Defective Function of Fas in Patients With Type 1 Diabetes Associated With Other Autoimmune Diseases

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Fas (CD95) triggers programmed cell death and is involved in cell-mediated cytotoxicity and in shutting off the immune response. Inherited loss-of-function mutations hitting the Fas system cause the autoimmune/lymphoproliferative syndrome (ALPS). We have recently shown that ALPS patients’ families display increased frequency of common autoimmune diseases, including type 1 diabetes. This work evaluates Fas function in type 1 diabetic patients without typical ALPS. Cell death induced by anti-Fas monoclonal antibody was investigated in T-cells from 13 patients with type 1 diabetes alone and 19 patients with type 1 diabetes plus other autoimmune diseases (IDDM-P). Moreover, we analyzed 19 patients with thyroiditis alone (TYR), because most IDDM-P patients displayed thyroiditis. Frequency of resistance to Fas-induced cell death was significantly higher in patients with IDDM-P (73%) than in type 1 diabetic (23%) or TYR (16%) patients or in normal control subjects (3%). The defect was specific because resistance to methyl-prednisolone–induced cell death was not significantly increased in any group. Fas was always expressed at normal levels, and no Fas mutations were detected in four Fas-resistant IDDM-P patients. Analysis of the families of two Fas-resistant patients showing that several members were Fas-resistant suggests that the defect has a genetic component. Moreover, somatic fusion of T-cells from Fas-resistant subjects and the Fas-sensitive HUT78 cell line generates Fas-resistant hybrid cells, which suggests that the Fas resistance is due to molecules exerting a dominant-negative effect on a normal Fas system. These data suggest that Fas defects may be a genetic factor involved in the development of polyreactive type 1 diabetes. Diabetes 50:483–488, 2001

Fas/Apo-1 (CD95) is a type I transmembrane molecule belonging to the tumor necrosis factor (TNF) receptor superfamily and interacts with the Fas ligand (FasL), a type II transmembrane protein belonging to the TNF cytokine superfamily. Fas ligation by mAbs or FasL induces programmed cell death in several cell lines (1,2). Fas plays a dual role in immune response. Lymphocytes can express Fas and be targets of cells expressing FasL. This Fas/FasL interaction is involved in shutting off immune responses, lymphocyte life span regulation, induction of peripheral tolerance, and exclusion of immune cells from immune privileged sites. Moreover, CTL, T<sub>h</sub>1, and NK cells express FasL, and their interaction with Fas expressed by target cells is one of the mechanisms they use to exert cytotoxic function.

Both Fas functions are involved in autoimmunity because 1) Fas-mediated cytotoxicity results in tissue damage in cell-mediated autoimmune diseases, and 2) defective Fas function alters the immune response shutting-off system and promotes autoimmunity. In fact, high levels of Fas and FasL are expressed in β-pancreatic islets from type 1 diabetic patients, thyroids from patients with Hashimoto thyroiditis, and multiple sclerosis lesions (3), whereas inherited loss-of-function mutations of the Fas gene seem to delay the disease onset in experimental models of type 1 diabetes and multiple sclerosis, i.e., in nonobese diabetic (NOD) mice and experimental autoimmune encephalomyelitis (4–7). In addition, decreased function of Fas is associated with the development of autoimmunity and lymphoproliferation. This pattern is typically displayed by MLR<sub>lpr/lpr</sub> and gld/gld mice and patients with the autoimmune/lymphoproliferative syndrome (ALPS) carrying inherited loss-of-function mutations of the Fas or FasL genes and developing hematological autoimmunities and nonmalignant lymphoproliferation with lymphadenopathy and/or splenomegaly and expansion of CD4<sup>+</sup>CD8<sup>+</sup>TCRβ (double negative [DN]) T-cells (1,2,8–10). Moreover, these mutations are not the only cause of decreased Fas function associated with autoimmunity, because we detected deficient Fas function in a group of pediatric patients with an ALPS-like
clinical pattern but no mutation of the Fas gene and no DN T-cell expansion (11,12). T-cells from these patients were also resistant to cell death induced by ceramide, which triggers a death pathway partially overlapping that used by Fas (13), and we suggested that the defect may be due to genetic alterations of the signaling pathway(s) used both by Fas and ceramide. This possibility has been recently confirmed in two patients with an ALPS-like clinical pattern who displayed mutations of caspase-10, which is involved in Fas signaling, in the absence of mutations of Fas and FasL (14). A recent classification named ALPS-Ia and -Ib the disease with mutations of Fas and FasL, respectively, and ALPS-II the disease without mutations of these genes (10). We have recently suggested that these inherited defects may also be predisposed to development of autoimmune diseases different from the rare ALPS pattern because an analysis involving 10 ALPS-II patients’ families showed that they displayed increased frequency of common autoimmune diseases, including type 1 diabetes (1). Moreover, defective Fas-induced T-cell death was also detected in a substantial proportion of patients with the multiple autoimmune syndrome (i.e., autoimmune patients from families displaying more than one case of autoimmunity within first- or second-degree relatives) (12). Also these families displayed several organ-specific and systemic autoimmune diseases, including type 1 diabetes. This observation created the possibility that decreased function of Fas may play a role in the development of type 1 diabetes, at least in some patients, and prompted this study, which evaluates Fas function in pediatric type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

A total of 32 patients with type 1 diabetes were evaluated: 13 of them displayed type 1 diabetes alone (type 1 diabetes), whereas 19 displayed type 1 diabetes plus thyroiditis and 6 patients with IDDM-P. This is not surprising because autoimmune thyroid diseases are the most frequent autoimmunities associated with type 1 diabetes (15). A total of 19 patients with thyroiditis alone (TYR) were also evaluated.

Clinical features of patients are summarized in Table 1. Informed consent was obtained from patients or their parents.

**Immune phenotypic analysis.** Expression of surface molecules was evaluated by direct immunofluorescence and flow cytometry (FACScan; Becton Dickinson, San Jose, CA). The following monoclonal antibodies (mAbs) were used: anti-CD3 (Leu-4), anti-CD4 (Leu-3a), anti-CD8 (Leu-2a), anti-CD80 (Becton Dickinson), anti-Fas (Immunotech, Marseille, France). CD4 and CD8 DN TCRαβ–positive cells were detected by two-color immunofluorescence, using fluorescein isothiocyanate (FITC)-conjugated anti-TCRαβ mAb and phycocerythrin (PE)-conjugated anti-CD4 and -CD8 mAbs. Fas was detected by two-color immunofluorescence on resting and activated T-cells using PE-conjugated anti-CD3 mAbs and FITC-conjugated mAbs to Fas (Chemicon, Temecula, CA). Nonspecific background fluorescence was established with the appropriate isotype-matched control mAb (Becton Dickinson). Antigenic density was expressed as median fluorescence intensity ratio (MFI-R) of total lymphocytes according to the following formula: MFI-R = MFI of sample histogram (arbitrary units)/MFI of control histogram (arbitrary units).

**Functional and genetic analysis of Fas.** Fas- or C2-ceramide–induced cell death was evaluated as previously reported (11) on T-cell lines obtained by activating peripheral blood mononuclear cells with phytohemagglutinin (PHA) at days 0 (1 μg/ml) and 15 (0.2 μg/ml) and cultured in RPMI 1640 plus 10% fetal calf serum (FCS) (PS) plus recombinant interleukin (IL)-2 (2.5 U/ml) (Biogen, Geneva, Switzerland). Fas function was assessed 6 days after the second stimulation (21 days of culture). Cells were incubated with control medium or anti-Fas mAbs (IgM isotype) (1 μg/ml) (Upstate Biotechnology, Lake Placid, NY) in the presence of recombinant IL-2 (1 U/ml) to minimize spontaneous cell death. Cell survival was evaluated after 18 h by counting live cells in each well by the trypan blue exclusion test. The same conditions were used to measure cell death induced by methyl-prednisolone (PDN) (100 μmol/l) (Upjohn, Poors, Belgium) or C2-ceramide (50 μmol/l) (N-acetyl-sphingosine) (Sigma, St. Louis, MO). Assays were performed in triplicate and analyzed by a blind observer. Cells from two normal donors were included in each experiment as a positive control. Results were expressed as percent relative cell survival, calculated as follows: (total live cell count in the assay well/total live cell count in the control well) × 100. In the control well (i.e., in the absence of apoptotic stimuli), spontaneous cell loss was always <10% of the seeded cells and similar in cultures from the patients and normal donors.

This protocol was chosen in preliminary experiments when several anti-Fas mAb concentrations (10, 1, and 0.1 μg/ml) and incubation times (1, 4, 8, 18, 48, and 72 h) were used to induce cell death in T-cell lines derived from normal donors cultured for 3, 6, 9, 15, 18, 21, and 24 days with PHA plus IL-2. Cell death was evaluated indirectly (by counting total surviving cells by the trypan blue exclusion test) or directly (by fluorescence-activated cell sorter [FACS] determination of the proportion displaying shrunken/hypergranular morphology or those stained by annexin V using the Annexin-V-Fluos kit [Boehringer Mannheim, Mannheim, Germany]). The protocol chosen was found to give the

| Patient | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
| IDDM-P  |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| Sex     | F | F | F | F | F | M | M | F | M | F | M | M | F | F | F | M | M | M | M |
| Age     | 17 | 15 | 15 | 17 | 7 | 18 | 16 | 13 | 17 | 13 | 14 | 14 | 6 | 15 | 10 | 12 | 10 | 17 | 6 |
| At study | 4 | 7 | 3 | 7 | 2 | 8 | 6 | 4 | 8 | 6 | 10 | 7 | 5 | 8 | 5 | 9 | 6 | 3 | 6 |
| Other autoimmune diseases† | T | T | T | T | T | T | T | T | T | T | T | T | T | T | A | RA | E | V | RA | RA | S | A | Thr |
| Type 1 diabetes | ● | ● | ○ | ● | ▲ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ |
| Sex     | F | F | F | F | M | M | M | M | F | F | M | M | M | M | M | M | M | M | M | M |
| Age     | 12 | 9 | 16 | 14 | 13 | 17 | 23 | 18 | 15 | 14 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| At diagnosis | 5 | 5 | 10 | 10 | 6 | 2 | 12 | 9 | 10 | 9 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 |
| TYR     | ● | ● | ○ | ● | ▲ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ | ▼ |
| Sex     | F | F | F | F | M | M | M | M | F | F | M | M | M | M | M | M | M | M | M | M |
| Age     | 17 | 11 | 8 | 10 | 14 | 16 | 13 | 18 | 18 | 8 | 15 | 14 | 16 | 12 | 20 | 12 | 13 | 16 | 17 | 17 | 17 | 17 |
| At diagnosis | 15 | 9 | 5 | 10 | 12 | 14 | 13 | 15 | 15 | 6 | 14 | 13 | 13 | 11 | 12 | 9 | 11 | 13 | 15 | 15 | 15 | 15 |

*Symbols used in Fig. 1. †A, alopecia; E, hepatitis; RA, juvenile rheumatoid arthritis; S, scleroderma; T, thyroiditis; Thr, thrombocytopenia; V, vitiligo.*
Fas-induced cell death was assessed in long-term T-cell lines derived from 19 patients with IDDM-P, 13 patients with type 1 diabetes, 19 patients with TYR, and 65 normal donors. We also evaluated the response to ceramide, whose pathway partially overlaps that of Fas (13), and to PDN, which does not directly involve the Fas system. Figure 1 and Table 2 show that 14 of 19 IDDM-P patients were resistant to Fas-induced cell death, whereas only 3 of 13 type 1 diabetic patients and 3 of 19 TYR patients were Fas-resistant. Resistance to ceramide was displayed by four IDDM-P, three type 1 diabetic, and two TYR patients, and five of them (three IDDM-P, one type 1 diabetic, and one TYR) were also resistant to Fas. Resistance to PDN was displayed by three IDDM-P and two type 1 diabetic patients, and three of them (two IDDM-P and one type 1 diabetic) were also resistant to Fas. In all Fas-resistant patients, Fas resistance was confirmed by evaluating Fas-induced apoptosis by annexin V staining (data not shown). Similarly to that in ALPS patients (11,12), Fas function was not abolished in Fas-resistant patients because cell death increased when incubation was prolonged to 48 h and/or Fas triggering was potentiated by anti-IgM antibodies (data not shown).

Statistical analysis showed that frequency of resistance to Fas was significantly higher in IDDM-P patients than in type 1 diabetic patients, TYR patients, and normal control subjects (Table 2). Moreover, frequency of resistance to ceramide was significantly higher in IDDM-P patients than in normal control subjects, whereas frequency of resistance to PDN was not significantly increased in any group. In the IDDM-P group, no difference was found between patients with type 1 diabetes plus thyroiditis (n = 13) (10 were Fas-resistant, 2 ceramide-resistant, and 2 PDN-resistant) and those with type 1 diabetes plus autoimmune diseases different from thyroiditis (n = 6) (4 were Fas-resistant, 2 ceramide-resistant, and 2 PDN-resistant).

Fas expression was evaluated in each T-cell line by direct immunofluorescence on the same day the cell death assay was performed and was always in the normal range (data not shown). A search for DN T-cells in fresh peripheral blood mononuclear cells by two-color immunofluorescence revealed expansion of these cells in only two IDDM-P patients—IDDM-P/19 and IDDM-P/5—with 10 and 13% DN cells, respectively.

To compare Fas function in Fas-sensitive and Fas-resistant individuals at different times of T-cell culture, peripheral blood T-cells from Fas-resistant IDDM-P/7, Fassensitive IDDM/1, and one normal donor were cultured with PHA plus IL-2, and Fas-induced cell death was assessed at days 1, 3, 6, 10, 18, and 21 of culture (cells were

The normal range of T-cell responses to Fas-, ceramide-, and PDN-induced T-cell death, defined as the mean ± 2 SD of data (percent cell survival) obtained from 65 normal donors, was 60 ± 22, 58 ± 32, and 49 ± 26, respectively.

In the hybrid cell lines, cell death induced by the anti-Fas mAb was detected by using the Annexin-V-Fluos kit and analyzed by flow cytometry. Long-term T-cell lines were treated with the indicated reagent, and survival was assessed after 18 h. Results are expressed as percent relative cell survival. The horizontal lines indicate the upper limit of the normal range, calculated as the mean + 2 SD from data obtained from 65 normal donors.

In the control groups (i.e., in the absence of apoptotic stimuli), spontaneous cell loss was always <10% of the seeded cells and similar in cultures from the patients and normal donors.

most reproducible results. It evaluates the overall cell survival at each time point and was found to be more sensitive than the other techniques, detecting the instantaneous proportion of dying cells at each time. Moreover, in a parallel experiment performed on 20 normal donors and 13 ALPS patients (10 ALPS-II and 3 ALPS-Ia), we found that the defective Fas function displayed by ALPS patients was better detected by evaluation of cell survival (by the trypan blue exclusion test) than by evaluation of cell apoptosis (by annexin V staining) (cell survival, mean ± SD: control 62 ± 10%, ALPS 90 ± 8%; cell apoptosis: control 16 ± 10%, ALPS 4 ± 3%) (12). Fas-induced cell death was always less striking in these polyclonal T-cell lines than in stabilized tumor cell lines because it was slower and more asynchronous.

In the hybrid cell lines, cell death induced by the anti-Fas mAb was detected by using the Annexin-V-Fluos kit and analyzed by flow cytometry. Hybrid cell lines were produced by polyethylene glycol-fusing PHA-activated CD4+ T-cells with the Fas-sensitive HUT78 cell line and by culturing fused cells in RPMI-1640 plus 10% FCS plus anti-Fas mAb (1 µg/ml). In these culture conditions, hybrid cells survive if Fas-resistant lymphocytes carry a dominant-negative factor inhibiting Fas function. By contrast, unfused HUT78 cells die after Fas triggering, and unfused CD4+ T-cells do not grow in the absence of appropriate stimuli. Control fusions between PHA-activated CD4+ T-cells from normal donors and HUT78 cells were performed in each experiment.

Mutation analysis of the Fas gene was performed by single-strand conformation polymorphism (SSCP) and cDNA sequencing, as previously reported (11).

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<td><strong>Fas-, ceramide-, and PDN-resistant subjects in normal control subjects and different patient groups</strong></td>
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<td>Subjects</td>
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<tr>
<td>Control subjects</td>
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<td>Type 1 diabetic patients</td>
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<td>TYR patients</td>
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<td>IDDM-P patients</td>
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Data are n (%). *Subjects displaying a specific cell survival higher than the mean + 2 SD displayed by the control subjects; †significantly different than IDDM-P (P < 0.05, Fisher’s exact test).
restimulated with PHA plus IL-2 at day 15). Figure 2 shows that T-cells from the normal donor and type 1 diabetes patient no. 1 were initially relatively resistant to Fas-induced cell death, but they became frankly sensitive after the second stimulation with PHA plus IL-2. In contrast, T-cell cultures from IDDM-P/1 were always Fas-resistant. These results are in line with those reported in previous works on ALPS patients (11,12) (see Research Design and Methods) and with the notion that Fas connection to the death signaling pathway is a late event in T-cell activation (1,2).

To evaluate the familial component of the decreased function of Fas, we evaluated susceptibility to cell death in the father and mother of the Fas-resistant patients IDDM-P/7, -P/10, and -P/11, and in the mother of patient IDDM-P/15 (the father was not available). All these subjects were healthy. Moreover, we evaluated two sisters of patient IDDM-P/10 and -P/15. These sisters displayed type 1 diabetes without other autoimmune diseases. We found that four of four mothers, two of three fathers, and one of two sisters were resistant to Fas-induced cell death. Moreover, the father with a normal response (the father of patient IDDM-P/10) was near the upper limit of the normal range. The mother of patient IDDM-P/10 and the father of patient IDDM-P/11 were also resistant to ceramide, whereas the mother of patient IDDM-P/11 and the father of patient IDDM-P/10 displayed a response to ceramide that was near the upper limit of the normal range (Fig. 3). The mothers of patient IDDM-P/11 and P/15 and the father of patient IDDM-P/7 were also resistant to PDN.

Decreased function of Fas has been reported in ALPS-Ia patients displaying mutations of the Fas gene and expansion of DN T-cells and in ALPS-II patients not displaying these features but displaying resistance to ceramide. Therefore, we searched for mutations of the Fas gene in four Fas-resistant IDDM-P patients (IDDM-P/1, -P/14, -P/18, and -P/19). SSCP analysis and sequencing of the whole coding region of the Fas gene did not detect any mutation (data not shown).

In ALPS patients, mutations often display a dominant-negative effect. To evaluate whether Fas-resistant patients carried factors capable of inhibiting a functional Fas system, we fused the Fas-resistant CD4+ T-cell lines derived from patient IDDM-P/10, the father and mother of patient IDDM-P/7 and -P/10, and the mother and sister of patient IDDM-P/15 with the human Fas-sensitive continuous HUT78 T-cell line and cultured these hybrid cells under the selective pressure of anti-Fas mAb. All these fusions gave rise to Fas-resistant hybrid cell lines (Fig. 4). The defect of Fas function was not due to loss of Fas expression in the hybrid cell lines, as detected by direct immunofluorescence (Fig. 4). By contrast, fusions of Fas-sensitive T-cell lines from 15 healthy donors with HUT78 cells did not give rise to any hybrid line.

**DISCUSSION**

These data show that a substantial proportion of IDDM-P patients display defective Fas-induced cell death. The death pathway triggered by ceramide was also defective in some patients, but less frequently. This defect may decrease the efficiency of the lymphocyte shutting-off system and may be involved in development of this polyreactive autoimmunity. The defect could be the outcome of a lymphocyte functional state induced by unknown environmental factors, although a genetic component is suggested by the observation that Fas function was decreased in most parents of Fas-resistant patients. Moreover, somatic fusion of T-cells from Fas-resistant subjects and a Fas-sensitive continuous T-cell line generates Fas-resistant hybrid cells, which suggests that Fas resistance is due to molecules exerting a dominant-negative effect on a normal Fas system. These molecules seem to be selectively expressed by Fas-resistant subjects because they are not detected in T-cells from Fas-sensitive normal donors.

In ALPS-II patients, defective Fas function is presum-
ably due to inherited mutations hitting the Fas signaling pathway downstream from Fas because 1) the Fas gene is not mutated, 2) ceramide-induced cell death is also defective, and 3) most ALPS-II patients’ parents display resistance to Fas- and/or ceramide-induced cell death in vitro (12). It is unlikely that our Fas-resistant IDDM-P patients carry mutations of the Fas gene because most of them did not display expansion of DN T-cells, which is usually displayed by ALPS-Ia patients (10). Moreover, the search for mutations of the Fas gene in four Fas-resistant patients did not detect any mutation. Therefore, the putative genetic defect(s) displayed by IDDM-P patients may be similar to those displayed by the ALPS-II patients. It must be underlined that our IDDM-P patients display two major differences from the ALPS-II patients: most of them did not display any sign of lymphoadenopathy and/or splenomegaly (only patient IDDM-P/19 displayed a moderate axillary lymphadenomegaly) and were normally sensitive to ceramide. However, this finding was not surprising because we found that ALPS-II patients’ families displayed a high frequency of common autoimmune diseases without signs of lymphoproliferation (12). Moreover, in a study involving autoimmune patients selected from families displaying a high frequency of autoimmunity, we found that a substantial proportion of them displayed resistance to ceramide-induced cell death, but only a few displayed resistance to ceramide-induced cell death (12), suggesting that these resistances may be inherited independently.

Type 1 diabetes is a cell-mediated autoimmune disease in which β-cells are destroyed by autoreactive cytotoxic T-cells and by an inflammatory response organized by autoreactive Th1 cells. Cytotoxic T-cells kill their targets by using perforin and granzymes, secreting cytotoxic cytokines, and triggering Fas expressed on target cells. Th1 cells produce cytokines inducing Fas expression in β-cells and express FasL, triggering apoptosis of Fas-positive targets (3,16–24). Moreover, the Fas/FasL interaction has been suggested to play a role in thyroiditis, in which Th1-induced inflammation induces expression of Fas and FasL in thyrocytes and causes tissue damage (3). Therefore, our suggestion that genetically based deficiencies of Fas may be predisposed to IDDM-P is in apparent contrast with this model and with the observation that the lpr character protects from autoimmunity in experimental models of type 1 diabetes (4,5). This model is debated by other authors (25–27). However, our data do not imply that decreased Fas function is a common cause of type 1 diabetes or thyroiditis because we detected it in most polyreactive patients, but only in a few with type 1 diabetes or TYR. Predisposition to autoimmune diseases is multifactorial and may involve factors controlling autoantigen expression, lymphocyte responsiveness, and several aspects of immune effector functions. Therefore, decreased function of the Fas system may protect from development of autoimmunity in certain genetic contexts and models of autoimmunity, but may be detrimental in others. Moreover, the expression pattern of the disease may be influenced by residual Fas function and function of other systems involved in apoptosis induction, such as TNF, TNF-related apoptosis-inducing ligands, and the granzymes systems, which use apoptotic pathways partially overlapping those of Fas (2,28,29).

In conclusion, our data suggest that defects of the immune response switching-off system may be involved in the development of polyreactive type 1 diabetes. Therefore, these defects may be novel genetic factors involved in the development not only of patterns of autoimmunity/lymphoproliferation but also of organ-specific autoimmunities. Moreover, the observation that a minority of type 1 diabetic and TYR patients display resistance to Fas- or ceramide-induced cell death suggests that these patients may develop other autoimmune manifestations and that evaluation of Fas function could be a valuable prognostic tool.

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