Elevated Serum IP-10 Levels Observed in Type 1 Diabetes

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OBJECTIVE — Although most patients with type 1 diabetes are considered to have T-cell–mediated autoimmune disease, a method of measuring of pancreatic β-cell–specific T-cell function in cases of type 1 diabetes has yet to be established. Here, we focused on interferon-inducible protein-10 (IP-10), a chemokine that promotes the migration of activated T-helper 1 (Th1) cells and measured serum IP-10 levels in patients with human type 1 diabetes, which is regarded as a Th1-mediated disease.

RESEARCH DESIGN AND METHODS — Serum samples were obtained from diabetic patients, and the levels of autoantibodies (GAD and insulinoma-associated protein-2 [IA-2]) and IP-10 were measured. Diabetic patients positive for either or both of the autoantibodies were classified as Ab$^+$ type 1, and those negative for both were classified as Ab$^-$ type 1. To evaluate islet antigen–specific responses, peripheral blood from patients stimulated with or without GAD was used, and intracellular cytokine staining for flowcytometry was performed.

RESULTS — The Ab$^+$ and Ab$^-$ type 1 groups both showed a significantly higher serum IP-10 level than the healthy subjects ($P < 0.001$ and $P < 0.05$, respectively), and the IP-10 level in the recent-onset Ab$^+$ subgroup was significantly higher than that in the established (longstanding) Ab$^+$ subgroup ($P < 0.002$). Furthermore, there was a significant positive correlation between the serum IP-10 level and the number of GAD-reactive γ-interferon–producing CD4$^+$ cells in the Ab$^+$ type 1 group ($P < 0.007$).

CONCLUSIONS — Our findings demonstrate that measurement of serum IP-10 concentrations is useful in patients with type 1 diabetes.

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M ost patients with type 1 diabetes are considered to have T-cell–mediated autoimmune disease. T-cell insulitis exists not only in cases of classical type 1 diabetes with an insulin-dependent state (1), but also in cases of type 1 diabetes with residual β-cell function, as we reported previously (2). However, a clinically applicable method of measuring pancreatic β-cell–specific T-cell function in cases of type 1 diabetes has yet to be established, probably because of the low frequency of these T cells among peripheral blood lymphocytes. Thus, measurement of autoantibodies against pancreatic β-cells, such as GAD and insulinoma-associated protein-2 (IA-2), is currently the only way to evaluate the autoimmune state in cases of type 1 diabetes (3). Although measurement of autoantibodies is an efficient way to screen for type 1 diabetes, the autoanti-

body titer itself does not consistently reflect the disease activity. For instance, a high GAD antibody titer can be seen in Stiffman's syndrome in nondiabetic patients (4). Therefore, it is important to establish a suitable marker of T-cell reactivity toward pancreatic β-cells in cases of type 1 diabetes. Chemokines, factors that induce directional migration of various types of cells including T lymphocytes, have recently been reported to be important for the regulation of immune and inflammatory reactions (5). We have focused on interferon-inducible protein-10 (IP-10), a chemokine that promotes the migration of activated T cells (T helper 1 [Th1] lymphocytes) (6–9), and we recently reported a correlation between the level of this chemokine and disease activity in cases of chronic active hepatitis in humans (10). In the present study, we measured serum IP-10 levels in patients with type 1 diabetes, which is considered to be a Th1-mediated disease, to evaluate whether measurement of serum IP-10 concentration is useful in cases of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Patients
Serum samples were obtained from 74 Japanese diabetic patients on admission, with informed consent from the patients and permission from the institutional review board. Samples were collected in the morning and frozen at −80°C until assay, and autoantibodies (GAD and IA-2), IP-10, and γ-interferon (IFN-γ) were measured as described below. Diabetic patients negative for both autoantibodies were designated the Ab$^-$ type 1 group ($n = 23$, 12 men, 11 women; age 40.4 ± 2.5 years; disease duration 5.6 ± 1.5 years). Those positive for either or both were designated the Ab$^+$ type 1 group (autoimmune diabetes; $n = 42$, 21 men, 21 women; age 36.9 ± 2.1 years; disease duration 5.0 ± 0.8 years). The diagnosis of Ab$^-$ type 1 diabetes was based on the criteria of the American Diabetes Association (ADA) for type 1 diabetes, with pan-

creatic β-cell destruction as the primary cause of the disorder and a tendency toward ketoacidosis (11,12). The definition of Ab[+] type 1 diabetes was made based on GAD and/or IA-2 antibody positivity and the requirement for insulin. Therefore, the latter category included Ab[+] diabetic patients with residual β-cell function, so-called latent autoimmune diabetes in adults (LADA) (13) or slowly progressive insulin-dependent diabetes (14) (*n* = 12). Classical type 1 diabetes with autoantibody (*n* = 30) met the criteria of the ADA for type 1 diabetes. None of the patients had inflammatory diseases, including hepatitis. Control serum samples were obtained from healthy subjects (*n* = 49, 28 men, 21 women; age 35.3 ± 1.8 years) and from Ab[−] type 2 diabetic patients (*n* = 9, 7 men, 2 women; age 48.1 ± 5.6 years; disease duration 5.0 ± 2.3 years).

Measurement of autoantibodies IP-10 and IFN-γ

The antibodies GAD65 (detection limit <0.4 u/ml; 100% sensitivity and 100% specificity of the assay in the GAD antibody proficiency test [Immunology of Diabetes Workshop]; Lab ID number 305) and IA-2 (detection limit <0.75 u/ml; M. Powell, S. Chen, H. Tanaka, M. Masuda, C. Beer, B. Rees Smith, J. Farmaniak, unpublished observations) were detected using a previously described radioligand binding assay (15). The normal range of the assay was considered to be the mean + 3 SD of the levels in healthy subjects.

IP-10 concentration was measured by enzyme-linked immunosorbent assay (ELISA) using two anti-human IP-10 monoclonal antibodies (αhIPb and αhIPd) as described previously (10). Briefly, 96-well ELISA plates were coated overnight at 4°C with αhIPb at 50 μl/well (20 μg/ml). After discarding the antibody, unbound sites were blocked with 100 μl Block-Ace for 2 h at room temperature (RT). After three washes with phosphate-buffered saline (PBS) containing 0.05% Tween-20, 50-μl aliquots of standard solutions of IP-10 or serum samples were added, and incubation was continued for 1 h. After three washes, 50 μl biotinylated αhIPd (0.3 μg/ml in PBS containing 1% bovine serum albumin [BSA]) was added as the second antibody. The plates were incubated for 1 h and washed three times, then 50 μl of streptavidin-conjugated β-D-galactosidase diluted 1/1,000 was added to each well.

After a 1-h incubation period, the plates were washed three times, and 50 μl of 0.01% 4-methyl-umbelliferyl-β-D-galactoside was added. After shaking for 10 min, 100 μl of 2 mol/l sodium carbonate was added to stop the reaction, and the fluorescence intensity of the samples in the wells was determined at 460 nm (excitation 355 nm) using a Fluoroscan II microplate fluorometer (Labsystems, Hampshire, U.K.). The amount of chemokine present was determined from a standard curve prepared using purified recombinant chemokine. The detection limit for IP-10 was <10 pg/ml. The intra- and interassay variations in the assay of IP-10 were <10% (6 and 9%, respectively).

IFN-γ concentration was measured by ELISA as previously described (16). Briefly, a flat-bottom 96-well plate was coated with anti–IFN-γ antibodies (NIB42; Pharmingen, San Diego, CA) in PBS with overnight incubation at 4°C. Serum samples and standard solutions (purified recombinant IFN-γ) were added, and incubation was continued for another 2 h. After washing with 0.1% Tween 20 PBS, biotinylated anti–IFN-γ antibodies (4S.B3; Pharmingen) were added, followed by another 1-h period of incubation. AB solution (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) was then added, and incubation was continued for 30 min. Finally, the color reagent 2,2′-azinobis (3-ethylbenz-thiazoline sulfonic acid [ABTS; Sigma, St. Louis, MO]) was added and the optical density was measured with an ELISA reader (Bio-Rad, Richmond, CA) at 405 nm. The amount of cytokine present was determined from a standard curve prepared using purified recombinant cytokine. The detection limit for IFN-γ was <20 pg/ml. Intra- and interassay variations in the assay of IFN-γ were <5% (3 and 4%, respectively). Because IL-4 was not detectable in the serum of any of the Ab[+] type 1, Ab[−] type 1, or Ab[−] type 2 diabetic patients, only sera from control subjects who also had IL-4 levels below the detection limit were used in this study. This allowed us to rule out the possibility of heterophile antibodies in serum (17).

Antigen-specific stimulation and intracellular cytokine staining for flowcytometry

To examine antigen-specific cytokine responses, 500 μl of heparinized whole blood was placed in 5-ml polystyrene round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ) containing 500 μl RPMI1640 medium (GibcoBRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL), penicillin/streptomycin (Gibco-BRL), and 1 μg anti-CD28 antibody (L293; Becton Dickinson, San Jose, CA), with or without 5 μg/ml recombinant GAD65 produced in yeast (RSR, Cardiff, U.K.). To confirm the system was working, the response to PPD (Japan BCG, Tokyo) was used as a positive control for subjects with a positive tuberculin test. The tubes were incubated at 37°C in a humidified 5% CO_2 atmosphere for a total of 72 h with the last 4 h including a final concentration of 10 μg/ml Brefeldin A (Sigma). After incubation, 300 μl of activated blood was transferred to other tubes. Then, 20 μl CD4-PC5 antibody (Coulter, Marseille, France) was added and the tubes were incubated at RT for 15 min. Then, 4 ml of FACS lysing solution (Becton Dickinson) was added, vortexed gently, and incubated at RT for another 10 min. After centrifuging the tubes at 1,600 rpm for 5 min, the supernatant was removed, and the cells were washed with 0.1% BSA-PBS. Then, 1.5 ml FACS permeabilizing solution (Becton Dickinson) was added and the tubes were incubated for 10 min at RT in the dark. After washing with 0.1% BSA-PBS twice, 20 μl antibody mixture (IFN-γ-FITC and IL-4-PE [phycoerythrin]; Becton Dickinson) or isotype control mixture (IgG2a-FITC and IgG1-PE; Becton Dickinson) was added and the tubes were incubated for 30 min at RT in the dark. After washing, the prepared cells were analyzed by Epics Altra (Coulter).

Statistical analysis

Results are presented as means ± SE. Differences in serum IP-10 levels between groups were analyzed using the Mann-Whitney *U* test for nonparametric unpaired observations. The correlation between serum IP-10 and IFN-γ levels, IP-10 and disease duration, IP-10 and age, and IP-10 and the number of GAD-reactive IFN-γ–producing CD4^{+} cells was analyzed by Spearman correlation analysis.
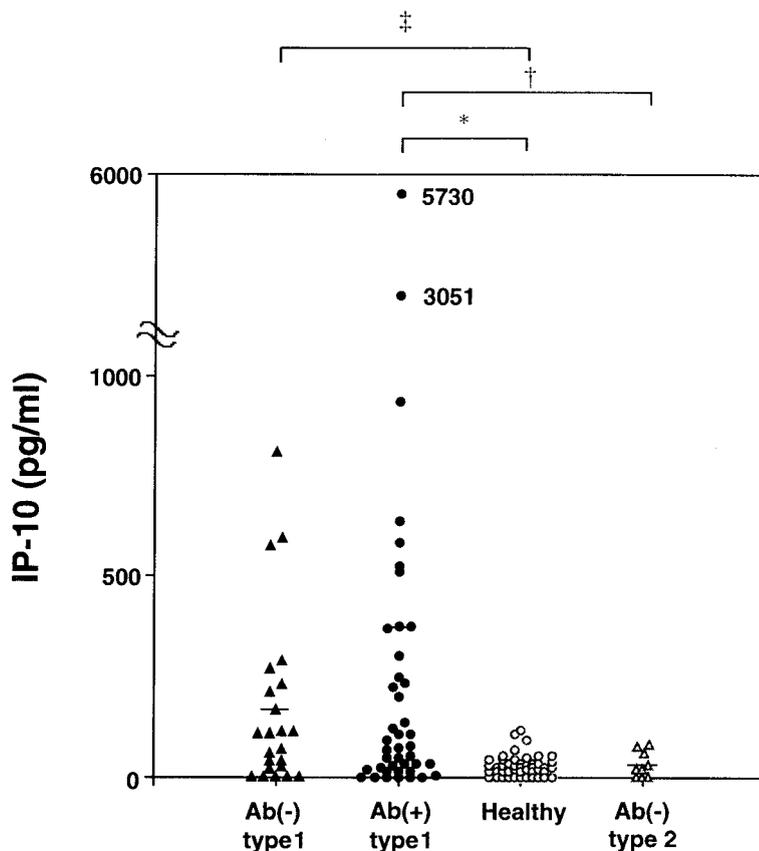
Figure 1—*Serum IP-10 levels in Ab⁻ type 1 and Ab⁺ type 1 diabetic patients, healthy subjects (Healthy), and Ab⁻ type 2 diabetic patients. *P < 0.001, †P < 0.02, ‡P < 0.05.*

RESULTS

Serum IP-10 is significantly elevated in autoimmune diabetes

Type 1 diabetes is regarded as a heterogeneous disorder, as suggested by a study of pancreatic biopsy specimens from insulin-dependent patients with type 1 diabetes (18), among whom insulitis was mainly observed in Ab⁺ cases and only rarely in Ab⁻ cases. Therefore, we divided the type 1 diabetic patients into the two following groups: Ab⁺ type 1 and Ab⁻ type 1.

First, we measured the serum levels of IP-10 in both the Ab⁺ and Ab⁻ type 1 groups and compared the levels with those in the controls. As shown in Fig. 1, both the Ab⁺ type 1 (mean 394.5 pg/ml) and Ab⁻ type 1 (mean 166.1 pg/ml) patients showed significantly elevated serum IP-10 levels as compared with the healthy subjects (mean 41.5 pg/ml; $P <$ 0.001 and $P < 0.05$, respectively). Moreover, the serum IP-10 level was significantly higher in the Ab⁺ type 1 (but not the Ab⁻ type 1) patients than in the Ab⁻

type 2 diabetic patients (mean 38.2 pg/ml; $P < 0.02$). Among the patients in the Ab⁺ type 1 group, both classical type 1 (mean 480.6 pg/ml) and LADA (mean 179.2 pg/ml) patients showed significantly elevated serum IP-10 levels compared with the healthy subjects ($P <$ 0.001 and $P < 0.03$, respectively). Moreover, no significant difference in IP-10 levels was observed between the classical type 1 and the LADA groups. Therefore, these two groups were combined as the Ab⁺ type 1 group in this study. Thus, the serum concentration of IP-10 is significantly elevated in cases of autoimmune diabetes (Ab⁺ type 1).

Positive correlation between serum IP-10 and IFN-γ in cases of autoimmune diabetes

IP-10 is thought to be a chemoattractant for Th1 lymphocytes (9), and IFN-γ is known to destroy pancreatic β-cells in vitro (19). Therefore, we examined the relationship between the serum levels of IP-10 and IFN-γ; the latter is likely to be an important Th1 type cytokine in cases

of autoimmune diabetes. A significant positive correlation between serum IP-10 and IFN-γ levels was observed in this group ($P < 0.02$). This correlation was still evident when we reanalyzed the data from only GAD antibody–positive or only IA-2 antibody–positive patients. However, there was no correlation between the autoantibody titer and the IP-10 level (data not shown). The correlation between serum IP-10 and IFN-γ levels was specific to the Ab⁺ type 1 group (autoimmune diabetes), because no such relationship was observed in the Ab⁻ type 1 group, the Ab⁻ type 2 diabetic patients, or the healthy control subjects (data not shown). Regarding absolute levels of serum IFN-γ, no significant difference was observed among the groups.

High serum IP-10 levels observed in cases of recent-onset autoimmune diabetes

If serum IP-10 reflects insulitis activity, one could argue that the level should be high in cases of recent-onset autoimmune diabetes because the β-cell mass would be reduced by β-cell destruction in cases of established (longstanding) type 1 diabetes. Therefore, we compared serum IP-10 (or IFN-γ) levels in the recent onset Ab⁺ type 1 (disease duration <3 years) and the established (long-standing) Ab⁺ type 1 groups (disease duration ≥3 years). As shown in Fig. 2, in the Ab⁺ type 1 group, the recent-onset patients showed a significantly higher IP-10 level than those with established diabetes (mean 622.6 vs. 88.5 pg/ml, $P < 0.002$). Moreover, there was a significant negative correlation between serum IP-10 levels and disease duration in this group ($P < 0.04$). Furthermore, we also analyzed the relationship between serum IP-10 levels and age in cases of autoimmune diabetes because higher insulitis activity is generally expected in younger subjects. There was a significant negative correlation between these two factors (i.e., IP-10 and age [$P < 0.03$]). These relationships were specific to autoimmune diabetes (Ab⁺ type 1), because no such correlation was observed in the other groups (data not shown). On the other hand, the level of IFN-γ, which is reported by some investigators to be significantly elevated in cases of recent-onset type 1 diabetes (20,21), was not significantly higher in the patients with recent-onset Ab⁺ type 1 diabetes than it was in
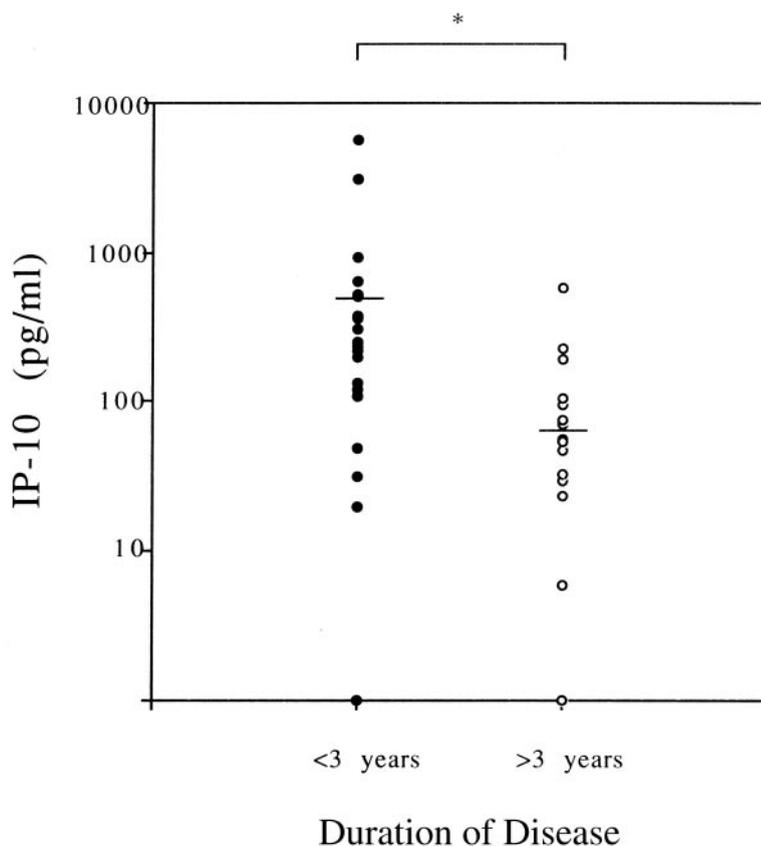
Figure 2—*Patients with a disease duration <3 years (recent onset) showed significantly higher IP-10 levels than patients with a disease duration of ≥3 years (established diabetes) among those with Ab$^+$ type 1 diabetes* (P < 0.002).

those with established Ab$^+$ type 1 diabetes (288.4 vs. 152.8 pg/ml; P = 0.16).

Positive correlation between serum IP-10 levels and number of GAD-reactive IFN-γ—producing CD4$^+$ cells

To confirm that serum IP-10 really reflects reactivity to pancreatic islets, we examined the correlation between serum IP-10 levels and the number of GAD-reactive IFN-γ— producing CD4$^+$ cells in cases of autoimmune diabetes and found a significant positive correlation between the two (P < 0.007). Moreover, GAD-reactive IFN-γ–producing CD4$^+$ cells were observed in all of the IP-10$^+$ (level greater than mean + 3 SD for healthy subjects) patients examined, but in only 21.4% of IP-10$^-$ (level less than mean + 3 SD for healthy subjects) Ab$^+$ type 1 diabetic patients examined (P < 0.002 using Fisher's exact test). Approximately (mean) 100 GAD-reactive IFN-γ– producing CD4$^+$ cells in 50,000 CD4$^+$ cells in peripheral blood from patients

with autoimmune diabetes were detected in this system.

CONCLUSIONS — In animal models of type 1 diabetes, such as the NOD mouse, we previously showed that a shift from a Th2-dominant to a Th1-dominant response is correlated with the onset of type 1 diabetes (22). Recently, lower IL-4 production (23) (less Th2 state = Th1-dominant state) and a negative correlation between cellular immunity and autoantibody response (24) have been reported in cases of human type 1 diabetes as well. Therefore, evaluation of T-cell reactivity in cases of type 1 diabetes is clearly important. Many investigators are struggling to establish a T-cell assay directed at pancreatic β-cell antigens, but this goal has been elusive. One reason may be the low frequency of autoreactive T cells in the periphery. As shown in this study, only (mean) 100 GAD-reactive IFN-γ– producing cells were identified among 50,000 CD4$^+$ cells in Japanese type 1 diabetic patients. In this situation, we no-

ticed the existence of IP-10, a CXC chemokine that selectively upregulates human T-cell cytokine synthesis with enhancement selectively targeted to promotion of Th1-like dominance (25). This chemokine is known to induce migration of Th1 cells to local lesions (9), raising the possibility of it being useful as a marker of Th1 activity in local (pancreatic islet) lesions of type 1 diabetes. In this study, we found the serum IP-10 level in the Ab$^+$ type 1 group (autoimmune diabetes) to be significantly higher than that in control subjects and that the serum IP-10 level was correlated positively with the serum IFN-γ level in this group. Moreover, in this study we observed that, with GAD stimulation, the number of IFN-γ– producing CD4$^+$ cells was positively correlated with serum IP-10 levels in Ab$^+$ type 1 diabetic patients. Antigen-driven IFN-γ responses have been shown to increase dramatically in the presence of very low IP-10 concentrations (25). Therefore, we speculate that serum IP-10 levels may reflect the Th1 type response (or at least the cellular immune response) against pancreatic β-cells. We have also demonstrated the existence of IP-10 in insulitis lesions in NOD mice and the upregulation of IP-10 in the pancreas after cyclophosphamide treatment (to induce diabetes) in this model (J. Morimoto et al., unpublished data). Thus, it seems that there is a correlation between IP-10 levels and insulitis activity. It would be of great interest if a correlation between serum IP-10 levels and the existence of insulitis lesions could be confirmed in humans as well. Such confirmation should be sought in future studies.

IP-10 levels were also higher in the Ab$^-$ type 1 group than in the healthy subjects, and there was no significant difference between the Ab$^+$ and Ab$^-$ type 1 groups in this study. One reason may be inadequate sensitivity of the autoantibody assay. If a more sensitive assay for detecting the autoimmune response against pancreatic β-cells could be established, some patients with autoimmune-related diabetes might be detected among the Ab$^-$ type 1 population. We are currently attempting to detect T-cell reactivity to GAD and/or IA-2 antigen using an intracellular cytokine assay focusing on the Ab$^-$ type 1 group and to determine whether there is a correlation with serum IP-10 levels. If we can confirm that assaying the serum level of IP-10 is a

more sensitive means of detecting an autoimmune response than assaying autoantibodies in patients with type 1 diabetes, this would further support the importance of measurement of IP-10 concentrations in cases of type 1 diabetes. Another possible reason for high IP-10 levels in the Ab$^-$ type 1 group is that this group may include some patients who have autoantibodies against pancreatic β-cells other than GAD and IA-2. However, we cannot exclude the possibility that high IP-10 levels in the Ab$^-$ type 1 group may reflect a pathophysiology other than the Th1-type response. More detailed analyses of this group are necessary to reach a conclusion.

Regarding the serum IFN-γ levels in patients with recent-onset type 1 diabetes as determined in previous studies, the results are inconsistent (20,21,26). The most recent report has shown significantly higher serum IFN-γ levels in recent-onset patients as compared with established type 1 diabetic patients (20). In the present study, we could not find any difference between the two. One explanation might be differences in age. In the previous report (20), the median age of the type 1 diabetic patients examined was 23 years, as compared with 37–40 years in our study. Moreover, children were included in their study but not in ours. Another possibility is differences between Caucasians and the Japanese. Unlike Caucasian type 1 diabetes, which includes many cases of abrupt-onset type 1 diabetes associated with insulin-dependence, Japanese type 1 diabetes includes many LADA patients. Because the possibility of a difference between childhood-onset and adult-onset type 1 diabetes remains, we must also investigate IP-10 levels in children to reach a conclusion. However, we consider that measurement of serum IP-10 concentration in diabetic patients, at least in adults, with autoimmune-related type diabetes to be useful.

Although mean serum IP-10 levels in classical type 1 diabetes were higher than those in LADA patients, we could not find a significant difference between the two. We cannot exclude the possibility of bias resulting from the relatively small number of samples, but even when we analyzed the "recent onset" (disease duration <3 years) Ab$^+$ type 1 group, still no significant difference was observed between the two (classical type 1 vs. LADA; mean 793

vs. 270.6 pg/ml). More cases should be accumulated to reach a conclusion.

One might argue that we should investigate serum IP-10 levels in prediabetics as well to further clarify the importance of measuring serum IP-10 levels in cases of type 1 diabetes. Unfortunately, however, we had no access to suitable samples. Therefore, we are planning to follow serum IP-10 levels to see if IP-10 predicts the insulin-dependent state in Ab$^+$ diabetic patients who were originally diagnosed with type 2 diabetes and who are not currently being treated with insulin, along with the IgE subclass of GAD antibody (GAD-IgE), the level of which reportedly decreases just before the onset of hyperglycemia in cases of type 1 diabetes (27). Although there was no correlation between serum IP-10 levels and GAD (or IA-2) antibody titers, attempting to assess the relationship between serum IP-10 and GAD-IgE might be worthwhile in a future study. At this moment, the serum IP-10 levels examined so far in Ab$^+$ diabetic patients who were originally diagnosed with type 2 diabetes and who are not currently being treated with insulin were negative (less than mean + 3 SD for healthy subjects), suggesting that disease (insulitis) may not be active in these patients. To evaluate whether measurement of serum IP-10 levels has predictive value for insulin dependency in these patients, close observation and follow-up of these patients, including periodical measurement of serum IP-10 levels, are necessary.

We previously reported a correlation between serum IP-10 levels and disease activity in cases of human chronic active hepatitis (10). The present results suggest that in cases of human autoimmune diabetes, elevated serum IP-10 levels may reflect active disease (or insulitis) status, because recent-onset patients (disease duration <3 years) showed significantly higher IP-10 levels than patients with established diabetes (disease duration ≥3 years), and a significant positive correlation between IP-10 levels and the number of GAD-reactive IFN-γ–producing CD4$^+$ cells was observed in cases of autoimmune diabetes. In addition to the above data, we also found that the proportion of IP-10$^+$ patients (level greater than mean + 3 SD for healthy subjects) in the recent-onset Ab$^+$ type 1 subgroup was significantly larger than that in the established Ab$^+$ type 1 subgroup (65.2%

vs. 15.8%, $P < 0.01$ using Fisher's exact test). These observations were not considered to be attributable to nonspecific inflammation, because other inflammatory diseases were excluded in this study and the serum IP-10 level was not elevated in patients with rheumatoid arthritis; in these patients the erythrocyte sedimentation rate is high, as we reported previously (10). Based on these findings, we conclude that measurement of serum IP-10 concentration is useful in cases of type 1 diabetes, and this approach is considered to be important in delineating the pathogenesis of autoimmune diseases.

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