Increased Concentrations of Total IgM at Clinical Onset of Type 1 (Insulin-Dependent) Diabetes: Correlation with IgM Binding to Cells

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Eighty patients with type 1 (insulin-dependent) diabetes (ages 0–39 years) were consecutively recruited by the Belgian Diabetes Registry. Sera obtained at clinically diagnosed onset (i.e., before start of insulin therapy or within 7 days of initial treatment) were analyzed for total IgM concentrations and for IgM binding to fixed rat splenocytes (IgM-LyAb) and permeabilized rat islet cells (IgM-ICAAb). Comparison of results with those in age- and sex-matched control subjects, by fluorescence-activated cell-sorter analysis, indicated greater concentrations of IgM-LyAb and IgM-ICAAb in sera from the patients. IgM antibodies reacted indiscriminately with islet β and islet endocrine non-β cells. The prevalence of IgM-ICAAb, but not of IgM-LyAb, was significantly (P < 0.05) higher in patients than in the control subjects. Of the ICA-Ab-positive patients, 54% were also LyAb-positive, whereas none of the control subjects were doubly positive. IgM-ICAAb and IgM-LyAb binding signals were positively correlated. Serum IgM concentrations were significantly (P < 0.001) greater in patients than in control subjects and were significantly correlated with IgM-LyAb (P < 0.001) and IgM-ICAAb (P < 0.01). The positivity for IgM binding was not, however, merely a reflection of total IgM, because no such correlation was found in sera from seven patients with Waldenström macroglobulinemia. Clinical onset of type 1 diabetes is apparently accompanied by increased production of IgM. The correlation between IgM concentrations and IgM binding to islet cells might reflect polyclonal activation or natural autoantibodies.

Additional Keyphrases: immunoglobulins - fluorescence-activated cell sorting - islet cells - lymphocytes - splenocytes - autoantibodies - autoimmunity - Waldenström macroglobulinemia

Clinical onset of type 1 (insulin-dependent) diabetes mellitus is often accompanied by anomalies of humoral and cell-mediated immunity (1–6). The prediagnosis occurrence of disease-specific IgG antibodies that recognize islet-cell components constitutes a potential marker for predicting the impending clinical manifestations of the disease (7–10). While researchers progressively unravel the molecular nature of the target antigens for these IgG antibodies (6, 11–13), it remains puzzling why, unlike immune responses induced by infectious agents, antigen-specific IgM antibodies are seldom encountered (14, 15). By analogy to other autoimmune diseases (16–18), polyreactive autoantibodies of the IgM class are observed at the clinical onset of type 1 diabetes (14, 15). Several authors speculate (16–18) that these antibodies reflect polyclonal activation potentially essential for the (auto)antigen-driven changeover to production of specific IgG autoantibodies. Alternatively, they might represent "natural" autoantibodies, implicated in constitutive immune network defenses against autoimmunity (18–21). Whatever their significance, a polyreactive IgM response might be more important for the pathogenesis or prediction of the disease by expressing an earlier event in the development of humoral autoimmunity than is the presence of IgG autoantibodies.

Using fluorescence-activated cell-sorter (FACS) analysis, we previously reported that 30% of type 1 diabetic patients with clinically diagnosed onset before age 20 years had IgM that bound with the surface antigens of viable rat splenocytes and anterior pituitary cells but not of rat islet cells (14, 15). For the present study we examined a larger group of patients, ages 0–39 years at clinical onset, and an equal number of age- and sex-matched control subjects. We adapted the cell-binding assay for use with fixed-cell suspensions to facilitate large-scale implementation. We also used it with islet cell suspensions that were first permeabilized with detergents to make intracellular antigens accessible for antibody binding. Further, we analyzed IgM binding to islet cells in terms of total IgM concentrations and of the characteristics of the subjects studied.

Subjects and Methods

Subjects

Serum samples were obtained from 80 newly diagnosed type 1 diabetic patients, before or within the first 7 days of insulin therapy, and from 80 age- and sex-matched control subjects. The newly diagnosed type 1 diabetics, younger than 40 years, were recruited by the
Belgian Diabetes Registry, a nationwide data and sample bank for type 1 diabetic patients. Each group (patients and controls) comprised 34 females and 46 males, mean age 22 years (range 2–39 years). None of the control subjects presented clinical signs of acute infection or had personal or familial antecedents with diabetes mellitus or other autoimmune disease. All had normal values for leukocyte counts and erythrocyte sedimentation rates.

Venous blood was collected in plastic tubes (Sarstedt, Essen, Belgium), allowed to clot at room temperature, and centrifuged within 1 h at 1000 × g for 15 min. Serum was stored in 200-μL portions at −80 °C. On the day of analysis, samples were thawed and ultracentrifuged at 100 000 × g for 15 min.

Cell Preparation for IgM Binding Assay

Rat pancreatic islet cells were obtained according to previously described procedures (22). Briefly, islets of Langerhans were isolated from collagenase-digested pancreata of adult male Wistar rats and dissociated into single cells. The cells were fixed in formaldehyde (20 mL/L) in phosphate-buffered saline (PBS), for 30 min at room temperature and kept at 4 °C until used in the flow-cytometric antibody assay (within 7 days). Treatment with Triton X-100 (Sigma Chemical Co., St. Louis, MO; 0.5 mL/L, 10 min, room temperature) permeabilized the islet cells as evidenced by specific cytoplasmic staining with anti-insulin and anti-glucagon antibodies.

Splenocytes were prepared from adult male Wistar rats and from piglets. The spleens were mechanically dispersed with a syringe and suspended in PBS containing 1 g of bovine serum albumin (BSA, Fraction V; Boehringer, Mannheim, FRG) and 0.1 g of sodium azide per liter. The cell suspension was centrifuged for 25 min at 800 × g on Lymphoprep medium (density 1.07 kg/L; Nycomed AS, Oslo, Norway), and the mononuclear cells were harvested at the interface between the supernate and the Lymphoprep cushion (23). The splenocyte suspension contained ~50% B lymphocytes, 45% T lymphocytes, and 5% macrophages. Human lymphocyte suspensions were prepared from healthy volunteers: venous blood was collected in potassium EDTA (Sarstedt), diluted with an equal volume of PBS-BSA, and layered on Lymphoprep. The cells were fixed as described for islet cells.

IgM Binding to Cells

IgM binding to rat islet cell antigens (IgM-ICAb) was determined with fixed and permeabilized islet cells. After fixation and storage, the cells were washed twice with 5 mL of assay buffer [PBS containing 5 g of BSA (Fraction V, RIA grade; Sigma) per liter] and then permeabilized by 10-min exposure to Triton X-100, 0.5 mL/L in assay buffer. After washing, the cells were incubated with 400-fold-diluted human serum for 60 min at 15 °C. The cells were again washed and then incubated for 30 min at 15 °C with rhodamine-labeled rabbit anti-human IgM (μ-chain) antiserum (Dakopatts, Glostrup, Denmark) diluted 200-fold in assay buffer.

Cell-bound fluorescence was analyzed by FACS analysis with an FACStar Consort 30 (Becton Dickinson, Sunnyvale, CA), with use of an argon ion laser (Model 2016; Spectra Physics, Mountain View, CA) at 415 nm (14, 15).

A similar procedure was used to identify the type of hormonal product involved: the cells were first incubated with mouse anti-insulin or antiguacagon antibodies, then with rhodamine-conjugated goat antimouse (IgG+IgM) antiserum (Jackson Immunoresearch Labs., West Grove, PA). Monoclonal mouse anti-insulin antiserum (final dilution 500-fold) and antiguacagon antiserum (diluted 100-fold) were kindly provided by Dr. M. Ziegler (Zentralinstitut für Diabetes, Karlsruhe, FRG).

Splenocytes and lymphocytes were first incubated with 100-fold-diluted serum in PBS–1 g/L BSA (Boehringer) for 60 min at 4 °C and then with anti-human IgM as described for islet cells. Each assay included one positive and one negative control serum, yielding respectively positive and negative immunofluorescence in fluorescence microscopy. FACS analysis of these sera allowed selection of the window settings for the fluorescent and nonfluorescent cell populations. The negative control serum consistently yielded <10% fluorescent particles vs 60–70% for the positive control sample. Omission of the incubation with human immunoglobulins invariably yielded <1% positive cells. For each unknown sample, binding of IgM antibodies to splenocytes or pancreatic cells was expressed as the percentage of particles in the window for increased cellular fluorescence. Interassay (n = 6) CVs for IgM-ICAb and IgM-LyAb were between 5% and 10%.

We also examined samples by fluorescence microscopy (Dialux 20 EB; Leitz, Wetzlar, FRG) to detect membrane-bound and intracellular fluorescence.

Total IgM and C-Reactive Protein in Serum

Concentrations of IgM and C-reactive protein (CRP) in serum were determined with a BNA rate nephelometer (Behring, Marburg, FRG), with use of commercial antiserum (OSAT 14/15 and OpμSV 10/11 for CRP; Behring) according to the manufacturer's specifications.

Statistical Analysis

Differences between groups were assessed by using the two-tailed unpaired Student's t-test for normally distributed data and the Mann–Whitney U test for not-normally distributed data. Analysis of variance (ANOVA) was used for comparisons between multiple groups for normally distributed data, the chi-square test for assessing the significance of differences in prevalence. Linear-regression analysis determined the degree of correlation between two variables. For all tests, a P-value <0.05 was considered significant. The mean ± 2 SD of the results for the control subjects was used as the cutoff point for positivity.

Results

IgM Antibodies to (Splenic) Lymphocytes and Islet Cells

Exposure of fixed rat splenocytes to sera from newly diagnosed type 1 diabetic patients yielded a signifi-
binding because binding percentages islet positive ticed only suspensions cells (right) Fig. 2.

For spleen cells, fluorescence was exclusively located at the cell membrane (left), whereas cytoplasmic staining predominated in permeabilized islet cells (right).

Fig. 1. Flow-cytometric detection of IgM binding to fixed rat spleen cells (left) and fixed rat pancreatic islet cells (right) in type 1 diabetic patients at onset and in healthy control subjects.

Fig. 2. Patterns of cell-bound rhodamine fluorescence as observed in cell suspensions of fixed rat spleen cells (left), and permeabilized rat islet cells (right) with fluorescence microscopy (410× magnification, 513 nm) after incubation with a positively reacting serum from a type 1 diabetic patient at clinical onset.

significantly higher fraction of fluorescence-labeled spleen cells than did exposure to sera from age- and sex-matched control subjects (P < 0.04) (Figure 1). Cell suspensions that were scored positive in the FACS assay displayed a distinct membrane-associated fluorescence pattern under the microscope (Figure 2). By contrast, only a few cells with membrane fluorescence were noticed after incubation with sera that reacted negatively in the FACS assay. Sera that were positive in this IgM binding assay with fixed rat splenocytes were also positive when freshly isolated rat splenocytes, fixed piglet splenocytes, or fixed human blood lymphocytes were used (results not shown).

When serum IgM fractions were reacted with living islet β and non-β cells at a final 100-fold dilution, no IgM binding to surface antigens was found (14). Use of permeabilized islet cells yielded highly variable percentages of fluorescent cells with negative control sera. Because this variability disappeared at a 400-fold dilution of serum, we selected this dilution for further binding studies with pancreatic islet cells. Microscopy of FACS-positive sera showed a positive cellular cytoplas-

mic fluorescence, with the intensity varying from cell to cell (Figure 2).

Sera from 80 newly diagnosed type 1 diabetic patients displayed greater IgM antibody binding to permeabilized islet cells (29.5 ± 24.0% of fluorescent cells) than did sera from the age- and sex-matched control subjects (21.5 ± 17.5%; P < 0.02) (Figure 1). During FACS analysis, contour graphs of the permeabilized islet cell suspensions revealed two distinct cell populations (Figure 3). The two subpopulations differed mostly by forward light scatter, a measure of cell size (24). Immunocytochemical analysis characterized the high-scatter population (solid lines) as being mostly (>90%) insulin-containing cells, whereas the low-scatter population (broken lines) corresponded mostly (>65%) to glucagon-containing cells. FACS analysis of an IgM-ICAb-positive sample (Figure 3) indicated that IgM binding invariably involved both insulin- and glucagon-containing cells.

The percentage of fluorescent cells detected in the IgM-ICAb assay (y) correlated significantly with that in the IgM-LyAb assay (x), both for control sera (y = 0.79x + 10.3; n = 80, r = 0.54; P < 0.001) and for patients' sera (y = 0.99x + 10.7; n = 80, r = 0.54; P < 0.001).

Prevalence and Concordance of IgM-LyAb and IgM-ICAb

In the group of diabetic patients, 8 of 80 (10%) sera tested at 100-fold dilution exceeded the cutoff value for IgM-LyAb-positivity, whereas 4 of 80 (5%) sera from the control subjects did; however, this difference in prevalence was not statistically significant, neither for the two groups as a whole nor when subdivided according to age (0–19 vs 20–39 years) or to sex. At 400-fold serum dilution, the prevalence of IgM-ICAb was 5% (4 of 80) in the control group and 16% (13 of 80) in the type 1 diabetic patients (P < 0.05; Table 1). The correlation between the occurrence of IgM-ICAb and IgM-LyAb was significant (P < 0.05) in the 80 diabetic patients, but not in age- and sex-matched healthy individuals. Type 1 diabetic patients positive for IgM-ICAb were positive for IgM-LyAb in 54% of the cases (Table 1). By contrast, none of the IgM-ICAb-positive control individuals were simultaneously positive for IgM-LyAb (Table 1). Double
positivity for IgM-ICAb and IgM-LyAb in the recent onset group appeared not to be sex-linked (four males, three females), but further analysis by age revealed that six of seven IgM double-positive patients were older than 20 years.

IgM Concentrations and Relation to IgM Autoantibodies

Total IgM concentrations were measured to investigate their possible relationship to the increased prevalence of IgM-ICAb among type 1 diabetic patients. In IgM-ICAb-positive patients, mean total IgM concentrations in serum (4.40 ± 2.60 g/L) were significantly greater (P < 0.05) than in IgM-ICAb-negative patients or in the IgM-ICAb-negative or -positive healthy individuals (Table 2). In addition, even in the IgM-ICAb-negative patients, mean total IgM titers were significantly greater than in the control subjects, whether IgM-ICAb-negative or -positive. Finally, in the patient group as a whole, mean total IgM concentrations in serum (2.80 ± 1.78 g/L) were significantly greater (P < 0.001) than for the age- and sex-matched control subjects (1.65 ± 0.74 g/L). This difference remained statistically significant when the patients were grouped according to age (0–19 and 20–39 years) or to sex. ANOVA revealed no sex- or age-related differences in IgM titters within either group, patients or control subjects. Of the 80 patients, 30 (37.5%) had IgM concentrations that exceeded the mean of the control group by >2 SD, as opposed to 4 of 80 (5%) of the control subjects (P < 0.001; chi-square test). CRP concentrations in patients and control subjects did not support a hypothesis for greater frequency of recent infections at onset because CRP was undetectable (<3 mg/L) in 79 of 80 control subjects and 78 of 80 recently diagnosed diabetic patients. CRP was increased in one control subject (64 mg/L) and two diabetic patients (30 and 45 mg/L). At variance with our observations for IgM, there were no differences in IgG or IgA titers between type 1 diabetic patients and the control subjects (results not shown).

Multiple-regression analysis showed that the degree of IgM binding to spleen cells and pancreatic islet cells was related to total IgM concentration, both in the control subjects (P < 0.005 for IgM-LyAb and IgM-ICAb) and in diabetic patients at onset (P < 0.001 for IgM-LyAb and P < 0.01 for IgM-ICAb).

We then examined whether positivity for IgM-ICAb or IgM-LyAb (or both) merely reflected an increased concentration of total IgM. We tested seven sera from patients with Waldenström macroglobulinemia, using the IgM-LyAb and IgM-ICAb assays. Although all seven patients had very high IgM titers (range 24.1–68.6 g/L), three of them presented no IgM binding to rat splenic lymphocytes and pancreatic islet cells in serum tested at 100-fold and 400-fold dilutions, respectively (Figure 4). Incubation of sera with pancreatic islet cells did show IgM-ICAb positivity in four of the seven samples, but only one IgM-ICAb-positive serum was positive for IgM-LyAb. All seven sera were tested again at a further 16-fold dilution, at which dilution the IgM concentration in the Waldenström samples was similar to that in samples from healthy control subjects and type 1 diabetic patients (range 1.50–4.30 g/L). For two of the four IgM-ICAb-positive samples from the Waldenström patients, the percentage of fluorescence-labeled islet cells (Figure 4) remained unchanged (even at 25 600-fold dilution). By contrast, IgM binding to islet cells decreased with further dilution for the other two IgM-ICAb-positive samples and was no longer detectable at 1600-fold dilution. Unlike the sera from patients or control subjects, the degree of IgM binding of sera from patients with Waldenström macroglobulinemia to rat islet cells and splenocytes was not related to total IgM concentration (P > 0.1). Upon serial dilution (100-, 400-, and 800-fold), all IgM-LyAb- or ICAb-positive samples from the control subjects (8 of 80) and the diabetic patients (14 of 80) yielded IgM binding values that decreased progressively.

Table 1. Prevalence of IgM-LyAb in Diabetic Patients and Control Subjects: Relation to IgM-ICAb Prevalence

<table>
<thead>
<tr>
<th>IgM-ICAb status, no. (and %)</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>76 of 80 (95)</td>
<td>4 of 80 (5)</td>
</tr>
<tr>
<td>IgM-LyAb-positive</td>
<td>4 of 78 (5)</td>
<td>0 of 4 (0)</td>
</tr>
<tr>
<td>Recent-onset diabetic patients</td>
<td>67 of 80 (84)</td>
<td>13 of 80 (16)</td>
</tr>
<tr>
<td>IgM-LyAb-positive</td>
<td>1 of 67 (2)</td>
<td>7 of 13 (54)</td>
</tr>
</tbody>
</table>

Table 2. IgM Concentrations in Diabetic Patients at Onset vs Control Subjects: Relation to IgM-ICAb Positivity

<table>
<thead>
<tr>
<th>IgM-ICAb status</th>
<th>Mean (SD) IgM, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>1.70 (0.70)ab</td>
</tr>
<tr>
<td>Onion of diabetes</td>
<td>2.00 (0.40)ab</td>
</tr>
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* P < 0.05 vs IgM-ICAb-positive diabetic patients at onset.

b P < 0.05 vs IgM-ICAb-negative diabetic patients at onset.

Fig. 4. Flow-cytometric detection of IgM binding to fixed rat spleen cells (left) and fixed rat pancreatic islet cells (right) in serum from patients with Waldenström macroglobulinemia, respectively tested at 100- and 400-fold dilutions (left columns in each panel).

To produce a final total IgM concentration in the assay mixture similar to those observed in control subjects and diabetic patients at onset, we resuspended sera at further dilutions of, respectively, 1600- and 8400-fold (right column in each panel). For each serum, the results obtained at different dilutions are connected with a line. The horizontal line represents the cutoff value for positivity determined as the mean + 2 SD for 80 healthy control subjects, assayed at dilutions of 100-fold (LyAb) and 400-fold (ICAb).
with increasing dilution and generated comparable dilution curves.

At the clinically diagnosed onset of diabetes, the total IgM concentration was not related to positivity for islet cell cytoplasmic antibodies (ICAb), as determined by indirect immunofluorescence in cryosections of pancreata from humans of blood group O. Binding of IgG, but not of IgM, to permeabilized dispersed islet cells was significantly greater in ICA-positive patients (results not shown).

Discussion

This report confirms and extends our previous observations that IgM antibodies from type 1 diabetic patients at clinical onset exhibit an increased binding to cellular antigens. Detection of comparable IgM binding to cells from different species (rats, humans, pigs) argues against the contention that this increase results from heterophilic antibodies (25). The IgM binding assay was adapted to fixed cell suspensions, which can be stored for at least 1 week; we applied it to a greater number of samples from type 1 diabetic patients and age- and sex-matched control subjects than we did in the previous assay involving freshly prepared cells. The present study confirms increased concentrations of IgM-LyAb in diabetic patients, but the prevalence of LyAb-positivity was lower than previously reported (15) and was no longer statistically different from that in the control group. On the other hand, the use of permeabilized and fixed rat pancreatic islet cells allowed the detection of IgM binding to islet cell antigens. The concentrations and prevalence of these IgM-ICAb were significantly increased at the clinically diagnosed onset of diabetes and were correlated with the findings for IgM-LyAb. Concomitant positivity for the two types of IgM reactivity was not seen in any control subjects but occurred in ~50% of the IgM-ICAb-positive patients at disease onset. Total concentrations of circulating IgM were also significantly increased at onset and correlated with IgM binding to lymphocytes and islet cells. However, the latter phenomenon cannot be a mere reflection of a hyper-IgM state, because no such correlation was seen in sera from patients with Waldenström macroglobulinemia, which reacted mostly negatively for IgM-LyAb or IgM-ICAb when tested at IgM concentrations similar to that in diabetic patients and control subjects.

This is the first report of increased IgM concentrations at clinical onset of diabetes. No increase in IgM has been noticed in patients who were not sampled at onset (26,27). The increased IgM concentration at onset is unlikely to reflect recent infections, given that CRP was undetectable in the vast majority of sera in both subject groups (28). Conceivably, however, earlier exposure to viruses could have elicited, perhaps through idiotypic-anti-idiotypic networks, the production of antibodies that cross-react with structurally related epitopes on cell membranes, cytoskeletal proteins, or nucleic acids, thereby conferring multiple-organ autoreactivity to these immunoglobulins (29–32). Anti-insulin-receptor autoantibodies of the IgM class have been noticed in 45% of untreated patients with type 1 diabetes of recent onset (33), but this observation requires confirmation (34).

By analogy to what is known for other autoimmune diseases, increases in IgM concentrations and IgM binding to cellular antigens might reflect a polyclonal activation of B lymphocytes (16–18). Whether such polyclonal activation reflects an environmental challenge, an endogenous disease process, or a genetically determined characteristic is unknown. Irrespective of its underlying mechanism, a state of IgM hyperreactivity may precede an antigen-driven switch to specific T or B cell autoreactivity, a hypothesis that is testable in family members of diabetic patients. Interestingly, increased IgM binding to cellular antigens was observed more frequently in nondiabetic first-degree relatives of type 1 diabetic patients than in control subjects (14,15).

The presence of polyreactive autoantibodies at the onset of type 1 diabetes could also correspond to an increased titer of natural autoantibodies (18–21). These antibodies are produced by CD5-positive B lymphocytes in response to exogenous antigens (e.g., during infections), and thus provide a nonspecific first-line defense committed to (auto)antigen clearance and (or) to binding the immune system for potentially cross-reacting autoantigens. Natural autoantibodies are known to react with diverse autoantigens such as hormones, serum proteins, cell-surface receptors, nuclear antigens, and cytoskeletal proteins (18–21). It is thus interesting to note that IgM antibodies reacting with cytokeratin in exocrine pancreatic cells are more numerous at an early stage of type 1 diabetes (35) and that CD5-positive cells are more numerous in diabetic patients than in control subjects (36). Expansion of natural antibody-producing cell lines is also relatively frequent in Waldenström macroglobulinemia, leading to a high frequency of polyreactive M components directed against (e.g.) cytoskeletal proteins (37). This might explain why some, but not all, of the Waldenström sera gave a positive response for IgM binding to lymphocytes and islet cells, even at very high dilutions.

The observation that six of the seven patients with IgM binding to islet cells and lymphocytes were older than 20 years may indicate an increasing frequency of circulating autoantibodies with age (38,39). It might also reflect the importance of repeated antigenic challenge in diabetic patients with later clinical onset of the disease.

In conclusion, clinical onset of type 1 (insulin-dependent) diabetes is accompanied by increased binding of IgM to fixed lymphocytes and permeabilized islet cells and by increased circulating concentrations of total IgM. The latter are positively correlated with cellular IgM binding in control subjects and in patients with diabetes but not in patients with Waldenström macroglobulinemia. Prospective follow-up studies in individuals at increased risk for diabetes will help establish the temporal and mechanistic relationship, if any, of polyreactive IgM autoantibodies to the development of the disease and to the elaboration of diabetes-specific IgG
autoantibodies. Measurement of total IgM concentrations may emerge as a simple, automateable, and quantitative test for IgM autoantibodies, in view of its close relationship with IgM-LyAb or IgM-ICAb.

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