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Cell surface antibodies in Type 1 (insulin-dependent) diabetic patients

II. Presence of immunoglobulins M which bind to lymphocytes

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Summary. A standardized cell surface antibody assay was used to measure binding of circulating human immunoglobulins to rat or piglet splenocytes. In 100-fold diluted serum fractions, lymphocyte surface antibodies were detected in 30% of Type 1 (insulin-dependent) diabetic patients under 20 years of age but in none of 33 control subjects. Binding occurred with T and B lymphocytes, appeared unrelated to Fc receptors or protein glycosylation and was not attributable to insulin or albumin antibodies. At clinical onset of the disease, the lymphocyte surface antibodies belonged primarily to the IgM-class. Their presence was positively correlated to that of IgM-pituitary cell surface antibodies and their absorption by anterior pituitary cells occurred as well as by splenocytes. Lymphocyte surface antibodies remained present during the first years of insulin treatment. They were also detected in first degree relatives of lymphocyte surface antibody-positive patients. It is unlikely that IgM-lymphocyte surface antibodies mark the destructive process in the pancreatic B cell population. They may, instead, express a state of immune reactivity which precedes the formation of IgG-autoantibodies and therefore be associated with an event in the development of diseases such as Type 1 (insulin-dependent) diabetes.

Key words: Type 1 (insulin-dependent) diabetes, autoantibodies.

At clinical onset of Type 1 (insulin-dependent) diabetes circulating IgG often bind to the surface of islet B cells and also of pituitary cells [1–5]. In a recent study we described the presence, in certain sera, of IgM which interact with surface antigens of rat anterior pituitary cells (APSA) [5]. These IgM autoantibodies were absorbed by pituitary cells but also by rat splenocytes suggesting polyreactive properties or recognition of common membrane antigens. To further evaluate the significance of these immunoglobulins, we examined whether IgM-lymphocyte surface antibodies (LYSA) exist in Type 1 diabetic patients and whether they are correlated to the presence of IgM-APSA. LYSA have already been detected in diabetic BB rats, often before clinical onset and in positive correlation with islet cell surface antibodies [6–7]. Lymphocytotoxic antibodies have been described in human diabetes [8–11] as well as in other autoimmune diseases [12-14], but little is known on their class and antigen specificity [11, 15, 16], nor on their significance as a marker for the disease or its aetiological factors. In the present study IgM and IgG fractions are examined separately for their binding to rat splenocytes using a standardized technique for the detection and quantification of circulating cell surface antibodies [5].

Patients, materials and methods

Subjects

Sera were collected from non-diabetic and diabetic individuals younger than 20 years. All diabetic patients presented the Type 1 form of the disease. The non-diabetic group comprised 33 normal control subjets and 18 first degree relatives of Type 1 diabetic patients [5].

Preparation of samples

Serum was obtained as described [5] and stored at −20 °C until fractionation by HPLC-gel filtration [5]. The IgM- and IgG-peaks were pooled, kept at 4 °C and used, within 2 weeks, in a cell surface antibody assay.

Cell isolation

Cells were isolated from spleens of adult male Wistar rats. Contaminating erythrocytes were lysed by hypo-osmolar shock; this step did not alter antibody binding properties of lymphocytes since similar results were obtained with splenocytes prepared on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient [17]. Splenocytes were also prepared from piglet spleens using the same procedure. In all experiments, the viability of the isolated cells exceeded 95% as judged by trypan blue exclusion.

Cell surface antibody assay

Isolated rat splenocytes (10^6 cells per 200 µl), were incubated for 60 min with IgG or IgM column fractions (at 4 °C and continuous shaking). They were then washed, further incubated with rhodamine labelled swine-anti-human immunoglobulin antiserum (Nordic, Tilburg, The Netherlands), washed again and finally fixed in 4% (volume/volume (v/v)) formaldehyde. Samples were analysed by fluorescence microscopy (Leitz dialux 20EB, Wetzlar, FRG) and by fluorescence-activated cell sorting (FACS)-analysis [5]. Fluorescence microscopy was used to determine the dilutions at which virtually no binding occurred with Ig-fractions from normal control subjects and to select negative and positive control samples. It was thus decided to perform the binding-assay with splenocytes with 10-fold diluted column fractions (i.e. 1:100 final dilution as compared to serum).

A Becton-Dickinson FACS IV apparatus or Facstar (Sunnyvale, Calif., USA) equipped with a 2025-05 or 2016-04 Argonlaser (Spectra Physics, Mountain View, Calif., USA) was used to determine the % fluorescent cells [5]. Each experiment included one blank, one positive control and minimally three negative control samples. The negative control samples were analysed first to set the windows for the fluorescent and non-fluorescent cell populations and to determine the mean percent fluorescent particles for the negative control samples (C). For each unknown sample X, the percent fluorescent cells was measured by cell sorter analysis and used to calculate its positivity ratio as

$$\frac{\%\text{ positive particles in X}}{\text{mean }\%\text{ positive particles in C}}$$

An unknown sample was considered positive if its positivity ratio exceeded the value determined by the mean positivity ratio in the normal control group by more than 3 SD. Each patient group was compared with the normal control group by assessing the statistical difference in the respective mean positivity ratios.

Characterization of target lymphocytes

A double labelling immunofluorescence technique was used [18, 19] to determine the subset of lymphocytes which is recognized by surface antibodies. Lymphocytes (10^6 per 200 µl) were first incubated for 1 h at 4 °C with the Ig-fraction at a final 1:100 dilution, then washed and further incubated in 180 µl of a 40-fold diluted fluorescein (FITC) labelled swine-anti-human immunoglobulin serum (Nordic). After two washings, 200 µl of a monoclonal mouse antibody against B or T lymphocytes (respectively ER12 and ER1, kindly donated by Dr. J. Rozing, TNO, Rijswijk, The Netherlands) was added to the cells at a final 1:100 dilution. Following a 1 h incubation at 4 °C the cells were again washed and exposed (30 min at 4 °C) to 20 µl of undiluted phyco-erythrin (PE) labelled monoclonal rat anti-mouse-kappa chain antibody (Becton-Dickinson Monoclonal Center, Mountain View, Calif., USA). Cells were fixed in 4% (v/v) formaldehyde and subjected within 24 h to FACS analysis. An excitation wavelength of 488 nm produced maximal fluorescence emission at 525 nm for FITC and at 575 nm for PE; both fluorescence intensities were measured simultaneously. No cross-reactivity was noted between the first and second antibodies.

Adsorption studies

Samples containing LYSA were re-analysed for LYSA after they had been incubated with lymphocytes or pituitary cells. Both APSA-positive and APSA-negative samples were used. The adsorption conditions have been described previously [5].

Statistical analysis

The statistical significance of differences between the mean positivity ratio's of two subject groups was assessed by two-tailed t-test. A chi-square test with Yates-correction was employed to investigate whether the occurrence of different types of autoantibodies was statistically correlated.

Results

Detection of lymphocyte surface antibodies (LYSA)

Under conditions wherein less than 5% of the lymphocytes were fluorescently labelled after incubation with negative control samples, markedly higher percentages of fluorescent cells were noted after exposure to samples from certain Type 1 diabetic patients. The fluorescent cell population could be separated from the non-fluorescent population by cell sorting, using the window settings described under methods (Fig. 1).

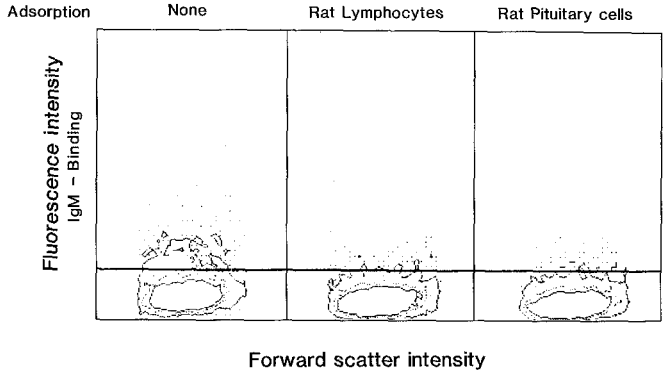

Fig. 1. Cell sorter analysis of IgM-binding to rat splenocytes. Cells were incubated with 100-fold diluted IgM fractions; cell-bound immunoglobulins were labelled with rhodamine-conjugated anti-human immunoglobulins. Each cell is plotted according to its forward scatter intensity and rhodamine-associated fluorescence intensity. The left panel represents a contourgraph for a sample containing lymphocyte surface antibodies (LYSA). More than 10% of the particles are recovered at a higher fluorescence intensity than for LYSA-negative samples. Sorting of the cells in the higher window yields cells with membrane fluorescence. The middle and right panels represent contourgraphs for the same IgM-fraction but after absorption by, respectively, rat splenocytes and rat anterior pituitary cells. In both conditions, the percent cells recovered in the window of higher fluorescence intensity has decreased to the values measured for LYSA-negative samples

More than 90% of the cells in the window of higher fluorescence intensity exhibited staining at their peripheral membrane. The percentage of cells recovered in this window can thus be used as a parameter for the presence of LYSA. This percentage is expressed as a ratio to that measured for negative control samples.

For IgM- and IgG-fractions from non-diabetic control sera, the mean positivity ratio was, respectively, 0.989 and 1.001 which is not statistically different from 1, the value set for negative controls. The upper limit of negativity was set at 3 SD above these mean positivity ratios, i.e. 1.56 for IgM and 1.31 for IgG. Samples with a higher positivity ratio were considered positive. None of the 33 IgM- and IgG-fractions prepared from non-diabetic control subjects contained LYSA as defined by these criteria.

Prevalence of LYSA in Type 1 diabetic patients and first degree relatives

In Type 1 diabetic patients at clinical onset, 8 of 30 (or 27%) presented circulating IgM which were positive in the LYSA assay (Table 1). This was also the case in 30% of patients under insulin treatment (Table 1). The mean positivity ratio calculated for the group of diabetic patients at onset differed significantly ($p < 0.001$) from that in normal control subjects (Fig. 2). A statistically significant difference ($p < 0.001$) was also found between the mean values in insulin-treated patients and normal control subjects (Fig. 2).

The group of Type 1 diabetic patients also contained individuals with IgG-LYSA but their number was low (7–18%) (Fig. 2 and Table 1). The mean positivity ratio calculated at onset did not differ significantly from that in normal control subjects (Fig. 2). A modest but significant ($p < 0.05$) increase was noted in patients treated for less than 3 years, but not in those with a longer treatment.

At diagnosis, 9 of 30 Type 1 diabetic patients presented LYSA and in 8 of 9 cases these surface antibodies were IgM (Table 1). For insulin-treated patients, the LYSA belonged also primarily to the IgM class (Table 1). In the Type 1 diabetic patients that were followed longitudinally, the LYSA-state at diagnosis remained constant over several years: the 5 LYSA-negative patients stayed negative over the 2 to 6 year follow-up, whereas the 5 LYSA-positive patients remained positive during the subsequent 3 to 7 years.

In first degree relatives of Type 1 diabetic patients; all younger than 20 years and non-diabetic, 6 of 18 individuals were LYSA positive (Table 1). This contrasts with the prevalence of the ICSA-positivity in first degree relatives (1 of 18 – [5]). In the normal control group no sera were LYSA-positive. For 5 of 6 LYSA-positive relatives, a LYSA positivity was also found in the diabetic sibling.

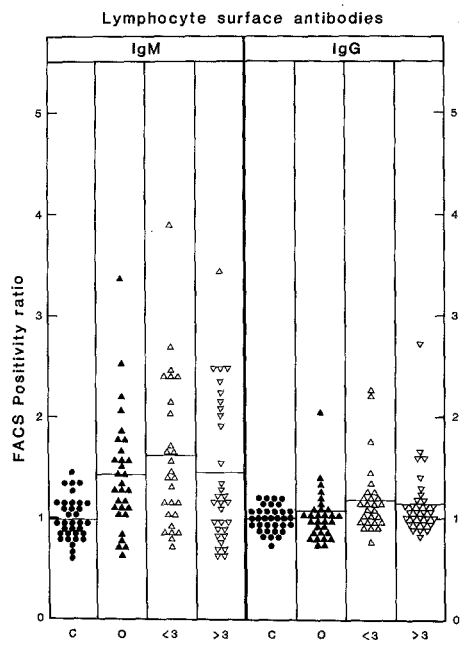

Fig. 2. Binding of rat splenocytes to IgM (left 4 columns) or IgG serum fractions (right 4 columns) at final 1/100 dilution. Immunoglobulin fractions were prepared from healthy control subjects (c), untreated Type 1 (insulin-dependent) diabetic patients at clinical onset (o), Type 1 diabetic patients treated for less than 3 years (<3) or for longer periods (>3). Mean positivity ratio's of patient groups are compared to the mean ratio of the control group and found to be significantly higher ($p < 0.001$) in all patient groups for IgM-LYSA as well as in patients within less than 3 years of insulin treatment ($p < 0.05$) for IgG-LYSA

Table 1. Prevalence of lymphocyte surface antibodies in Type 1 (insulin-dependent) diabetic patients[a]

Ig-class	Non-diabetic control subjects	Type 1 diabetic patients		Non-diabetic 1st degree relatives
		At onset	Insulin (>3 year)	
IgM	0/33 (0%)	8/30 (27%)	10/31 (32%)	3/18
IgG	0/33 (0%)	2/30 (7%)	5/31 (16%)	3/18
IgM- and/ or IgG	0/33 (0%)	9/30 (30%)	13/31 (42%)	6/18

[a] Assay at 1:100 dilution

Characteristics of Ig-binding to lymphocytes

Both LYSA-containing IgM and IgG-fractions were re-evaluated for their binding to lymphocytes after pre-treatment of the fractions or the cells. The Ig-binding remained detectable in lymphocytes that had been exposed to the same trypsin concentrations (11 μg/ml) as employed during the preparation of endocrine cells [20]. Trypsinization seems therefore not to be an obstacle in using dissociated endocrine cells in surface antibody assays, or in comparing the results in dissociated cell preparations with those obtained in spleno-

cytes. The positivity of LYSA-containing IgM or IgG-fractions did not disappear after preincubating the lymphocytes with heat-inactivated rat or calf serum, a condition which is expected to saturate the cellular Fc-receptors. The LYSA-positivity is therefore not attributable to binding of patient immunoglobulins to Fc receptors.

Pretreatment of the Ig-fractions with insulin (10^{-6} mol/l, 60 min at 4 °C) did not remove the LYSA-positivity indicating that it is unrelated to insulin antibodies. The LYSA were detectable at 4 °C as well as at 15 and 37 °C suggesting that they are not identical with the cold agglutinins which have been previously described in undiluted sera [8-11]. They appeared not to be induced by excessive glycosylation since in vitro glycosylation [5, 21] of LYSA-negative serum fractions failed to generate LYSA-positivity. In addition, no significant correlation was found between the FACS-positivity ratio's and the HbA_{1c} patient values.

Samples that were positive in the LYSA-assay on rat splenocytes, were also positive when tested on splenocytes from adult pigs. An increase in the number of rat splenocytes from $5 \cdot 10^4$ to 10^6 cells per test tube did not alter the positivity or negativity of the samples. Lowering the albumin concentration of the medium from 1 to 0.2% slightly increased the percentage of positive cells in positive control samples; an increase to 5% slightly decreased it. The modifications described for APSA [5] also improved the sensitivity of the LYSA assay.

Characterization of LYSA-binding lymphocytes

A double labelling technique was used to identify the lymphocytes to which the IgM and IgG serum fractions of Type 1 diabetic patients were bound. FACS-analysis of the cellular FITC- and PE-related fluorescence intensity indicated the percentage of B and T cells that were positive for LYSA. The initial splenocyte suspensions contained approximately 60% B and 40% T lymphocytes. For 8 of 10 tested LYSA-positive Ig column fractions (5 IgM and 5 IgG), the lymphocyte binding immunoglobulins appeared to bind with both B and T lymphocytes (Fig. 3). For 2 of 10 LYSA-positive IgG fractions, binding occurred almost exclusively with B lymphocytes (Fig. 3).

Correlation of IgM-LYSA and IgM-APSA in Type 1 diabetic patients

The group of Type 1 diabetic patients presenting circulating IgM-LYSA, had been previously found to contain IgM-APSA. A strong positive correlation ($p < 0.001$) was noted in the respective occurrence of IgM-LYSA and of IgM-APSA.

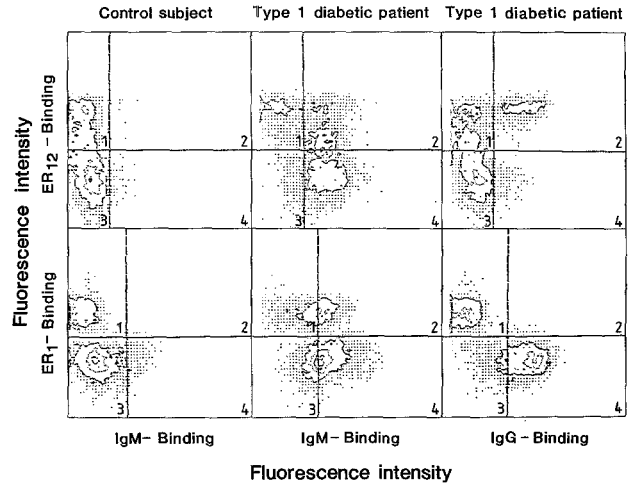

Fig. 3. Contour plots after cell sorter-analysis of lymphocytes labelled with fluorescein for surface-bound immunoglobulins (LYSA-fluorescence intensity on horizontal axis) and with phyco-erythrin for their subset markers (fluorescence intensity on vertical axis). Binding with three serum fractions was analysed: an IgM-LYSA negative sample from a non-diabetic control subject (left panels), an IgM-LYSA positive sample from a Type 1 (insulin-dependent) diabetic patient (middle panels) and an IgG-LYSA positive sample from another Type 1 diabetic patient (right panels). The control sample produced no fluorescein-positive cells (IgM-binding). The first Type 1 diabetic patient (middle panels) contained IgM-LYSA which were detected on both ER12 positive cells (B-lymphocytes) and ER1 positive cells (T lymphocytes). The second Type 1 diabetic patient (right panels) exhibited IgG-binding with ER12 positive cells (B lymphocytes) but not with ER1 positive cells (T lymphocytes)

Adsorption of LYSA to lymphocytes and pituitary cells

A preincubation of LYSA-positive IgM fractions with rat splenocytes reduced the LYSA-positivity of all tested samples by 30 to 100% ($n = 10$) (Fig. 1). When preincubation occurred with pituitary cells, the decrease in LYSA-positivity was noted in 6 of 10 samples; the four samples with unaltered LYSA-positivity were those with the lowest adsorption on splenocytes.

Discussion

The present report documents the occurrence of LYSA in Type 1 diabetic patients as well as in their first degree relatives. The immunoglobulins have been detected in a standardized assay which has been previously used to quantify islet (ICSA) and pituitary (APSA) cell surface antibodies [5]. The procedure involves incubation of splenocytes with diluted IgM- or IgG fractions, followed by fluorescent labelling of surface bound immunoglobulins and electronic measurement of the percent fluorescent cells.

The immunoglobulin binding to rat lymphocytes was not caused by antibodies against rat specific antigens, since similar results were obtained with piglet splenocytes. Binding to human lymphocytes could not

be tested as a result of their interaction with the second antihuman Ig antibody. The binding to rat or piglet lymphocytes was not affected by an excess of insulin or albumin, and appeared unrelated to glycosylation of circulating proteins. It was not attributable to an interaction with Fc receptors since immunoglobulins from normal sera failed to prevent binding of the positively scored Ig-fractions.

The LYSA were found to bind to T and B lymphocytes. Whether they play a role in vivo is unknown. It has been suggested that autoreactive B cell clones can impair suppressor T cell function [16, 22, 23], but no experiments have so far been conducted to test this hypothesis in Type 1 diabetes.

Under the selected assay conditions, LYSA were detected in 30% of Type 1 diabetic patients and in none of the 33 non-diabetic control subjects. They belonged predominantly to the IgM class. Their prevalence did not decrease with insulin treatment. In a limited longitudinal study, the LYSA-positivity or negativity was found to be maintained over periods up to 7 years. In first degree relatives of Type 1 diabetic patients, LYSA were markedly more prevalent than in non-diabetic control subjects. They appeared more frequently in relatives of LYSA-positive patients, but the number of subjects was too small to calculate the correlation. The occurrence of LYSA in first degree relatives and the constancy of the LYSA positivity after clinical onset, make it unlikely that LYSA mark the islet destructive process. They may rather express a state of immune autoreactivity which occurs in Type 1 diabetes but which is not necessarily associated with this disease.

Lymphocyte antibodies have been previously described in a variety of autoimmune diseases [12–14, 16], but also in viral [24] and parasitic [22] infections. They were mostly detected as HLA-unrelated lymphocytotoxins in unfractionated serum at low dilution. The present study examined the presence of cell surface binding immunoglobulins in fractionated serum at 100-fold dilution. It can be considered as a more sensitive screening for surface antibodies with higher affinity. These assay conditions allowed us to discern IgM-surface antibodies in Type 1 diabetic patients but not in control subjects. This does not exclude the possibility that similar antibodies occur in the normal state but at a lower concentration or affinity. Several studies have demonstrated that healthy subjects also present IgM-autoantibodies [25]. They are produced by the CD5 $(Leu\text{-}1)^+$ subset of B lymphocytes and exhibit a polyreactivity, binding with different affinities to a variety of exogenous and endogenous antigens [26]. It is conceivable that the presently described IgM-LYSA belong to this class of natural autoantibodies. First, IgM-LYSA were detected in some normal control sera tested at a 1:10 dilution instead of 1:100. Second, the IgM-LYSA were as well absorbed by pituitary cells as by lymphocytes. Their binding to lymphocytes was strongly correlated with the IgM-binding to pituitary cells, and the IgM-APSA were also absorbed by lymphocytes. The possibility should thus be examined that the detected IgM-LYSA and IgM-APSA express a stimulation of $CD5^+$-B lymphocytes which are known to produce natural autoantibodies. Their detection may help identify the conditions leading to this stimulated state and selecting subjects wherein a switch to monoreactive IgG-autoantibodies is more likely to occur.

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