An Association of Autoantibody Status and Serum Cytokine Levels in Type 1 Diabetes

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At onset of type 1 diabetes, the islet autoantibody status of patients has been reported to predict progression of the disease. We therefore tested the hypothesis that the systemic immunoregulatory balance, as defined by levels of circulating cytokines and chemokines, is associated with islet autoantibody status. In 50 patients with recent-onset type 1 diabetes, antibodies to GAD and insulinoma-associated antigen 2 (IA-2) were analyzed by radioimmunoassay; cytoplasmic islet cell antibodies were determined by indirect immunofluorescence. Cytokine and chemokine concentrations were measured by rigidly evaluated double antibody enzyme-linked immunosorbent assay. Of four classically defined Th1/Th2 cytokines (γ-interferon, interleukin [IL]-5, IL-10, IL-13), none showed an association with multiple autoantibody positivity. Of six mediators mainly produced by innate immunity cells, three were associated with multiple autoantibody status (IL-18 increased, MIF and MCP-1 decreased) and three were unaffected (IL-12, MIP-1β, IP-10). GAD and/or IA-2 antibody titers negatively correlated with systemic concentrations of MIF, MIP-1β, and IL-12. Combining the data of several cytokine and chemokine levels made it possible to predict islet antibody positivity in individual patients with 85% sensitivity and 94% specificity. These data suggest a close association of islet antibody status with systemic immunoregulation in type 1 diabetes. *Diabetes* 52: 1137–1142, 2003

Type 1 diabetes results from immune-mediated destruction of insulin-producing β-cells in pancreatic islets of Langerhans (1,2). The recent observation of type 1 diabetes occurring in a patients who lack B-cells and autoantibodies (3,4) demonstrated that the disease process might not involve humoral immunity, yet the majority of patients show humoral autoimmunity at diagnosis of insulin dependence (5–7). Prominent islet autoantibody types include glutamic acid decarboxylase antibodies (GADA), insulinoma-associated antigen 2 antibodies (IA-2A), and islet cell antibodies (ICA), a mixture of various autoantibodies binding to islet cell cytoplasm constituents in cryostat sections of human pancreas (8,9). In addition, insulin autoantibodies are prominent in young patients (5,10).

The presence of islet autoantibodies in serum of first-degree relatives indicates increased risk of developing type 1 diabetes (5–7). An important finding was that the highest risk is associated with the presence of two or more different islet autoantibody species (5,11). This observation suggests that the islet autoantibody status may distinguish mild from strong disease activity. After clinical onset of the disease, different characteristics have been noted for patients who were either positive or negative for islet autoantibody (12–20). In addition, high and multiple islet autoantibody titers may be an early sign of a rapid disease progression in terms of loss of residual β-cell function during the first years of disease (21–26). The latter association was not found in all studies (27,28).

Taken together, these findings suggest that the islet autoantibody status in recent-onset type 1 diabetes is linked to disease activity. We therefore tested the hypothesis that the islet autoantibody status in recent-onset type 1 diabetes reflects the immunoregulatory state. As surrogate of the immunoregulatory milieu, we analyzed systemic levels of circulating immunoregulatory mediators, i.e., cytokines and chemokines.

RESEARCH DESIGN AND METHODS

Patients. All patients received a diagnosis of type 1 diabetes during 1999–2001. In adults, type 1 diabetes was distinguished from type 2 diabetes by the presence of lean BMI in combination with hyperglycemia, ketonuria, and short duration of symptoms such as polyuria, polydipsia, and weight loss.

Informed consent was obtained from all patients before blood sampling. At the time of blood withdrawal, patients were in stable metabolic control and were on insulin for at least 3 days but not longer than 2 weeks after diabetes diagnosis. In all patients, metabolic control was stabilized within 24 h of diagnosis by intravenous insulin infusion, followed by at least an additional 7 days of hospitalization. All samples were obtained while patients were still in the hospital, with intense insulin treatment and strict dietary guidelines. None of the patients showed symptoms of acute infectious disease. Serum was isolated within 1 h of blood sampling and stored at −20°C until further use.

Islet autoantibody determination. Cytoplasmic ICAs were detected by the indirect immunofluorescence test on unfixed cryostat sections of human pancreas from an organ donor with blood group 0 as described (23) The cutoff of our assay was 2.5 Juvenile Diabetes Foundation units. In the Immunology of Diabetes Workshop ICA Proficiency Program, our laboratory achieved values of 100% for sensitivity, specificity, validity, and consistency (Lab ID #116). Autoantibodies to full-length GAD65 (GADA) and the intracytoplasmic domain of the tyrosine phosphatase-like protein IA-2 (IA-2A) were determined by radioligand assays as described previously (23). Antibody levels were expressed as arbitrary units calculated as follows: U = (cpm [test serum] − cpm [negative standard serum])/(cpm [positive standard serum] − cpm [negative standard serum]) × 100. Cutoff levels were 6.5 GADA units and 3.4 IA-2A units, respectively (99th percentile of normal controls). In the Com-

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ELISA, enzyme linked immunosorbent assay; GADA, glutamic acid decarboxylase antibody; IA-2A, insulinoma associated antigen 2 antibody; ICA, Islet cell antibody; IL, interleukin; IP, interferon-inducible protein; MCP, monocyte chemoattractant protein; MIF, macrophage migration inhibition factor; MIP, macrophage inflammatory protein; OD, optical density.

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bined Autoantibody Workshop, the adjusted diagnostic sensitivity for the GAD and IA-2 antibody assays was 81 and 74% with a specificity of 99% for both assays (29).

Cytokine determination. Concentrations of cytokines and chemokines in the sera were measured by double-sandwich enzyme linked immunosorbent assay (ELISA) using matched antibody pairs for interleukin (IL)-10, interferon-inducible protein (IP)-10 (Pharmingen, San Diego, CA), γ-interferon (IFN-γ; Endogen, Woburn, MA), and macrophage migration inhibition factor (MIF) (R&D Systems, Wiesbaden, Germany) or commercially available sets for IL-5, IL-12 (p70), IL-13 (OptEIA Set, Pharmingen), and IL-18 (Biozol, Eching, Germany). Precoated plates (Endogen) were used to measure monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1β following the manufacturer's instructions.

ELISA conditions. Ninety-six-well microtiter plates were coated with capture antibody overnight at 4°C and were blocked with PBS (0.2 mol/l) containing 10% (vol/vol) FCS, which was used also as assay diluent. Samples and standards were incubated overnight on a shaker at 4°C. After washing, detection antibodies and streptavidin-horseradish peroxidase conjugate (Biosource) were each incubated for 1 h or 30 min, respectively, at room temperature. Manufacturer's recommendation was followed for measuring IL-5, IL-12, (p70) and IL-13. Color reaction was monitored after adding substrate tetramethyl benzidine and stopped by adding stop solution (3 N SO_4H_2). The intensity of color was quantified by measuring absorbance at 450 nm within 30 min after stopping the reaction. Detection levels were 2 pg/ml for IL-10 and IFN-γ, 3 pg/ml for IL-5, 4 pg/ml for IL-12 (p70) and IL-13, 5 pg/ml for IP-10, 6 pg/ml for MCP-1, 10 pg/ml for MIP-1β, 15 pg/ml for IL-18, and 76 pg/ml for MIF.

ELISAs were established to meet the following criteria: linearity of signal for the standard curve was between optical density (OD) 0.05 and 2.0. Detection level was the mean pg/ml corresponding to the lowest OD 450 of linear range of the standard curve that was not 0. For the purpose of log transformation, the calculated 0 pg/ml values were corrected to 0.1 pg/ml, which is within the 10% variation of the assay. Differences between expected and measured signal in spiking experiments were <15%, mean intra-assay variation below 10%, mean interassay variation <10%, loss of signal after freezing and thawing of sera three times <20%. When sera gave signals above OD 2.0, measurements were repeated with more dilute samples. All sera were analyzed in duplicate. Measurements were repeated when there was >20% difference between the two parallel measurements. For avoiding the interference of heterophile antibodies (as a source for false-positive signals) FCS was added to the assay diluents as recommended (30). In addition, any serum that gave similar readings at 450 nm in several assays was remeasured with a nonmatched second antibody. In all cases, this led to background levels of OD, excluding interference by heterophile antibodies.

For assessing the biological variation of cytokine or chemokine levels, 15 healthy adults without signs of acute infection (8 male, 7 female; mean age, 27.5 ± 3.2 years) were analyzed for cytokine and chemokine levels in serum on two occasions, 3 months apart. When comparing the median of concentrations measured at baseline and at 3 months, the mean differences were 2% for IFN-γ, 4% for IL-5 or IL-10, 27% for IP-10, 40% for IL-18, and 50% for MIF.

Statistics. Because individual cytokine or chemokine levels were not normally distributed, mean and median concentrations were calculated per group. Differences between groups were assessed by Kruskal-Wallis followed by Mann-Whitney *U* test, two-sided. $P < 0.05$ was considered significant. Significant influences of age and sex were evaluated with multiunivariate linear regression analysis. All *P* values given are adjusted for these possible confounders.

The correlation of cytokine levels was evaluated using Spearman rank correlation. For identifying combinations of chemokines and cytokines predictive of subjects who were either negative or positive for autoantibody, discriminant analysis was carried out. We used both linear and quadratic methods, and we report leave-one-out-estimated classification rates.

To classify individuals into antibody-positive or antibody-negative groups, we performed discriminant analysis to find a set of prediction equations based on cytokine and chemokine levels as independent variables. Data were screened on the basis of the criteria described elsewhere (31). Excel spreadsheet (Microsoft, Seattle, WA), NCSS 2000 (Number Cruncher Statistical Systems, Keysville, UT) and GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA) were used for statistical analysis.

TABLE 1
Islet autoantibody status of patients

Patient no.	Age* (years)	Sex (m/f)	Islet autoantibody titer†		
			ICA	GADA	IA2-2A
1	17	f	0	1.5	0.1
2	20	f	0	1.3	0.1
3	28	f	0	1.3	0.1
4	28	m	0	1.5	0.1
5	30	m	0	0.23	0.1
6	30	m	0	1.7	0.1
7	31	m	0	0.31	0.1
8	32	m	0	0.1	0.1
9	32	f	0	2.2	0.1
10	34	m	0	0.4	0.1
11	35	m	0	1.8	0.1
12	39	f	0	0.81	0.1
13	43	m	0	0.1	0.1
14	44	m	0	2.8	0.1
15	5	m	40	1.7	0.1
16	21	m	40	5.5	0.2
17	30	m	5	0.46	1.6
18	35	m	3	2	0.5
19	23	m	0	84.1	0.1
20	27	f	0	34.2	0.1
21	31	f	0	122.2	0.1
22	32	m	0	102	0.1
23	38	f	0	43	0.1
24	42	f	0	114.1	0.1
25	49	f	0	17.4	1.5
26	7	m	10	124.5	1.9
27	15	m	>80	16.8	0.1
28	22	f	>80	28.6	0.1
29	23	f	>80	111.2	0.1
30	30	m	>80	7.8	0.1
31	30	m	>80	81.9	0.1
32	31	f	40	107.1	0.1
33	32	m	>80	116.4	0.1
34	33	f	3	33	0.1
35	33	m	20	7.6	0.1
36	34	f	3	154.8	2.6
37	36	m	20	38.9	0.1
38	36	m	40	109.9	0.1
39	43	m	5	116.5	0.1
40	52	f	3	61	0.1
41	32	m	>80	4.5	94.3
42	11	m	>80	47.7	99.2
43	11	f	40	17.6	101.6
44	12	f	40	184.6	94.1
45	12	m	10	13.7	96.4
46	12	f	40	53.4	101.5
47	15	f	5	93.4	92.7
48	22	f	>80	43.2	92.1
49	24	m	10	49.7	11.4
50	58	f	40	120.6	47.2

*At diagnosis of type 1 diabetes. †The threshold for antibody positivity was 2.5 Juvenile Diabetes Foundation units/ml for ICA, 6.5 units/ml for GADA, and 3.4 units/ml for IA-2A. The dotted lines distinguish no from single, double, or triple islet autoantibody positivity.

RESULTS

Antibody status of patients with recent-onset type 1 diabetes. Of 50 subjects who received a diagnosis of type 1 diabetes during 1999–2001, 14 had no detectable ICA, GADA, or IA-2A (Table 1). Eleven patients were positive for one islet autoantibody type (4 for ICA, 7 for GADA), and 25 subjects had detectable serum levels of at least two autoantibodies (Table 1). The three subgroups did not differ significantly for mean age or distribution of sex.

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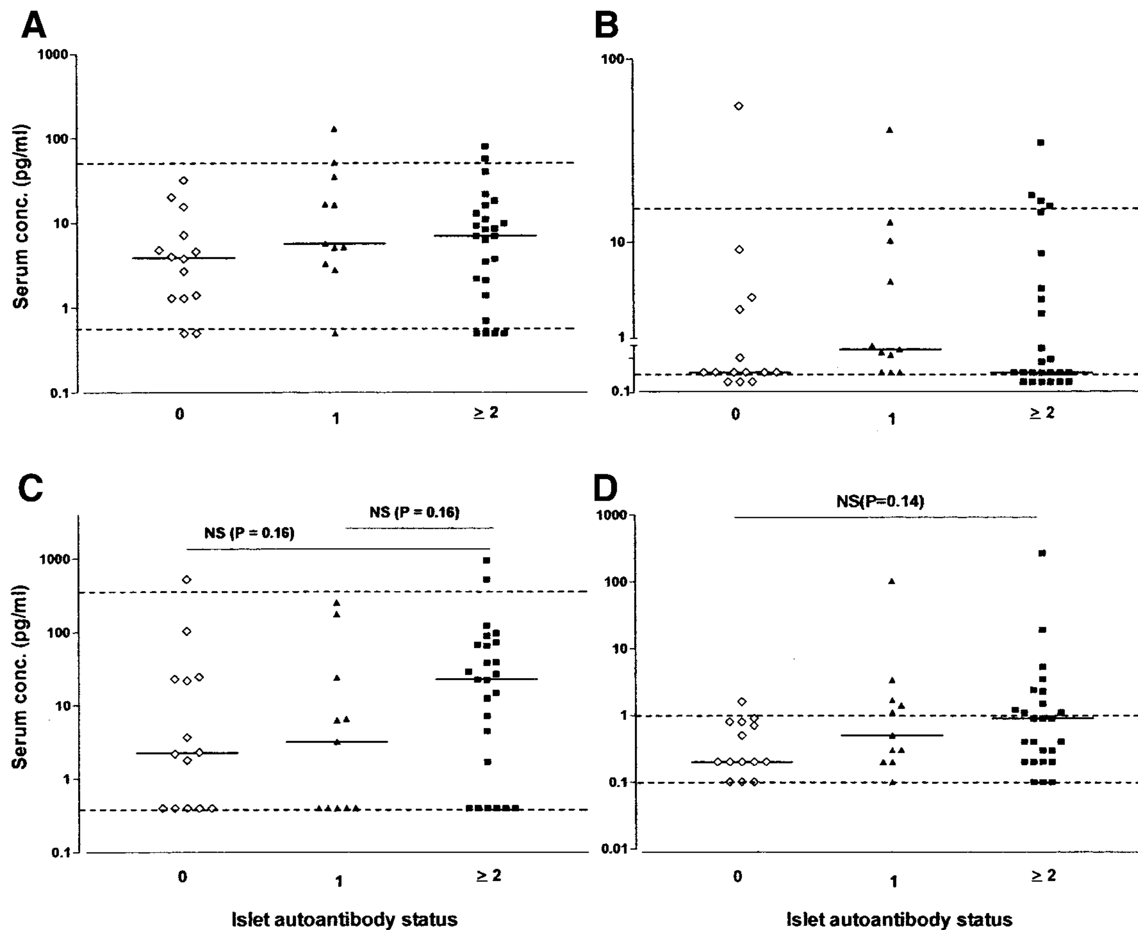

FIG. 1. No association of classic Th1 or Th2 cytokines with islet autoantibody status. IFN-γ (*A*), IL-13 (*B*), IL-5 (*C*), and IL-10 (*D*) serum levels. Each symbol depicts an individual patient. The horizontal bar indicates the median cytokine concentration per group. Dotted lines show the normal range.

Association of antibody status with classic Th1- or Th2-type cytokines. The lead cytokine of Th1 cells, IFN-γ, did not differ in median serum concentrations between islet autoantibody-negative and single- or multiple-positive patients (Fig. 1*A*). For the lead Th2 cytokine IL-4, we rarely found serum levels above detection limit. We instead determined systemic concentrations of IL-13, which shares receptor elements with IL-4. As shown in Fig. 1*B*, median cytokine levels were not significantly affected by antibody status.

Next, we analyzed systemic levels of IL-5 and IL-10. For both cytokines, the higher median levels in the multiple autoantibody-positive group were not significantly different from the autoantibody-negative group (Fig. 1*C* and *D*). There was significant correlation of individual IL-5 serum levels with those of IL-10 ($r = 0.56$, $P < 0.001$).

Association of islet autoantibody status with innate immunity cytokines. Mean serum concentrations of IL-18 significantly correlated with autoantibody status in that levels were lower in the absence of detectable islet autoantibody versus the presence of only one ($P < 0.05$) or more than two autoantibodies ($P < 0.05$; Fig. 2*A*). It is interesting that levels of IL-12, which shares Th1-promoting activity with IL-18, were not elevated in autoantibody-positive groups (Fig. 2*B*).

Chemokines associate with islet autoantibody status. Both median MIF and MIP-1β serum levels were lower in the multiple islet autoantibody-positive versus -negative patients (significant for MIF; Fig. 3). Serum IP-10 levels were slightly elevated in single but not in multiple autoantibody-positive patients (Fig. 4*A*). By contrast, levels of the antagonistic chemokine MCP-1 were decreased in patients with islet autoantibodies (Fig. 4*B*).

Association of islet autoantibody status with cytokine/chemokine pattern. An additional approach was to use the pattern of all cytokines and chemokines measured to discriminate between islet autoantibody-negative and -positive groups. By applying discriminant function analysis, sera could be predicted as being islet antibody-negative with 84.6% sensitivity (11 of 13 negative sera identified) and 69.0% specificity. Conversely, islet autoantibody-positive sera were predicted with 85.3% sensitivity (29 of 34 positive sera identified) and 93.5% specificity (Table 2). It is interesting that individual concentrations of only three immune mediators were required for classifying sera as antibody-positive or -negative, with the specificity and sensitivity as described in Table 2. These were MCP-1, MIF, and IL-18. As evident from Figs. 1–3, cytokine and chemokine levels poorly distinguished between single and multiple islet autoantibody positivity.

Association of serum cytokine/chemokine levels with islet autoantibody type or titer. These analyses were performed with GADA and IA-2A but not ICA because the last represents a mixture of antibodies against various islet

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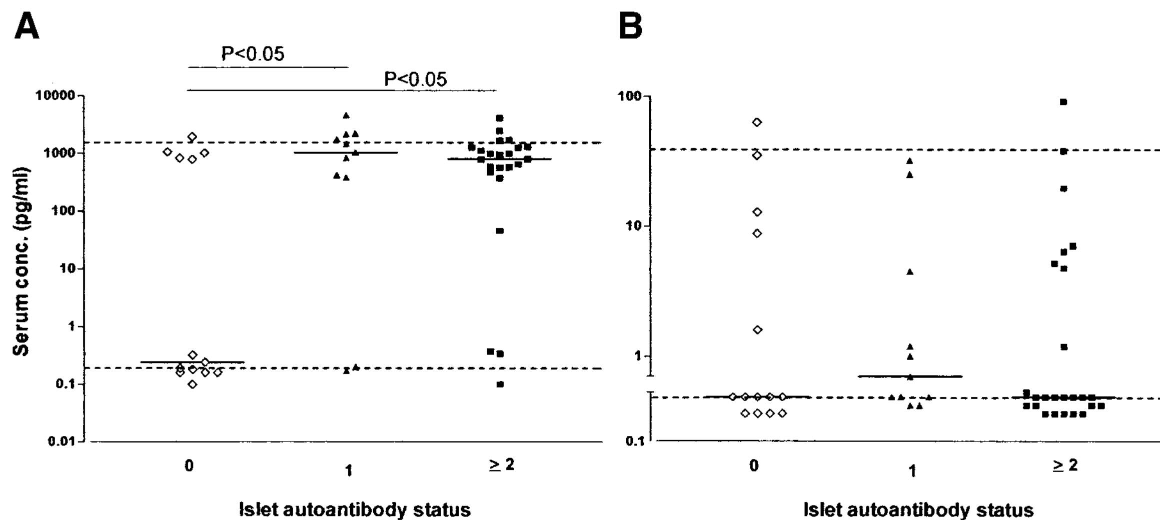

FIG. 2. Association of innate immunity cytokines with islet autoantibody status. IL-18 (*A*) and IL-12 (p70) (*B*) serum levels. Each symbol depicts an individual patient. The horizontal bar indicates the median cytokine concentration per group. Dotted lines show the normal range.

cell antigens, including GAD and IA-2. A comparison of systemic cytokine/chemokine levels for patients positive for GADA with those positive for IA-2A did not show significant differences in median concentrations for any of the mediators ($P > 0.05$). There was some correlation between individual cytokine/chemokine levels and antibody titer. MIF levels were associated with both GADA and IA-2A titers. Serum levels of MIP-1β and IL-12 correlated with IA-2A titers but not with concentrations of GADA (Table 2). It is interesting that all three mediators showed a negative correlation with antibody titers.

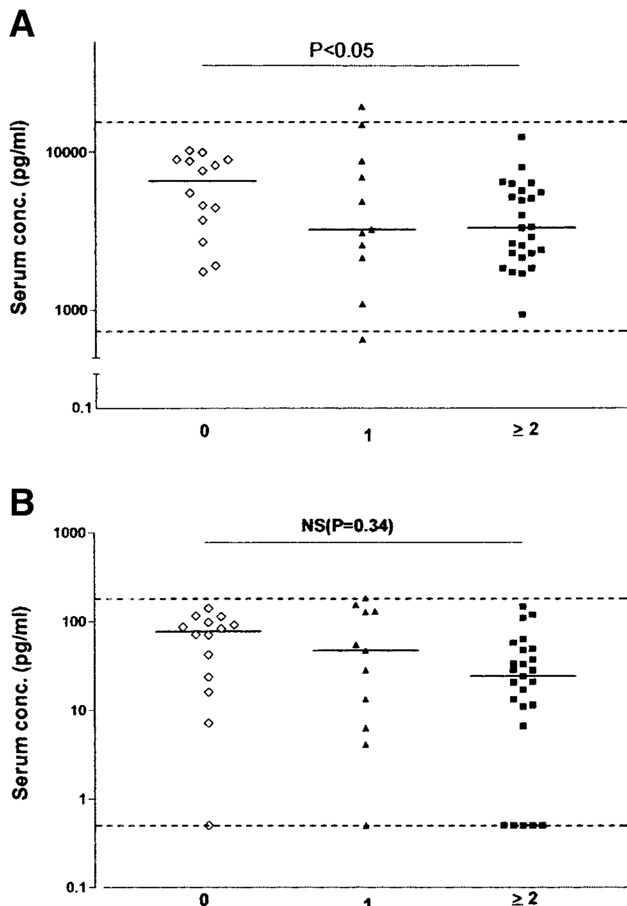

FIG. 3. Association of MIF and MIP levels with islet autoantibody status. *A*: MIF. *B*: MIP-1β. Each symbol depicts an individual patient. The horizontal bar indicates the median cytokine concentration per group. Dotted lines show the normal range.

DISCUSSION

The study focused on GADA, IA-2A, and ICA as the three major islet autoantibodies in type 1 diabetes (5–7). A fourth islet autoantibody species, insulin autoantibody, is prominent only at young age (5,10). Because almost all patients studied were adolescents or adults, the last autoantibody type could not be included.

Evidence has been accumulating that the islet autoantibody status at diagnosis of type 1 diabetes reflects disease quality, in that patients with islet autoantibodies exhibit faster loss of endogenous β-cell function during the next years (12–20). In addition, multiple autoantibody positivity and titer seem relevant (21–26). It therefore seems relevant that multiple islet autoantibody-positive patients can be clearly distinguished from autoantibody-negative patients on the basis of systemic cytokine/chemokine concentrations. No differences were observed at the level of classic Th1/Th2 defining cytokines. Of four cytokines analyzed, none showed an association. Of six mediators that are mainly produced by innate immune cells, three showed significantly different median levels between multiple autoantibody-positive and -negative groups.

Because islet autoantibody status has not been studied in parallel with systemic cytokine/chemokine levels before, the data cannot be related to previous reports. However, two findings seem to be consistent throughout the present study. The first is that classic cytokines, mainly T-cell–derived Th1 type (IFN-γ) or Th2 type (IL-5, IL-10, IL-13), did not show an association with islet autoantibody status. By contrast, there was an association of autoantibody status with chemokines, three of six mediators were significantly associated. This link between products of the innate immune system and islet autoantibody production

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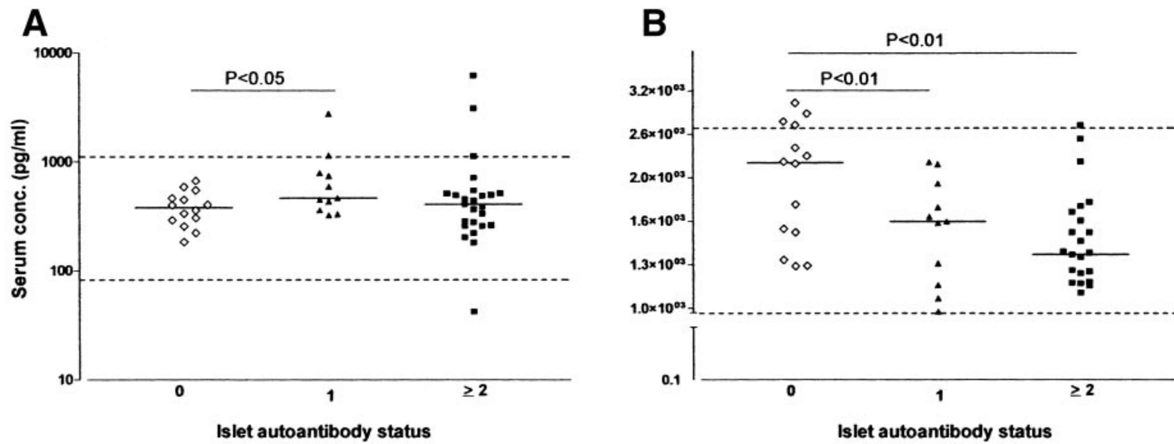

FIG. 4. Association of IP-10 and MCP-1 levels with islet autoantibody status. IP-10 (*A*) and MCP-1 (*B*) serum levels. Each symbol depicts an individual patient. The horizontal bar indicates the median cytokine concentration per group. Dotted lines show the normal range.

is novel and has to await confirmation in independent studies.

A second, unexpected finding was that there was no association between multiple islet autoantibody positivity and an enhanced systemic Th1 bias. Of the Th1-associated mediators IFN-γ, IL-18, IL-12, MIF, and IP-10, only IL-18 was increased and MIF levels were lower in multiple autoantibody-positive patients. The distribution of IL-18 levels in individual sera was almost bimodal with either high or low concentrations. Such an uneven distribution of IL-18 serum levels has also been reported in patients with cardiovascular disease (32). Th2 associated mediators were not affected by autoantibody status (IL-13), tended to be increased (IL-5, IL-10), or were decreased in serum levels (MCP-1). These findings do not bear on the bias toward Th1-type cytokines in type 1 diabetes as reported by many groups (33–42). However, among patients with type 1 diabetes, a multiple islet autoantibody positivity does not associate with a further enhanced systemic Th1 bias.

In a separate analysis we compared individual GAD and IA-2 autoantibody titers with cytokine/chemokine levels. It is interesting that, again, only mediators of the innate immune system were found associated. MIP-1β, MIF, and IL-12 were found to be inversely correlated with GADA and/or IA-2A. These findings extend the association of autoantibody and systemic cytokines/chemokines.

Final support for a nonrandom association of systemic cytokine/chemokine levels with islet autoantibody status came from the attempt of combining data for several mediators to distinguish between antibody-positive and -negative patients, at the individual level. A standard approach for this purpose is discriminant function analysis. We found that the cytokine/chemokine pattern could predict autoantibody positivity, with 85% sensitivity and 94% specificity. Data of only three mediators were required for staging of sera. It is interesting that all three mediators, MCP-1, MIF, and IL-18, came from the innate immune system rather than from T-cells.

TABLE 2
Prediction of islet autoantibody status from cytokine/chemokine pattern by discriminant function analysis

Islet autoantibody	No. of patients*	Identification by chemokine/cytokine pattern	
		Sensitivity (%)	Specificity (%)
None	13	84.6	69.0
>1	34	85.3	93.5

*Of three patients, IL-18 levels were not available; these patients were excluded from the analysis.

The association of islet autoantibody status and cytokine/chemokine levels in serum provides further evidence for the clinical relevance of systemic concentrations of immune mediators. Serum cytokine/chemokine patterns clearly are nonrandom and may help with monitoring disease activity as has been reported for diabetes development in autoimmune diabetic NOD mice (43). Similarly, as reported for the autoantibody status at the onset of type 1 diabetes, cytokine and chemokine levels may be useful for predicting the loss of residual C-peptide secretion.

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