Spontaneous in vitro immunoglobulin secretion at the diagnosis of insulin-dependent diabetes

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Abstract. Blood mononuclear cells obtained from 17 newly diagnosed insulin-dependent diabetic (IDDM) patients treated with insulin for 5–7 days were assessed for the number of spontaneous and pokeweed mitogen (PWM)-stimulated immunoglobulin-secreting cells in a reverse haemolytic plaque assay. The spontaneous in vitro immunoglobulin secretion was evanescent and decreased in individual patients within 1–4 months of insulin treatment. Compared to matched controls, 53% (9/17) of the IDDM patients had an elevated spontaneous secretion of immunoglobulin, 41% (7/17) for IgG, 35% (6/17) for IgM, and 35% (6/17) for IgA. The quantities of PWM-stimulated IgG, IgM, or IgA secreting cells in IDDM were comparable to the controls. The IDDM patients with spontaneous immunoglobulin secreting cells had higher fasting C-peptide levels compared to the patients with immunoglobulin-producing cells within the normal range (P < 0.05). The average titre of islet cell cytoplasmic antibodies was 1:26 in (9 out of 9 were positive) patients with, compared to 1:1 in patients (4 out of 8 were positive) without spontaneous secretion (P = 0.025). These results suggest that the clinical onset of IDDM is associated with a polyclonal B lymphocyte activation and that higher levels of fasting C-peptide islet cell antibodies are associated with this immunoregulatory abnormality.

Insulin-dependent diabetes mellitus (IDDM) in young individuals is associated with a high incidence of circulating antibodies reacting not only with pancreatic islet cells (Bottazzo et al. 1974; Irvine et al. 1977; Lernmark et al. 1978; Doleans et al. 1980) but also with other endocrine cells (MacCuish & Irvine 1975; Nerup & Lernmark 1981), non-endocrine cells (Serjeantson et al. 1981) or subcellular constituents such as single-stranded DNA and double-stranded RNA (Huang et al. 1981). The prevalence of islet cell antibodies has been found to be highest close to the diagnosis of the disease with a decline thereafter. Furthermore, increased levels of Ia antigen bearing T lymphocytes have been noted in the blood of newly diagnosed IDDM patients (Jackson et al. 1982). Although controversial (Jackson et al. 1982; Mascart-Lemone et al. 1982), such patients often exhibit a decrease in the number of OKT8 (cytotoxic/suppressor cells) reactive peripheral lymphocytes (Galluzzo et al. 1982; Buschard et al. 1983; Horita et al. 1982) and a depressed suppressor cell activity (Buschard et al. 1980; Fairchild et al. 1982). These abnormalities in cell-mediated immunity appeared transient as they could not be detected 2–6 months after the clinical onset of IDDM (Buschard et al. 1980, 1983; Horita et al. 1982; Fairchild et al. 1982). Thus, an immunoregulatory abnormality may be associated with higher numbers of antibody-producing B lymphocytes. To test this hypothesis we have subjected peripheral blood mononuclear cells from newly diagnosed insulin-dependent diabetic patients to the in vitro plaque forming cell (PFC) assay to test their ability to produce IgM, IgG or IgA, spontaneously and after polyclonal stimulation with pokeweed mitogen (PWM).
Materials and Methods

Patients and clinical parameters
We studied 21 IDDM patients, 10 females and 11 males (mean age 21 years, range 12–30 years). The patients were either newly diagnosed (n = 17) or had suffered from IDDM for 1–4 months (n = 4). At the clinical onset the patients were treated with highly purified porcine insulin for 5–7 days before blood sampling for analysis. For patients examined at onset (n = 17) blood samples were withdrawn only after near-normoglycaemia (fasting blood glucose < 9 mm) at least 5 days after the beginning of insulin treatment. In the remaining 4 patients and in 5 of the newly diagnosed IDDM patients, blood samples were obtained at routine clinical visits 1 or 4 months after discharge from the hospital. All patients have remained insulin-dependent for up to 18 months after the initiation of this study. None of the patients showed clinical signs of any other autoimmune disease but candidiasis infection was detected in 4 patients. The normal controls were 10 healthy individuals, 5 females and 5 males (mean age 19 years, range 16–25 years), who were unrelated to the patients and asked to participate during the same time of the year as the IDDM patients were diagnosed.

Isolation of mononuclear cells and cell culture
Mononuclear cells from heparinised venous blood samples were prepared by gradient sedimentation through Ficoll-Hypaque (Böyum 1968). The culture conditions were the same as previously described (Agger et al. 1982). Briefly 5 × 10⁶ peripheral mononuclear cells (PMC) were suspended in 10 ml RPMI 1640 supplemented with 10% v/v heat-inactivated foetal calf serum, 2 mmol/l glutamine, and 20 µg/ml gentamycin and placed at 37°C in an upright culture flask (No. 3013, Falcon, Oxnard CA) in a humidified atmosphere with 5% CO₂. The cultures were stimulated with pokeweed mitogen (PWM) (Gibco, Paisley, Scotland) at a final dilution of 1/250. After 6 days without changing the medium, cells were harvested and washed once in minimum essential medium (Flow, Irvine, Scotland).

Plaque-forming cell (PFC) assay
The number of class specific immunoglobulin (Ig) secreting cells was determined in a reverse haemolytic plaque-forming cell (PFC) assay as previously described (Agger et al. 1982). Spontaneous Ig secreting cells were determined from PMC suspensions immediately after separation from peripheral blood (day 0). PFC from stimulated cultures were determined on day 6, at which day the normal controls yielded the maximum number of Ig secreting cells. Briefly, mononuclear cells were mixed with protein A-conjugated sheep red blood cells, rabbit anti-human IgM, IgG, or IgA, guinea-pig complement and agar kept at 45°C. Agar drops were placed under cover slips and incubated for 4 h before counting the plaques (Agger et al. 1982).

Assays for organ-specific antibodies
The patients’ sera were checked for anti-cytoplasmic islet cell antibodies by the indirect immunofluorescence method as described by Marner et al. (1983). In brief, serum samples were applied onto frozen sections of type 0 human pancreas and incubated at room temperature for 45 min. The specimens were extensively washed and a 1/10 dilution of rabbit anti-human IgG (fluorescein-isothiocyanate conjugated) antiserum (DAKO, Copenhagen, Denmark) was added. Following a 30 min incubation and washing the samples were scored by two independent observers for islet cell cytoplasmic fluorescence on a Zeiss fluorescence microscope.

Thyroglobulin and thyroid microsomal antibodies were determined in a standard haemagglutination assay (Medicinsk Laboratorium ApS, Copenhagen, Denmark).

Assays for C-peptide and HbA₁c
Circulating fasting plasma C-peptide was determined by radioimmunoassay (Binder & Faber 1978). The normal range (mean + 2 × sd) in this assay is 0.15–0.60 pmol/ml; the lower detection limit being 0.05 pmol/ml.

HbA₁c was determined on the fraction of haemoglobin which was not rapidly glycosylated (Svendsen et al. 1980).

Statistical evaluation
Differences between controls and diabetic patients or between diabetic patients with or without spontaneous antibody-secretion were tested with the Mann-Whitney U-test.

Results
Blood samples were first obtained at the time of discharge from the clinic, when each patient had been treated with insulin for 5–7 days and the blood sugar levels were normal or near-normal at an insulin dosage of 0.50–0.25 IU/kg (range 0.15–0.96; mean ± sd). Nine out of 17 (53%) IDDM patients had spontaneous secretion of immunoglobulin (IgG, IgM, or IgA) exceeding the mean + 2 × sd of the controls (Fig. 1). The spontaneous secretion of immunoglobulins among the controls conforms with what is routinely being found in the present test (Agger et al. 1982). Spontaneous secretion of IgG was observed in 6/17 (35%), IgM in 5/17 (19%), and IgA in 4/17 (24%). In 3 of the 9 IDDM patients with enhanced spontaneous secretion all three classes were increased while the remaining 6 patients showed an increase in only one of the classes.
The peripheral mononuclear cells from 11 of the IDDM patients and controls when stimulated with PWM had quantities of IgG, IgM, and IgA secreting cells comparable to the healthy control individuals (Table 1). The IDDM patients tended to have a lower degree of PWM stimulation at diagnosis compared to controls, the difference, however, did not reach statistical significance at the 5% level. The PFC analysis was also carried out in 9 patients at subsequent visits to the out-patient clinic 1–4 months after discharge (Fig. 1). It was found that 3/9 (33%) of these IDDM patients had increased spontaneous IgG and IgA secretion while only one patient showed an elevated IgM secretion. PWM-stimulated values were not different from the controls (Table 1).

In 5 patients the in vitro PFC assay was performed both at discharge and at a subsequent visit to the out-patient clinic 1–4 months later (Fig. 2). In all 3 patients with increased numbers of immunoglobulin secreting cells at discharge, there was a normalization or a decrease to near-normal values at the follow-up test.

It was tested whether the patients shown in Fig. 1 either without or with spontaneous immunoglobulin secretion at discharge differed in metabolic and immunologic parameters (Table 2). Patients with spontaneous immunoglobulin secretion had higher fasting C-peptide levels at admission compared to

**Table 1.**

Immunoglobulin-secreting cells in peripheral blood from insulin-dependent diabetic (IDDM) patients and controls after pokeweed mitogen (PWM) stimulation.

<table>
<thead>
<tr>
<th>Length of insulin treatment</th>
<th>Number of subjects</th>
<th>Plaque forming cells 10⁶ (PFC)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>5–7 days</td>
<td>11</td>
<td>4057 ± 2436</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(377–7169)</td>
</tr>
<tr>
<td>1–4 months</td>
<td>9</td>
<td>5601 ± 3827</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1201–12 469)</td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>5296 ± 2107</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2858–8691)</td>
</tr>
</tbody>
</table>

* Results are given as PFC per 10⁶ originally cultured peripheral mononuclear cells.
Mean values ± sd. Range is shown with parentheses.
Spontaneous plaque forming cells (PFC) secreting IgG, IgM, or IgA in 5 insulin-dependent diabetic patients both at discharge after 5–7 days of insulin treatment and at a subsequent visit to the out-patient clinic 1–4 months later. The hatched lines represent the mean $\pm 2 \times$ SD for 10 matched healthy controls as described in the legend to Fig. 1.

Table 2.
Comparison of clinical parameters in insulin-dependent diabetic patients with or without spontaneous immunoglobulin production.

<table>
<thead>
<tr>
<th></th>
<th>IDDM patients spontaneous immunoglobulin production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With (n = 9)</td>
</tr>
<tr>
<td>Female/male</td>
<td>5/4</td>
</tr>
<tr>
<td>Age at admission (years)</td>
<td>19 $\pm$ 6</td>
</tr>
<tr>
<td>Fasting C-peptide (pmol/ml) at admission</td>
<td>0.23 $\pm$ 0.09</td>
</tr>
<tr>
<td>HbA1c (%) at admission</td>
<td>12.6 $\pm$ 2.8</td>
</tr>
<tr>
<td>Insulin dosage (IU/kg) at discharge</td>
<td>0.46 $\pm$ 0.22</td>
</tr>
</tbody>
</table>

Islet cell cytoplasmic antibodies

<table>
<thead>
<tr>
<th></th>
<th>With (n = 9)</th>
<th>Without (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>9/9 (100%)</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>Range</td>
<td>1:1–1:81</td>
<td>neg–1:3</td>
</tr>
<tr>
<td>Average titre of positives</td>
<td>1:27</td>
<td>1:1**</td>
</tr>
<tr>
<td>Thyroglobulin antibodies</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>Thyroid microsomal antibodies</td>
<td>negative</td>
<td>negative</td>
</tr>
</tbody>
</table>

Mean values $\pm$ SD are shown. *$P < 0.05$ (Mann-Whitney U-test). **$P < 0.025$. 

Fig. 2.
patients with immunoglobulin secreting cells within the normal range (P < 0.05). Insulin dosage and HbA1c levels did not differ. While none of the patients had thyroglobulin or thyroid microsomal antibodies, the indirect immunofluorescence test for islet cell cytoplasmic antibodies was positive in 77% (13/17) of the patients. Reciprocal dilutions in repeated tests of the antibody positive sera showed that the IDDM patients without spontaneous immunoglobulin secretion had an average titre of 1:1 compared to 1:26 for the patients with spontaneous secretion (P = 0.025).

Discussion

The reverse haemolytic plaque assay used in the present study revealed that blood mononuclear cells in many patients with newly diagnosed IDDM had increased numbers of B lymphocytes spontaneously secreting IgG, IgM, as well as IgA. This derangement was most evident at the time of discharge from the diabetes clinic after 5–7 days of careful diabetes control with near-normalization of blood glucose. Our observations support the hypothesis that alterations of the normal immunoregulation, reflected in a decreased proportion of T suppressor cells at the onset of IDDM (Galluzzo et al. 1982; Buschard et al. 1980, 1983; Horita et al. 1982; Fairchild et al. 1982; Pozzilli et al. 1983), would lead to higher numbers of antibody-producing B lymphocytes. On the other hand, the increased spontaneous immunoglobulin-secretion could possibly reflect an effect of the short-term (5–7 days) insulin treatment. Non-treated controls are obviously not available in man, however, streptozotocin-induced diabetes in mice showed increased numbers of T suppressor cells and the state of functional immune suppression could be transferred to syngeneic mice (Ptak et al. 1980). It has also been shown that antigen-specific, non-cytotoxic murine lymphocyte clones express insulin receptors upon antigen exposure (Braciale et al. 1982). If applicable to man it is therefore difficult to see how the short-term insulin treatment would lead to an increase in the number of PFC in some patients but not in others.

The abnormalities observed at the clinical onset of IDDM tended to revert to normal levels. Increases in numbers of immunoglobulin secreting cells have been interpreted as an indication of an in vivo polyclonal activation (for review, see Waldmann & Broder 1982). Similar increases have been reported in patients with autoimmune diseases e.g. systemic lupus erythematosus (Ginsburg et al. 1979), multiple sclerosis (Goust et al. 1980), rheumatoid arthritis (Al-Balaghi et al. 1982) and concomitant IDDM and Hashimoto’s thyroiditis (Horita et al. 1982). The increases in spontaneous immunoglobulin secretion which often parallel the activity of the disease may represent an immunoregulatory dysfunction in such individuals (Ginsburg et al. 1979).

In contrast to the measurements of spontaneous B lymphocyte activity in our patients we did not observe any difference between controls and IDDM patients after incubating the PMC with PWM for 6 days, a time of incubation previously found to yield maximal cellular activity (Agger et al. 1982). The response to PWM in various autoimmune diseases is, however, not at all uniform. PWM produced a significantly reduced response in lupus patients (Fauci et al. 1978) while an enhanced response was observed in rheumatoid arthritis (Tsoukas et al. 1980) or Hashimoto’s thyroiditis (McLachlan et al. 1981). However, it cannot be excluded that our use of maximally stimulated cultures might have obliterated a possible quantitative difference between IDDM patients and controls.

After the completion of our measurements Horita et al. (1982) reported no evidence of an increased number of immunoglobulin-secreting cells in the peripheral blood of IDDM patients except in 7 out of 10 patients with concomitant IDDM and Hashimoto’s thyroiditis. The discrepancy is likely to be explained by the shorter period of clinical IDDM in our study. Thyroid disease was not found in our patients who were all thyroid antibody negative. The presence of infectious diseases is also unlikely to explain our findings. We observed that 4/17 patients had candidiasis, however, only 2 of them showed spontaneous antibody secretion while the other 2 were within normal range.

Several lines of evidence suggest that the development of IDDM is associated with a number of immunopathological phenomena. At the clinical onset of IDDM which occurs primarily in HLA-DR 3 and/or 4 positive individuals, most patients (60–90%) have circulating islet cell antibodies detectable in a variety of assay systems (for a review see Brogren & Lernmark 1982). Out of the present
17 IDDM patients, 15 (76%) had islet cell cytoplasmic antibodies titrated on frozen sections of human pancreas in an indirect immunofluorescence test (Marner et al. 1983). Many patients with IDDM of short duration also have signs of increased cellular hypersensitivity against pancreatic antigens (Nerup et al. 1971). Although the number of patients studied was limited we found that the IDDM patients with increased spontaneous immunoglobulin production had higher fasting C-peptide levels at the time of admission (Table 2).

In the 5 patients studied serially, one patient with increased immunoglobulin production at diagnosis developed lower C-peptide values while the other 4 remained unchanged. We also observed that the highest titres of islet cell cytoplasmic antibodies were detected in patients with spontaneous secretion. It is therefore less likely that the increased spontaneous secretion is simply explained by a lack of insulin which causes an altered immune response. An increased duration of IDDM is in fact known to be associated with a progressive loss of the ability of the endocrine pancreas to release C-peptide (Faber & Binder 1976). Although it remains to be established to what extent fasting C-peptide reflects the number of residual pancreatic B cells, it will be of interest to study the in vitro B lymphocyte function, not only in terms of total immunoglobulin classes, but particularly in terms of antibodies against specific islet cell autoantigens (Bøkkeskov et al. 1982). In Graves’ disease and Hashimoto’s thyroiditis the plaque forming cell assay has revealed thyroglobulin antibody producing cells particularly by thyroid-derived lymphocytes (Weetman et al. 1982). The specific immunoregulatory abnormalities detected in the present system may assist in the identification before the clinical onset of individuals known by heritage and/or HLA-DR typing to be susceptible to developing IDMM (Gorusch et al. 1981; Srikantha et al. 1983).

Acknowledgments
We wish to thank Bodil Andreassen, Jane Falk, Gitte Bille, and Anne Ambjørnsen for expert technical assistance and Stig Waldorff for statistical advice. This study was supported in part by the National Institutes of Health (Grant AM 26190) and by the National Danish Association against the Rheumatic Diseases (Grant 233-379).

References


Received on September 28th, 1983.