$ standard deviation) of triplicate values of a representative experiment. Each experiment was done at least three times

clone in vitro. Sulphatide did not inhibit non-specific T-cell proliferation. This was confirmed using PHA stimulated primary PBMC cultures in which addition of sulphatide had no effect (data not shown). Antigen specific proliferation was affected by sulphatide to-

gether with insulin but not by sulphatide alone, in one T-cell clone specific to secretory granule protein, but not in two T-cell clones with different specificities (imogen-38 [22], hsp65; data not shown). Thus, a potential inhibition of proliferative response by local sulphatide-insulin aggregates in the insulitic lesion need not be limited to insulin-specific T-cell clones, but is certainly not universal. A variety of T-cell clones specific for different autoantigens have been isolated from Type I diabetic patients [26]. The apparently limited inhibitory effect of sulphatide on certain T-cell reactivities could be related to particular HLA restriction as well as the specific nature of the autoantigens that are recognized. Although there are no other insulin-specific T-cell clones at present, it would be important to validate our findings with different insulin-specific T-cell clones with different HLA restriction characteristics.

The amount of sulphatide in human pancreas is found to be 84 nmol/g pancreas which is all present in the islets [2]. Correspondingly, the insulin content is estimated to be 1.4 IU (medium immunoreactive insulin) value of 32 human pancreases [27]) equiva-

lent to 50 µg or 8.3 nmol per g pancreas, which gives an overall mol:mol relation between sulphatide and insulin of 10:1. This molar ratio, as well as an equimolar ratio, was tested in our study. Sulphatide is expressed at the surface of the islet cells at least partly due to exocytosis of the secretory granules containing sulphatide [28]. Note, insulin appears also at the outer plasma membrane of the beta cells in a dot-like fashion at the sites of exocytosis [29].

The dynamic light scattering analyses have shown that sulphatide forms aggregates of sizes up to 300–400 nm but to a lesser degree when insulin is present. Like insulin [30], sulphatide [3] has hydrophilic and hydrophobic regions, which is the physical explanation for its particle forming properties. We hypothesize that insulin and sulphatide interact and that attachment due to different ion charge and hydrophobic attraction is most conceivable.

The absence of an inhibitory effect of sulphatide on non-specific T-cell proliferation suggests that the suppression could be related to processing and presentation of insulin molecules. This is further supported by our data showing that the T-cell response to the peptide-epitope of insulin (B11–27), which does not require processing, was unaffected by coculture with sulphatide. Inhibition of macrophage priming has been described by sulphatide SL-I [31, 32] which, however, is structurally not identical with sulphatide (3'-sulphogalactosylceramide) used in the present study. Recently, it has been found that the phagocytosis of *Staphylococcus aureus* by polymorphonuclear cells and by monocytes is facilitated by coating with sulphatide [33]. Furthermore, sulphatide interfered with phagosome-lysosome fusion [33]. Uptake of sulphatide-containing liposomes of phosphatidylcholine by macrophages was statistically significantly greater than the uptake of liposomes made without sulphatide [34] whereas sulphatide inhibited the uptake of phosphatidylserine liposomes by macrophages to a level of approximately 15% of control values [34]. The interference of sulphatide with macrophages is suggested to be mediated through anionic charging modifying organelle membranes [32] or to be due to hydrophobic forces [35].

Our data suggest that sulphatide in combination with insulin is able to inhibit T-cell reaction in the local environment of the islets. This could contribute to the chronic character of islet destruction during diabetes development which often takes several years, and is consistent with the notion of persistent beta-cell function in some people having autoantibodies as well as T-cells directed against beta-cell autoantigens including insulin.

Several studies, both human and experimental, favour active beta cells being more vulnerable than the passive ones [14]. Among the most important indications for this concept is the hyperproduction of insulin seen in people at risk of developing diabetes

[36]. Even for NOD mice beta-cell rest is described to be of importance [37]. A finding of note in our study was that low glucose concentration upregulates the sulphatide content. In contrast, a lower sulphatide content is seen in diabetes-prone BB rats (87 ± 20 pmol per 100 islets) compared with both diabetes-resistant Wistar Furth rats (181 ± 50 pmol) and Lewis rat islets (410 ± 50 pmol) [2]. Note, NK-cells – present in the insulinitis lesion – are described to express arylsulphatase on their surface [38] which is able to desulphate sulphatide making gal-cer. Theoretically, the presence of insulitic NK-cells can lead to ineffective modulation of T-cell autoreactivity by reduced sulphatide concentrations. Biases of NK-cells in diabetes have been reported [39].

Insulin is an important antigen in Type I diabetes. Antibodies against insulin are amongst the first antibodies seen in pre-Type I diabetes patients [40]. Furthermore, peripheral T-cell reactions directed against insulin are common in both preclinical and overt Type I diabetic patients and non-diabetic controls [41]. Our study shows that sulphatide, found together with insulin in the secretory granules and at the surface of beta cells, is capable of modifying the immunogenicity towards insulin and thereby possible influencing local T-cell autoreactivity in the microenvironment of the islets.

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